MESENCHYMAL STROMAL CELLS: PRECLINICAL AND CLINICAL CHALLENGES

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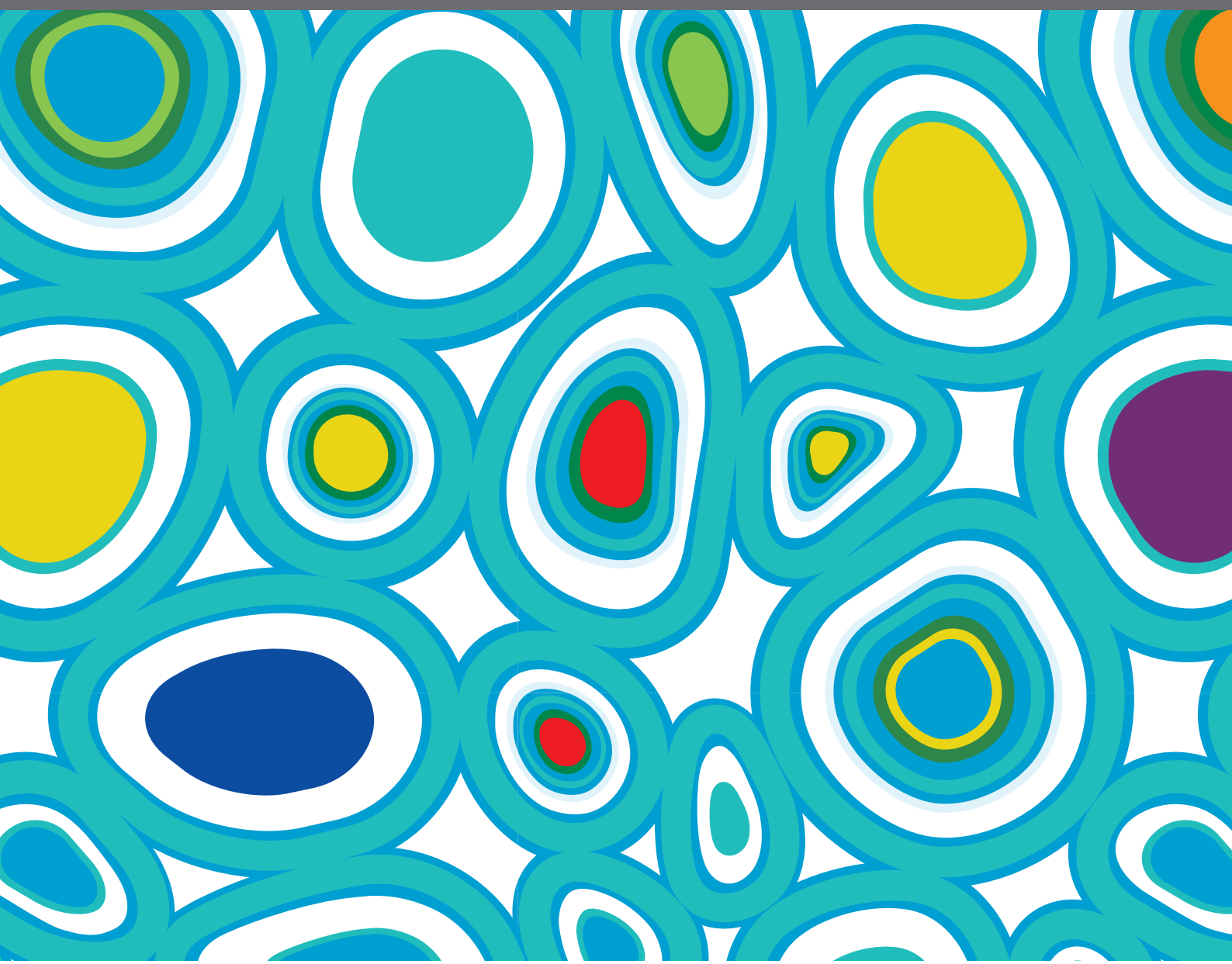

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MESENCHYMAL STROMAL CELLS: PRECLINICAL AND CLINICAL CHALLENGES

Topic Editors:
Joan Oliva, Emmaus Lifes Sciences, Inc., United States
Josep M. Canals, University of Barcelona, Spain
Mayasari Lim, Fujifilm Irvine Scientific, Inc., United States
Simone Pacini, University of Pisa, Italy

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EDITORIAL
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Editorial: Mesenchymal Stromal Cells: Preclinical and Clinical Challenges

Joan Oliva[1]*, *Simone Pacini*[2], *Josep M. Canals*[3] and *Mayasari Lim*[4]

[1]Department of Clinical Research, Emmaus Life Sciences, Torrance, CA, United States, [2]University of Pisa, Pisa, Italy, [3]Laboratory of Stem Cells and Regenerative Medicine, Department of Biomedical Sciences, Creatio- Production and Validation Center of Advanced Therapies, Faculty of Medicine and Health Sciences, University of Barcelona, Barcelona, Spain, [4]Fujifilm Irvine Scientific, Inc, Santa Ana, CA, United States

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Editorial on the Research Topic

Mesenchymal Stromal Cells: Preclinical and Clinical Challenges

For the past 20 years, mesenchymal stromal cells (MSCs) has become the most studied cell population in the development of adult cell therapies for disease treatment (Keshtkar et al., 2018; Han et al., 2019). MSCs can differentiate into many types of cells (neurons, hepatocytes, myoblast . . .) and this is one reason why MSCs hold great promise to the treatment of many immune diseases, cardiac and neurological injuries, and tissue regenerative applications (Hwang et al., 2009). Although MSC research discoveries brought new information, the road for cell therapy approval is still at its dawn. Due to the insurgence of MSC therapies, federal agencies with regulatory oversight to healthcare such as the Food and Drug Administration, European Medical Agency, Pharmaceuticals and Medical Devices Agency, Federal Service for Surveillance in Healthcare, etc. have adapted and continue to update their guidelines as needed (Mendicino et al., 2014; Corbett et al., 2017; Pigeau et al., 2018; Stroncek et al., 2022). Similarly, cell therapy manufacturers and suppliers had to adapt quickly in establishing and adopting best practices that ensure safety, quality, and reproducibility of products and raw materials destined to be used in cell therapy manufacturing. Even so, many questions have surfaced around preclinical tests, scalability of MSC production and clinical application, reproducibility of the results, better characterization of the MSCs, the need for development of defined culture media and GMP compliant animal-free components, ancillary materials, and the development of 3D structures mimicking the tissue organization.

Friedenstein reported the first fibroblastic-like and spindle-shaped cells to differentiate into other type of cells: chondrocyte and osteoblast (Friedenstein et al., 1968; Friedenstein et al., 1974). MSCs have been isolated from different tissues: adipose tissue, dental pulp, periosteum, Wharton's jelly, umbilical cord (Zuk et al., 2001; Nagamura-Inoue and He, 2014). Due to terminology discrepancies, one of the first task will be to use the exact terminology of MSCs depending on their functional attributes (Bhartiya, 2018). In addition of the Dominici minimal criteria, additional criteria were added for the MSC characterization like MSC responsive to INF-g, TNF-a, indoleamine 2,3 dioxygenase *etc* (Dominici et al., 2006; Bhartiya, 2018), thanks to the formation of international consortium among expert in MSCs. As mentioned by Najar *et al*, Wright et al. and Jayaraman et al., the identification of additional MSC markers will strongly support consistency of data obtained from studies. However, *in vitro,* and *in vivo* results obtained in clinical trials are still not consistent due to lot-to-lot variations, quality of the cells, variability among the donors. To decrease the variability among studies and to increase the reproducibility, standardization of the isolation, identification of additional surface markers, methodology of MSC expansion must be established (Stroncek et al., 2020). A recent study showed that, even if the laboratories use MSCs from the same material source, MSCs showed different behavior in terms of viability after thawing and different transcriptome due to different methodologies used in the lab and donor source

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Valerie Kouskoff,
The University of Manchester, United Kingdom

***Correspondence:**
Joan Oliva
joliva@emmauslifesciences.com

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(Stroncek et al., 2020). Standardization of MSC isolation, expansion, and characterization, like harmonizing the guidelines for clinicals trials is a major goal for the scientific community. Publications in this Research Topic hopefully will be part of the discussion to help understanding the challenges due to the failure to establish standardized protocols. After the characterization of the MSCs, one of the most important task and challenge encountered is to establish protocols to expand MSCs, that are in harmony and compliance with federal agencies.

There is still a debate around effective delivery and use of MSCs in treating diseases, by injection, by transplantation or by using the secreted extracellular vesicles. The cheapest and fastest methodology is the injection of isolated cells. Injection of cells showed encouraging results, but the long-term effects of MSCs are unknown in terms of treating the diseases, or impairing organ function due to random anchoring of the MSCs. In addition, the survival of injected MSCs is very low (Gyöngyösi et al., 2008), which could explain why treatment of patients is more complicated and not very efficient. Different approaches have been developed to overcome the low efficacy of injected MSCs: increasing the number of injected cells or increasing the number of injections, but it will require a larger scale manufacturing of MSCs, priming the cells, finding the optimal route of administration (Kurtz, 2008; Noronha et al., 2019). The development of cell sheets is an option to control the targeting of the cells in the organs. Myoblast cell sheets were transplanted on heart damaged areas, after heart failure, increasing the period of free events, increasing of survival, and decreasing of death rate. Characterization and the establishment of release criteria before transplantation is a major concern. Actually, only visual observation is used to determine when a cell sheet is ready, and the high variability in outcome is largely due to human dependency based on their experience and knowledge. A more rigorous approach as reported in by Ochiai et al., is to utilize physical characteristics (strength, optical) of the cell sheets to standardize the cell sheets release criteria in GMP facilities.

In addition, the formation of cell sheets modified the production of cytokines by the MSC (Bou-Ghannam et al., 2021). Also, it was noticed that the effect of MSCs in *vivo* studies cannot be explained by the number of cells that reach their target (Bou-Ghannam et al., 2021) indicating that paracrine factors, released by the MSCs, could be the major factor. It is well known that MSCs can modulate the immune system by secreting paracrine factors (Ferreira et al., 2018). MSCs influence inflammation through paracrine factors, which lead to the study of mechanism of action. Many publications, in this research topic, mentioned the importance of EV characterization and variability produced by MSCs, for a beneficial effect on treating different diseases such as spinal cord injury, amyotrophic lateral sclerosis, wound healing, pancreatic cancer, heart failure. As reported by Sykova et al., Johnson et al., Najar et al. and Fernandez-Gonzalez et al, the content of these EV can be altered due to genetic engineering, by priming the MSCs or by using them as a drug transporter, which allow the manipulator to "guide" the EV in a way to have an optimal curative property.

MSCs are used in clinical trials, reported by Garcia-Bernal et al., Sykova et al., Harman and Wiese et al., but as mentioned by Najara, MSCs are used to treat diseases and widely used in clinical trials, but the effect of MSCs on one of the most frequent diseases is still controversial. It is still unclear why MSCs can promote or repress tumors growth/survival. To better understand such opposite effects, retrospective analysis of hundreds of clinical trials is necessary but because reported data are incomplete, the data analysis will be challenging to explain MSC influence on tumors, as mentioned in this research topic by Zhao et al.

Due to the lack of knowledge and experience in MSC clinical applications, federal agencies had to update their guidelines, and keep improving them in parallel to increased pre-clinical and clinical experiences. Food and Drug Administration is a perfect example of how the federal agencies are updating and adding complementary guidelines in the translational field of Cellular and Gene Therapies (Food and Drug Administration, 2022). From 1998 to 2014, FDA released 11 guidelines, but from 2015 to 2022, FDA released 22 guidelines, underlining the importance for agencies to improve their guidelines due to the increase of pre-clinical and clinical studies (Couto et al., 2017; Kabat et al., 2020). However, challenges are still around the corner because many clinical trials published on *clinicaltrials.gov* are still failing to provide detailed information about the patient's population, the manufacturing of the MSCs (that require a large-scale manufacturing). In addition to the topics mentioned, other crucial subjects about MSCs are presented and discussed in the research topic like priming by Berglund et al., alleviation of ischemia injuries by Arjmand et al. and Chua et al., development of tendinopathy animal model for MSC treatment by Meeremans et al., discovery of new drugs to accelerate MSC differentiation into osteoblast by Wang et al., use of immortalized MSC model for hormonal by Kulebyakin et al., tissue specificity of mesangiogenic progenitors cells by Barachini et al., transcriptional characterization of MSCs by Fan et al. and the impact of MSC senescence state used in clinical trials by Alves-Paiva et al. In order to continue progressing MSC field in clinical applications, academics, clinicians, and industry partners need to continue collaborating, sharing both knowledge and best practices that help to advance this field.

AUTHOR CONTRIBUTIONS

JO wrote the first draft of the editorial. ML, SP and JMC edited and revised the editorial.

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HS-5 and HS-27A Stromal Cell Lines to Study Bone Marrow Mesenchymal Stromal Cell-Mediated Support to Cancer Development

Annalisa Adamo[1,2†], Pietro Delfino[3†], Alessandro Gatti[1], Alice Bonato[1], Paul Takam Kamga[1,4], Riccardo Bazzoni[1], Stefano Ugel[2], Angela Mercuri[1], Simone Caligola[2] and Mauro Krampera[1*]*

[1] Stem Cell Research Laboratory, Section of Hematology, Department of Medicine, University of Verona, Verona, Italy, [2] Department of Medicine, Section of Immunology, University of Verona, Verona, Italy, [3] Department of Diagnostic and Public Health, University of Verona, Verona, Italy, [4] EA4340-BCOH, Biomarker in Cancerology and Onco-Haematology, UVSQ, Université Paris Saclay, Boulogne-Billancourt, France

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Edited by:
Simone Pacini,
University of Pisa, Italy

Reviewed by:
Qi Gao,
Stanford University, United States
Gianluca Carnevale,
University of Modena and Reggio Emilia, Italy

***Correspondence:**
Mauro Krampera
mauro.krampera@univr.it
Annalisa Adamo
annalisa.adamo@univr.it;
annalisa.adamo@gmail.com

[†] These authors have contributed equally to this work

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In this study, we compared the overall gene and pathway expression profiles of HS-5 and HS-27A stromal cell lines with those of primary bone marrow MSCs to verify if they can be considered a reliable alternative tool for evaluating the contribution of MSCs in tumor development and immunomodulation. Indeed, due to their easier manipulation *in vitro* as compared to primary MSC cultures, several published studies took advantage of stromal cell lines to assess the biological mechanisms mediated by stromal cells in influencing tumor biology and immune responses. However, the process carried out to obtain immortalized cell lines could profoundly alter gene expression profile, and consequently their biological characteristics, leading to debatable results. Here, we evaluated the still undisclosed similarities and differences between HS-5, HS-27A cell lines and primary bone marrow MSCs in the context of tumor development and immunomodulation. Furthermore, we assessed by standardized immunological assays the capability of the cell lines to reproduce the general mechanisms of MSC immunoregulation. We found that only HS-5 cell line could be suitable to reproduce not only the MSC capacity to influence tumor biology, but also to evaluate the molecular mechanisms underlying tumor immune escape mediated by stroma cells. However, HS-5 pre-treatment with inflammatory cytokines, that normally enhances the immunosuppressive activity of primary MSCs, did not reproduce the same MSCs behavior, highlighting the necessity to accurately set up *in vitro* assays when HS-5 cell line is used instead of its primary counterpart.

Keywords: mesenchymal stromal cells, stromal cell lines, tumor biology, immunomodulation, tumor escape

INTRODUCTION

Mesenchymal stromal cells (MSCs) are a heterogeneous cell population representing the progenitors of stromal tissues and containing multipotent cells capable of differentiating *in vitro* and *in vivo* into mesodermal tissues, such as osteoblasts, chondrocytes, and adipocytes (Campagnoli et al., 2001; Im et al., 2005; da Silva Meirelles et al., 2006). In addition, MSCs are provided

with immunomodulatory functions that are elicited by the presence of an inflammatory microenvironment. This phenomenon, called "MSCs licensing," induces MSCs to become strongly inhibitory towards different immune effector cells (IECs) of both innate immunity, such as neutrophils, monocytes and natural killer (NK) cells, and adaptive immunity, such as T cells, B cells and dendritic cells (Krampera, 2011; Di Trapani et al., 2016). MSC-mediated immunosuppression has been confirmed by several preclinical and clinical studies related to a large spectrum of inflammatory and autoimmune diseases, such as Graft-versus-Host Disease, Crohn's disease, sepsis, colitis, acute kidney injury, autoimmune encephalomyelitis, and other disorders (García-Olmo et al., 2005; Le Blanc et al., 2008; Gonzalez-Rey et al., 2009; Patel and Genovese, 2011; Ciccocioppo et al., 2012; Ciccocioppo and Corazza, 2016; Dal Collo et al., 2020). The well-known molecular mechanisms involved in MSC-mediated immunosuppression are represented by the up-regulation of several immunosuppressive molecules, including IDO1 and PD-L1 (Krampera, 2011; Di Trapani et al., 2016). Moreover, the role of FasL expression on MSCs cell surface has been recently reported to induce Fas-mediated T cell apoptosis (Akiyama et al., 2012).

In the last years, MSCs have been further recognized as crucial facilitators of tumor development in the context of both solid and liquid cancers. Emerging data suggest that MSCs can promote different tumor processes, including malignant transformation, angiogenesis, metastasis formation, cancer cell survival and chemoresistance (Nwabo Kamdje et al., 2017; Ridge et al., 2017; Galland and Stamenkovic, 2020). Last, but not least, the immunosuppressive properties of MSCs play a crucial role in mediating the mechanisms of immune escape in the context of tumor (Galland and Stamenkovic, 2020). Therefore, MSCs can be recruited within the tumor environment and establish dynamic interactions with tumor cells and other cellular elements, including IECs, by paracrine or contact-mediated communication (Galland and Stamenkovic, 2020; Le Naour et al., 2020). On the other hand, MSCs can also influence tumor growth by endocrine signals through the release of bioactive factors, including extracellular vesicles (Adamo et al., 2019a,b).

Several recent studies have tried to characterize the molecular mechanisms underlying the interactions amongst IECs, cancer cells and MSCs (Whiteside, 2018; Adamo et al., 2019a; Wei et al., 2019). These efforts may allow to identify novel potential therapeutic targets not only in the field of inflammatory and autoimmune disease, but also in the context of solid tumors and hematological malignancies. Considering the heterogeneity of MSC populations and that they may be potentially difficult to source, the use of commercially available bone marrow-derived cell lines, such as HS-5 and HS-27A, may have some advantages to obtain reproducible disease models *in vitro,* with low variability of the results obtained in presence of stromal cells. Therefore, several research groups take advantage of such commercially available cell lines to study the mechanisms mediated by MSCs in influencing immune responses and tumor progression (Garrido et al., 2001; Windus et al., 2013; Bar-Natan et al., 2017). HS-5 is a fibroblast-like cell line secreting significant levels of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage-CSF (GM-CSF), macrophage-CSF (M-CSF), Kit-ligand (KL), macrophage-inhibitory protein-1 alpha, interleukin-6 (IL-6), IL-8, and IL-11. Furthermore, HS-5 supports the proliferation of hematopoietic progenitor cells when co-cultured in serum-deprived media without exogenous factors (Roecklein and Torok-Storb, 1995). HS-27A cell line shows an epithelioid morphology with much larger cell size as compared to HS-5, poorly secreting growth factors and not supporting the proliferation of isolated hematopoietic progenitor cells in co-cultures. Similarly, HS-27A-derived conditioned medium fails to support the growth of myeloid colonies (Roecklein and Torok-Storb, 1995). Therefore, it is likely that HS-5 and HS-27A might represent functionally distinct components of the bone marrow stromal microenvironment (Roecklein and Torok-Storb, 1995). However, further and detailed comparison is still missing concerning the capability of such cell lines to reproduce typical functional properties of primary bone marrow MSCs, including immunoregulatory functions. Theoretically, the use of immortalized cell lines in experimental procedures might have a number of advantages to evaluate the molecular mechanisms underlying tumor immune escape, due to their easier manipulation *in vitro* as compared to primary cultures. On the other hand, it is necessary to assess carefully whether mesenchymal cell lines may accurately reproduce the physiological properties of primary MSCs, considering that the immortalisation process could profoundly alter gene expression profile, and consequently biological characteristics.

MATERIALS AND METHODS

Datasets, Expression Profiles and Statistical Analysis

NCBI Gene Expression Omnibus (GEO) database was searched for datasets with publicly accessible datasets with the keywords MSC, HS-5 and HS-27A. We selected four microarray datasets, GSE9593 (Wagner et al., 2008), GSE10595 (Iwata et al., 2014a), GSE48302 (Paul et al., 2013) and GSE53199 (Iwata et al., 2014b) containing samples that were eligible for our analysis. The details of the datasets and samples used are reported in **Table 1**. The following procedure was employed to account for the batch effect differences across the datasets and make the expression profiles comparable. First, platform-specific normalized data were downloaded with the *GEOquery Bioconductor* R package (Davis and Meltzer, 2007); multiple probes mapping to the same gene were collapsed by mean values; each dataset was subsetted to the samples indicated in **Table 1**; each dataset was individually quantile normalized using the function *normalize.quantiles.use.target* from the *Bioconductor* package *preprocessCore*, using as target distribution the quantile normalization vector available at https://api.refine.bio/v1/qn_targets/homo_sapiens, prepared by the refine.bio project[1]; all the four datasets were then merged and the dataset batch

[1]https://www.refine.bio

TABLE 1 | Details of the datasets and samples used for the expression profiles comparison.

GEO ID	Technology	PMID	Samples Used	Platform ID	Samples ID
GSE9593	Microarray	18493317	MSC	HG-U133_Plus_2	GSM242651, GSM242652, GSM242653, GSM242666, GSM242667, GSM242668, GSM242669, GSM242672, GSM242673, GSM242674, GSM242675
GSE10595	Microarray	24131213	HS-5, HS-27A	HG-U133_Plus_2	GSM267077, GSM267078, GSM267081, GSM267082
GSE48302	Microarray	24090675	HS-5, HS-27A	Illumina HumanHT-12 V3.0	GSM1174437, GSM1174438, GSM1174439, GSM1174440
GSE53199	Microarray	25275584	HS5	Illumina HumanHT-12 V4.0	GSM1287201, GSM1287202

effect was removed with the *removeBatchEffect* function from *limma* package (Ritchie et al., 2015). Gene sets collections were obtained from MSigDB database (Subramanian et al., 2005) and to obtain gene sets/pathways expression levels we employed the *GSVA Bioconductor* package (Hänzelmann et al., 2013) and the *gsva* function, applied to the merged expression matrix. GSVA scores were used to compare the pathways expression levels between cell lines and the *eBayes* function from *limma* was used to compute moderated t-statistics after linear model fitting. Statistical significance was set at FDR < 0.05 and all the *p*-values reported in the boxplots represent adjusted *p*-values. All statistical analyses were performed with *R* software environment version 3.6.2.

Cell Cultures

Primary MSCs were isolated from BM aspirates of healthy donors under informed consent, as approved by Ethical Committee of Azienda Ospedaliera Universitaria Integrata Verona (N. 1828, May 12, 2010 "Institution of cell and tissue collection for biomedical research in Onco-Hematology") and characterized as already described (Di Trapani et al., 2016; Adamo et al., 2019a). HS-5 and HS-27A human stromal cell lines were obtained from ATCC® (ATCC® CRL-11882™ and ATCC® CRL-2496™, respectively). Both primary MSCs and cell lines were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2% L-Glutamine (all from Sigma Aldrich). All experiments were performed between passages 2 and 7 of primary MSCs. Cells at 80% confluence were treated or not for 48 h with 10 ng/mL IFN-γ and 15 ng/mL TNF-α (R&D Systems) to induce inflammatory priming. PBMCs were isolated from human blood using Lymphoprep (Stem cells Technologies). B, T, and NK lymphocytes were isolated from PBMCs using immunomagnetic negative selection (Miltenyi Biotec) with at least 95% cell purity, as evaluated by flow cytometry. PBMCs were stimulated with 5 μg/ml of phytohemagglutinin (PHA) (Sigma-Aldrich) for 4 days in IMDM supplemented with 10% pooled human AB serum, 1% penicillin-streptomycin and 2% L-Glutamine (all from Sigma-Aldrich). T cells were activated with 0.5 μg/mL cross-linking anti-CD3 and anti-CD28 antibodies (Sanquin) for 6 days in RPMI supplemented with 10% human AB serum, 1% penicillin-streptomycin and 2% L-Glutamine (all from Sigma-Aldrich). B cells were activated with 5 μg/mL antihuman IgM+IgA+IgG (F(ab')2, Jackson Immunoresearch), 50 IU/mL rhIL-2 (Novartis), 50 ng/mL polyhistidine-tagged CD40 ligand, 5 μg/mL anti-polyhistidine antibody (R&D Systems), and 0.5 μg/mL CpG ODNs (Invitrogen), in RPMI medium supplemented with 10% FBS, 1% penicillin-streptomycin and 2% L-Glutamine (all from Sigma-Aldrich). The identity of HS-5 and HS-27A was checked for the presence of mesenchymal markers. Cell suspension were stained using the antibody anti-human CD73-PE, CD90-PE, CD105-PE, CD14-PE, CD31-PE, CD34-PE, and CD45-PE, HLA-ABC-PE, HLA-DR-PE, Fas-FITC, FasL-PE (BD Bioscience). The inflammatory immunophenotype was established using the anti-human CD54-PE, CD106 PE, HLA-ABC-PE, HLA-DR-PE, CD274-PE monoclonal antibodies (BD Bioscience). Peripheral blood mononuclear cells (PBMCs) were characterized using the anti-human CD3-FITC, CD16/56-PE, CD45-PerCP, CD19-APC, CD4-APC-H7, and CD8-PECy7 monoclonal antibodies (BD Bioscience). All data were collected through flow cytometry (FACS Canto II, BD Bioscience) and analyzed with FlowJo software (TreeStar). The expression of MSC markers was analyzed on living cells by using TO-PRO™-3 Iodide (Thermo Fisher) and normalized on FMO (fluorescence minus one) control. Osteogenic and adipogenic differentiative ability of primary MSC and γ-irradiated HS-5 and HS-27A cells (20 Gy – ^{137}Cs as source of γ-radiation) was evaluated has already described (Di Trapani et al., 2016; Adamo et al., 2019a). Primary MSCs and the cell lines were negative for mycoplasma.

Immunological Assays

Standardized assays were carried out to assess the inhibitory functions of primary MSCs and cell lines on different IECs, as previously described by our group (Di Trapani et al., 2016). Either primary MSCs or cell lines at resting and inflammatory-primed conditions were cultured in presence of activated PBMCs or purified T, B, NK cells previously stained with 5 μM carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies). HS-5 and HS-27A were plated at 80% confluence. After cell adhesion, 2×10^5 PBMCs, 2×10^5 T cells, 2×10^4 B cells, or 2×10^4 NK cells were added. At the end of the co-culture, cells were harvested at the stained with mouse anti-human CD45-PerCP-Vio700 (Miltenyi Biotec), and TOPRO-3 Iodide (Life Technologies). The proliferation was assessed on viable TOPRO-3 negative and CD45 positive cells by FlowJo software (TreeStar) by using the CFSE Geometric Mean of proliferating cells. The same experimental procedures were carried out using γ-irradiated HS-5 and HS-27A cells (20 Gy – ^{137}Cs as source of γ-radiation).

RESULTS

HS-5 and HS-27A Cell Lines Display the Typical Markers Expression Profile of Primary MSCs, but With Different Intensity

According to the minimal criteria for defining MSCs established by the International Society for Cellular and Gene Therapy (ISCT), primary bone marrow MSCs and HS-5 and HS-27A cell lines were positive for CD73, CD90, CD105, and HLA-ABC, with no expression of CD14, CD31, CD34, CD45, and HLA-DR surface molecules (**Figures 1A,B**). However, the expression intensity of the positive surface markers was significantly different among the different cell types. CD73, CD90, CD105, and HLA-ABC displayed a uniform expression on primary MSCs, regardless of the different healthy donors considered (**Figure 1A**). HS-5 showed a statistically significant higher expression of CD73

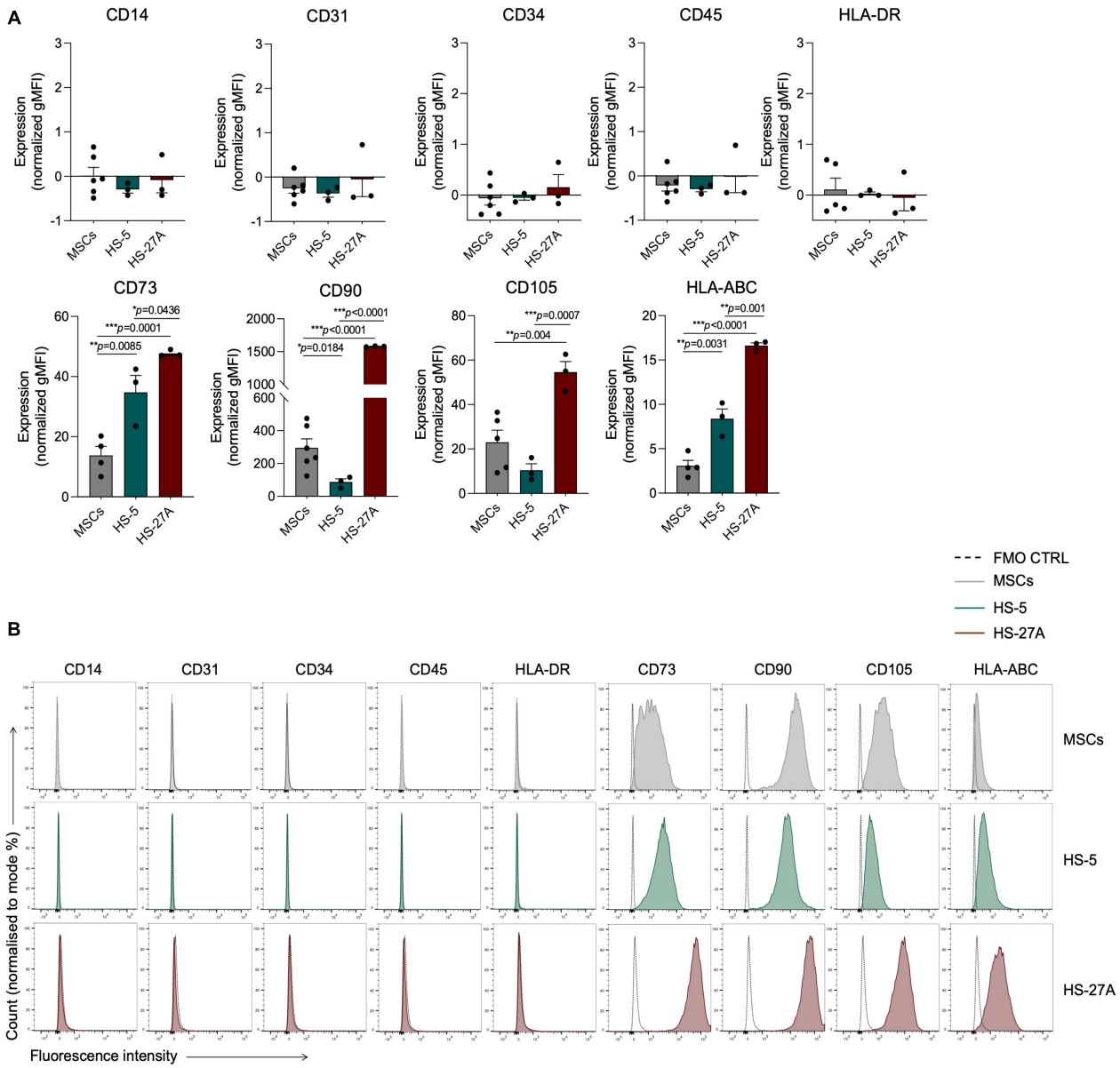

FIGURE 1 | Markers expression profile of primary MSCs, HS-5, and HS-27A cell lines. **(A)** Flow cytometry immunophenotypic analysis of primary MSCs, HS-5, and HS-27A cell lines showing the expression profile of specific hematopoietic markers (CD14, CD31, CD34, CD45), mesenchymal stromal cell markers (CD73, CD90, CD105), and MHC class I and II molecules (HLA-ABC and HLA-DR, respectively). Data are represented as mean of gMFI (geometric mean fluorescence intensity) normalized on FMO (fluorescence minus one) control ± SEM. **(B)** Representative histograms showing the expression profile of hematopoietic markers (CD14, CD31, CD34, CD45), mesenchymal stromal cell markers (CD73, CD90, CD105), and MHC class I and II molecules (HLA-ABC and HLA-DR, respectively) on MSCs, HS-5 and HS-27A cell lines.

and HLA-ABC and a lower expression of CD90 compared to primary MSCs (**Figures 1A,B**). HS-27A displayed a significantly higher expression of all the positive surface markers compared to both primary MSCs and HS-5 (**Figures 1A,B**). Overall, these data confirm the preservation of the well-defined MSCs immunophenotypic profile in HS-5 and HS-27A cell lines. To further characterize stromal cell lines, we tested the ability of irradiated HS-5 and HS-27a to differentiate into osteoblasts and adipocytes. Both HS-5 and HS-27A were partially able to differentiate into osteoblasts, while they did not show any adipogenic differentiation properties (**Supplementary Figure 1**).

HS-5 Cell Line Recapitulates the General Expression Pattern of Primary Bone Marrow MSCs

We first compared the overall gene and pathway expression profile of primary bone marrow MSCs and HS-5 and HS-27A cell lines. As the comparison was based on a multi-datasets level, we applied a strategy to reduce the batch effect of the different datasets and we were able to control the datasets differences, as shown in the PCA and boxplot in **Supplementary Figures 2A,B**, respectively. From the PCA plot no clustering of the samples based on datasets was appreciable, while the boxplot of the normalized and quantile-transformed expression values outlined no evident differences across datasets. Indeed, exploring the variability of the cell lines based on PCA of the 500 top variable genes, we observed a clustering of the HS-5 sample cluster closer to MSCs, while HS-27A was confined and distinct from the other two cell types (**Figure 2A**). To further compare the overall differences between primary MSCs and cell lines, we took advantage of Gene Set Variation Analysis (GSVA) by exploring the Molecular Signatures Database (MSigDB), a collection of annotated gene sets[2]. For the initial evaluation of potential differences or similarities between primary MSCs and cell lines, we considered two general MSigDB gene set collections covering a good portion of the human cellular and biological pathways, i.e., C2 and C5 collections. C2 collection includes several gene sets deriving from various sources, i.e., online pathway databases and the biomedical literature. The C2 collection is divided into two sub-collections: Chemical and genetic perturbations (CGP) and Canonical pathways (CP). The majority of the CGP sets came from the biomedical literature, thus identifying different signatures of biological and clinical states, such as cancer metastasis, stem cell characteristics, etc. The CP sub-collection includes several pathway gene sets from commonly used online databases, including BioCarta, KEGG, Matrisome Project and others. C5 collection consists of gene sets derived from Gene Ontology (GO) annotations. Therefore, the C5 collection is based on GO terms, belonging to the three GO ontologies [molecular function (MF), cellular component (CC) or biological process (BP)], and their associations to human genes. Considering both C2 and C5 collection, the PCA analysis on differentially expressed pathways (DEP) clearly showed two distinct clusters. HS-5 cell line and primary MSCs clustered together, whereas HS-27A cell line represented

[2]https://www.gsea-msigdb.org

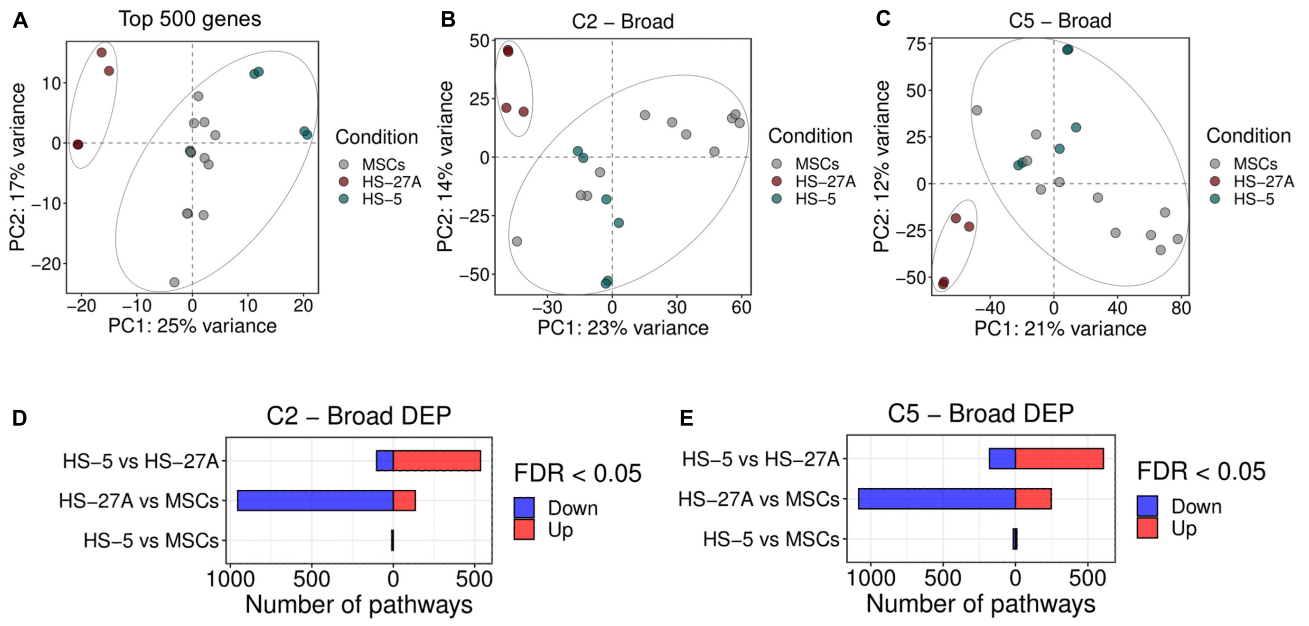

FIGURE 2 | Overall gene expression profile of primary MSCs, HS-5 and HS-27A cell lines. **(A)** Score plot of the first two PCs calculated following the application of PCA on top 500 genes expressed by primary MSCs, HS-5, and HS-27 cell lines. **(B,C)** Score plot of the first two PCs following the application of PCA on DEP included in C2 **(B)** and C5 **(C)** gene sets collections between primary MSCs, HS-5 and HS-27 cell lines. **(D,E)** Number of significantly down- and up-regulated pathways included in C2 **(D)** and C5 **(E)** gene sets collections between primary MSCs, HS-5, and HS-27 cell lines. (*n* MSCs, HS-5, HS-27A = 11, 6, 4). PCs, principal components; PCA, principal component analysis; DEP, differentially expressed pathways.

a distinct group (**Figures 2B,C**). Surprisingly, HS-27A cell line displayed a substantial number of differentially expressed pathways compared to primary MSCs, considering both C2 and C5 collection (1091 and 1331, respectively) (**Figures 2D,E**). On the other hand, only 10 and 26 pathways included in C2 and C5 collections, respectively, resulted significantly modulated in HS-5 cell line as compared to primary MSCs (**Figures 2D,E**). Consequently, a high number of pathways were differentially expressed in the two cell lines (639 and 787 included in C2 and C5 collections, respectively) (**Figures 2D,E**). The lists of differentially expressed pathways in MSCs and cell lines by using C2 and C5 collections are available in **Supplementary Tables 1**, **2**.

Taken together, these data indicate that HS-5, but not HS-27A, represents an immortalized cell line with a general expression pattern similar to the one observed in bone marrow-derived MSCs and, consequently, might be a reliable model to reproduce the biological properties mediated by MSCs.

HS-5 Cell Line Recapitulates the Ability of Primary MSCs to Affect Tumor Biology

In the last years, MSCs have been extensively recognized as crucial players during the processes of tumor development in the context of both solid and liquid cancers. Emerging data suggest that MSCs can promote malignant transformation, angiogenesis, metastasis formation, cancer cell survival and chemoresistance (Ridge et al., 2017; Adamo et al., 2019b). Several reports took advantage of immortalized HS-5 and HS-27A cell lines to characterize such properties and to discover the molecular mechanisms underlying the dynamic interactions between MSCs and cancer cells. In order evaluate the reliability of using HS-5 and HS-27A as an alternative tool for the characterisation of primary MSC regulatory properties in the context tumor processes, we compared primary MSCs and immortalized cell lines as far as the expression profile of different available gene sets involved in tumor biology is concerned. In detail, we took advantage of Hallmark collection in MSigDB, which includes 50 gene sets. Among these, several signatures have been reported as crucial pathways responsible for the pro-tumor activity mediated by MSCs. PCA analysis on all the Hallmark gene sets clearly showed two distinct clusters related to primary MSCs and cell lines, as previously shown considering the C2 and C5 general dataset collections. HS-5 cell line and primary MSCs clustered together, whereas HS-27A cell line represented a distinct group (**Figure 3A**). Twenty-four pathways were differentially expressed in HS-27A as compared to primary MSCs. Among these, 19 resulted down-regulated and 5 up-regulated (**Figure 3B**). Several pathways that have been reported to be involved in MSC-dependent pro-tumor activity displayed a strong up-regulation in primary MSCs compared to HS-27A cell line, including "angiogenesis," "Wnt/β catenin signaling," "KRAS signaling," "PI3K-AKT-mTOR signaling," and many others (Wang et al., 2015; El-Badawy et al., 2017; Poggi et al., 2018; Adamo et al., 2019a; **Figure 3D**). Conversely, HS-5 cell line and primary MSCs displayed a similar pathway expression profile. Only the "Protein secretion" gene set was significantly down-modulated in HS-5 compared to primary MSCs (**Figure 3B**). As expected, the comparison between HS-5 and HS-27A cell lines revealed 12 DEP (**Figures 3B,C**). Overall, these data suggest that HS-5 is a more appropriate model to reproduce the typical MSC expression pattern responsible for the pro-tumor activity. Therefore, HS-5 cell line could be a reliable alternative to primary MSCs to deeply characterize the molecular interactions between stromal and cancer cells. The list of differentially expressed pathways in MSCs and cell lines according to Hallmark collection is available in **Supplementary Table 3**. Considering the well-established properties of MSCs to promote angiogenic processes, we reported in **Supplementary Figure 3** the expression of all genes included in "angiogenesis" pathway from Hallmark MSigDB (Molecular Signature Database) in MSCs and stromal cell lines. As expected, several genes involved in such pathway resulted equally expressed in MSCs and HS-5, suggesting a similar ability in promoting angiogenic processes.

HS-5 Cell Line Recapitulates the Ability of Primary MSCs to Affect Immune Responses

MSCs possess broad immunomodulatory functions affecting both innate and adaptive immune responses (Gao et al., 2016). In order to define the immunological expression profile of stromal cell lines in comparison to primary MSCs, we took advantage of C7 immunological signature collection in MSigDB, consisting of several gene sets involved in the regulation of the immune system. PCA analysis on all the immunological signatures in primary MSCs and cell lines confirmed the presence of two distinct groups. HS-5 cell line clustered within the group of primary MSCs, whereas HS-27A cell line represented a distinct group (**Figure 4A**). A higher number of immunological signatures resulted significantly different in HS-27A, rather than in HS-5, in the comparison with primary MSCs, i.e., 1785 and 74 DEP, respectively (**Figure 4B**). Moreover, the GO gene sets, including a wide list of genes implicated in the regulation of both adaptive and innate immune responses, resulted significantly up-regulated in primary MSCs as compared to HS-27A cell line, thus confirming that HS-5 is suitable to reproduce the immunological properties of primary MSCs (**Figure 4C**). To further compare primary MSCs and immortalized cell lines in terms of immunological properties, we analyzed the expression profile of the well-established GO immunological signatures responsible for the immunosuppressive activity mediated by MSCs. The synthesis and subsequent release of chemokines and cytokines by MSCs play a crucial role in regulating immune responses. Here, we showed that the processes involved in the biosynthesis of chemokines were strongly up-regulated in primary MSCs and HS-5 as compared to HS-27A (**Figure 4D**). In particular, the release of IL-10 and IL-12 positively correlated with MSCs suppressive effects (De Miguel et al., 2012; Kyurkchiev et al., 2014; Nakajima et al., 2017). As expected, the GO immunological signatures related to the release of IL-10 were up-regulated in both primary MSCs and HS-5 compared to HS-27A (**Figure 4D**). In addition, the GO immunological signatures

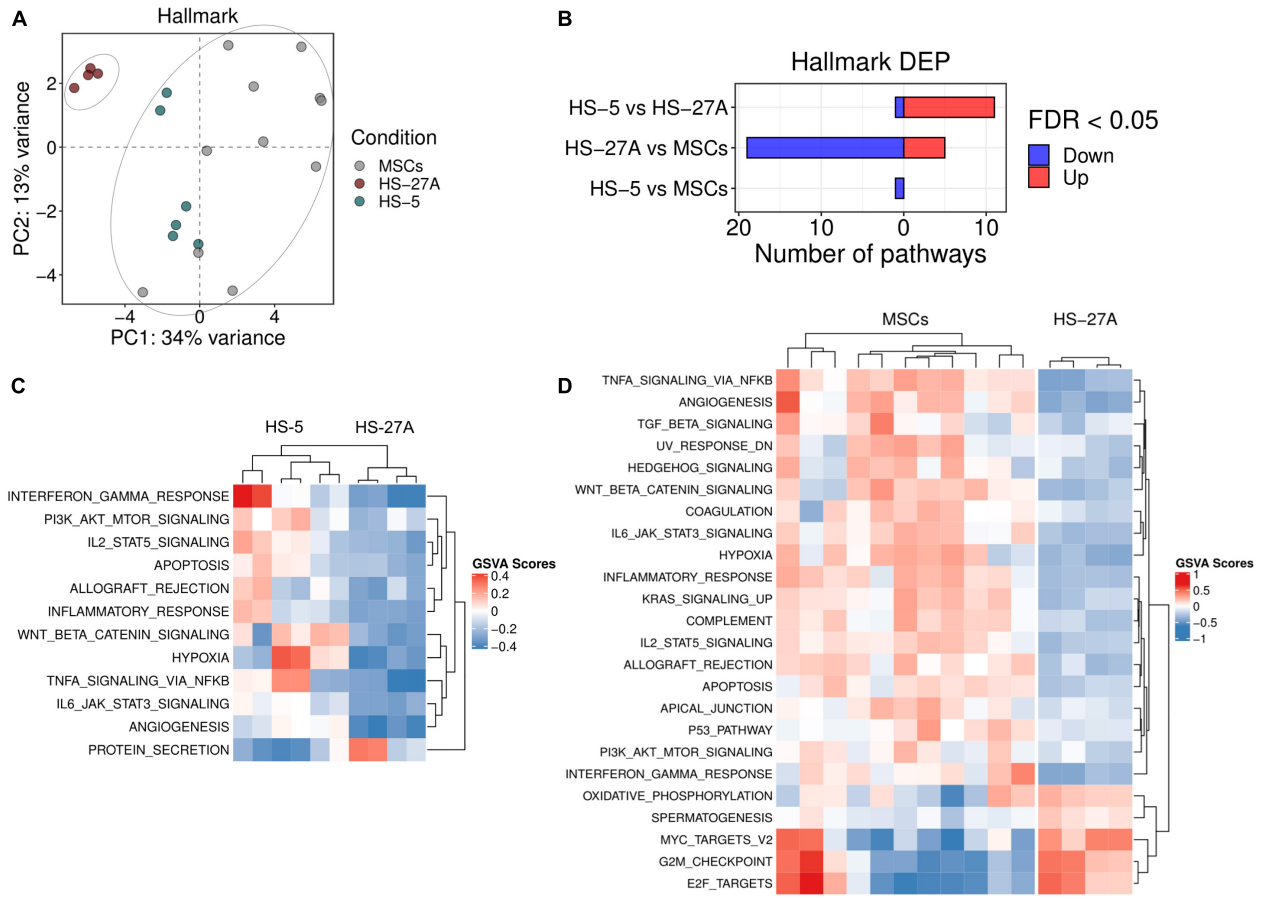

FIGURE 3 | Tumor-affecting pathways expression on primary MSCs, HS-5 and HS-27A cell lines. **(A)** Score plot of the first two PCs following the application of PCA on DEP included in Hallmark gene sets collection between primary MSCs, HS-5, and HS-27 cell lines. **(B)** Number of significantly down- and up-regulated pathways included in Hallmark gene sets collection between primary MSCs, HS-5, and HS-27 cell lines. **(C)** Heatmap summarizing significantly DEP between HS-5 and HS-27 cell lines. **(D)** Heatmap summarizing significantly DEP between primary MSCs and HS-27 cell lines. (*n* MSCs, HS-5, HS-27A = 11, 6, 4). PCs, principal components; PCA, principal component analysis; DEP, differentially expressed pathways.

related to the release of IL-12 was higher in primary MSCs compared to HS-27A, whereas we did not detect any difference between MSCs and HS-5 cell line (**Figure 4D**). Furthermore, we also investigated the expression of two pathways normally overexpressed during the immunosuppression mediated by MSCs. MSC inflammatory priming with IFN-γ enhances the immunosuppressive pathways responsible for the inhibition of different IECs (Krampera et al., 2006; Carvalho et al., 2019). Therefore, the IFN-γ-mediated signaling pathway can be considered as an essential signature of MSC-mediated immunosuppression. Such pathway was significantly enhanced in both primary MSCs and HS-5 compared to HS-27A (**Figure 4E**). The same trend was observed for "Toll-like receptor (TLR) signaling pathways" (**Figure 4E**), an additional biological system that may increase the immunosuppressive phenotype of MSCs (Najar et al., 2017; Shirjang et al., 2017). Overall, these data indicate that HS-5 is a more appropriate cell line to reproduce the immunological expression patterns responsible for the immunosuppressive activity of MSCs. The list of differentially expressed pathways in MSCs and cell lines according to C7 immunological signatures is available in **Supplementary Table 4**.

Immunological Characterisation of HS-5 and HS-27A Cell Lines

In order to validate our meta-analysis, we applied standardized assays to evaluate the immunological properties of immortalized cell lines. As previously reported by our group, the presence of inflammatory cytokines makes primary MSCs acquire an inflammatory phenotype characterized by increased expression of CD54 (I-CAM), CD106 (V-CAM), HLA-ABC, and HLA-DR (MHC-II), and CD274 (PD-L1) (Dal Collo et al., 2020). Furthermore, the inflammatory microenvironment induces a strong inhibitory effect on primary MSCs (primed MSCs, pMSCs), leading to the inhibition of immune responses mediated by different IECs (Di Trapani et al., 2016). In order to evaluate the immunological activity of HS-5 and HS-27A, we first assessed their phenotype in presence or not of inflammatory cytokines. Both HS-5 and HS-27A cell lines were capable of acquiring the typical phenotype of activated MSCs, except for

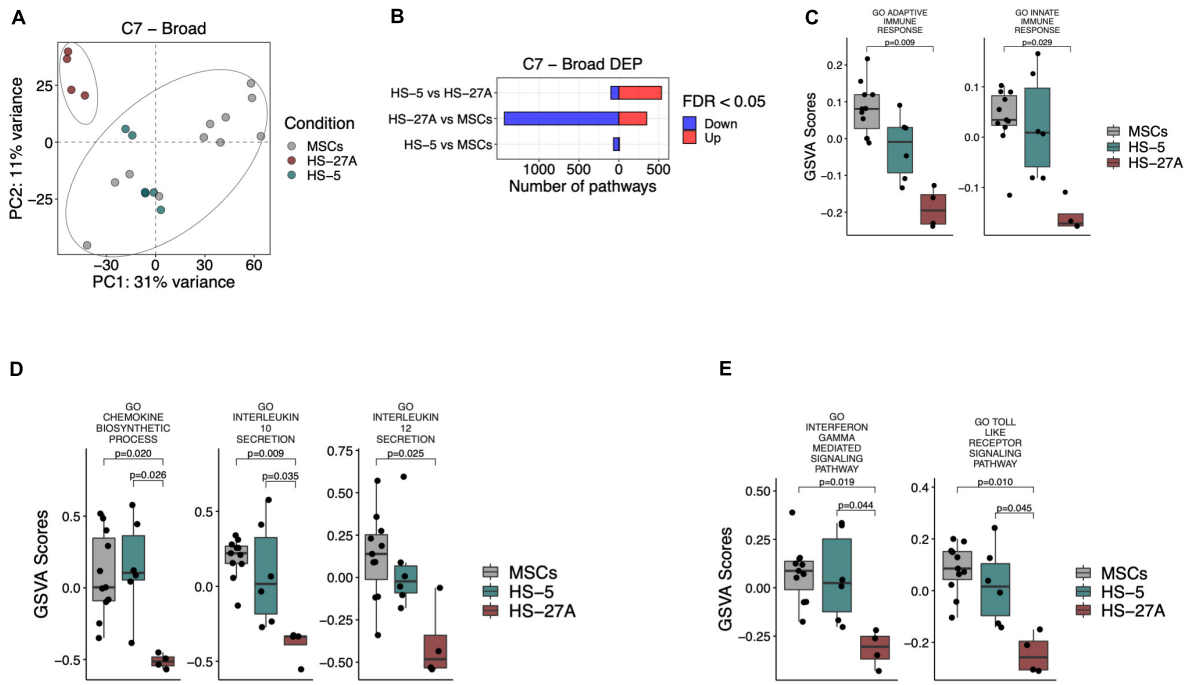

FIGURE 4 | Immunological signatures on primary MSCs, HS-5, and HS-27A cell lines. **(A)** Score plot of the first two PCs following the application of PCA on DEP included in C7 immunological signatures collection between primary MSCs, HS-5 and HS-27 cell lines. **(B)** Number of significantly down- and up-regulated pathways included in C7 immunological signatures collection between primary MSCs, HS-5 and HS-27 cell lines. **(C–E)** GSVA scores related to the expression of selected immunological signatures responsible for the immunoregulatory activity mediated by MSCs on primary MSCs, HS-5 and HS-27 cell lines. (*n* MSCs, HS-5, HS-27A = 11, 6, 4). PCs, principal components; PCA, principal component analysis; DEP, differentially expressed pathways; GSVA, gene set variation analysis.

the expression of CD106 on HS-5 that resulted absent both at resting and primed condition (**Figure 5A**). We also evaluated the expression of Fas and FasL in primary MSCs and stromal cell lines both in resting and primed condition (**Supplementary Figures 4A,B**). The expression of FasL by murine MSCs has been recently reported to be involved in Fas-mediated T cell apoptosis (Akiyama et al., 2012). In our cell models we observed a higher expression of FasL in stromal cell lines compared to primary MSCs (**Supplementary Figure 4A**) but the presence of inflammatory cytokines did not induce an up-regulation of the protein on the cell surface (**Supplementary Figure 4B**). As already reported (Yang et al., 2016; Martínez-Peinado et al., 2018), MSCs were able to significantly inhibit the proliferation of activated PBMCs, with a more pronounced effect when MSCs were pre-treated with inflammatory cytokines (**Figures 5B,E**). As expected, resting HS-5 led to a significant reduction of PBMC proliferation, as observed for primary MSCs, thus confirming its capability to reproduce the immunosuppressive activity mediated by MSCs towards PBMCs at resting conditions. However, the pre-treatment with inflammatory cytokines (pHS-5) did not affect PBMC proliferation (**Figures 5C,E**). HS-27A did not show any effect on PBMCs proliferation either at resting or primed conditions (**Figures 5D,E**). We observed similar results when HS-5 and HS-27A were γ-irradiated before the co-culture to prevent cell proliferation, and the immunosuppressive effect mediated by resting HS-5 was not observed any longer (**Supplementary Figures 5A,B**). Considering these data, we excluded the intrinsic ability of the two cell lines to induce the proliferation of resting PBMCs. HS-5 and HS-27A as well as primary MSCs were not able to activate resting PBMCs (**Supplementary Figure 5C**). Taken together, our data confirm a higher similarity of HS-5 to primary MSCs in terms of immunological activity.

HS-5 Cell Line Reproduces the MSC Immunosuppressive Activity on Activated T, B, and NK Cells at Resting Conditions

We further investigated HS-5 cell line immunological properties towards purified T, B, and NK cells by using standardized immunological assays. As already published by our group (Di Trapani et al., 2016), resting MSCs displayed a more significant suppressive effect on T cells as compared to other lymphocyte subsets (**Figure 6A**). These differences were partially related to the level of inflammatory cytokines released by activated IECs, which promoted the enhancement of MSC licensing. Accordingly, B and NK cell division was not inhibited by resting MSCs, due to their inability to make them acquire significant immunosuppressive activity (**Figure 6A**). Following IFN-γ and TNF-α pre-treatment, MSCs dramatically lowered T, B and NK cell proliferation by more than 80% (**Figure 6A**). As observed for primary MSCs, the co-culture with resting HS-5 induced a significant reduction of T cell proliferation, whereas we did not

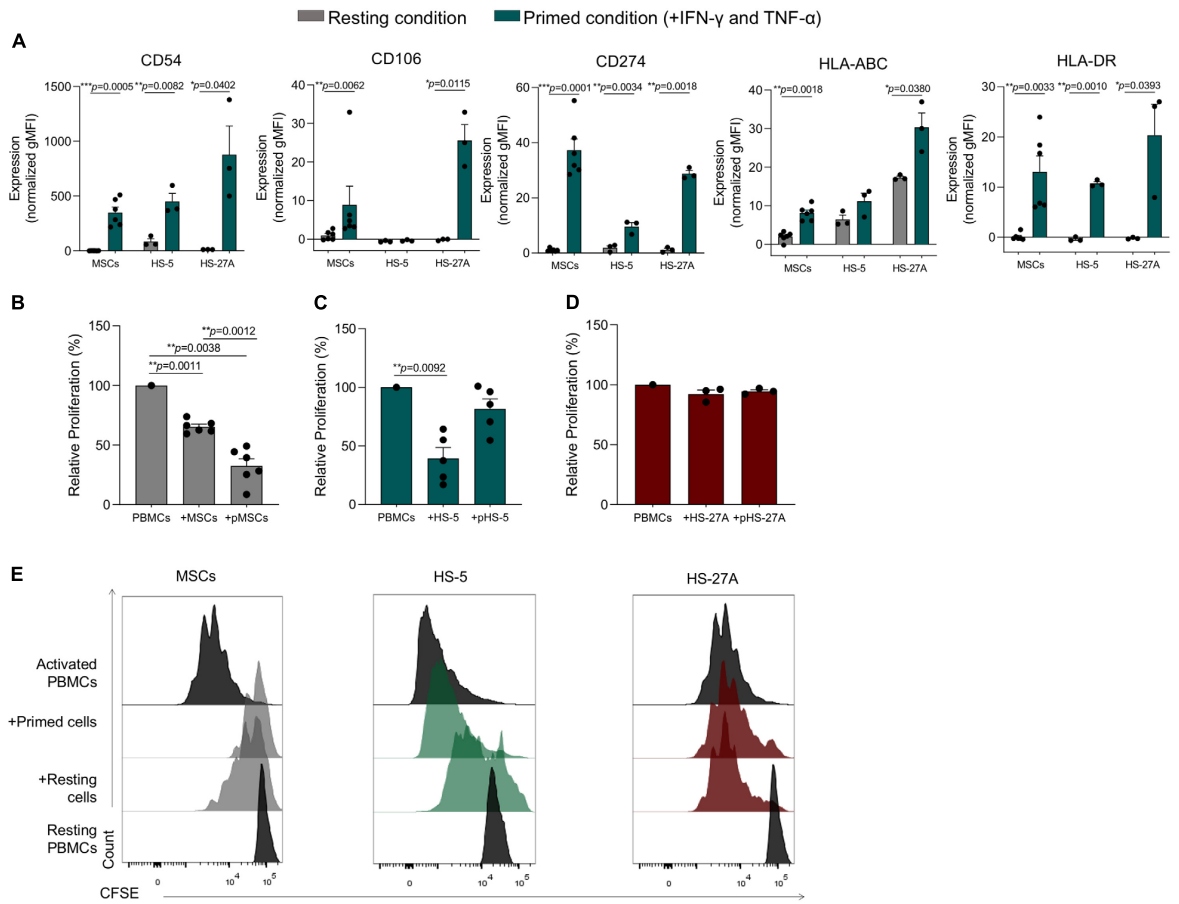

FIGURE 5 | Immunological characterization of HS-5 and HS-27A cell lines. **(A)** FACS immunophenotypic analysis of primary MSCs, HS-5, and HS-27A cell lines showing the expression profile of CD54, CD106, CD274, HLA-ABC, and HLA-DR in resting or primed condition. Data are represented as mean of gMFI (geometric mean fluorescence intensity) normalized on FMO (fluorescence minus one) control ± SEM. **(B–D)** Relative PBMCs proliferation following 4 days of co-culture with resting or primed MSCs, HS-5 or HS-27A, respectively. PBMCs proliferation was calculated on living $CD45^+$ cells according to CFSE dilution method by measuring CFSE gMFI and normalized on activated PBMCs cultured in absence of stromal cells. Data are represented as mean ± SEM. **(E)** Representative proliferation peaks of living $CFSE^+CD45^+$ PBMCs following the co-culture with resting or primed MSCs (gray), HS-5 (green), and HS-27A (dark red).

observe any effect on B and NK cell proliferation (**Figure 6B**). Conversely, the treatment of HS-5 cell line with inflammatory cytokines neither increased its immunosuppressive activity on T cell proliferation nor induced cell proliferation arrest of both B and NK cells (**Figure 6B**). As previously reported in the experimental setting of PBMCs, we did not observe any intrinsic ability of both HS-5 and primary MSCs to promote resting T, B, and NK cell proliferation (data not shown). Taken together, these data showed the capability of HS-5 cell line to reproduce the typical inhibitory effect of MSCs on T cell proliferation. However, the presence of inflammatory cytokines was not able to further enhance this phenomenon by using standardized immunological assay set up with primary MSCs.

DISCUSSION

The therapeutic potential of MSCs has been increasingly studied in the field of inflammatory and autoimmune diseases due to the ability of these cells to strongly suppress the immune responses. The well-established MSC immunomodulatory functions can be ascribed to their dynamic interactions with IECs mediating both adaptive and innate immune responses, through cell-to-cell contact and paracrine activity via soluble factors and extracellular vesicle release (Adamo et al., 2019a; Li et al., 2019; Zhou et al., 2019). The inflammatory microenvironment dramatically increases MSC immunosuppressive activity by influencing such interactions both *in vitro* and *in vivo* (Le Blanc et al., 2004; García-Olmo et al., 2005; Ciccocioppo et al., 2012; Di Trapani et al., 2016). MSC capability of affecting the immune responses plays a crucial role not only in the field of inflammatory disorders, but also in the context of tumors (Galland and Stamenkovic, 2020). In fact, MSCs can establish direct and indirect dynamic interactions with immune cells and favor the complex mechanisms of immune evasion. Furthermore, MSCs can influence a variety of tumor processes, directly promoting malignant transformation, angiogenesis, metastasis formation, cancer cell survival and chemoresistance

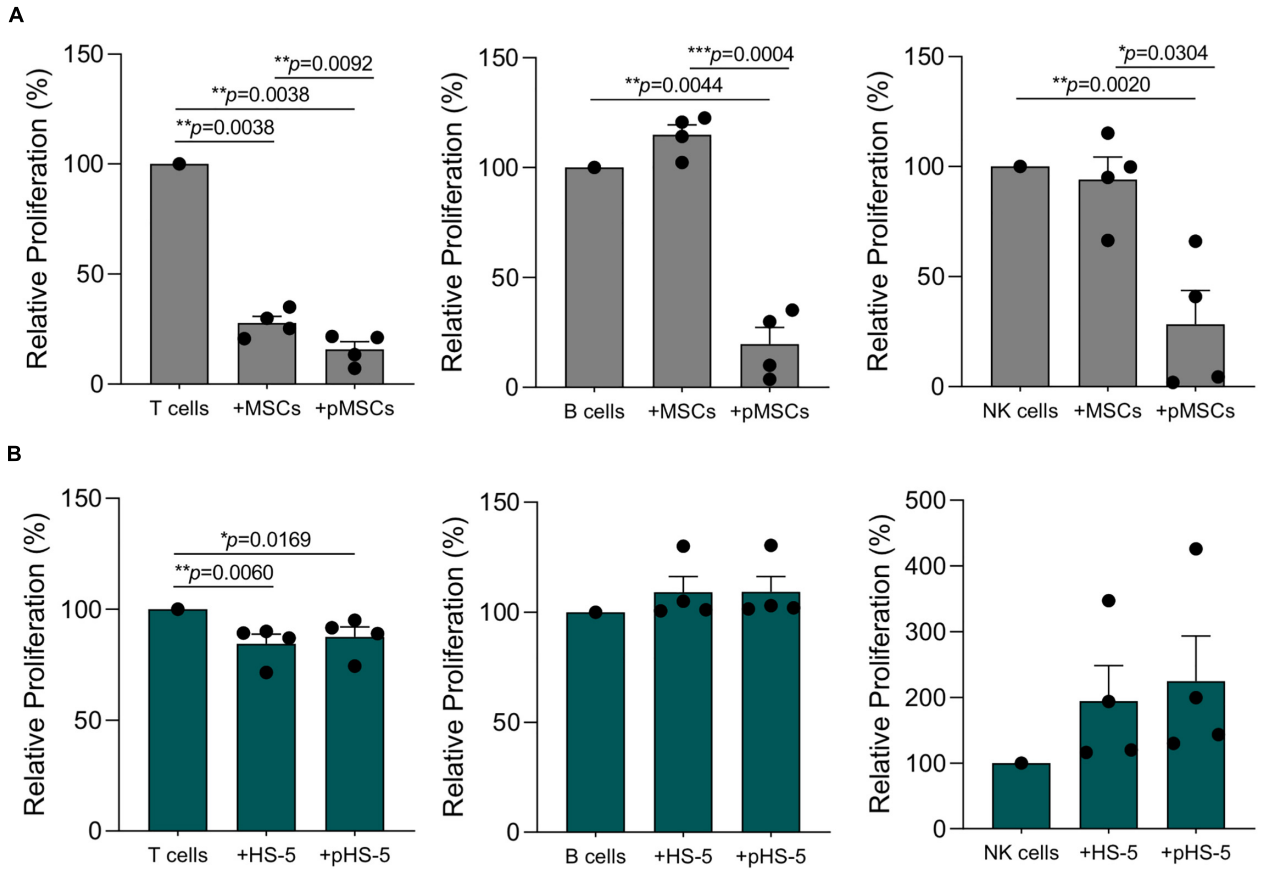

FIGURE 6 | HS-5 cell line immunosuppressive activity on T, B, and NK proliferation in resting and primed condition. **(A,B)** Relative T, B, and NK proliferation following the co-culture with resting or primed MSCs (gray) and HS-5 (green), respectively. Cell proliferation was calculated on living $CD45^{+}$ cells according to CFSE dilution method by measuring CFSE gMFI and normalized on activated IECs cultured in absence of stromal cells. Data are represented as mean ± SEM.

(Nwabo Kamdje et al., 2017; Adamo et al., 2019b; Galland and Stamenkovic, 2020; Le Naour et al., 2020). Consequently, the characterisation of the molecular mechanisms underlying the interactions amongst IECs, cancer cells and MSCs may help to identify novel potential therapeutic targets. Immortalized cells are frequently used to describe the molecular mechanisms underlying the interactions between MSCs and various target cells. Some of these cell lines can support hematopoietic cell survival and proliferation, similarly to primary MSCs (Roecklein and Torok-Storb, 1995). However, little is known about the reproducibility and reliability of using mesenchymal cell lines in the field of immunomodulation and tumor biology. Here, we compared the overall expression profile of primary bone marrow MSCs with that of bone marrow-derived HS-5 and HS-27A cell lines. The aim of our study was to evaluate if HS-5 and HS-27A cell lines may represent standardized and reproducible cellular models to employ for the assessment of the molecular mechanisms underlying the reciprocal interactions of MSCs with IECs and cancer cells.

In our hands, only HS-5 cell line displayed a general expression pattern similar to the one observed in bone marrow-derived MSCs; instead, HS-27A did not. Consequently, HS-5 cell line could be a reliable model to reproduce the biological properties mediated by MSCs. This hypothesis was further and more strongly confirmed when we studied the pathways involved in tumor progression. We did not detect any differentially expressed pathway in primary MSCs and HS-5, except for the "Protein secretion" signature, thus suggesting that HS-5 cell line could help to characterize the molecular interactions between MSCs and cancer cells. Instead, HS-27A cell line could represent the negative control, as the majority of the gene signatures involved in the pro-tumor activity mediated by primary MSCs resulted down-modulated in this cell line, such as those regulating "angiogenesis," "Wnt/β catenin signaling," "KRAS signaling" and "PI3K-AKT-mTOR signaling" (Wang et al., 2015; El-Badawy et al., 2017; Poggi et al., 2018; Adamo et al., 2019a). The significant differences between HS-5 and HS-27A cell lines support the reliability of our method of comparison.

HS-5 cell line shared also the immunological signatures and the pathways responsible for the immunosuppressive activity of MSCs. In fact, we found that the processes involved in the biosynthesis of chemokines were strongly up-regulated in both primary MSCs and HS-5 cell line. As expected, the GO immunological signatures related to IL-10, IL-6, and IL-12 release

were up-regulated in both primary MSCs and HS-5 cell line as compared to HS-27A. Similar findings were found as far as the expression of "IFN-γ-mediated signaling pathway" and "Toll-like receptor (TLR) signaling pathways" is concerned, two pathways strictly related to MSC immune regulatory effect. All these data were then confirmed by the functional assays we performed on activated PBMCs and purified IECs, although HS-5 pre-treatment with inflammatory cytokines, that normally enhances the immunosuppressive activity of primary MSCs, did not affect PBMC proliferation. This difference requires further investigation, because it could reflect either a different sensitivity of HS-5 cell line to inflammatory priming or a persistent status of intracellular activation.

Taken together, these data indicate that HS-5 cell line is suitable to reproduce not only the MSC capacity to influence tumor biology, but also to evaluate the molecular mechanisms underlying tumor immune escape mediated by stroma cells, with a number of advantages due to its easier manipulation *in vitro* as compared to primary MSC cultures. However, we strongly highlight and recommended to accurately set up the immunological assays when HS-5 cell line is used instead of its primary counterpart.

The pronounced differences between HS-5 and HS-27A reported in this work is further supported by Li et al. (2013) who showed that HS-27A, differentially from HS-5, can be co-injected in NSG mice with $CD34^+$ cells isolated from myelodysplastic syndrome patients to promote the engraftment of clonal hematopoietic precursor. Furthermore, human $CD34^+$ precursors harvested from bone marrow and spleen of primary murine recipients, when combined with HS-27A cells, were also engrafted successfully in secondary NSG recipients, showing the persistence of the original clonal characteristics (Li et al., 2013). The authors suggested that HS-27A stromal cells "traveled" in direct contact with hematopoietic precursors and enabled their propagation. An essential signal for engraftment appears to be CD146, which is prominently expressed on HS-27A cells compared to HS-5 (Li et al., 2013). Therefore, the higher levels of specific MSCs markers on HS-27A cell surface compared to HS-5 might probably be responsible for that capacity. In this light, HS-5 could represent a suitable model to study the immunoregulatory and tumor-promoting properties mediated by MSCs. On the other hand, HS-27A could be a reliable model to evaluate the role of MSCs in engraftment processes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Azienda Ospedaliera Universitaria Integrata Verona; N. 1828, May 12, 2010 "Institution of cell and tissue collection for biomedical research in Onco-Hematology". The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AA designed, performed the laboratory work, and wrote the manuscript. PD performed statistical and gene expression profile analyses and wrote the manuscript. AG, AB, AM, PT, RB, and SC performed the laboratory work and contributed to manuscript writing. SU critically revised the manuscript. MK coordinated the research plan, wrote and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.584232/full#supplementary-material

Supplementary Figure 1 | Osteoblasts and adipocytes differentiation of primary MSCs and irradiated stromal cell lines HS-5 and HS-27A.

Supplementary Figure 2 | Overall gene expression profile of primary MSCs, HS-5, and HS-27A cell lines following batch effect normalization. **(A)** Score plot of the first two PCs calculated by the application of PCA on top 500 genes expressed by primary MSCs, HS-5, and HS-27 cell lines following batch effect normalization. **(B)** Normalized gene expression intensities of primary MSCs, HS-5, and HS-27 cell lines within the four different GSE datasets. (*n* MSCs, HS-5, HS-27A = 11, 6, 4).

Supplementary Figure 3 | Z-scores related to the expression of all genes included in "angiogenesis" pathway from Hallmark MSigDB (Molecular Signature DataBase) in MSCs, HS-5 and HS-27 cell lines.

Supplementary Figure 4 | Evaluation of Fas/FasL expression in primary MSCs, HS-5 and HS-27A cell lines. **(A)** Fas/FasL expression in primary MSCs, HS-5 and HS-27A cell lines in resting condition. **(B)** Fas/FasL expression in primary MSCs, HS-5 and HS-27A cell lines in resting and primed condition.

Supplementary Figure 5 | Immunological characterization of HS-5 and HS-27A cell lines. **(A,B)** Relative PBMCs proliferation following 4 days of co-culture with γ-irradiated resting or primed HS-5 **(A)** or HS-27A **(B)**. PBMCs proliferation was calculated on living $CD45^+$ cells according to CFSE dilution method by measuring CFSE gMFI and normalized on activated PBMCs cultured in absence of stromal cells. Data are represented as mean ± SEM. **(C)** Representative proliferation of living $CFSE^+CD45^+$ non-activated PBMCs following the co-culture with resting or primed MSCs, HS-5, and HS-27A.

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A Rapid and Highly Predictive *in vitro* Screening Platform for Osteogenic Natural Compounds Using Human Runx2 Transcriptional Activity in Mesenchymal Stem Cells

*Li-Tzu Wang[1], Yu-Wei Lee[2], Chyi-Huey Bai[3,4], Hui-Chun Chiang[1], Hsiu-Huan Wang[2], B. Linju Yen[2,5] and Men-Luh Yen[1]**

[1] Department of Obstetrics and Gynecology, National Taiwan University (NTU) Hospital and College of Medicine, Taipei, Taiwan, [2] Regenerative Medicine Research Group, Institute of Cellular and System Medicine, National Health Research Institutes, Zhunan, Taiwan, [3] School of Public Health, College of Public Health, Taipei Medical University, Taipei, Taiwan, [4] Department of Public Health, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, [5] Department of Obstetrics and Gynecology, Cathay General Hospital Shiji, New Taipei City, Taiwan

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Joan Oliva, Emmaus Medical Inc., United States

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***Correspondence:**
Men-Luh Yen
mlyen@ntu.edu.tw

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The rapid aging of worldwide populations had led to epidemic increases in the incidence of osteoporosis (OP), but while treatments are available, high cost, adverse effects, and poor compliance continue to be significant problems. Naturally occurring plant-based compounds including phytoestrogens can be good and safe candidates to treat OP, but screening for osteogenic capacity has been difficult to achieve, largely due to the requirement of using primary osteoblasts or mesenchymal stem cells (MSCs), the progenitors of osteoblasts, to conduct time-consuming *in vitro* and *in vivo* osteogenic assay. Taking advantage of MSC osteogenic capacity and utilizing a promoter reporter assay for Runx2, the master osteogenesis transcription factor, we developed a rapid *in vitro* screening platform to screen osteogenic small molecules including natural plant-based compounds. We screened eight plant-derived compounds from different families including flavonoids, polyphenolic compounds, alkaloids, and isothiocyanates for osteogenic capacity using the human RUNX2-promoter luciferase reporter (hRUNX2-luc) transduced into the mouse MSC line, C3H10T1/2, with daidzein—a well-studied osteogenic phytoestrogen—as a positive control. Classical *in vitro* and *in vivo* osteogenesis assays were performed using primary murine and human bone marrow MSCs (BMMSCs) to validate the accuracy of this rapid screening platform. Using the MSC/hRUNX2-luc screening platform, we were able not only to shorten the selection process for osteogenic compounds from 3∼4 weeks to just a few days but also simultaneously perform comparisons between multiple compounds to assess relative osteogenic potency. Predictive analyses revealed nearly absolute correlation of the MSC/hRUNX2-luc reporter platform to the *in vitro* classical functional assay of mineralization using murine BMMSCs. Validation using human BMMSCs with *in vitro* mineralization and *in vivo* osteogenesis assays also demonstrated nearly absolute

correlation to the MSC/hRUNX2-luc reporter results. Our findings therefore demonstrate that the MSC/hRUNX2 reporter platform can accurately, rapidly, and robustly screen for candidate osteogenic compounds and thus be relevant for therapeutic application in OP.

Keywords: osteoporosis, Runx2, luciferase promoter assay, mesenchymal stem cells, drug screening platform

INTRODUCTION

With the aging and increased life expectancy of worldwide populations, the incidence of osteoporosis (OP) in both men and postmenopausal women is reaching epidemic proportions. A progressive systemic disease in which bone mineral density is decreased, OP significantly increases fracture risk, with risks of OP-related fracture in women and men over 50 years old estimated to be 30 and 20%, respectively (Siris et al., 2001; Cooper et al., 2011). Among the most serious OP-related fracture are vertebral and hip fractures, which have devastating economic as well as health consequences with mortality as high as one in three patients within 1 year after a hip fracture in some estimates (Burge et al., 2007; Katsoulis et al., 2017). Women in particular are at risk for OP decades before men, due to the loss of estrogen at menopause (Rosen, 2005). The simplest therapy for postmenopausal OP therefore has been to replace the lost endogenous sex hormone with exogenous estrogen or hormone replacement therapy (HRT). However, long-term use of HRT is now known to be associated with increasing breast cancer and vascular disease risk (Hulley et al., 1998; Rossouw et al., 2002), necessitating other therapeutic options. The most common treatments act on bone-resorbing osteoclasts to mitigate bone loss, such as bisphosphonates and denosumab. While effective, such agents are slow to act, taking several months to years to see significant effects. Bone anabolic agents including selective estrogen receptor modulators (SERMs) and teriparatide, a form of parathyroid hormone, target bone-producing osteoblasts and induce bone growth more rapidly, but all OP treatments are difficult to adhere to due to numerous side effects which range from discomfort to cancer risk to—ironically—atypical fracture risk (Black and Rosen, 2016). In addition, many of the newer therapies are prohibitively expensive, bringing into question widespread use especially in developing nations where OP is increasing rapidly (Handa et al., 2008; Mailankody and Prasad, 2014). Given the global reach of OP, more affordable therapeutic options are clearly necessary.

Naturally occurring plant-based compounds have been known to harbor therapeutic properties including osteogenesis. Phytoestrogens, which are naturally occurring non-steroidal plant compounds, are especially good candidates given their ability to exert beneficial agonistic effects on alleviating menopausal symptoms and bone loss (Dutertre and Smith, 2000; Lv et al., 2018; Geng et al., 2019; Zhou et al., 2020), without increasing estrogenic cancer risks (Adlercreutz, 2002; Horn-Ross et al., 2003; Anderson et al., 2013; Yang et al., 2019). Reports on the osteogenic agonism of individual phytoestrogens have been varied, ranging from strong agonism to antagonism with induction of adipogenesis even for the same compound despite the structural similarity to estrogen (Dang and Lowik, 2005). Moreover, classical osteogenesis assays are time-consuming, with both *in vitro* and *in vivo* functional assays requiring several weeks of time to complete. In addition, all these assays are performed with primary cells whether it be osteoblasts, pre-osteoblasts, or mesenchymal stem cells (MSCs) which are the progenitors of osteogenic cells (Zheng et al., 2018, 2019; Abdelrazik et al., 2019; Lavrentieva et al., 2020), all which require primary isolation and can lose osteogenic capacity when senescent after prolonged *in vitro* passaging (Moerman et al., 2004; Ho et al., 2013; Liu et al., 2020). Thus, development of a robust and rapid screening platform for selection of phytoestrogens and other plant-based compounds with strong osteogenic agonistic properties is sorely needed.

Runx2 is the master transcription factor controlling osteogenesis (Ducy et al., 1997; Komori et al., 1997), and strong osteogenic agonists including estrogens, Wnt/β-catenin, bone morphogenic proteins (BMPs), and sirtuins consistently upregulate the activity of this critical gene (Lee et al., 2000; McCarthy et al., 2003; Hill et al., 2005; Zainabadi et al., 2017). We therefore developed a rapid and robust *in vitro* osteogenesis MSC-based screening platform using a luciferase reporter of the human RUNX2 promoter (hRUNX2-luc) transduced into an immortalized but non-cancerous murine MSC line. Classical *in vitro* and *in vivo* osteogenesis assays using primary murine and human MSCs were performed to validate the findings of this *in vitro* platform. High correlation of the cell-based hRUNX2-luc *in vitro* screening system was found with both *in vitro* and *in vivo* mineralization assays, demonstrating this method to be a feasible and robust platform for rapid selection of phytoestrogens and osteogenic natural compounds.

Abbreviations: OP, osteoporosis; HRT, hormone replacement therapy; SERMs, selective estrogen receptor modulators; MSCs, mesenchymal stem cells; BMPs, bone morphogenic proteins; hRUNX2-luc, human RUNX2 promoter; C3H, C3H10T1/2; BMEM, Basal Medium Eagle medium; BM, bone marrow; CM, complete medium; DMEM, Dulbecco's Modified Eagle's medium; DMSO, dimethyl sulfoxide; CAPE, caffeic acid phenylethyl ester; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pCMV-β-gal, CMV-driven β-galactosidase construct plasmid; OM, osteogenic medium; AM, adipogenic medium; pNPP, p-nitrophenylphosphate; ALP, Alkaline phosphatase; AR, Alizarin Red; H&E, hematoxylin and eosin.

MATERIALS AND METHODS

Cell Culture and Differentiation Studies

Mouse C3H10T1/2 (C3H) mesenchymal progenitor/stem cells (Taylor and Jones, 1979) were obtained from American Type Culture Collection (ATCC, Manassas, VA, United States)

and maintained in Basal Medium Eagle medium (BMEM) (Invitrogen-Thermo Fisher Scientific, MA, United States) with 10% FBS (Hyclone-Thermo Fisher Scientific), 100 U/ml of penicillin/streptomycin, 2 mM L-glutamine (all from Gibco-Thermo Fisher Scientific). Murine bone marrow (BM)-derived MSCs were isolated from C57BL/6J strain (National Laboratory Animal Center, Taipei, Taiwan), and human BMMSCs were obtained from commercial sources (Promocell, Heidelberg, Germany) and cultured in complete medium (CM) consisting of low-glucose Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen-Thermo Fisher Scientific) with 10% FBS, 100 U/ml of penicillin/streptomycin, and 2 mM L-glutamine. MSC mesodermal differentiation assays were performed as previously reported (Tseng et al., 2011; Yen et al., 2011). Adipogenic differentiation medium (AM) consisted of in CM with 0.5 mM isobutyl-methylxanthine, 1 μM dexamethasone, and 0.1 μM insulin (all obtained from Sigma-Aldrich, St Louis, MO, United States), whereas osteogenic differentiation medium (OM) consisted of CM with 0.2 mM ascorbate, 10 nM dexamethasone, and 10 mM β-glycerophosphate (all from Sigma-Aldrich). For promoter assays, cells were cultured in either CM, AM, or OM for 2 days after transfection. For functional osteogenic differentiation and assays, cells were cultured in OM for 3 weeks and replaced with fresh medium every 3 days. Isolated natural-occurring compounds were dissolved in various vehicles (which were also used as controls) as recommended by the manufacturer in the following manner: daidzein (purity ≥ 95%) and apigenin (purity ≥ 98%) were dissolved in dimethyl sulfoxide (DMSO), while baicalein (purity ≥ 95%), caffeic acid phenylethyl ester (CAPE, purity ≥ 98%), capsaicin (purity ≥ 95%), curcumin (purity ≥ 90%), epicatechin (purity ≥ 90%), naringenin (purity ≥ 98%), and sulforaphane (purity ≥ 98%) were dissolved in ethanol. Doses of each compound were added as indicated, and all compounds were purchased from Cayman Chemical (Ann Arbor, MI, United States).

Cytotoxicity Assay

All compounds were tested at various doses for their cytotoxic effect on C3H cells by colorimetric analyses of cell viability with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (GoldBio, St Louis, MO, United States) according to the manufacturer's recommendations.

Promoter Luciferase Reporter Assay

The hRUNX2-luc reporter plasmid was constructed as we previously reported (Tseng et al., 2011). Briefly, the upstream of human RUNX2 promoter (−1,557/ + 32) was constructed into pGL3-basic Luc (Promega, San Luis Obispo, CA, United States). Murine MSC line C3H cells (4×10^4/well) were maintained in 24-well plates and then co-transfected with a 1 μg plasmid mixture of hRUNX2-luc and CMV-driven β-galactosidase construct plasmid (pCMV-β-gal) at a 9:1 ratio using DNAFect LT transfection reagent (ATGCell, Edmonton, Alberta, Canada) according to the manufacturer's recommendation. After 24 h, the media were replaced with fresh CM, osteogenic medium (OM) or adipogenic medium (AM) and tested compounds for another 48 h. Cell extractions were prepared, and luciferase activity was measured in a microplate luminometer using the Promega Luciferase Assay System (Promega, Madison, WI, United States) standardized against β-galactosidase activity.

Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity assay was performed as previously reported (Tseng et al., 2011). Briefly, cells were lysed by protein lysis buffer without protease inhibitor, and cellular ALP activity was assessed by incubating the protein lysates with substrate p-nitrophenylphosphate (pNPP, Sigma-Aldrich) at 37°C for 30 min, with the colorimetrical reaction measured by absorbance at 405 nm and normalized to corresponding protein amounts.

Alizarin Red Staining

Alizarin Red (AR) staining and quantification was performed to analyze calcium deposition as previously described (Tseng et al., 2011). Briefly, cells were fixed with 100% methanol for 30 min, washed with boric acid buffer (0.1 M, pH 4.0), and stained with AR solution (40 mM, pH 4.2, Sigma-Aldrich) for 30 min. Unbound AR stain was washed with boric acid buffer twice, and then distilled water until calcium deposits were visualized. To quantify mineralization, elution of AR stain with 10% cetylpyridinium chloride (Sigma-Aldrich) was performed and quantified using spectrophotometric analysis by reading absorbance at 520 nm.

Quantitative PCR

Quantitative PCR (qPCR) was performed as previously reported (Ho et al., 2013). RNA was extracted with TRIzol reagent (Invitrogen-Thermo Fisher Scientific), and cDNA synthesis was performed with ReverTra Ace set (TOYOBO, Osaka, Japan) according to the manufacturer's protocols. qPCR was carried out using the SYBR Green Supermix Taq Kit (Bio-Rad Laboratories, Hercules, CA, United States) and performed on the ABI Real-time PCR 7500 System according to the manufacturer's instructions (Applied Biosystems Inc., Carlsbad, CA, United States). Primers for amplifying human osteogenic and adipogenic genes are as follows: GAPDH (internal control), forward primer 5′-GTGGACCTGACCTGCCGTCT-3′, reverse primer 5′-GGAGGAGTGGGTGTCGCTGT-3′; RUNX2, forward primer 5′-CCAGATGGGACTGTGGTTACTG-3′, reverse primer 5′-TTCCGGAGCTCAGCAGAATAA-3′; C/EBPβ, forward primer 5′-AAACTCTCTGCTTCTCCCTCTG-3′, reverse primer 5′-GTTGCGTCAGTCCCGTGTA-3′.

In vivo Ectopic Bone Formation Assay

Animal experimentation was performed using protocols approved by the Institutional Animal Care and Use Committee. Human BMMSCs (2×10^6 cells) were treated with various compounds for 2 days then mixed with 300 μl Matrigel matrix (BD Biosciences, San Jose, CA, United States) for subcutaneous injection into the dorsal surface of 6-week-old NOD/SCID mice as previously reported (Reinisch et al., 2015). After 5 weeks, the implants were harvested and fixed with 10% formalin overnight. Then, paraffin embedding was performed and 5-μm sections

were prepared for histological analyses with hematoxylin and eosin (H&E) staining.

Statistical Analysis

For comparisons between two groups, Student's *t*-test was used for analyses, and for comparisons between multiple groups, ANOVA was used for analyses. Data was presented as mean ± SD. Goodness of fit (with Chi square value) was used to assess the predictive ability of the MSC/hRUNX2-luc screening results to the data of the functional assays. $p < 0.05$ was defined as statistically significant. Analyses were performed using GraphPad Prism software (San Diego, CA, United States).

RESULTS

Development of an MSC-Based Rapid Screening Platform Using hRUNX2 Promoter Activity for Selection of Osteogenic Natural Compounds

To establish an *in vitro* platform for screening osteogenic compounds, we utilized the hRUNX2-luc reporter vector which was transfected into a well-documented MSC line, C3H MSCs (Taylor and Jones, 1979). To demonstrate that hRUNX2 promoter activity is strongly elicited during osteogenesis, we cultured hRUNX2-luc-transfected C3H cells in CM, AM, or OM conditions. We found that not only is hRUNX2 transcriptional activity significantly activated in OM compared to CM conditions but that the activity is significantly decreased in AM compared to CM conditions (**Supplementary Figure 1**). To determine the optimal doses of the tested compounds, we performed dose-dependent cytotoxicity assays with all compounds at various concentrations on C3H cells cultured in CM and OM (**Supplementary Figure 2**). The phytoestrogen daidzein has been well documented in *in vitro* studies to have strong osteogenic inductive properties via interactions with estrogen receptors given its structural similarities to estrogen (Dang and Lowik, 2004; Schilling et al., 2014). We confirmed these previous results, finding that daidzein at the concentrations of 5, 10, and 20 μM significantly induced hRUNX2-luc activity in C3H cells cultured under OM conditions for 48 h (**Figure 1A**), and therefore used this estrogenic isoflavone as a positive osteogenic compound in subsequent assays. We then used this MSC-based platform to screen the osteogenic efficacy of a wide variety of plant-based compounds with reports of medicinal efficacy: the flavones apigenin and baicalein; the polyphenolic compounds caffeic acid phenethy ester (CAPE) and curcumin; the flavonols epicatechin and naringenin; capsaicin, an alkaloid; and sulforaphane, an isothiocyanate. We found that both apigenin (**Figure 1B**) and baicalein (**Figure 1C**) significantly increased hRUNX2-luc activity in C3H cells in a dose-dependent fashion. On the other hand, CAPE (**Figure 1D**), capsaicin (**Figure 1E**), curcumin (**Figure 1F**), epicatechin (**Figure 1G**), and naringenin (**Figure 1H**) did not activate hRUNX2-luc. Sulforaphane unexpectedly decreased hRUNX2-luc activity significantly in a dose-dependent manner (**Figure 1I**). These findings demonstrate that hRUNX2 transcriptional activity in MSCs was rapidly and strongly upregulated by daidzein, a known potent osteogenic inducer, and was also significantly upregulated by apigenin and baicalein while decreased by sulforaphane.

Validation of hRUNX2-Promoter Activation With *in vitro* ALP Activity, an Early-Stage Assay for Osteogenesis

To validate this rapid and MSC-based osteogenic compound screening platform, we performed a number of classical *in vitro* and *in vivo* osteogenesis functional assays using primary isolated murine and human BMMSCs. We first assessed the induction of *in vitro* cellular ALP activity, an early osteogenic biomarker, with primary murine BMMSCs. Using daidzein again as a positive control, we found that addition of this phytoestrogen to OM-cultured BMMSCs after 1 week significantly increased ALP activity in a dose-dependent fashion over that of BMMSCs cultured in OM only (**Figure 2A**). Addition of apigenin at the dose of 20 μM (**Figure 2B**) or baicalein at doses of 1 and 10 μM (**Figure 2C**) to OM also significantly increased ALP activity in BMMSCs over OM-only culture; these findings are indicative of osteogenic differentiation and consistent to the activation of hRUNX2 promoter activity by these two compounds (**Figures 1B,C**). Capsaicin was the only other compound which significantly increased BMMSC ALP activity (**Figure 2E**), which is in contrast to its lack of hRUNX2 promoter activation (**Figure 1E**). All other tested compounds either decreased ALP activity in OM-cultured BMMSCs, including CAPE (**Figure 2D**), curcumin (**Figure 2F**), epicatechin (**Figure 2G**), and sulforaphane (**Figure 2I**), or had no effect like naringenin (**Figure 2H**); these five compounds also did not activate hRUNX2 promoter activity. Collectively, these results show that daidzein, apigenin, and baicalein increase both ALP activity and hRUNX2 transcriptional activity, while sulforaphane decrease activities of both osteogenic assays, while the other five compounds demonstrate either no or inconsistent effects in both assays.

Validation of hRUNX2-Promoter Activation With *in vitro* Mineralization, a Late-Stage Assay for Osteogenesis

The most definitive *in vitro* functional assay for osteogenesis is calcium deposition and mineralization, a late-stage event in osteogenesis which typically requires several weeks of time to perform (Gregory et al., 2004). We therefore assess the capacity of all compounds to induce *in vitro* mineralization in primary isolated murine BMMSCs undergoing osteogenesis using AR staining with subsequent quantification. Using daidzein as a positive control, we found that addition of this compound at any dose to OM-cultured BMMSCs after 3 weeks' time led to significantly increased calcium deposition, compared to BMMSCs cultured in OM only (**Figure 3A**). Addition of either apigenin at 10 and 20 μM (**Figure 3B**) or baicalein at 1 and 10 μM (**Figure 3C**) to OM-cultured BMMSCs also significantly increased the levels of AR staining compared to BMMSCs cultured in OM only, with both compounds

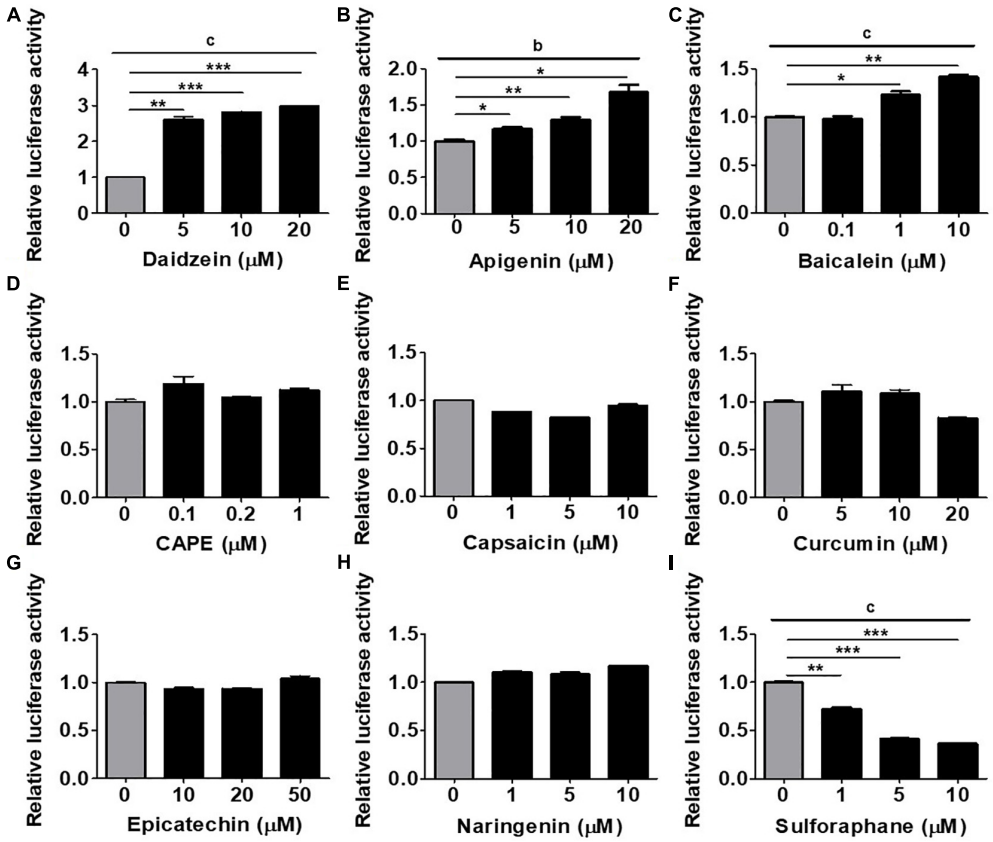


FIGURE 1 | Assessment of an MSC-based rapid screening platform using the human RUNX2 (hRUNX2) proximal promoter activity for selection of osteogenic natural compounds. **(A)** hRUNX2-promoter activity in murine C3H10T1/2 (C3H) mesenchymal stem cell (MSC) line cultured under osteogenic conditions as assessed by measurement of luminometric luciferase signals 48 h after treatment with or without **(A)** daidzein (5–20 μM), **(B)** apigenin (5–20 μM), **(C)** baicalein (0.1–10 μM), **(D)** CAPE (0.1–1 μM), **(E)** capsaicin (1–10 μM), **(F)** curcumin (5–20 μM), **(G)** epicatechin (10–50 μM), **(H)** naringenin (1–10 μM), or **(I)** sulforaphane (1–10 μM). All data were normalized to the luminometric luciferase signal in osteogenic medium (OM)-culturing C3H MSCs without compound treatment ($n = 3$ for each group). Data are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ as analyzed by Student's *t*-test; a, $p < 0.05$; b, $p < 0.01$; and c, $p < 0.001$ as analyzed by ANOVA.

eliciting a more robust mineralization response and in a dose-dependent fashion than daidzein. These results are in line with the ALP activity and hRUNX2-luc activity data for these three compounds. No other compounds added to OM led to increased calcium deposition by BMMSCs over OM-only conditions (**Figures 3D–I**). Interestingly, addition of either curcumin (**Figure 3F**) or sulforaphane (**Figure 3I**) actually resulted in significant suppression of calcium deposition in a dose-dependent fashion, which correlates with the hRUNX2-luc activity for these two compounds (**Figures 1F,I**). These results demonstrate that apigenin and baicalein exert potent *in vitro* functional osteogenic properties extending to mineralization similar to daidzein, a known osteogenic phytoestrogen.

hRUNX2-luc Reporter Activity but Not ALP Activity Is Highly Correlated With *in vitro* Mineralization Assay

To assess the robustness of the rapid *in vitro* hRUNX2 transcriptional activity screening platform, we compared the outcome in all three *in vitro* osteogenesis assays for all nine tested compounds: the hRUNX2-luc promoter assay conducted in C3H MSCs, ALP activity conducted with primary BMMSCs, and mineralization conducted with primary BMMSCs. Using a heatmap graph to visualize these comparisons, we found that the best correlation was between the hRUNX2 transcriptional activity and mineralization assay, with nearly all tested compounds demonstrating similar trends between the two assays except for the compound naringenin (**Figure 4A**). Surprisingly, ALP activity correlated poorly with the other two assays: only four out of nine compounds—daidzein, apigenin, baicalein, and sulforaphane—yielded ALP activity results that were similar to hRUNX2-luc activity and/or calcium deposition assay. ALP activities of four other compounds—CAPE, capsaicin, curcumin, epicatechin—trended in the opposite direction of the other two assays; only with one compound, naringenin, was there some correlation of ALP activity to hRUNX2 transcriptional activity. To examine the predictive ability of hRUNX2-luc transcriptional activity for osteogenesis, we further calculated the correlation based on the trends of compound effects on

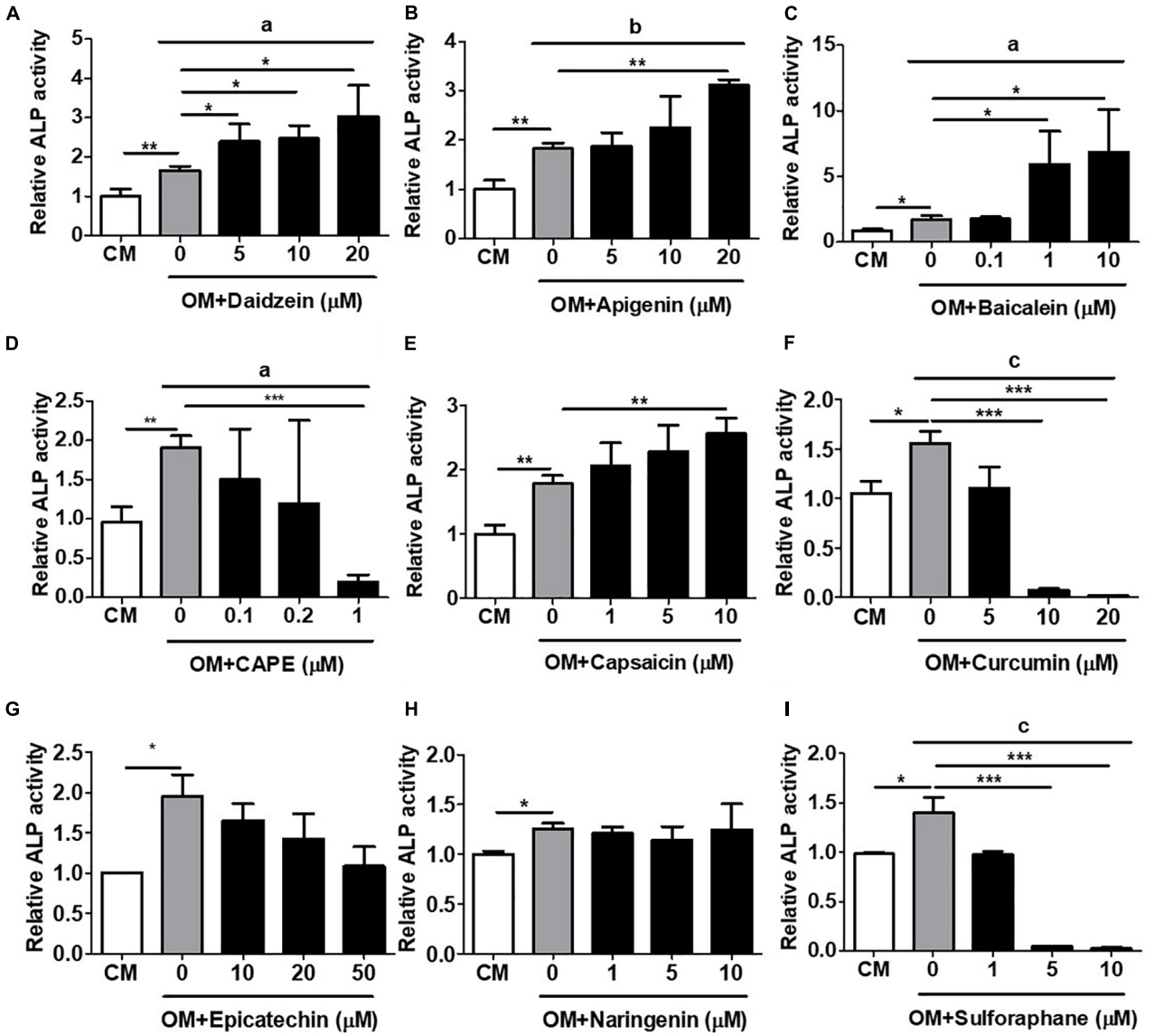


FIGURE 2 | Validation of hRUNX2-promoter activation with *in vitro* alkaline phosphatase (ALP) activity, an early-stage assay for osteogenesis. ALP activity was assessed in primary murine bone marrow (BM) MSCs at 8 days cultured in complete medium (CM) or OM with or without treatment of **(A)** daidzein (5–20 μM), **(B)** apigenin (5–20 μM), **(C)** baicalein (0.1–10 μM), **(D)** CAPE (0.1–1 μM), **(E)** capsaicin (1–10 μM), **(F)** curcumin (5–20 μM), **(G)** epicatechin (10–50 μM), **(H)** naringenin (1–10 μM), or **(I)** sulforaphane (1–10 μM). All data were normalized to the enzymatic activity of cellular ALP in CM-cultured BMMSCs ($n = 3$ for each group). Data are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ as analyzed by Student's *t*-test; a, $p < 0.05$; b, $p < 0.01$; and c, $p < 0.001$ as analyzed by ANOVA.

MSC osteogenesis with the results in mineralization (quantified AR staining), which is the most finite functional assay for *in vitro* bone development, and found that results in hRUNX2-luc reporter assay had a significantly positive correlation with AR staining at 96.3%, which was better than the correlation of ALP activity—a functional, early marker of osteogenesis—with AR staining which was only 77.8% (**Figure 4B**). These results demonstrate that the rapid *in vitro* hRUNX2-luc activity screening platform is highly correlated with the more definitive and late-stage functional assay of mineralization, while the early osteogenic event of ALP activity poorly correlated with either the hRUNX2 transcriptional activity or mineralization assay.

Compounds Screened Through the Murine C3H-hRUNX-Luc System Strongly Induce *in vitro* and *in vivo* Osteogenesis in Human BMMSCs

To validate whether results from the C3H-transduced hRUNX2-luc reporter system and primary murine BMMSCs were relevant in the human system, we assessed the screened

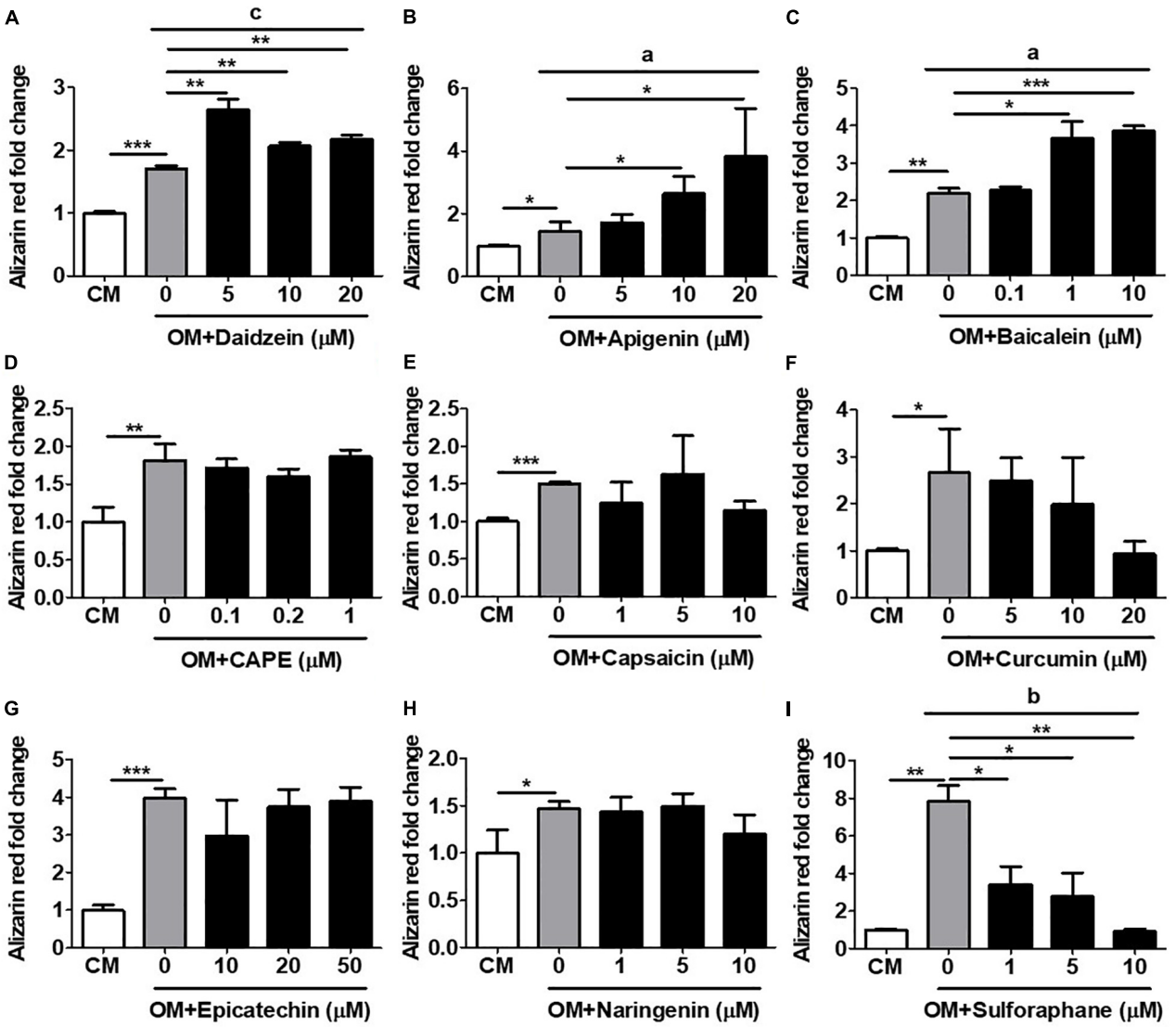
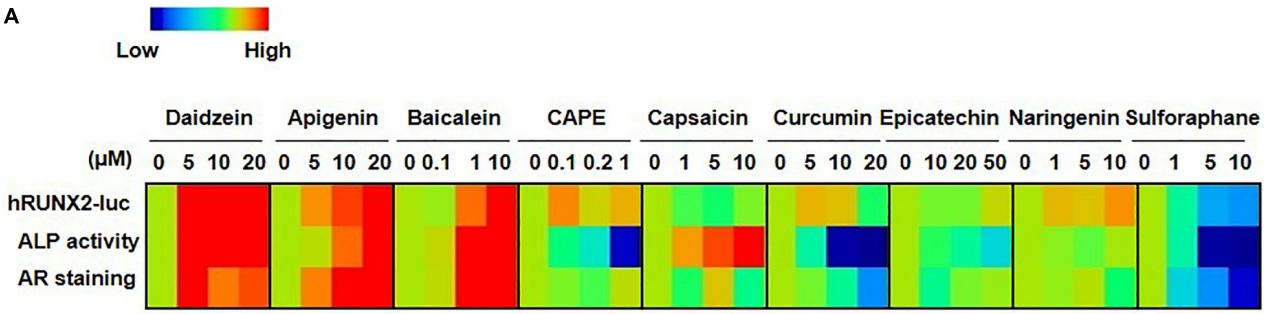


FIGURE 3 | Validation of hRUNX2-promoter activation with *in vitro* mineralization, a late-stage assay for osteogenesis. *In vitro* calcium deposition and mineralization in mouse BMMSCs was assessed by Alizarin Red (AR) staining with elution for quantification after 3 weeks of culture in CM or OM with addition of **(A)** daidzein (5–20 μM), **(B)** apigenin (5–20 μM), **(C)** baicalein (0.1–10 μM), **(D)** CAPE (0.1–1 μM), **(E)** capsaicin (1–10 μM), **(F)** curcumin (5–20 μM), **(G)** epicatechin (10–50 μM), **(H)** naringenin (1–10 μM), or **(I)** sulforaphane (1–10 μM). All data were normalized to AR levels in CM-cultured BMMSCs ($n = 3$ for each group). Data are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ as analyzed by Student's *t*-test; a, $p < 0.05$; b, $p < 0.01$; and c, $p < 0.001$ as analyzed by ANOVA.

compounds for the ability to induce RUNX2 gene expression and mineralization in primary isolated human BMMSCs. We selected the most potent osteogenic compounds screened through the C3H-hRUNX2-luc assay and validated in the murine BMMSC mineralization assay—apigenin and baicalein—along with daidzein as the positive control, and also included sulforaphane as a negative osteogenic compound based on the murine MSC data. We found that addition of apigenin or baicalein, as well as daidzein, significantly increased RUNX2 gene expression levels in OM-cultured human BMMSCs at 48 h compared to OM-only conditions, while sulforaphane significantly decreased RUNX2 expression levels to below OM-only levels (**Figure 5A**). In contrast, addition of either of the three osteogenic compounds resulted in significantly decreased expression of C/EBPβ, one of the earliest transcription factors of adipogenesis (Guo et al., 2015), in AM-cultured human BMMSCs at 48 h to basal levels; interestingly, sulforaphane significantly increased C/EBPβ expression levels to levels above AM-only conditions (**Figure 5B**). *In vitro* mineralization assay using human BMMSCs demonstrated that addition of daidzein, apigenin, or baicalein significantly enhanced calcium deposition in OM-cultured human BMMSCs compared to OM-only conditions, while sulforaphane significantly suppressed mineralization to below OM-only levels and nearly down to undifferentiated conditions (**Figure 5C**, representative data; and **Figure 5D**, pooled quantitative data).

A



B

Assay / Con. / Compound	hRUNX2-luc activity			ALP activity			AR staining		
	L	M	H	L	M	H	L	M	H
Daidzein	↑	↑	↑	↑	↑	↑	↑	↑	↑
Apigenin	↑	↑	↑	-	-	↑	-	↑	↑
Baicalein	-	↑	↑	-	↑	↑	-	↑	↑
CAPE	-	-	-	-	-	↓	-	-	-
Capsaicin	-	-	-	-	-	↑	-	-	-
Curcumin	-	-	-	-	↓	↓	-	-	-
Epicatechin	-	-	-	-	-	-	-	-	-
Naringenin	-	-	-	-	-	-	-	-	-
Sulforaphane	↓	↓	↓	-	↓	↓	↓	↓	↓
Accuracy (compare to AR staining)	96.3%			77.8%					
Chi-square	4.1033								
p-value	0.042798								

FIGURE 4 | Comparisons of hRUNX2-luc reporter activity in C3H MSCs to *in vitro* functional assays demonstrate that hRUNX2-luc reporter activity but not ALP activity is highly correlated with *in vitro* mineralization assay. **(A)** Heatmap analysis of expression levels of hRUNX2-luc reporter activity in compound-treated C3H MSCs, and ALP activity as well as AR staining in primary murine BMMSCs treated with tested compounds. **(B)** Correlation analyses of expression trends in all three *in vitro* assays to mineralization assay for each tested compound. When compared to OM-cultured MSCs without compounds, significant increases in specific assay endpoints in compound-treated conditions were labeled as "↑," while significant decreases were labeled as "↓," and no changes (non-significant increases or decreases) are labeled as "–." $p < 0.05$ using goodness of fit.

To assess the *in vivo* relevance of these collective *in vitro* findings, we assessed for induction of ectopic bone formation using human BMMSCs in immunocompromised mice (**Figure 5E**). We found that subcutaneously transplantation of human BMMSCs pretreated with daidzein, apigenin, or baicalein improved osteoid formation compared to vehicle-treated BMMSCs, whereas pretreatment with sulforaphane enhanced adipogenesis rather than osteogenesis (**Figure 5F**).

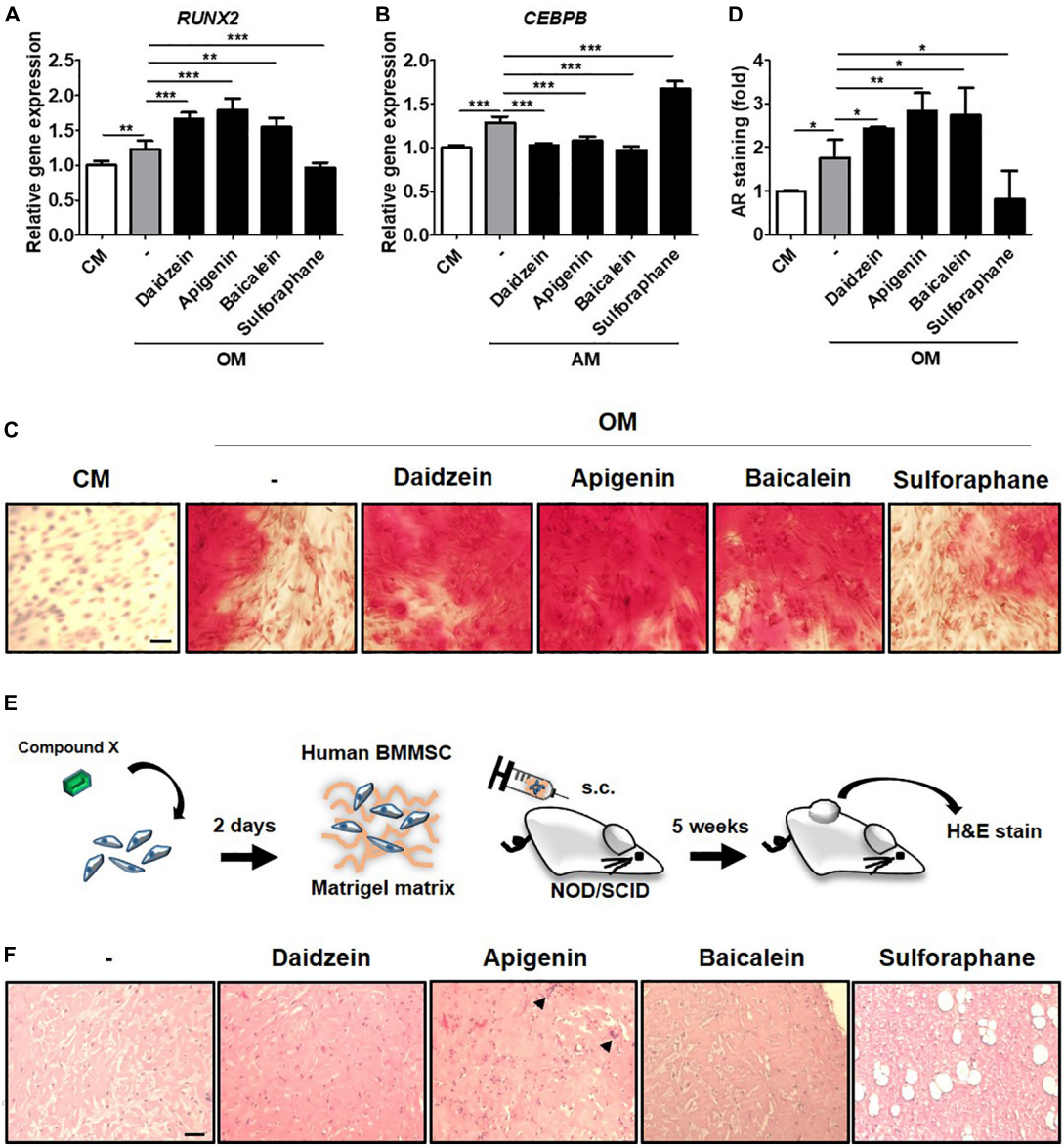


FIGURE 5 | Compounds screened through the murine C3H-hRUNX-luc system strongly induce *in vitro* and *in vivo* osteogenesis in human BMMSCs. **(A)** Quantitative PCR (qPCR) analyses of gene expression of osteogenic master gene, RUNX2, was assessed in human BMMSCs under CM or OM with or without daidzein (10 μM), apigenin (20 μM), baicalein (10 μM), or sulforaphane (10 μM). Gene expression of each condition was normalized to the gene level in CM-culturing human BMMSCs (n = 5 for each group). **(B)** qPCR analysis of early adipogenic gene, CCAAT/enhancer-binding protein beta (CEBPB), was assessed in human BMMSCs under CM or adipogenic medium (AM) with or without daidzein (10 μM), apigenin (20 μM), baicalein (10 μM), or sulforaphane (10 μM). Gene expression of each condition was normalized to the gene level in CM-culturing human BMMSCs (n = 5 for each group). **(C)** and **(D)** Representative and pooled quantitative data (n = 4 for each group), respectively, of calcium deposition in human BMMSCs were assessed by staining for AR. Human BMMSCs were cultured in CM or OM with daidzein (10 μM), apigenin (20 μM), baicalein (10 μM), or sulforaphane (10 μM) for approximately 3 weeks, and AR staining was performed to assess extracellular mineralization by image examination **(C)** as well as AR elution and then quantification with normalized to the AR amount in CM-culturing human BMMSCs **(D)**. Scale bar, 50 μm. Data are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **(E)** Schematic diagram illustrating the *in vivo* ectopic bone formation: human BMMSCs were treated with selected compounds for 2 days and then mixed with Matrigel matrix for subcutaneous transplantation in immunodeficient NOD/SCID mice with H&E analyses of the implants at 5 weeks. **(F)** Assessment of osteoid formation by H&E staining; scale bar, 100 μm. Arrows denote multinucleated osteoclast-like cells.

Interestingly, apigenin pretreatment seem to induce more mature bone/osteoid formation, with multinucleated osteoclast-like cells seen in sections. These results show that apigenin and baicalein are potent plant-derived osteogenic compounds for human BMMSCs, whereas sulforaphane inhibits osteogenesis and may be an inducer of BMMSC adipogenesis. Collectively, these findings demonstrate that the hRUNX2-luc cell-based *in vitro* platform is a highly predictive and robust system for rapid screening of osteogenic compounds (**Figure 6**).

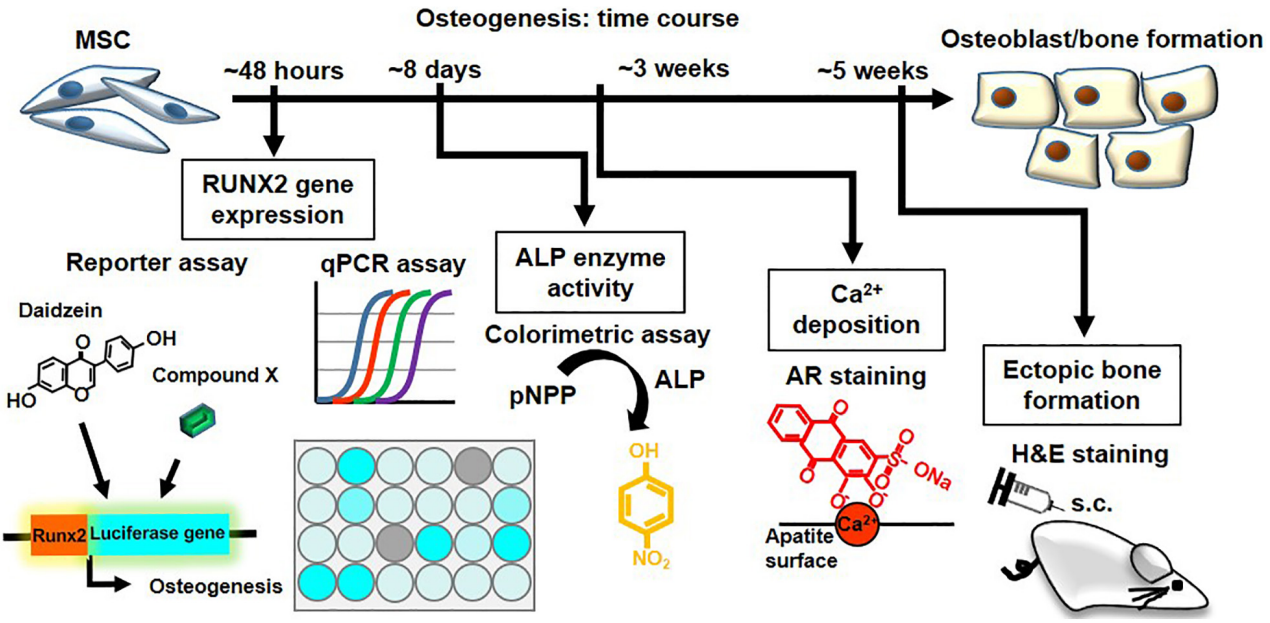


FIGURE 6 | The hRUNX2-luc MSC-based *in vitro* platform is a rapid and robust system for screening of osteogenic compounds. Summary and time required of various osteogenic assays performed in the study: rapid screening for osteogenic phytoestrogens and natural plant-derived compounds using C3H-RUNX2-luc platform at 48 h with qPCR confirmation for RUNX2 gene expression. Validation using classical functional assays of osteogenesis using primary murine and human BMMSCs was performed for the early-stage assay of *in vitro* ALP activity which required 8 days, late-stage assay for *in vitro* mineralization assay which required 3 weeks, and *in vivo* ectopic bone formation which required 5 weeks.

DISCUSSION

The increasing incidence of OP globally have been accelerating, and while a number of treatments are available, compliance continues to be a major problem due to slow efficacy and rare but serious side effects (Warriner and Curtis, 2009; Hiligsmann et al., 2019). Human dietary studies have demonstrated phytoestrogens and other natural plant-derived compounds to have minimal safety concerns, but while the chemical structure of individual compounds is helpful for inference of estrogenic effects, dose-related differences in potency and efficacy has been difficult to predict, hampering drug discovery and development (Dang and Lowik, 2005; Vitale et al., 2013). Such challenges are further compounded in screening for osteogenic effects by the long process required for both *in vitro* and *in vivo* functional assays—which range from several days for the ALP assay to several weeks and even months for mineralization assays—with the additional requirement of using primary cells with the capacity for osteogenesis. Indeed, one major reason for the conflicting reports of agonistic/antagonistic osteogenic properties of many compounds may be due to the quality of the primary MSCs used, since it is well documented that MSC senescence is related to a loss of osteogenic differentiation capacity while increasing adipogenic capacity (Nuttall and Gimble, 2000; Moerman et al., 2004; Ho et al., 2013; Conley et al., 2020). We therefore sought to develop a rapid and robust *in vitro* platform for drug screening of osteogenic compounds based on our previous work on the proximal human Runx2 promoter, the master osteogenesis transcription factor, introduced into an immortalized, non-cancerous MSC cell line. Validation performed with primary isolated BMMSCs, the progenitors of osteoblasts, of classical *in vitro* functional assays including ALP activity and mineralization as well as *in vivo* osteogenesis demonstrated high fidelity of the hRUNX2-luc assay to accurately select osteogenic compounds with dose-dependent information at a fraction of the time and effort required for osteogenic functional assays.

Given the high incidence of OP worldwide and severe adverse complications, there has been surprisingly only two reports on developing *in vitro* platforms to screen for osteogenic compounds (Hojo et al., 2008; Li et al., 2009). Strangely, these two previous reports used rodent promoters of the osteogenic genes collagen one and BMP2 as screening platforms, rather than the corresponding human promoters. The choice of collagen one as a screen for osteogenesis is likely too non-specific, since this protein is the most abundant proteins in humans (Di Lullo et al., 2002). Conversely, while BMP2 is clearly a strong osteogenic agonist and has been therapeutically available as a recombinant protein for many years, there has been a number of clinical reports on the considerable side effects associated with this molecule (Carragee et al., 2011). Moreover, major pathways involved in osteogenesis including BMPs—as well as estrogens/sex hormones, Wnt/β-catenin, and sirtuins—are clearly also important in other biological processes (Varga and Wrana, 2005; Bartoli-Leonard et al., 2018); thus, screening platforms based on these pathways can lead to non-osteogenic effects. Since all of these major osteogenic pathways have been found to converge on RUNX2 for osteogenic effects,

the use of transcriptional activity of this osteogenic master transcription factor as a screen for drug discovery would appear to be highly efficacious. An additional benefit of using Runx2 activity as an outcome may be the ability to predict adipogenic differentiation—as was seen with the compound sulforaphane (**Figures 1I**, **2I**, **3I**, and **5F**)—which has confounded the therapeutic value of a number of phytoestrogens for osteogenesis (Dang and Lowik, 2005; Vitale et al., 2013). Our use of the Runx2 promoter as a screening tool, therefore, has the strong advantage of capturing compounds acting as agonists in any of the major osteogenic pathways as well as excluding compounds that induce adipogenesis.

Data obtained from this screening platform likely have strong translational implications since we have diligently performed several *in vitro* functional assays using murine as well as human primary isolated BMMSCs, along with an *in vivo* assay using human BMMSCs, which previous reports have surprisingly not done, bringing to question the robustness of those screening platforms. Interestingly, we found that the ALP assay was not as consistent or robust as the *in vitro* mineralization assay or the *in vivo* osteogenesis assay, the latter two assays which arguably offer more definitive information by evaluating osteogenesis to the outcome of mineralization. The wide distribution of ALP in many organs/tissues is well known and may be reason for the less specific correlation of this assay to mineralization assays. Given that the data from the hRUNX2-luc platform correlate best with the mineralization assays rather than the ALP assay, it appears that the hRUNX2-luc screening platform is robust at selecting osteogenic compounds.

In summary, we developed a rapid *in vitro* platform for screening of phytoestrogens and other natural plant-derived compounds using the hRUNX2-luc reporter transduced into an MSC line. Multiple functional assays both *in vitro* and *in vivo* and using primary isolated BMMSCs from both murine and human systems were performed for validation, with results demonstrating robust correlation from this *in vitro* screening platform. These findings implicate the contributions of a rapid and highly predictive *in vitro* screening platform using MSCs toward discovery of potent therapeutic candidates in the global fight against OP.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Laboratory Animal Center, National Health Research Institutes.

AUTHOR CONTRIBUTIONS

M-LY: conceptualization and funding acquisition. L-TW, Y-WL, C-HB, H-CC, H-HW, and BY: methodology. L-TW, Y-WL, H-CC, and H-HW: investigation. L-TW, BY, and M-LY: writing—original draft. L-TW and M-LY: writing—review and editing. All authors contributed to the article and approved the submitted version.

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TGF-β2 Reduces the Cell-Mediated Immunogenicity of Equine MHC-Mismatched Bone Marrow-Derived Mesenchymal Stem Cells Without Altering Immunomodulatory Properties

Alix K. Berglund[1,2†], Julie M. Long[1], James B. Robertson[3] and Lauren V. Schnabel[1,2†]*

[1] Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, United States, [2] Comparative Medicine Institute, North Carolina State University, Raleigh, NC, United States, [3] Office of Research, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, United States

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Josep M. Canals,
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Essen University Hospital, Germany

***Correspondence:**
Lauren V. Schnabel
lvschnab@ncsu.edu

†ORCID:
Alix K. Berglund
orcid.org/0000-0001-8913-7924
Lauren V. Schnabel
orcid.org/0000-0002-1993-8141

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Allogeneic mesenchymal stem cells (MSCs) are a promising cell therapy for treating numerous diseases, but major histocompatibility complex (MHC)-mismatched MSCs can be rejected by the recipient's immune system. Pre-treating MSCs with transforming growth factor-β2 (TGF-β2) to downregulate surface expression of MHC molecules may enhance the ability of allogeneic MSCs to evade immune responses. We used lymphocyte proliferation assays and ELISAs to analyze the immunomodulatory potential of TGF-β2-treated equine bone marrow-derived MSCs. T cell activation and cytotoxicity assays were then used to measure the *in vitro* cell-mediated immunogenicity. Similar to untreated MSCs, TGF-β2-treated MSCs inhibited T cell proliferation and did not stimulate MHC-mismatched T cells to proliferate. Additionally, similar quantities of prostaglandin E2 and TGF-β1 were detected in assays with untreated and TGF-β2-treated MSCs supporting that TGF-β2-treated MSCs retain their strong immunomodulatory properties *in vitro*. Compared to untreated MSCs, TGF-β2-treated MSCs induced less T cell activation and had reduced cell-mediated cytotoxicity *in vitro*. These results indicate that treating MSCs with TGF-β2 is a promising strategy to reduce the cell-mediated immunogenicity of MHC-mismatched MSCs and facilitate allogeneic MSC therapy.

Keywords: mesenchymal stem cell, TGF-β2, cytotoxicity, immunogenicity, major histocompatibility complex, allogeneic

INTRODUCTION

Mesenchymal stem cells (MSCs) are currently being investigated in clinical trials for the treatment of musculoskeletal, immune-mediated, and degenerative diseases (Squillaro et al., 2016; Galipeau and Sensébé, 2018). The mechanism by which MSCs exert their therapeutic effect appears to be primarily through the secretion of paracrine factors, which inhibit immune responses,

promote angiogenesis, reduce apoptosis, and support the recruitment and differentiation of local progenitor cells (da Silva Meirelles et al., 2009; Caplan and Sorrell, 2015). MSCs are strongly immunomodulatory *in vitro*, which initially led investigators to conclude that MSCs were immune privileged and that allogeneic cells could be used without risk of rejection (Le Blanc et al., 2003). However, subsequent *in vivo* studies have demonstrated that major histocompatibility complex (MHC)-mismatched MSCs are in fact recognized and rejected by the recipient immune system (Eliopoulos and Stagg, 2005; Nauta et al., 2006; Badillo et al., 2007; Zangi et al., 2009; Isakova et al., 2014; Pezzanite et al., 2015). Donor MHC I-specific $CD8^+$ T cell and cytotoxic alloantibody responses have been detected following *in vivo* administration of allogeneic MSCs (Zangi et al., 2009; Berglund and Schnabel, 2017). Rejection of donor MSCs may lead to increased risk of adverse events and decreased therapeutic potential and must be prevented to realize the full clinical potential of allogeneic MSC therapy (Berglund et al., 2017b).

While *in vivo* studies support that the immunomodulatory properties of MSCs alone cannot prevent allorecognition and rejection *in vivo*, MSCs are rejected more slowly than non-immunomodulatory cells like fibroblasts (Zangi et al., 2009) so they can be considered immune evasive (Ankrum et al., 2014). Additionally, allogeneic MSC therapy is still attractive over autologous therapy as the age and health status of a donor can greatly affect the quality of the cells (Nie et al., 2010; Fafián-Labora et al., 2015). MHC-matching is a labor-intensive process and is not practical in most clinical settings. Manipulation of MHC surface expression on MSCs is a promising strategy for enhancing the immune evasive qualities of MHC-mismatched MSCs and has been shown to promote persistence of allogeneic MSCs in mouse models (de la Garza-Rodea et al., 2011; Huang et al., 2016). We recently published that treatment of equine bone-marrow derived MSCs with 1 ng/ml transforming growth factor-β2 (TGF-β2) significantly downregulated constitutive MHC I surface expression and inhibited IFN-γ-induced MHC I and MHC II expression without altering MSC phenotype or secretion of endogenous TGF-β1 or TGF-β2 (Berglund et al., 2017a). The degree to which MHC I surface expression must be downregulated to prevent T cell activation and rejection of MHC-mismatched MSCs *in vivo* remains unclear.

Mixed lymphocyte reactions (MLRs) and other lymphocyte proliferation assays have traditionally been used to measure MSC immunogenicity (Le Blanc et al., 2003; Tse et al., 2003), but more recent studies have demonstrated that the ability of MSCs to avoid T cell allorecognition and suppress proliferation *in vitro* does not necessarily correlate with the ability to avoid allorecognition *in vivo* (Nauta et al., 2006; Poncelet et al., 2007; Zangi et al., 2009). However, mixed cell cultures or other modified T cell proliferation assays are still useful for measuring the immunomodulatory capabilities and mechanisms of MSCs. For predicting the *in vivo* cell-mediated immunogenicity of MSCs, *in vitro* cytotoxicity assays are more appropriate (Berglund et al., 2017b). As both the immunomodulatory and immune evasive properties are critical for the therapeutic potential of allogeneic MSCs, the goal of this study was to characterize the immunomodulatory properties and cell-mediated immunogenicity of allogeneic TGF-β2-treated equine MSCs. Horses, like humans, are an outbred species and are one of the best available translational models for assessing MSC therapy efficacy for musculoskeletal diseases (Patterson-Kane and Rich, 2014; Kol et al., 2015). Therefore, understanding the immunogenicity of equine MSCs is important for furthering allogeneic MSC therapy in human medicine.

MATERIALS AND METHODS

Horses and MHC-Haplotyping

A total of eight horses were used in this study. All animals were between the ages of 6 and 18 years of age, free of systemic disease as determined by routine physical examinations and bloodwork, free of medication for 48 h prior to use, and non-pregnant. The MHC haplotype of each horse was determined by microsatellite testing as previously described (**Table 1**; Tallmadge et al., 2010; Tseng et al., 2010). The Institutional Animal Care and Use Committee of North Carolina State University approved the use of horses in these studies.

MSC Isolation and Culture

Bone marrow aspirates were collected aseptically from the sternum of horses by using 11-gage Jamshidi bone marrow biopsy needles under standing sedation with local anesthesia. Up to 40 ml of bone marrow was obtained from two sternebrae from each horse and purified via Ficoll-Paque Plus (GE Healthcare, Chicago, IL, United States) gradient centrifugation as previously described (Radcliffe et al., 2010). Untreated and TGF-β2-treated MSCs were cultured as previously described (Berglund et al., 2017a). Isolated bone marrow cells from each horse were plated onto 100 mm tissue culture plates at a density of 300,000 cells/cm^2. Half of the plates were cultured in normal culture expansion media and half were cultured in media with 1 ng/ml human recombinant TGF-β2 (BioLegend, San Diego, CA, United States). Culture media consisted of 1 g/dl glucose DMEM, 10% fetal bovine serum (FBS) (Atlanta Biolgicals, Flowery Branch, GA, United States), 2 mM L-glutamine, 100 U/ml penicillin and streptomycin, and 1 ng/ml basic fibroblast growth factor (bFGF) (Corning, Inc., Corning, NY, United States). Media were exchanged every 48 h. Cells were passaged at approximately 80% subconfluency by using Accutase cell dissociation solution (Innovative Cell Technologies, Inc., San Diego, CA, United States) and plated at a density of 10,000 cells/cm^2 for all subsequent passages. All MSCs used in this study were between passages 2 and 4.

Major histocompatibility complex I surface expression on untreated and TGF-β2-treated MSCs from each horse used in this study was compared using an LSRII flow cytometer (BD Biosciences, San Jose, CA, United States). MSCs were labeled with anti-equine MHC I antibody (clone cz3, Antczak Laboratory) at a 1:10 dilution and an APC-conjugated anti-mouse IgG secondary antibody (BD Biosciences) at 1:100 dilution as previously described (Berglund et al., 2017a). MSCs labeled with secondary antibody alone were used as negative controls.

TABLE 1 | MHC haplotypes of horses.

		Intra-MHC microsatellite alleles							
		Class I		Class II					
Horse	ELA haplotype	UMNJH-38	COR110	ABGe9030	EQMHC1	COR112	COR113	UM011	COR114
A	Unclassified	156	221	206	192	243	270	172	249
	Unclassified	156	221	205	194	256	270	172	249
B	A3a	163	207	211	192	254	260	172	243
	A3a	163	207	211	192	254	260	172	243
C	A9a	156	217	215	190	264	272	168	255
	A9a	156	217	215	190	264	272	168	255
D	A2	156	211	209	192	262	268	174	234
	Unclassified	156	221	211	192	262	268	176	247
E	A2	156	211	209	192	262	268	174	234
	Unclassified	156	221	211	192	262	268	176	247
F	A3b	163	207	211	192	262	268	176	247
	A3b	163	207	211	192	262	268	176	247
G	A2	156	211	209	192	262	268	174	234
	Unclassified	156	211	207	190	237	266	179	241
H	A19	156	211	212	190	262	270	184	245
	A9a	156	217	215	190	264	272	169	255

Equine leukocyte antigen (ELA) haplotypes were assigned by performing PCR amplification of eight polymorphic microsatellite loci and analyzing the length of each amplified fragment.

MSCs from each horse showed downregulation of MHC I surface expression when treated with TGF-β2 similar to our previously published results (**Supplementary Figure 1**).

T Cell Proliferation Assays

Peripheral blood leukocytes (PBLs) were isolated from venous blood by carbonyl iron treatment and Ficoll-Paque Plus gradient centrifugation. Isolated PBLs were re-suspended in lymphocyte media containing RPMI 1640, 10% FBS, 100 U/ml penicillin and streptomycin, and 0.1 mM molecular grade 2-mercaptoethanol.

Modified one-way MLRs were performed in duplicate using responder PBLs with MHC-matched (autologous) or MHC-mismatched stimulator MSCs as previously described (Tse et al., 2003; Schnabel et al., 2014). All MSC stimulator cells used in this assay were MHC II negative. MHC-matched stimulator PBLs were used as negative controls to determine background T cell proliferation and MHC-mismatched stimulator PBLs as positive controls. Untreated and TGF-β2-treated stimulator MSCs were plated at 5×10^4 cells/well in a 24-well plate in standard or TGF-β2 MSC media 24 h before adding responder PBLs. MSC wells were washed twice with phosphate buffered saline (PBS) to remove any exogenous TGF-β2 prior to the addition of responder PBLs. Stimulator PBLs were irradiated with 9 Gy at 60 cGy/min using a Varian Novalis TX linear accelerator and 1×10^6 cells/well were added immediately before the addition of responder PBLs. Responder PBLs were labeled with 5 μM 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) and 2×10^6 cells were added to each well. Cultures were maintained for 5 days in lymphocyte media at 37°C and 5% CO_2.

After culture, PBLs were aspirated from the wells and stained with primary mouse anti-horse CD3 antibody (clone UC-F6G, 1:20 dilution, Laboratory of Dr. J. Scott, University of California-Davis, Davis, CA, United States) and a secondary goat anti-mouse IgG-APC antibody (1:100 dilution, BD Biosciences, Franklin Lakes, NJ, United States). 4′,6′-diamidino-2-phenylindole (DAPI) was added to each sample at a concentration of 500 ng/ml 15 min prior to analysis. Samples were analyzed via flow cytometry using an LSRII (BD Biosciences). Proliferation was calculated using CFSE attenuation and the division index of live, $CD3^+$ cells in FlowJo v10 (FlowJo LLC, Ashland, OR, United States). All results were reported as the log fold change relative to the negative control.

ELISAs

Supernatant from each well of the MLRs was frozen and stored at −80°C prior to use. ELISAs for human TGF-β1 (Promega, Madison, WI, United States) and prostaglandin E2 (PGE2) (Enzo Life Sciences, Inc., Farmingdale, NY, United States) were performed per manufacturer's instructions. For PGE2 analysis, supernatant from MLRs were diluted 1:2 for reactions with PBL stimulator cells or 1:100 for reactions with MSC stimulator cells in reagent diluent.

T Cell Activation Assays

Major histocompatibility complex-specific effector cells were generated in mixed leukocyte reactions as previously described (Noronha and Antczak, 2012). Briefly, 50×10^6 PBLs were irradiated with 9 Gy and co-cultured with 100×10^6 responder PBLs in a T175 flask upright for 7 days. Cultures were maintained in media containing 60% AIM-V, 30% RMPI 1640, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and streptomycin, 1X non-essential amino acids, 0.5X sodium pyruvate, 0.1 mM

molecular grade 2-mercaptoethanol, and 50 U/ml human recombinant IL-2 (PeproTech, Rocky Hill, NJ, United States) at a concentration of 2.5×10^6 cells/ml. On day 7, cultures were re-stimulated with fresh irradiated stimulator cells that were plated at half the density of the surviving responder cells. All cultures were maintained for a total of 10 days and half of the culture media was exchanged for fresh media every 48 h.

2×10^6 effector cells and 5×10^4 MSC target cells were added to 24-well plates. Co-cultures were incubated for 24 h in effector cell media at 37°C and 5% CO_2. Effector cells were then labeled with primary anti-CD3 antibody as described under T Cell Proliferation Assays or anti-equine CD8 (clone CVS8, 1:80 dilution, Bio-Rad, Hercules, CA, United States).

In vitro Cell-Mediated Cytotoxicity

Major histocompatibility complex-specific effector cells were generated as described above. Untreated and TGF-β2-treated MSC target cells were labeled with 50 μl of chromium-51 (Cr-51) (PerkinElmer, Boston, MA, United States) for 30 min at 37°C and 5% CO_2. Labeled targets were plated to give effector/target ratios of 50:1 in 200 μl final volume in 96-well round-bottom plates. Spontaneous release control wells contained only target cells and media. 10% Triton X-100 was added to maximum release control wells. All tests were carried out in duplicate. The plates were incubated at 37°C and 5% CO_2 for 6 h and then centrifuged at 309 × *g* for 3 min. A total of 110 μl of supernatant was harvested from each well and mixed with Ultima Gold scintillation cocktail (PerkinElmer). Cr-51 activity was measured with a Tri-Carb 2900 TR scintillation counter (PerkinElmer) as counts per minute (cpm) over 2 min. Percent cytotoxicity was calculated as % = (experimental cpm−spontaneous cpm)/(maximum cpm−spontaneous cpm) × 100. The percent cytotoxicity for the duplicate wells was then averaged and reported.

Statistical Analysis

Data from the T cell proliferation assays were normalized by log transformation and analyzed with analysis of covariance (ANCOVA) with horse as covariate. When ANCOVA indicated significant differences ($p < 0.05$), a Tukey's test was used for multiple comparisons of individual means. Differences in CD3 and CD8 surface expression and percent cytotoxicity were analyzed using paired *t*-tests on the response to treatments matched by donor horse and MHC-matched or mismatched with a null hypothesis of no difference. All analyses were performed using JMP Pro 14 (SAS Institute Inc., Cary, NC, United States) or R (R Core Team, Vienna, Austria).

RESULTS

TGF-β2-Treated MSCs Inhibit MHC-Mismatched T Cell Proliferation *in vitro*

Inhibition of allogeneic T cell proliferation *in vitro* is an established characteristic of MSCs, but this ability can be influenced by expression of immunogenic surface molecules and secretion of cytokines (Rasmusson et al., 2005; Carrade Holt et al., 2014; Schnabel et al., 2014). As immunomodulation is critical for both the immune evasive and therapeutic properties of MSCs, the ability of untreated and TGF-β2-treated MSCs to avoid T cell allorecognition and inhibit T cell proliferation was measured using modified one-way MLRs. Significantly fewer live $CD3^+$ cells were recovered from assays with MSC stimulator cells compared with the PBL stimulator cells, although there was variation between horses (**Figures 1A,B**). Background T cell proliferation was detected in the negative control (MHC-matched PBL stimulator cells) by the presence of live $CD3^+CFSE^{low}$ cells and as expected, an increased number of live $CD3^+$ $CFSE^{low}$ cells were detected in the positive control (MHC-mismatched with PBL stimulator cells) (**Figure 1C**). There were fewer $CFSE^{low}$ $CD3^+$ cells in the MHC-matched and MHC-mismatched lymphocyte cultures with MSC stimulator cells even compared to the negative control (**Figure 1C**). T cell proliferation was significantly reduced in all MLRs with MSC stimulator cells compared to assays with MHC-mismatched PBL stimulator cells as measured by both the relative division index (**Figure 1D**) and relative GMFI of proliferating cells (**Figure 1E**). The relative division index and CFSE GMFI in all MLRs with MSC stimulator cells were lower compared to the negative control so neither untreated nor TGF-β2-treated MSCs induced T cell proliferation and both inhibited non-specific background proliferation. Although there was more variation in the number of live $CD3^+$ cells recovered from MHC-mismatched MLRs with TGF-β2-treated MSC stimulator cells, the number of $CD3^+$ cells in these assays did not correlate with increased proliferation. There was no significant difference between the untreated or TGF-β2-treated MSC treatment groups for either the relative division index or relative CFSE GMFI demonstrating that untreated and TGF-β2-treated MSCs display similar abilities to evade T cell allorecognition and inhibit proliferation *in vitro*.

Untreated and TGF-β2-Treated MSCs Secrete Similar Amounts of PGE2 and TGF-β1

Prostaglandin E2and TGF-β1 have previously been identified as the major immunomodulatory cytokines secreted by equine MSCs and are also important for the therapeutic properties of MSCs (da Silva Meirelles et al., 2009; Carrade et al., 2012; Carrade Holt et al., 2014). PGE2 concentrations were significantly higher in all cultures with MSC stimulator cells than in wells with PBL stimulator cells and there was no significant difference between PGE2 concentrations in the supernatant from cultures with untreated or TGF-β2-treated MSCs (**Figure 2A**). There was no significant difference in the concentration of TGF-β1 in the supernatant of any of the MLR treatment groups (**Figure 2B**). Although we cannot definitively determine if the TGF-β1 is produced by the MSCs or responder PBLs in this assay, these findings are consistent with our previous study that showed TGF-β2-treated MSCs produce similar quantities of TGF-β1 compared to untreated MSCs when cultured alone (14). These results

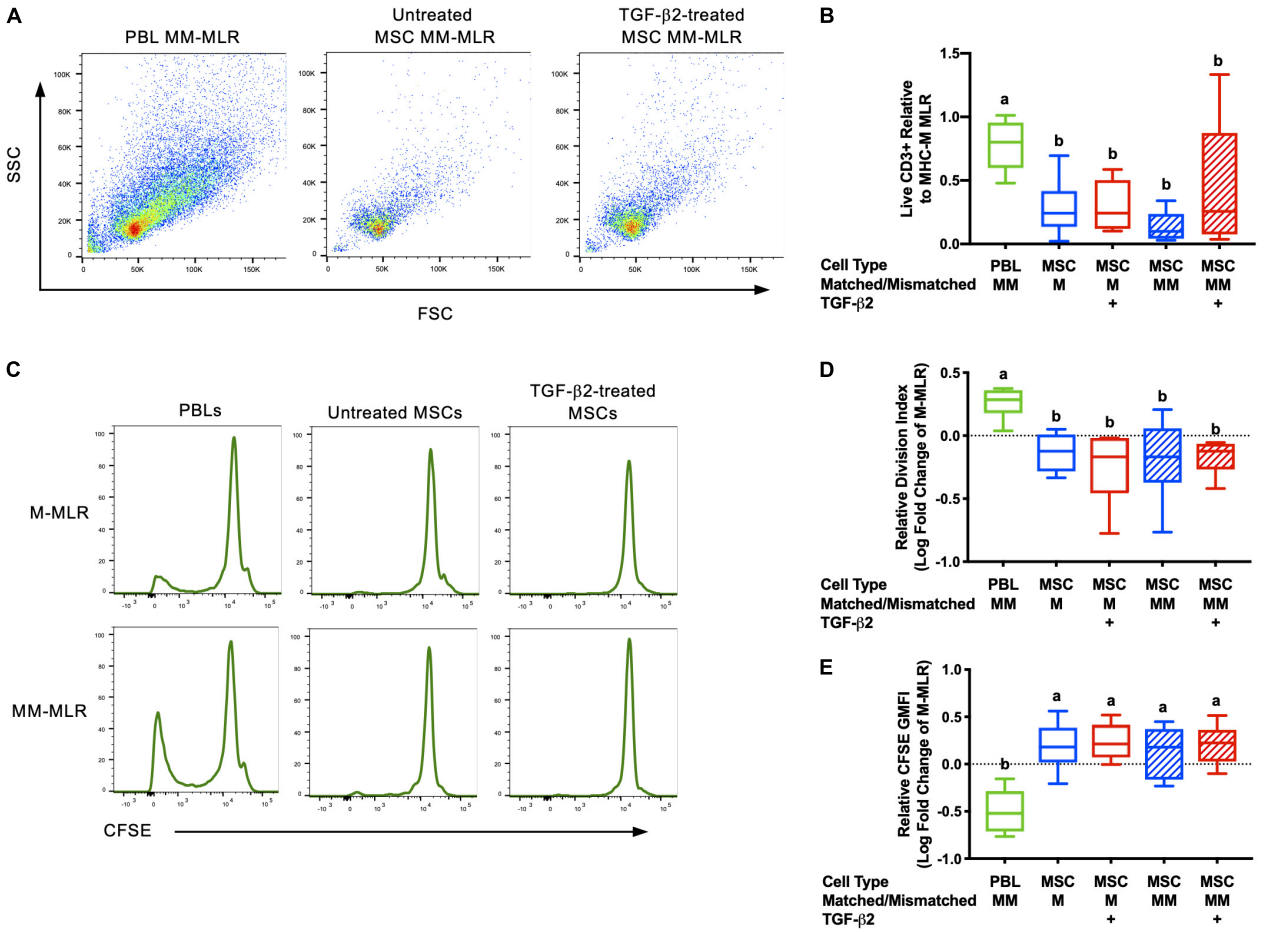

FIGURE 1 | Untreated and TGF-β2-treated MSCs inhibit proliferation of MHC-mismatched T cells *in vitro*. **(A)** Representative dot plots of live, $CD3^+$ cells from MLRs with MHC-mismatched PBL, untreated MSC, and TGF-β2-treated MSC stimulator cells. **(B)** Number of live $CD3^+$ cells recovered from MLRs relative to the MHC-M MLR with PBL stimulator cells. Data shown are mean ± SD of $n = 6$, $p = 0.0046$ by ANCOVA. Superscript letters indicate significant difference between groups by Tukey's test. **(C)** Representative histograms depicting CFSE attenuation in responder PBLs cultured with MHC-matched (M) and MHC-mismatched (MM) PBL, untreated, and TGF-β2-treated MSC stimulator cells. **(D)** Proliferation of live, $CD3^+$ responder T cells shown as the average log fold change in division index relative to the MHC-M MLR with PBL stimulator cells. Data shown are mean, minimum, and maximum values of $n = 6$, $p = 0.0025$ by ANCOVA. **(E)** Proliferation of live, $CD3^+$ responder T cells shown as the average log fold change in CFSE geometric mean fluorescent intensity relative to the MHC-M MLR with PBL stimulator cells. Data shown are mean, minimum, and maximum values for $n = 6$, $p = 0.0001$ by ANCOVA.

support that TGF-β2 treatment does not affect production or secretion of the main immunomodulatory factors produced by equine bone marrow-derived MSCs.

TGF-β2-Treated MSCs Induce Less T Cell Activation *in vitro*

Activation of $CD8^+$ T cells by MHC I molecules induces downregulation of CD3 and CD8 and secretion of cytolytic effector proteins (DiSanto et al., 1989; Valitutti et al., 1996). The degree of downregulation of CD3 is known to be dependent on the level of T cell receptor engagement (Valitutti et al., 1996). To determine if TGF-β2 treatment prevents MHC-mismatched MSCs from activating effector T cells, untreated and TGF-β2-treated MSCs were co-cultured with effector cells generated in a standard mixed leukocyte reaction prior to analysis of CD3 and CD8 surface expression. Downregulation of both CD3 and CD8 surface expression was detected when effector lymphocytes were cultured with untreated MHC-mismatched MSCs, but not untreated MHC-matched MSCs (**Figures 3A,C**). Both CD3 and CD8 expression were significantly higher in effector lymphocytes cultured with TGF-β2-treated MSCs compared with untreated MSCs relative to effector cells alone (**Figures 3B,D**). Changes in CD3 or CD8 surface expression could not be detected with every combination of responders and stimulators demonstrating the natural variation in cell-mediated alloimmune responses. Although not every combination of donors may result in activation of lymphocytes, these results support that T cells were less likely to be activated *in vitro* by TGF-β2-treated MHC-mismatched MSCs compared with untreated MSCs.

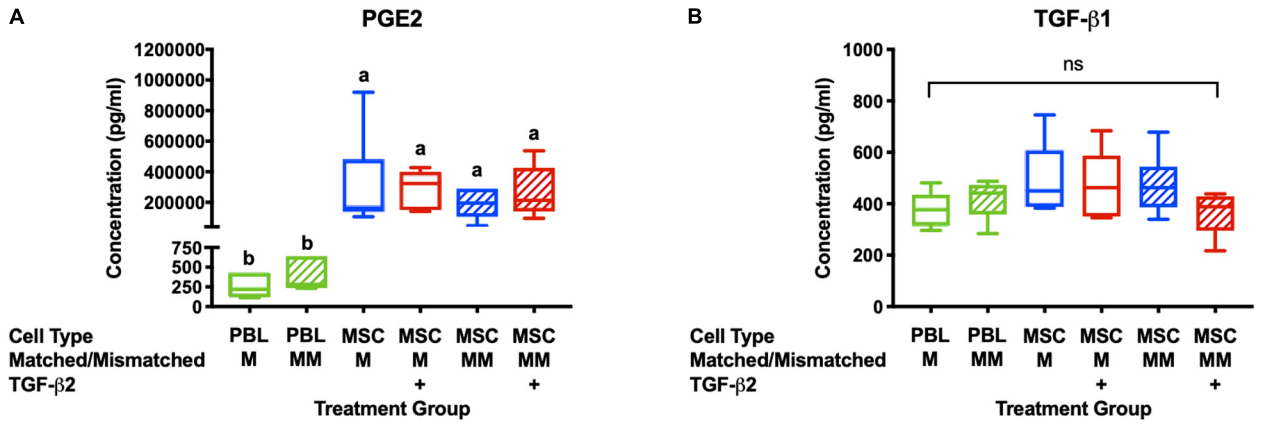

FIGURE 2 | PGE2 and TGF-β1 are secreted in MLRs with PBL and MSC stimulator cells. **(A)** PGE2 concentrations in the supernatant of MLRs as measured by ELISA. Data shown are mean ± SD of $n = 6$, $p < 0.0001$ by ANCOVA. Superscript letters indicate significant difference between groups. **(B)** TGF-β1 concentrations in MLR supernatant as measured by ELISA. Data shown are mean, minimum, and maximum values for $n = 6$, non-significant by ANCOVA.

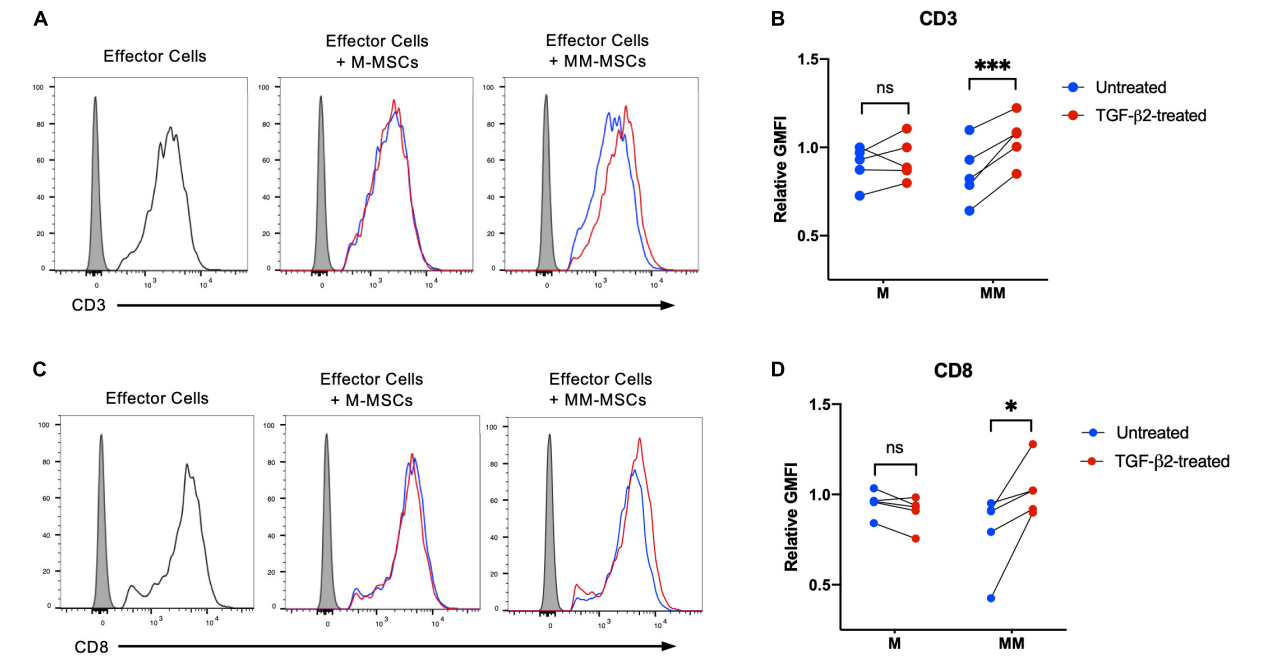

FIGURE 3 | TGF-β2 treatment of MHC-mismatched MSCs prevents activation of T cells. **(A)** Representative histograms of CD3 expression on effector lymphocytes from one horse cultured with MHC-matched or MHC-mismatched untreated or TGF-β2-treated MSCs. **(B)** CD3 expression on effector lymphocytes relative to effector cells incubated without MSCs. MSC donor-paired samples are represented by connected lines for $n = 5$, $^{***}p = 0.0016$ by a one-sample *t*-test on each pair. **(C)** Representative histograms of CD8 expression on effector lymphocytes from one horse cultured with MHC-matched or MHC-mismatched untreated or TGF-β2-treated MSCs. **(D)** CD8 expression on effector lymphocytes relative to effector cells incubated without MSCs. MSC donor-paired samples are represented by connected lines for $n = 5$, $^{*}p = 0.0223$ by a one-sample *t*-test on each pair.

TGF-β2-Treated MSCs Have Reduced T Cell-Mediated Cytotoxicity *in vitro*

In a previous study, human MSCs were effective *in vitro* at suppressing activation and proliferation of non-activated T cells, but were unable to inhibit the cytotoxicity of activated cytotoxic T cells (Rasmusson et al., 2003). Untreated and TGF-β2-treated MSC target cells were co-cultured with effector cells and percent cytotoxicity was measured using a Cr-51 release assay. As expected, there was no significant difference in the percent cytotoxicity between untreated and TGF-β2-treated MHC-matched MSCs co-cultured with effector cells (**Figure 4**). However, percent cytotoxicity was significantly lower for TGF-β2-treated MSCs compared with pair-matched untreated MSCs co-cultured with MHC-mismatched effector cells. As with the

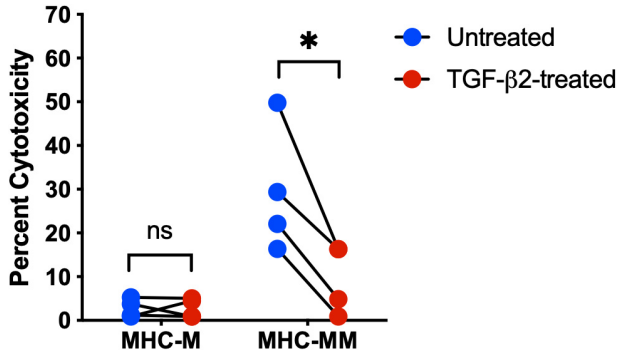

FIGURE 4 | TGF-β2 treatment decreases the cytotoxicity of MHC-mismatched MSCs. Percent cytotoxicity in a standard 6-h chromium-51 release assay for MHC-matched and MHC-mismatched untreated and TGF-β2-treated MSCs. MSC donor-paired samples are represented by connected lines for $n = 4$, $^*p = 0.0119$ by a one-sample *t*-test on each pair.

T cell activation assays, cytotoxicity was not detected with some combinations of MHC-mismatched effector and target cell donors (**Table 2**). Although sample size was limited to four MHC-mismatched effector and target cell combinations that demonstrated cytotoxicity, TGF-β2-treatment reduced the cytotoxicity of MSCs from each donor demonstrating that TGF-β2-treated MHC-mismatched MSCs have reduced cell-mediated cytotoxicity compared to untreated MSCs.

DISCUSSION

The purpose of this study was to investigate the immunomodulatory properties and cell-mediated immunogenicity of TGF-β2-treated equine bone marrow-derived MSCs. TGF-β2-treated MSCs have significantly reduced MHC I surface expression so we hypothesized that MHC-mismatched MSCs treated with TGF-β2 would retain their immunomodulatory properties and have reduced cell-mediated immunogenicity compared to untreated MHC-mismatched MSCs.

We demonstrated that TGF-β2-treated MHC-mismatched MSCs induce less T cell activation and have significantly reduced cell-mediated cytotoxicity compared to untreated MHC-mismatched MSCs. Additionally, this study supports that untreated MSCs are not innately immune privileged and can activate and be killed by cytotoxic T cells. Previous studies have reported that MSCs inhibit the generation of cytotoxic $CD8^+$ T cells, but cannot block the cytotoxic effects of pre-activated effector cells (Rasmusson et al., 2003, 2007). Once injected *in vivo*, MSCs are exposed to the effector and memory $CD8^+$ T cells of the recipient's immune system that do not require co-stimulatory molecules for activation and have a high frequency of cross-reactivity against allogeneic MHC molecules (Whitelegg and Barber, 2004). T cell activation and T cell-mediated killing is dependent on the level of occupancy of the T cell receptor with MHC I surface molecules on the surface of the target (Valitutti et al., 1996). This is consistent with our findings that TGF-β2-treated MHC-mismatched MSCs, which have reduced MHC I surface expression, induce less T cell receptor downregulation and cytotoxicity than untreated MHC-mismatched MSCs *in vitro*. One limitation of this study is that although we determined TGF-β2-treated MSCs had reduced cell-mediated immunogenicity, we cannot definitively state that this was due to decreased MHC I surface expression. It is possible that the TGF-β2 treatment affects other unidentified surface molecules or secretion of cytokines other than PGE2 and TGF-β1 and follow-up studies are in process. An investigation to determine if pre-treatment of MSCs with TGF-β2 is sufficient to delay or prevent rejection of donor MSCs by the recipient immune system *in vivo* is also currently in development.

Major histocompatibility complex -specific effector lymphocytes could not be generated by every combination of MHC-mismatched horses as indicated by the lack of cytotoxicity when effector lymphocytes were co-cultured with untreated MHC-mismatched MSCs. There were also observable differences in the degree of downregulation of CD3 and CD8 and cytotoxicity between responders. We believe both of these observations are most likely due to the natural variation in T cell responses to alloantigens and the MHC haplotypes of

TABLE 2 | MHC haplotypes of responder and stimulator horses for cytotoxicity assays.

Responder ID	Responder haplotype	Stimulator ID	Stimulator haplotype	Cytotoxicity
C	A9a/A9a	E	A2/unclassified	+
E	A2/unclassified	C	A9a/A9a	–
B	A3a/A3a	D	A2/unclassified	–
D	A2/unclassified	B	A3a/A3a	–
A	Unclassified/unclassified	F	A3b/A3b	–
F	A3b/A3b	A	Unclassified/unclassified	–
H	A19/A9a	D	A2/unclassified	+
D	A2/unclassified	H	A19/9a	–
C	A9a/A9a	F	A3b/A3b	+
F	A3b/A3b	C	A9a/A9a	–
H	A19/A9a	G	A2/unclassified	+

(+) Indicates cytotoxicity was detected for that combination and (–) indicates no cytotoxicity was detected in vitro.

the horses used in this study. The affinity and avidity of T cell receptors for MHC molecules is dependent on an individual's unique T cell repertoire so each responder or recipient may respond more strongly to some MHC haplotypes than others (Obst et al., 2000; Hornell et al., 2003; Stone et al., 2011). Like graft rejections, the degree of an immune response to MSCs is also dependent on the degree of MHC-mismatch between the responder and donor (Qureshi, 1997; Isakova et al., 2014) so *in vivo* studies are needed to compare the immunogenicity of TGF-β2-treated MSCs in a larger and more diverse population. Although cytotoxicity could not be detected from every haplotype combination *in vitro*, this does not support that cytotoxic effector cells against MSCs would not be able to generated *in vivo*. As MSCs are not innately immune privileged and can activate and be killed by lymphocytes, is critical that future pre-clinical and clinical studies using allogeneic MSCs MHC haplotype both donors and recipients and include analysis to detect alloimmune responses.

Modified one-way MLRs or other T cell proliferation assays involving MSCs are noted to be poor predictors of *in vivo* immunogenicity, but are still valuable for measuring the immunomodulatory functions of MSCs. PGE2 and TGF-β1 have both been shown to be important mediators of immune modulation MSCs (English et al., 2009; Carrade Holt et al., 2014). PGE2 suppresses T cell activation and proliferation through inhibition of IL-2 production and transferrin receptor expression (Chouaib et al., 1985) while TGF-β1 also inhibits IL-2 production and is important for the induction of T regulatory cells by MSCs (Brabletz et al., 1993; Hong et al., 2017). The importance of PGE2 to MSC therapeutic efficacy has been demonstrated in models of colitis (Yang et al., 2018), traumatic brain injury (Kota et al., 2017), contact hypersensitivity (Liu et al., 2020), and arthritis (Bouffi et al., 2010). Fewer studies have been conducted investigating the direct therapeutic effects of TGF-β secretion by MSCs, but TGF-β is known to contribute to the proliferation and differential of local stem/progenitor cells, tissue remodeling, extracellular matrix production, and wound healing (Verrecchia and Mauviel, 2002; Xu et al., 2018). Although PGE2 and TGF-β have been identified as particularly important to immunomodulation by MSCs, dozens of other cytokines and paracrine factors contribute to the MSC secretome and may play roles in the immunomodulatory and regenerative properties of MSCs.

It is important to note that while TGF-β2 treatment did not affect the ability of equine MSCs to suppress T cell proliferation in this study, treatment with TGF-β isoforms may negatively affect the immunomodulatory abilities of MSCs in other species. Co-culturing murine MSCs and stimulated lymphocytes in the presence of exogenous TGF-β1 or TGF-β2 reversed the immunosuppressive ability of the murine MSCs due to downregulation of iNOS (27). It is unknown if TGF-β2 affects production of immunomodulatory cytokines in human MSCs, however, targeted strategies other than TGF-β2 treatment could be used to downregulate MHC expression to achieve the same effect in humans without undesirable off target effects. Other strategies that have been published include knocking out MHC I using CRISPR or transfection with viral proteins, although these have their own unintended consequences including susceptibility to NK cell lysis (de la Garza-Rodea et al., 2011; Soland et al., 2012; Shao et al., 2020). Additionally, a dual strategy to reduce MHC I surface expression and increase production of immunomodulatory cytokines via inflammatory cytokine licensing may further enhance the immune evasive properties of MSCs and facilitate allogeneic use.

CONCLUSION

In summary, we observed that treating equine bone marrow-derived MSCs with TGF-β2 prior to co-culturing with lymphocytes did not affect the ability of MSCs to suppress T cell proliferation and reduced the cell-mediated immunogenicity of MHC-mismatched MSCs *in vitro*. The results of this study demonstrate a promising approach to reducing the immunogenicity of allogeneic MSCs and improving the safety and efficacy of clinical allogeneic MSC therapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of North Carolina State University.

AUTHOR CONTRIBUTIONS

AB and LS contributed to study conception and design and performed interpretation of the data. AB and JL contributed to the acquisition of the data. AB and JR performed the statistical analysis. AB wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.628382/full#supplementary-material

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REVIEW
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Therapeutic Use of Mesenchymal Stromal Cells: The Need for Inclusive Characterization Guidelines to Accommodate All Tissue Sources and Species

Adrienne Wright[1], *Marne L. Arthaud-Day*[2] *and Mark L. Weiss*[1,3]*

[1] *Department of Anatomy and Physiology, Kansas State University, Manhattan, KS, United States,* [2] *Department of Management, Kansas State University, Manhattan, KS, United States,* [3] *Midwest Institute of Comparative Stem Cell Biotechnology, Kansas State University, Manhattan, KS, United States*

Following their discovery over 50 years ago, mesenchymal stromal cells (MSCs) have become one of the most studied cellular therapeutic products by both academia and industry due to their regenerative potential and immunomodulatory properties. The promise of MSCs as a therapeutic modality has been demonstrated by preclinical data yet has not translated to consistent, successful clinical trial results in humans. Despite the disparities across the field, MSC shareholders are unified under one common goal—to use MSCs as a therapeutic modality to improve the quality of life for those suffering from a malady in which the standard of care is suboptimal or no longer effective. Currently, there is no Food and Drug Administration (FDA)-approved MSC therapy on the market in the United States although several MSC products have been granted regulatory approval in other countries. In this review, we intend to identify hurdles that are impeding therapeutic progress and discuss strategies that may aid in accomplishing this universal goal of widespread therapeutic use.

Keywords: MSC, clinical translation challenge, metrology and characterization, commercialization, biotherapeutic development

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***Correspondence:**
Mark L. Weiss
mlweiss@k-state.edu

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INTRODUCTION

Multipotent mesenchymal stromal cells (MSCs) are a heterogeneous population that when expanded *in vitro* includes stem, progenitor, and differentiated cells. MSCs have been implicated as a therapeutic modality in tissue injuries, chronic degenerative disorders, and inflammatory diseases on account of their regenerative potential and anti-inflammatory properties (Friedenstein et al., 1968, 1970; Galipeau and Senséb, 2018). Although therapeutic use in humans is the end goal, preclinical research relies on animal models for proof of concept and technique development, and thus animal applications cannot be overlooked. The first isolation and culture of MSCs were performed using bone marrow from guinea pigs (the 1970s) and then extended to rats in the 1980s (Friedenstein et al., 1987; Owen and Friedenstein, 1988). Isolation and culture of human MSCs did not begin until the early 1990s (Haynesworth et al., 1992; Lazarus et al., 1995; Pittenger et al., 1999). Since then, MSCs have become a widely studied experimental therapeutic product tested in over 1300 registered clinical trials (clinicaltrials.gov "mesenchymal" 6/5/20) (Galipeau and Senséb, 2018). In human clinical trials, allogeneic MSCs have been consistently shown to be safe but have

not been able to replicate the large effect sizes predicted from preclinical research. For this reason, small and large trials have failed to meet efficacy endpoints (Li et al., 2016; Galipeau and Senséb, 2018).

A vast preclinical dataset, from both *in vitro and in vivo* animal studies, supports the notion that MSCs are a potent cellular therapeutic agent. Here, we will review the *in vitro* preclinical data, but reviews of the *in vivo* preclinical data can be found here (Vu et al., 2014; Squillaro et al., 2016; Lukomska et al., 2019; Dave et al., 2020). Why is there such a gap between the expectations set by preclinical data and human MSC trials? The inconsistent results could be due to product irregularities, transferability across species, or poor estimation of effect size from preclinical data leading to insignificant findings. Our thesis here is that to move forward strategically, the MSC field needs to recognize and address shortcomings that have been given little consideration in the rush toward clinical development. Preclinical data needs to be strengthened in regards to its ability to be translated. Instead of continuing to produce inconsistent preclinical *in vitro* and *in vivo* data that poorly translates, effort should be placed on determining the root of the transferability issues so that consistent, reliable data can be generated allowing for replication across research laboratories. In addition, although the potential of MSCs remains undisputed, questions remain concerning the mechanisms-of-action (MOAs), how *in vitro* testing correlates to *in vivo* activity, the number of cells in a dose, the route of administration, and how all of this relates to the therapeutic effects for the various indications (Mendicino et al., 2014).

To do this, we believe that first, characterization guidelines need to be updated to accommodate different MSC populations. This includes addressing variations in the literature that may obscure rather than explain MSC's physiological effects that impact therapeutic response. These inconsistencies include, but are not limited to, MSC tissue source and species-to-species differences. Second, along with updated characterization guidelines, improved standardization in the field would help to eliminate product and lot-to-lot variation as well as address the concern of purity vs. potency. Lastly, to properly address these concerns, more research funding is required. With federal funding on research and development (R&D) declining, and businesses spending over three times the amount of the federal government on R&D, it is clear that industry-sponsored research is critical. Businesses are more prone to fund research that has commercial applicability rather than research that simply addresses a question (Sargent, 2020). By focusing research efforts on areas with commercial potential, not only could this increase research funding but also could decrease time to market.

CHALLENGES FOR CLINICAL TRANSLATION OF MESENCHYMAL STROMAL CELLS

Outdated Characterization Guidelines

In the early 1990s, Arnold Caplan was the first to use the term "mesenchymal stem cell" to describe the cells involved in embryonic bone and cartilage formation as well as repair and maintenance in the adult (Caplan, 1991). Following this discovery, many researchers argued that there was no feasible way to prove whether the *in vitro* cultured MSCs contained stem cells and, because of this, suggested alternative terms to label these cells. Although we still see the term "mesenchymal stem cells" used in literature more than 25 years later, the ISCT released a position piece in 2005 stating that the proper designation for these cells should be a multipotent mesenchymal stromal cell, seeing as they are a heterogeneous population in which not all cells have stem-like properties (Horwitz et al., 2005).

Following the nomenclature article, the ISCT's MSC working group released "minimal criteria" that should be demonstrated before a cell can be considered or referred to as an MSC (Dominici et al., 2006). These simplified guidelines include (1) Tissue culture plastic adherent; (2) Positive ($\geq$95%) for surface antigen markers CD105, CD90, and CD73 while also negative ($\leq$2%) for CD45 (pan-leukocyte), CD34 (hematopoietic and endothelial cells), CD14 or CD11b (monocytes and macrophages), CD79α or CD19 (B cells), and HLA-DR; and (3) Capable of differentiation to adipocytes, chondroblasts, and osteoblasts (Dominici et al., 2006). This definition is 14 years old and yet still widely used today. Although many researchers do go beyond this minimal definition, many also DO NOT meet this minimum.

The lack of uniformity has contributed to inconsistencies within the field. As noted by Mendicino et al., the current MSC guidelines used for characterization are not distinctive and therefore may not adequately define the cells and their biological function (Mendicino et al., 2014). Furthermore, this simplified definition does not consider species differences, tissue source, and passage of cells at the time of characterization, pointing to the need for refinement or updating of the "minimal criteria." In 2013, the ISCT amended the MSC definition to include a bioassay of immunosuppressive properties, but it did not refine the original definition. In 2019, ISCT updated their MSC definition to suggest (1) including the tissue origin of cells, (2) use of stromal cell nomenclature unless rigorous evidence for stemness is shown, and (3) including functional assays to define therapeutic mechanism of action, but no tissue-specific guidelines were addressed (Viswanathan et al., 2019). Although the ISCT suggestions exist, there has been no enforcement of the issue by academic journals. We suggest that the ISCT follow the International Society for Extracellular Vesicles (ISEV) and the Functional Genomics Data Society (FGED) and establish their own unique set of minimally accepted publication criteria (Brazma et al., 2001; Théry et al., 2018).

Biological Variability Translates to MSC Inconsistencies

To simply focus research on commercial use is only part of the picture. Science, either basic science or translational research, depends upon the ability to replicate published work, and hopefully, to extend that work. This includes observational research and hypothesis-driven research. As such, science depends upon the control of experimental variables, and

minimizing experimental error. One issue in biology is that certain variables are inherently "variable" due to the complexity of the system, and this adds intricacy to the metrology (the science of measurement).

Historically, problems associated with cell culture have had a significant impact on the field of biology. Issues such as misidentification, the use of contaminated cell cultures (e.g., mycoplasmas), or the effects of phenotypic drift have led to the creation of guidelines that not only highlight the problems, but also provide guidance on how to avoid or eliminate the issues. In some countries, legislation or codes of practice govern research since it interacts with both ethical and scientific boundaries. For example, in stem cell research, the production of new human embryonic cell lines was restricted in the US, forcing science institutions, many which were federally-funded, to use only existing embryonic lines. The result of these sanctions was that researchers were only able to use a handful of preexisting lines that were easy to propagate and make available, thus forcing standardization of the industry. Although this means of standardization was extreme, it still allowed the field to conform thereby inducing reproducible research. Although standardization is not required by the FDA for clinical use, MSC stakeholders should support standardization efforts as it would benefit the field by allowing for more meaningful comparisons among studies, thus allowing for a smoother clinical translation (Mendicino et al., 2014). Further, replication as a result of standardization would allow for more efficient research, consequently transferring to cost savings.

Regulatory Gaps in MSC Therapy

Currently, there are ten approved MSC therapies worldwide (**Table 1**) on the market for various indications, yet not a single FDA-approved product for use in the United States (Pereira Chilima et al., 2018; Levy et al., 2020; Shammaa et al., 2020). Differences in regulatory approvals around the globe have left gaps where some countries have approved products that have been on the market for over 10 years and other countries still have yet to grant approval to an MSC product. All countries with approved MSC products have a governing body, similar to the FDA, that has regulatory oversight of cell therapy products. Although similar, each country governs their own unique set of regulations and approval processes. These processes are reviewed in depth here (Ancans, 2012; Choi et al., 2015; Ridgway et al., 2015; Nagai and Ozawa, 2017; Tiwari and Desai, 2018; Mendicino et al., 2019; O'Sullivan et al., 2019). To alleviate gaps, some have suggested that the World Health Organization (WHO), an agency within the United Nations (UN), is a logical choice to develop guidelines and recommendations for the Member States (Petricciani et al., 2017). Although not a regulatory authority, WHO has a mandate to advance and advocate for international standards involving biological and pharmaceutical products, and many countries look to WHO for guidance in developing guidelines (Petricciani et al., 2017).

In the US, culture-expanded MSC-like cells are considered to be a more-than-minimally-manipulated cellular and gene therapy (CGT) product regulated by section 351 of the Public Health Service (PHS) Act 42 U.S.C.262 (Galipeau et al., 2016). Due to this designation, MSC-like cells require an Investigational New Drug (IND) application and approval from the FDA to be used in a clinical trial (Galipeau et al., 2016). Under this regulation, a test to measure potency as part of the release criteria is required although standardization among the field and ISCT minimal criteria are not required (Food and Drug Administration, 2011b; Galipeau et al., 2016). The FDA has released guidelines for CGT products, regulated under the Code of Federal Regulations (CFR) 210, 211 that outline release testing. The guidance released by the FDA includes: demonstration of biological activity (potency); quantitative data; pre-defined acceptance and/or rejection criteria; employment of appropriate standards, controls, and reference materials; documentation of accuracy, sensitivity, specificity, and reproducibility of test methods; ingredient strength and identity; dating periods; and labeling requirements (Food and Drug Administration, 2011a; Galipeau et al., 2016).

Similarly, in Europe, clinical MSCs are considered an advanced therapy medicinal product (ATMP) in accordance with the European Medicines Agency (EMA) regulation 1394/2007 of the European commission (EC) (European Commission, 2007; Ancans, 2012; Rojewski et al., 2019). Under the ATMP, the identity and impurities of the MSCs must be described using the ISCT minimal criteria or a modification to the criteria (Horwitz et al., 2005; Dominici et al., 2006; European Commission, 2007; Wuchter et al., 2015; Rojewski et al., 2019). In addition, release criteria, which vary by type of clinical trial and requirements from other national competent authorities, are also governed under the ATMP and include contamination screening (microbial, endotoxin, and mycoplasma), viability, clonogenicity, identity, purity, and functional tests (European Commission, 2007; Ancans, 2012; Rojewski et al., 2019). Europe's regulatory approval process for cell therapy products is reviewed more thoroughly here (Ancans, 2012; Blasimme and Rial-Sebbag, 2013). Although, the ISCT made a point to clarify that their 2006 proposed guidelines should not be confused with final product release criteria, the ATMP regulations, along with the literature and FDA regulation submissions point to the fact that they may be seen as synonymous by some (Mendicino et al., 2014).

Although the FDA has released recommendations for developing tests to measure potency of the MSC product, the FDA does not provide recommendations regarding which specific assay should be used. Currently, each IND application is reviewed based on individual product attributes and is not compared to other MSC products (Galipeau et al., 2016; Galipeau and Senséb, 2018). Due to the biological nature and limited amount of the MSC product, hurdles exist that make development of assays and standardization difficult. Galipeau and Senséb (2018) review these challenges thoroughly and they list a number of problems such as variability of raw materials, limited product for testing, absence of appropriate standards, and *in vivo* fate of the product. For "biologics" (i.e., biologically-derived therapeutics) such as MSC-based therapeutics to be successfully manufactured at large scale, they must meet four criteria: (1) a stable and well-defined cell line; (2) a good manufacturing practice (GMP)-grade supply chain with a process control plan that has set variability values that produce a product

TABLE 1 | MSC products with regulatory approval (Pereira Chilima et al., 2018; Levy et al., 2020).

MSC product (company)	Approval granted (year)	Indication	Product type
Queencell (Anterogen Co. Ltd.)	South Korea (2010)	Subcutaneous tissue defects	Autologous human AT-MSC
Cellgram-AMI (Pharmicell Co. Ltd.)	South Korea (2011)	Acute myocardial infarction	Autologous human BM-MSC
Cartistem (Medipost Co. Ltd.)	South Korea (2012)	Knee articular cartilage defects	Allogeneic human UC-MSC
Cupistem (Anterogen Co. Ltd.)	South Korea (2012)	Crohn's fistula	Autologous human BM-MSC
Prochymal, remestemcel-L (Osiris Therapeutics Inc., Mesoblast Ltd.)	Canada (2012) New Zealand (2012)	GvHD	Allogeneic human BM-MSC
Neuronata-R (Corestem Inc.)	South Korea (2014)	Amyotrophic lateral sclerosis	Autologous human BM-MSC
Temcell HS (JCR Pharmaceuticals)	Japan (2015)	GvHD	Allogeneic human BM-MSC
Stempeucel (Stempeutics Research PVT)	India (2016)	Critical limb ischemia	Allogeneic human BM-MSC
Alofisel (TiGenix NV/Takeda)	Europe (2018)	Complex perianal fistulas in Crohn's disease	Allogeneic human AT-MSC
Stemirac (Nipro Corp)	Japan (2018)	Spinal cord injury	Autologous human BM-MSC

with the desired therapeutic effect; (3) a standardized procedure that allows for process changes while maintaining product consistency; and (4) integrated redundancy and flexibility to allow for adaptation without sacrificing product consistency (Melsheimer et al., 2018). Even with these criteria met, biologics are still produced from living organisms and this variability causes product changes (e.g., quality, behavior, safety) that in turn affect the clinical use (Melsheimer et al., 2018).

An analysis of FDA IND applications by Mendicino et al. (2014) revealed variability in MSC tissue sources, manufacturing methods, and MSC characterization. Interestingly, it was noted that only 7 of the 9 ISCT-recommended MSC markers were ranked in the top 20 markers used by applicants to characterize human MSCs (Mendicino et al., 2014). In addition, they discovered that applications were submitted with MSC-characterization markers reported well below the 95% proposed by the ISCT, e.g., submissions with CD105 reported at only ∼80%, although it is unclear whether this impacts MSC function or not (Mendicino et al., 2014). This data brings the ISCT guidelines into question. If the end goal is clinical use as an FDA-approved therapeutic, yet the FDA does not require the proposed criteria, and they are not consistently demonstrated by applicants, what purpose are they serving related to that goal? If applicants are struggling to meet these guidelines, how well are the guidelines serving the human MSC product? Further, how can it be expected that nonhuman MSCs will adhere to these standards? To combat MSC product inconsistencies and ensure successful clinical translation, variability in the process and product must be realized, described, and managed.

Additionally, as noted in a review from the FDA, MSC manufacturing reflects a broadening of MSC characterization release criteria that are associated with phased clinical testing (Mendicino et al., 2014). This is the opposite of what the FDA expects and is a double-edged sword—allowing cells which fail to meet MSC criteria in the released MSC product may have secondary consequences of reduced potency and increased lot-to-lot variation. It should be noted that although MSC characterization is not required by the FDA, generating a consensus MSC definition would benefit all MSC shareholders as it would enable comparison across studies and enable therapeutic use by producing more consistent effect sizes (Mendicino et al., 2014).

MSC-Based Products Also Suffer From Lack of Standardization

MSCs being a product-by-process has implications that challenge the field, and it is a barrier to the idea that an MSC is a defined cell type. First, it implies that a process is necessary to generate or enrich cells of interest. Note that a similar notion is applied to pluripotent stem cells (PSCs), where the cells of interest are unnatural artifacts of the culture process and the culture conditions required to maintain them as immortal cells are known. In contrast, MSCs are mortal cells since the culture conditions needed to render MSCs as immortal cells are unknown. The product-by-process, together with the mortality of MSCs, implies that different MSC products are obtained at different times. Further, measures may reflect processes, and thus parse rather than unify.

The product-by-process assumption implies that prospective identification of MSCs is irrelevant since the product requires processing to be revealed. It also implies that different products are produced by altering the process. For example, "priming" MSCs by exposure to inflammatory cytokines can cause significant changes to MSCs such as inducing expression of MHC II (Romieu-Mourez et al., 2007; Tang et al., 2008). Moreover, the product-by-process focuses on *in vitro* and not the *in vivo* functionality of MSCs, and this is a key shortcoming to clinical translation.

If we embrace the product-by-process notion for MSCs, like we do PSCs, we can perhaps refocus efforts on what we can control and measure. For example, of the methods used to define MSCs, flow cytometry is the best method of cellular-level measurement that lends itself to metrology, i.e., a reference measurement system with traceability to the SI or other internationally agreed-upon units. In contrast, tri-lineage differentiation assays cannot be considered metrology as they lack defined measurands and reference materials. Therefore, we suggest that the MSC field develop and require measurable differentiation assays for publication.

It was once believed that the primary mechanisms of action for MSCs was contact-dependent signaling and engraftment into tissues, based on their potential for differentiation (Ankrum et al., 2014). In the past few years, it has become more widely accepted that MSCs' primary mechanism of action is through a paracrine effect. Through the paracrine effect, MSCs can secrete biologically active molecules, such as cytokines, chemokines, growth factors, extracellular matrix, and extracellular vesicles (EVs) (Liang et al., 2014). These molecules act therapeutically to stimulate tissue regeneration and angiogenesis as well as to modify inflammation, apoptosis, and fibrosis (Chen et al., 2009; Meirelles Lda et al., 2009; Ankrum and Karp, 2010; Linero and Chaparro, 2014). Due to their regenerative potential, EVs derived from MSCs (MSC-EVs) have become a target for therapeutic use. Preclinical data indicates that MSC-EVs may possess therapeutic behaviors similar to their parent cell of origin but with the additional benefit of using a cell-free product (Tögel et al., 2007; Yeo et al., 2013; Park et al., 2019). Although promising, the issue at hand is that without a consensus on the guidelines for characterizing an MSC, how can we logically move forward with MSC-based products? EVs isolated from conditioned media come with their own unique inconsistencies that can be due to parent cell of origin, the health of the cell donor, isolation and separation method, and storage condition (Li et al., 2019; Ludwig et al., 2019). Taken together with MSCs, the inconsistencies between the two products can only multiply when MSCs are used to manufacture EVs. Establishing guidelines for MSCs would further benefit EV research by allowing scientists to focus efforts on EVs rather than attempting to parse out inconsistencies from both sources.

Tissue Source Differences

MSC-like cells have been found in many tissues but due to the fact that MSCs were first described in the bone marrow (BM), BM-MSCs have dominated the field and are the focus for the defining criteria. BM harvest is a painful and invasive procedure. BM-MSCs isolated from elderly donors have been shown to be less "stemmy," and difficult, or sometimes impossible, to expand since they rapidly senesce (Pittenger et al., 1999; Stolzing et al., 2008). Here, "stemmy" is referring to cells within the MSC population with stem cell-like properties. Other adult tissue-derived MSCs such as adipose tissue (AT); dental pulp; muscle; and extra-embryonic tissues, such as the umbilical cord stroma, umbilical cord blood, and placenta, are also rich sources of MSCs (Wright et al., 2020). Some of these tissues, such as AT and extra-embryonic tissues, can be harvested rather easily secondary to routine or elective procedures. Furthermore, extra-embryonic tissues represent a painlessly-collected, virtually inexhaustible resource for MSC isolations. Consequently, they may represent an ideal source for MSCs because they are easily and painlessly obtained from donors of a consistent young age, hence minimizing the potential effects of aging or prior health conditions on the MSC pool.

Research groups may have a strong preference regarding which MSC tissue source they study and strong beliefs lead to claims of perceived superiority of a particular tissue source. Although there is consensus that MSCs derived from various tissues are not identical, the differences regarding characterization, and other behaviors, are often overlooked or perhaps exaggerated. The strongest evidence for this fact comes from the joint statement put out from the International Federation for Adipose Therapeutics (IFATS) and the ISCT in 2013 establishing an amended set of minimal guidelines for characterization of the uncultured stromal vascular fraction (SVF) and cultured stromal cells both derived from adipose tissue (Bourin et al., 2013). Importantly, these guidelines acknowledge that SVF can be CD34+ and adds CD44 (positive) and CD31 (negative) to the panel for cultured adipose-derived MSCs (Bourin et al., 2013). Interestingly, tissue-specific guidelines do not exist for other sources.

The literature highlighting tissue-specific MSC differences is vast but can often be conflicting and difficult to interpret. For example, umbilical cord-derived (UC-MSCs) and adipose-derived (AT) MSCs have been shown to have a higher proliferative capacity when compared to BM-MSCs (Kern et al., 2006; Lu et al., 2006; Baksh et al., 2007; Chen et al., 2009; Wu et al., 2009; Hass et al., 2011; Yu et al., 2018). Lu et al. (2006) reported a constant population doubling time (PDT) for human UC-MSCs passage 1−10 of ∼24 h compared to a PDT of ∼40 h for BM-MSCs, which increased significantly after passage 6. Peng et al. (2008) not only reported different PDTs of rat AT-MSCs compared to BM (45.2 h compared to 61.2 h, respectively) but also noted that BM-MSCs are morphologically larger than AT-MSCs. In regards to differentiation potential, BM-MSCs have been shown to have increased osteogenic potential and decreased adipogenic potential compared to AT-MSCs (Danisovic et al., 2009; Xu et al., 2017). Chen et al. (2009) demonstrated that although human BM- and UC-MSCs have similar adipogenic, chondrogenic, and osteogenic potential, UC-MSCs have a higher endothelial differentiation potential making them ideal for neovascularization of engineered tissues. Work reported from gene expression pathway analysis suggests that MSCs derived from human UC and amniotic membrane may possess an increased immunomodulatory capacity compared to BM-MSCs, while BM-MSCs have a higher potential for neuronal differentiation and development (Wegmeyer et al., 2013). Interestingly, in human placenta-, UC-, and amniotic membrane-derived MSCs, CD105, and CD29 expression was found to be negatively correlated to maternal age (Alrefaei et al., 2015, 2019). In equines, gene expression data found significant differences in CD44, CD90, CD29, and CD34 between BM and AT-MSCs (Ranera et al., 2011).

Species Differences

The ISCT's MSC definitions were based upon human BM-MSCs yet a large portion of MSC preclinical work is done in other species. Similar to pluripotent stem cells (PSCs), human MSCs are likely to have different characteristics than MSCs derived from other animals. To further complicate the matter, human MSCs also share some defining characteristics with animal MSCs, as shown in the case of human PSCs compared to rat and mouse PSCs (Schnerch et al., 2010). These similarities and differences between MSCs across species should be embraced to gain consensus and uniformity in the

field (Tropel et al., 2004; Hu et al., 2018; Uder et al., 2018). Additionally, availability and reliability of many antibodies against key surface markers are disparate across species, making it difficult to find reliable information for MSC characterization (Wright et al., 2020). Hence, it can be difficult to determine whether characterization differences are true differences or an artifact of antibody selection/performance.

Further, the tri-lineage differentiation potential of MSCs derived from nonhuman species is similar but not identical (Chamberlain et al., 2007; Uder et al., 2018). Scuteri et al. (2014) showed that BM-derived rat MSCs vary in their differentiation potential compared to BM-derived human MSCs in standard culture conditions. In terms of osteogenic and chondrogenic differentiation, the time required for differentiation was different between rat and human MSCs, while in adipogenic differentiation, human MSCs had a greater capacity than rat MSCs (Scuteri et al., 2014). In the canine MSC literature, it has been proposed that differentiation to two lineages is sufficient for characterization rather than three (Chamberlain et al., 2007; Neupane et al., 2008; Djouad et al., 2009; Vieira et al., 2010; Wood et al., 2012). In our review of 46 canine MSC papers, 22 (48%) demonstrated differentiation to three lineages. Of the remaining papers, 11 (24%) demonstrated differentiation to 2 of the lineages, and 10 (22%) papers did not address differentiation of the MSCs in any capacity (Wright et al., 2020). Of those, the most common lineage not shown, or not successful, was chondrogenic, which can be difficult (Zhang et al., 2015).

One similarity that all species seem to share is that differentiation potential decreases as cumulative population doublings increase. This attribute appears to be consistent among all lineages, species, and tissue sources (Requicha et al., 2012; Volk et al., 2012; Sasao et al., 2015; Marín-Llera and Chimal-Monroy, 2018). This evidence indicates that a true property of MSCs perhaps is a loss of potency, or "stemness," with time in culture. Despite this common feature, no priority has been placed on developing a standardized quantitative assay to measure differentiation or setting a standard number of cumulative population doublings at which differentiation potential should be assessed. In many cases, that information is not provided in MSC literature.

Mouse BM-derived MSCs have been shown to vary notably from human MSCs in their surface marker expression, specifically in the instance of CD34 (Chamberlain et al., 2007). Hu et al. (2018) demonstrated that BM-MSCs from C57BL/6 mice expressed high levels of CD34 but lacked CD90 as well as noted slight strain differences in surface marker expression. In our laboratory, canine MSCs derived from the UC require different culture conditions with regard to attachment factors, media formulation, and lifting agents compared to human UC-derived MSCs (Smith et al., 2017; Wright et al., 2020). Further, we have demonstrated that canine UC-MSCs express CD34 and CD90, albeit CD90 expression is not as high as human UC-MSCs (Wright et al., 2020). While others have also shown that canine MSCs express CD34, this finding raises concerns about the similarities of MSCs from different species (Kang et al., 2008; Ryu et al., 2009; Russell et al., 2016). AT-derived MSCs from rhesus monkeys and horses were shown to have related biological properties to human MSCs but differ in expression of surface markers and proliferation rates (Izadpanah et al., 2006; Ranera et al., 2011; Uder et al., 2018). AT-derived MSCs from rats and mice have also been shown to exhibit similar yet different surface marker expression compared to human AT-MSCs (Taha and Hedayati, 2010; Jeong et al., 2014; Uder et al., 2018).

As shown in **Figure 1**, in the canine MSC literature, there is a problem with demonstrating surface marker expression of all 3 classic MSC markers designated by the ISCT (CD73, CD90, and CD105). Some researchers believe that positive expression of CD44 and CD90 along with the negative expression of CD34, CD45, CD80, CD86, or MHC II is sufficient to characterize canine MSCs (Chamberlain et al., 2007; Neupane et al., 2008; Djouad et al., 2009; Vieira et al., 2010; Wood et al., 2012). Of the 46 papers reviewed, 41 (89%) either had negative results or did not report results for CD73, while only 4 (9%) had positive results (generously defined as >50% surface marker expression), and 1 (2%) had moderate expression (as defined as ≥5%—<50%). Note here the discrepancy in "positive" expression. The ISCT definition dictates that the MSCs should have ≥95% surface marker expression to be deemed positive yet instances exist of researchers stating positive results in populations with <50% expression. While CD90 expression was most consistently reported, only 27 (57%) of papers reviewed had positive expression. For CD105 expression, 37 (79%) of the papers reviewed had negative or unreported results. Bearden et al. (2017) reported that not only was CD105 expression more variable in canine MSCs than seen in humans, but it was also variable among canine MSC tissue sources. In the flow cytometric analysis of canine MSCs isolated from adipose, bone marrow, and synovium at the same passage, CD105 expression in MSCs derived from adipose (∼60%) and synovium (∼46%) was significantly higher than from bone marrow (∼17%) (Bearden et al., 2017).

Although some researchers report that MSCs are positive for a certain surface marker, what designates a positive expression is not clear and can be seen as subjective. The ISCT standards state that MSCs should be ≥95% expression for humans and other species are often held to this same standard (Dominici et al., 2006). We, and others, have only been able to demonstrate positive expression by approximately half, or even less, of the population (Radcliffe et al., 2010; Hermida-Gómez et al., 2011; Kisiel et al., 2012; Takemitsu et al., 2012; Screven et al., 2014; Escalhão et al., 2017; Kovac et al., 2017; Liu et al., 2018; Long et al., 2018; Wright et al., 2020). In the canine literature, this seems to be an issue with CD90 in particular (Kisiel et al., 2012; Takemitsu et al., 2012; Screven et al., 2014; Liu et al., 2018; Long et al., 2018; Wright et al., 2020). Further, in earlier published work, we demonstrated that there was no difference in expression between an antibody raised specifically to canine for CD90 and a human antibody with canine cross-reactivity (Wright et al., 2020). Either there is lower expression of CD90 in canine MSCs or there are issues with antibody specificity.

In a review of MSCs derived from other species, all species noted some difficulties exhibiting expression of the 3 classic MSC markers. In the equine literature, CD73 and CD105 are most often unreported or negative (de Mattos Carvalho et al.,

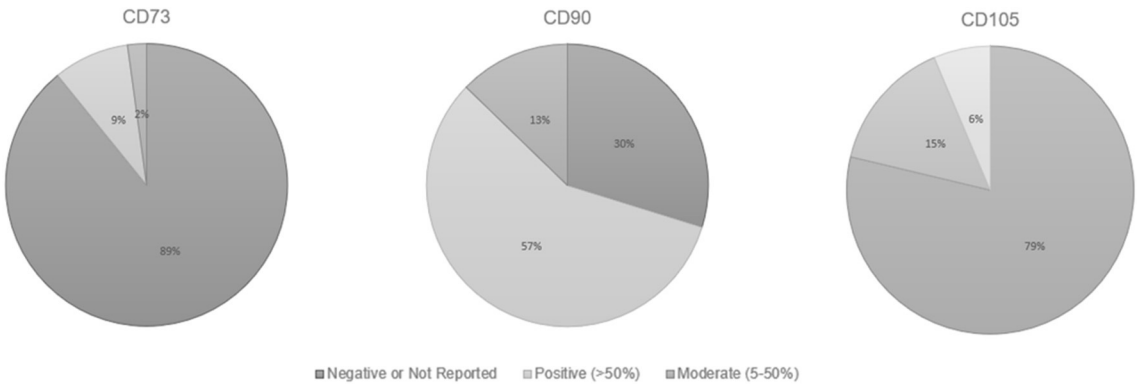

FIGURE 1 | Canine Expression of MSC Markers in the Literature. Positive expression is defined here as >50% surface marker expression, moderate is defined as ≥5%—<50%, and anything <5% is considered to be a negative result. Data derived from Wright et al. (2020).

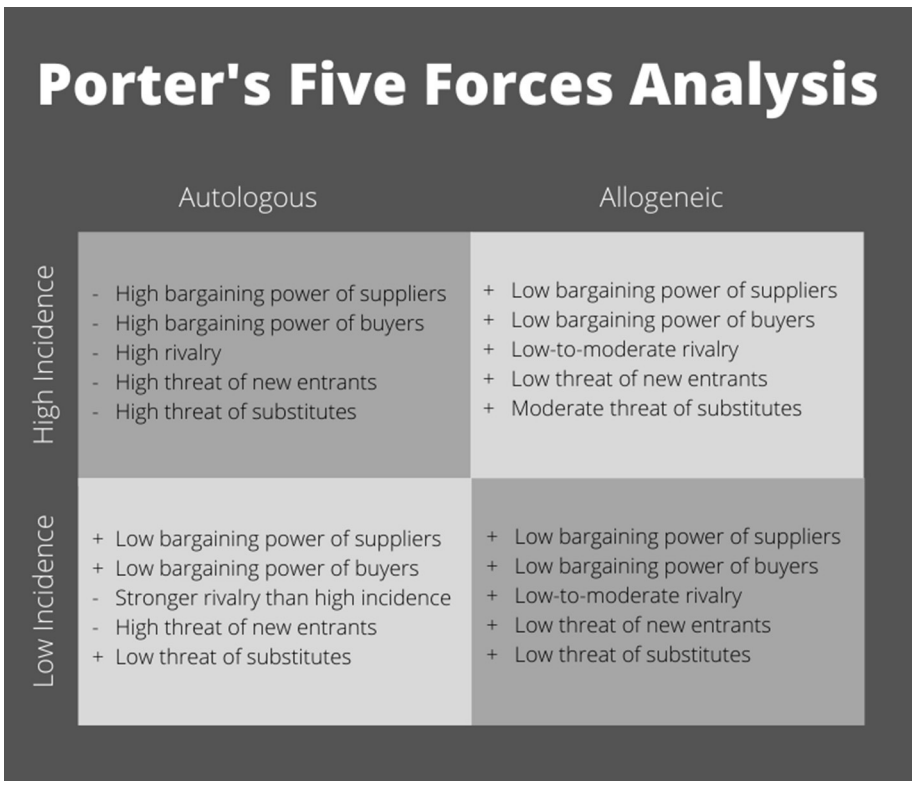

FIGURE 2 | Porter's five forces analysis of competitive environment within MSC clinical use.

2009; Radcliffe et al., 2010; Ranera et al., 2011; Maia et al., 2013; Barberini et al., 2014; Alipour et al., 2015; Zahedi et al., 2017; Gale et al., 2019; Kamm et al., 2019; Lepage et al., 2019). In mouse literature, there are several examples of researchers being able to demonstrate one marker and not the other two, but no clear pattern as to which marker is shown to have positive expression (Meirelles Lda and Nardi, 2003; Anderson et al., 2013; Hosseinzadeh Shirzeily et al., 2013; Deng et al., 2014; Li and Niyibizi, 2016; Ahmed et al., 2017). In rat literature, there are also several examples of researchers being able to demonstrate one

marker and not the other two, with all of the examples including CD105 as one of the two surface markers missing or negatively expressed (Rui et al., 2010; Meric et al., 2013; Sobh, 2014; Sarvandi et al., 2015; Suto et al., 2017). Porcine (Ock et al., 2010; Brückner et al., 2013; Lee et al., 2015; Pérez-Serrano et al., 2017; Wiater et al., 2018), ovine (Fadel et al., 2011; Czernik et al., 2013; Ji et al., 2016), rabbit (Lee et al., 2013; Xia et al., 2013; Kovac et al., 2017; Xiao et al., 2018), bovine (Corradetti et al., 2013; Gao et al., 2014; de Moraes et al., 2016; Yue et al., 2018), buffalo (Ghosh et al., 2015), and chickens (Bai et al., 2013) also demonstrate negative or missing classic MSC surface marker expression with no clear pattern or rationale. Interestingly, Kamm et al. (2019) noted significantly higher CD90 cell surface expression in MSCs derived from universal blood donor Standardbred equines compared to non-blood donor Standardbreds.

There is no way to know for certain if the negative results are true negatives, alluding to the fact that surface marker expression of MSCs varies by species, or if the antibody availability is limited for other species causing false negatives. There is evidence for both claims leading us to believe that it is a combination of the two. Researchers have demonstrated that these markers are present at the mRNA level, even if the protein expression is negative or not strongly positive (Requicha et al., 2012; Crain et al., 2019; Wright et al., 2020). Although not equal to showing surface marker protein expression, the fact that researchers feel compelled to demonstrate classic MSC markers at the mRNA level, yet cannot produce ISCT-standard flow cytometric data, brings the surface marker panel for MSC characterization into question. By holding MSCs from nonhuman species accountable for human characterization criteria, are we excluding valuable data from the field? Instead, we should be working toward a new consensus that makes accommodations for non-human MSCs.

MSC Heterogeneity

When considered jointly, the definition of an MSC and the ISCT minimal defining criteria contradict one another. On one hand, there is the definition of MSCs—a heterogeneous population that includes stem, progenitor, and differentiated cells. On the other hand, there are the guidelines for demonstrating that these cells are indeed MSCs, which includes plastic-adherence, tri-lineage differentiation, and a panel of positive and negative surface markers in which the positive should be expressed in $\geq$95% of the population (Dominici et al., 2006). Where did 95% come from? It may be unrealistic to assume that a heterogeneous population of cells, derived by different methods, from different tissues and species, may be able to demonstrate such high expression of a single marker, let alone an entire panel. Perhaps in the journey to reach a consensus on what an MSC is, the actual intent has been lost.

In addition, the definition of an MSC includes those cells from all tissues, yet the guidelines were established for human BM-MSCs. Researchers have been liberal with applying these guidelines to MSCs from many tissue sources and species. This act alone implies that MSCs isolated from different tissues and species are phenotypically and functionally similar. MSCs are not uniform and to insist that they are is unnecessarily forcing a round peg into a square hole. There is considerable evidence pointing to differences in MSCs derived from different culture conditions, different tissue sources, different aged donors, and different species. These differences are exhibited in MSC surface marker expression, their culture requirements, their longevity in culture, their transcriptome, their response to stimulation, and their growth rate. Taken together, this alludes to the fact that a simple definition might not properly serve all MSCs.

Purity vs. Potency

The issue remains that the characterization guidelines are nonspecific and, as discussed above, MSCs are a heterogenous population of cells with different gene expression profiles, differentiation and proliferation potential, and phenotype, which are all influenced by donor age, tissue source, species of origin, isolation procedure, and culture conditions (de Wolf and van de Bovenkamp, 2017). It is still unclear whether surface marker characterization, which is meant to assess the purity of the population, is correlated to functional activity, or potency of the MSCs. To combat this, most researchers use a functional assay to demonstrate potency of the cells. The assay should relate to the intended therapeutic MOA, but assays are left to the discretion of the researcher. At this time, it is still unclear whether *in vitro* functional assays correlate to *in vivo* activity, and that assumption is a major flaw with potency measures.

Need for an Expanded Surface Marker Characterization Panel

Even with the species variations considered, there are surface markers that are more uniformly expressed on MSCs of all species that are often included in flow cytometric panels (even in commercially available kits), and are thought of as "standard" MSC markers– yet they are not included in the ISCT characterization guidelines. Expression of CD44 and CD29 should be considered as logical additions to the MSC surface marker panel and adding them may give researchers working with nonhuman species additional options for MSC characterization.

CD44 is a hyaluronic acid receptor and a critical adhesion molecule. CD44 has been found to be highly expressed on MSCs derived from human (Hu et al., 2003; Le Blanc et al., 2003; Wexler et al., 2003; Brooke et al., 2008; Park and Patel, 2010; Lee et al., 2011; Aldridge et al., 2012; Liu et al., 2012; Guan et al., 2014; Qu et al., 2014; Secunda et al., 2015; Katsiani et al., 2016; Van Pham et al., 2016; Smith et al., 2017; Togarrati et al., 2018; Kaviani et al., 2019), canine (Filioli Uranio et al., 2011; Choi et al., 2013; Screven et al., 2014; Ivanovska et al., 2017; Zhang et al., 2018; Wright et al., 2020), equine (de Mattos Carvalho et al., 2009; Radcliffe et al., 2010; Maia et al., 2013; Barberini et al., 2014; Alipour et al., 2015; Sasao et al., 2015; Zahedi et al., 2017; Kamm et al., 2019; Lepage et al., 2019), mouse (Meirelles Lda and Nardi, 2003; Valorani et al., 2010; Deng et al., 2014; Fujita et al., 2015; Ahmed et al., 2017; Naik et al., 2017), rat (Rui et al., 2010; Yang et al., 2010; Meric et al., 2013; Sobh, 2014; Sarvandi et al., 2015; Li et al., 2020), rabbit (Lee et al., 2013; Xia et al., 2013; Kovac et al., 2017; Xiao et al., 2018), buffalo (Ghosh et al., 2015; Deng et al., 2018), bovine (Corradetti et al., 2013; Gao et al., 2014; de Moraes et al., 2016; Yue et al., 2018), porcine (Brückner et al.,

2013; Lee et al., 2015; Pérez-Serrano et al., 2017; Wiater et al., 2018), ovine (Fadel et al., 2011; Czernik et al., 2013; Chen et al., 2018), and chickens (Bai et al., 2013). CD44 expression is often associated with cell proliferation and migration (Yang et al., 2010; Azghadi et al., 2016; Ouhtit et al., 2020). It has been reported that CD44 expression in MSCs, both human and mice, is a product of *in vitro* culture as freshly isolated MSCs do not express CD44 until after cultured (Qian et al., 2012). On the contrary, some have demonstrated that CD44+ primary isolates are present (Hachisuka et al., 2007; Radcliffe et al., 2010; Fujita et al., 2015; Marín-Llera and Chimal-Monroy, 2018). Many researchers have documented increased CD44 expression on MSCs of multiple species with time in culture (Park and Patel, 2010; Radcliffe et al., 2010; Qian et al., 2012; Marín-Llera and Chimal-Monroy, 2018) with only minimal evidence of CD44 expression decreasing as time in culture increases (Sasao et al., 2015). Since flow cytometry assesses cell surface markers, the dissociation of MSCs using trypsin is also problematic due to cleavage or disruption of antigens. For example, trypsin dissociation significantly reduces CD44 expression, as well as other MSC surface markers, on human MSCs compared to other dissociation agents such as TrypLE (Tsuji et al., 2017). Further, CD44 expression may also affect the chondrogenic differentiation of human MSCs via the Smad 2/3 and ERK ½ signaling pathway (Xu et al., 2020). In UC blood-derived MSCs, Kwon et al. (2019) demonstrated that CD44 has an immunoregulatory role as evidenced by the induction of macrophage polarization via CD44 expression by the proteoglycan, decorin.

CD29, integrin beta-1, is a cell surface receptor that is involved in cell adhesion. CD29 has been found to be "highly" expressed (≥95%) on MSCs derived from human (Hu et al., 2003; Le Blanc et al., 2003; Wexler et al., 2003; Brooke et al., 2008; Pruszak et al., 2009; Park and Patel, 2010; Aldridge et al., 2012; Al-Nbaheen et al., 2013; Guan et al., 2014; Yang et al., 2014; Alrefaei et al., 2015; Katsiani et al., 2016; Van Pham et al., 2016; Togarrati et al., 2018; Kaviani et al., 2019), rat (Wu et al., 2009; Walker et al., 2010; Song et al., 2014; Davies et al., 2015; Suto et al., 2017), equine (Ranera et al., 2011; Alipour et al., 2015; Esteves et al., 2017; Zahedi et al., 2017; Gale et al., 2019; Lepage et al., 2019), canine (Filioli Uranio et al., 2011; Choi et al., 2013; Ivanovska et al., 2017), mouse (Meirelles Lda and Nardi, 2003; Ahmed et al., 2017), porcine (Ock et al., 2010; Lee et al., 2015; Wiater et al., 2018), buffalo (Deng et al., 2018), rabbit (Lee et al., 2013; Kovac et al., 2017), bovine (Corradetti et al., 2013; de Moraes et al., 2016), and chickens (Bai et al., 2013). Evidence suggests that CD29 expression may be involved with MSC migration along with CD73 (Ode et al., 2011). CD29 and CD105 expression has been found to be negatively correlated with maternal age on human placenta- and UC-derived MSCs and was proposed as a marker for quality control (Alrefaei et al., 2015, 2019). Both CD29 and CD44 expression were found to be involved with MSC adhesion, migration, and engraftment in the diseased liver (Aldridge et al., 2012).

A total of 72% of canine papers demonstrated either a single alternative MSC marker (CD29 or CD44) or both, which is more consistent than any of the classic MSC markers (**Figure 1**). This remains true with all other species examined here. All species noted here were able to demonstrate expression of either CD29, CD44, or both as a positive surface marker and at levels >50% of the population. Because of this, we believe that both CD29 and CD44 are logical additions to the MSC markers for all species, due to their demonstrated high expression levels and inclusion within all species. Although both CD29 and CD44 are expressed on epithelial cells, epithelial cells do not express the classic MSC markers CD105, CD90, and CD73, hence CD31 could be added as a negative marker for MSC characterization (Seeberger et al., 2009; Togarrati et al., 2018). The addition of CD44 and CD31 has already been done in the IFATS guidelines for cultured adipose-derived MSCs (Bourin et al., 2013).

Other markers, such as Stro-1, CD271, CD362, and ABCB5, are also considered as MSC markers by some researchers and even used for MSC flow sorting (Ning et al., 2011; Álvarez-Viejo et al., 2015; Ballikaya et al., 2020; Gonzalez et al., 2020). However, in our review we did not find these antibodies to be as available for other species or as well-demonstrated in the literature as CD29 and CD44. For those reasons we suggest CD29 and CD44 as the next logical additions to the MSC panel. Perhaps attempting to make generalized criteria to define MSCs from any tissue source, any species, and any culture conditions is too simplistic. Rather, an updated species- and tissue-specific set of criteria could better serve the field of MSC research given that they are specific and reproducible (Keating, 2012). Further, MSCs may represent different products, and treating them as homogeneous may impede new work in the field.

Metrology Standards

It is recognized that the MSC definition casts a "wide net" as it does not rely upon a single cell surface marker or activity assay that can prospectively identify the stemmy population within the mixed population. In lieu of a single surface marker, a surface marker analysis panel, consisting of both positive and negative markers, is one key element to defining MSCs. There is a vast amount of literature that addresses the flow cytometric analysis of MSCs, and it is quite challenging to compare the results between laboratories (Uder et al., 2018).

In response to this issue, some experts have proposed that MSC lines be generated and highly characterized to serve as "gold standard" lines for calibration (Viswanathan et al., 2014; Tanavde et al., 2015). Others have suggested the use of dedicated laboratories to serve as characterization centers for MSCs to enable standardized characterization in the field, as has been done with certain diagnostic tests. We find that both of these proposals come with their own advantages and disadvantages. A third, and perhaps more realistic consideration might be to forgo the simplified definition of an MSC in favor of guidelines that are specific to the species and tissue used to generate the MSCs. Generating a consensus sponsored by the ISCT around authentication methods and materials, e.g., specific monoclonal antibody clones, protocols, and criteria regarding positive and negative staining, as well as a consistent presentation of results, would enable reproducibility and comparison across laboratories.

Since the National Institutes of Health (NIH) and National Science Foundation (SF) require authentication of biological reagents, we suggest that cellular metrology

standards be set, just as they have been for other biologicals such as microbiology strains, bacteria, and cancer cell lines. Standards set by the community should provide guidance for publication, reproducibility requirements, and authentication standards. It is our belief that the ISCT should establish MSC metrology guidelines by species and tissue source; generate a consensus-gathering list of available and acceptable resources for characterization by species and tissue source; and enumerate guidelines that dictate the minimal information required for published MSC studies that includes characterization, methodology, and reproducibility requirements.

RESEARCH DRIVEN BY COMMERCIAL APPLICABILITY

Despite the nuances, a shared trait among all MSCs is that they possess unique and tissue-specific differences in immunomodulatory properties and regenerative potential. To simply take advantage of these unique features and push MSCs to market for therapeutic use is not feasible. Questions remain concerning the mechanism of action, how *in vitro* testing correlates to *in vivo* activity, the number of cells in a dose, the route of administration, and how all of this relates to the therapeutic effects for the various indications (Mendicino et al., 2014). To properly address these concerns, more research funding is required.

In the United States, R&D is primarily funded through the federal government, state governments, businesses, academia, and nonprofit organizations. From historical data dating back to 1953, businesses and the federal government combined have accounted for over 90% of the R&D expenditures (Sargent, 2020). While the federal government suffered 7 consecutive years of declines in funding (2009-2016), businesses have increased funding since 1953 (Sargent, 2020). In the most recent data for the fiscal year 2018 released this year, the federal government spent $127.3 billion on R&D while businesses spent $404.2 billion and state governments, academia, and nonprofit organizations spent a combined $48.5 billion (Sargent, 2020). Although it cannot be parsed out exactly where these funds were distributed, the point can be made that businesses are spending 2-4x more money on R&D than the US government. In a search of sponsored clinical trials in the United States (clinicaltrials.gov, search MSC, all trials, US, 7/29/20), other sponsors (individuals, universities, and organizations) accounted for almost half of the 1,195 total registered clinical trials (578), while industries sponsored 368, and NIH and other federal agencies accounted for 279, the smallest pool.

Research supported by federally-funded grants is fundamentally different from industry-sponsored research. While both are critical to moving science forward, federally-funded research addresses questions aiming to fill a void of knowledge. Industry-sponsored research is more focused on topics with a clear commercial application and an established large market share (Fabbri et al., 2018). For example, work examining biomedical research funding in the United States from the early 2000s found that industries were more likely to sponsor research centered around diseases projected to afflict areas of higher income as opposed to NIH funding targeting diseases with a global burden (Dorsey et al., 2009; Fabbri et al., 2018). MSCs represent an attractive research topic because they have applicability for numerous indications with widespread prevalence, an established market share, and the potential to outperform many standard of care therapies. Research focused on the big picture, i.e., commercial use of MSCs, could attract more industries looking to enter the MSC market, thus leading to increased research funds from industry sponsors. Here, we will compare the market of allogeneic and autologous MSC therapy. We should note that there are many other factors to take into consideration such as shipping logistics, cryopreservation, culture conditions, and manipulations to alter therapeutic effect (e.g., priming) that are not addressed here.

Allogeneic MSC Therapy Represents a Viable Business Model

MSCs can be used therapeutically in either an autologous or allogeneic manner and both have their own unique set of benefits and limitations. Autologous MSC therapies are considered a lower risk than allogeneic therapies for humans with intact immune systems. The two types of therapies are not synonymous and the results cannot be compared across clinical trials. Further, within allogeneic and autologous therapies, other factors such as preparative regimen, administration method, disease models, the dosage of MSCs administered, and the use of either culture-expanded or cryopreserved cells should also be carefully considered before comparing results, as they possibly impact therapeutic effectiveness of MSCs and the cells' ability to meet primary endpoints.

Autologous MSCs are a form of personalized medicine and are of less risk immunologically since they are one's cells. However, autologous MSCs typically require *in vitro* culture-expansion to produce enough cells to constitute a therapeutic dose. Hence, they are limited to situations in which time is not a critical factor and collection is feasible. Turnaround times from harvest to patient administration can vary widely due to the variable proliferation rates among patients and the number of cells required for a therapeutic dose. Further, MSCs have been shown to be less efficacious when harvested from elderly donors, thus limiting the potential patient pool (Lepperdinger, 2011; Alt et al., 2012). The high cost of autologous MSC therapy coupled with the lack of insurance coverage makes it unattainable for the majority of possible recipients. Despite causing heavy criticism and providing risky services that claim to provide unproven results, unregulated "stem" cell clinics around the world demonstrate that the market demand for cell therapy exists. In fact, the global market demand for MSCs is expected to reach $7.5 billion USD by 2022, with the US expected to have the largest market share (34.3%) despite the fact that the US has yet to grant approval to an MSC product (Pereira Chilima et al., 2018). It should be noted that unregulated stem cell clinics operate using a "minimally-manipulated" product or a homologous lipoaspirate [21 CFR 1271.10(a)(1) and 21 CFR 1271.10(a)(2), respectively]. It is unclear whether or not this will

continue to be an exempt product in the future. It should be noted that MSCs are not considered minimally manipulated since they require *in vitro* expansion and thus are not exempt.

Industry sponsors have funded the majority of advanced phase clinical trials (Ankrum and Karp, 2010; Galipeau and Senséb, 2018). Without industry support, getting MSC products approved for use is cost prohibitive. To gain industry backing, a clear path to profitability must be established in a manufacturing market that is driven by margins. To explore potential markets, let us apply a standard business model used to analyze industry profitability (**Figure 2**). Michael Porter's "five forces" approach to industry analysis examines the broader industry structure to determine the overall attractiveness of an industry for investment (Porter, 2008). In addition to interfirm rivalry, profit potential is determined by the threat of new entrants, the availability of attractive substitutes, and the power of suppliers and buyers, respectively.

The most logical pathway to commercialization is to target a sizable indication with a high incidence rate (**Figure 2**). Applying Porter's five forces model, autologous cell therapy does not appear to have the ability to produce an adequate profit pool. The industry is fully reliant on donors' willingness and suitability to provide the key input (autologous cells) as well as their desire and ability to pay (e.g., high buyer and supplier power). Due to the nature of the manufacturing process for autologous cells, production processes are not scalable. Large batch manufacturing is not cost effective; as a result, production remains dominated by small, local laboratories. Without economies of scale to serve as an entry barrier, autologous MSC therapy has a high risk of new entrants, making for a highly competitive environment. Further, it is worth clarifying that there are two patent pathways: cell line and production/differentiation techniques. With autologous MSC therapy, cell lines, although more easily patentable and marketable, are moot and this leaves process patents. As evidenced in iPSC technology, process patents come with unique challenges such as a low number of approvals compared to applications (e.g., only 11% of applications approved by the European Patent Office with 89% waiting to be reviewed), differences in international intellectual property laws, and small patent portfolios distributed among several entities (Zachariades, 2013; Roberts et al., 2014). Particularly, patents are an issue in Europe where exemptions to patentability exist that may affect stem cell therapeutics, specifically the "use of human embryos for industrial or commercial purposes" (Zachariades, 2013). Additionally, patents can be seen as risky since the regulatory approval process to get cell therapies to market is quite long; patents may expire before the technology can be utilized commercially (Roberts et al., 2014). Because of this, many companies rely on trade secrets, which allow for processes to be improved and protected from common knowledge, but alleviate the concern of expired patents, making trade secrets a more viable alternative to intellectual property (Roberts et al., 2014). So, in the case of autologous cell therapy, you're left with a splintered landscape of patents and trade secrets where companies are forced to "brand" their technology to convince the market that they have any sort of strategic competitive advantage. This leaves a perfectly competitive market of a wide range of technological advances where it is difficult for brands to build brand recognition and demand a premium price compared to other competitors. Autologous MSC therapy also has a high risk of substitution. A majority of high incidence indications already have an existing standard of care produced using efficient manufacturing processes that have been refined over the years. To even be considered, autologous MSCs would have to demonstrate therapeutic benefits and safety beyond currently approved modalities to justify the higher cost. In this context, multiple, small firms would be forced to compete based on price alone (i.e., perfect competition), narrowing profit margins even further and making autologous cell therapy an unattractive industry to enter.

In contrast, allogeneic MSCs have the potential to be a readily-available product that can serve in instances of acute disorders where time to culture-expand cells is not feasible, or as an option for patients who are not able to serve as their own donor. Because allogeneic MSCs may be produced from a wider pool of "qualified" donors, producers have much greater control over their supply chain. Meanwhile, manufacturing processes for allogeneic cell therapies are more closely related to other noncellular pharmaceuticals and biologics. Based on these similarities, protocols for culture-expansion of cells in smaller batches could easily be scaled up using existing technologies and equipment. Particularly if automated, large-scale manufacturing of allogeneic cell therapies would spread the cost of goods, labor, and quality control across more samples, thus lowering the cost of production per sample, making this option ideal to treat large numbers of patients. Due to economies of scale, allogeneic MSCs would face a lower risk of new entrants and fewer overall competitors. As an off-the-shelf product, allogeneic MSCs must be licensed and approved for treatment by the FDA. The time and costs associated with regulatory licensing as well as the high costs of capital (e.g., equipment, facilities, and trained staff) needed to manufacture allogeneic MSCs at a large scale represent additional barriers to market entry. Allogeneic cell therapy has a substitution risk but due to the lower cost, it may be able to compete effectively with existing standard of care therapies, especially if it can demonstrate superior safety and efficacy. Marketing these cells under a brand name, utilizing the pharmaceutical industry's sophisticated marketing capabilities, could help allogeneic MSCs to build brand recognition, thus commanding a price premium. The ability to differentiate based on quality combined with cheaper costs of production would increase firms' power over "buyers," who be more willing to pay a price premium for an approved therapy. From this standpoint, allogeneic MSCs represent a viable business venture.

An alternative "industry" to consider is an indication with a low incidence in which a standard of care may not exist or one with nonresponsive patients (**Figure 2**, bottom half). Autologous MSCs could be an option for treatment, if not time-constrained, due to the lack of available substitutes. Without the ability to scale, manufacturing costs would still be high, but buyers would be less price-sensitive and willing to pay a premium for a product with demonstrated efficacy, especially given the lack of a standard treatment option. By definition, an

indication with a low incidence would have a small market size. Because of the low entry barriers, new laboratories could still join the industry, but the lack of growth potential would result in an increased level of rivalry. While this scenario is modestly improved compared to the high incidence quadrant, allogeneic MSCs again represent the more commercially viable option. With their broader pool of donors (suppliers), allogeneic MSCs can increase production to meet demand, thus benefiting from economies of scale. Due to the lack of substitutes and decreased price-sensitivity of buyers, firms could demand a premium price for a product with demonstrated efficacy, increasing profits. Again, with an allogeneic product, higher market entry barriers exist due to licensing and the costs of startup at scale. The ability to differentiate products decreases both the intensity of rivalry and the threat posed by new entrants. Although the overall market size is notably smaller, allogeneic MSCs still represent an attractive industry in terms of profitability.

Biologics have been successful on the market—over 250 products are available and they account for seven of the top 10 selling drugs globally—and several companies have already taken advantage of the allogeneic MSC model to produce clinical therapeutics (Melsheimer et al., 2018). There are well-established companies such as, JCR Pharmaceuticals [Japan], Mesoblast [Australia], and Osiris Therapeutics [United States], with new biotechnology companies opening worldwide regularly. Prochymal from Osiris Therapeutics was granted conditional licensing approval to treat children suffering from acute graft vs. host disease (GvHD) in Canada in 2012 (Galipeau and Senséb, 2018; Chisholm et al., 2019). It was revealed in 2016 that Prochymal had not been utilized because it could not get reimbursed (David Gagnon, 2016; Galipeau and Senséb, 2018). On the other hand, JCR Pharmaceuticals has had financial success with its product, TEMCELL®, which was approved for use in acute GvHD in 2015 (Galipeau and Senséb, 2018). From JCR Pharmaceuticals' financial reports, they have reported revenue of ¥86.6 billion (∼817,400,000 USD) from fiscal years 2016–2019, with revenue increasing annually, and an operating income of ¥14.4 billion (∼135,919,000 USD) (JCR Pharmaceuticals Co L, 2017, 2018, 2019, 2020). Collectively, these data indicate that allogeneic MSC therapy represents the clearest path to profitability. By focusing research efforts on this modality, industry-sponsored funding may increase.

CONCLUSION

The cell therapy market is expected to grow to $61 billion by 2022 (Pereira Chilima et al., 2018). MSCs are an attractive cellular therapeutic product backed by promising preclinical data in animal models. There are currently ten MSC therapeutics with regulatory approval worldwide. Despite the positive preclinical data, in the US clinical trials have failed to meet efficacy endpoints, pointing to issues with translation from preclinical studies to clinical trials. Because of this, an FDA-approved MSC therapeutic product still does not exist. Unified under the common goal of widespread therapeutic use of MSCs, stakeholders should focus efforts on strengthening preclinical data so that it can be translated into safe and effective therapies, replicated among researchers, and compared across laboratories. To accomplish this, characterization guidelines should be updated to accommodate MSC populations from all tissue sources and species. Second, improved standardization that has both general characteristics and specific characteristics for each MSC population should be generated to decrease product variability. To accomplish this, research with commercial applicability should be prioritized to attract industry research funds. Without established consistency among MSCs, both MSCs and MSC-based products, such as EVs, will suffer from a lack of standardization, increasing the time to market as a licensed therapeutic.

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AW and MW conceived and wrote the manuscript. MA-D contributed to the commercialization portion of the manuscript. MW generated financial support for the research. All authors approved the final version submitted for consideration.

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Cord Lining Mesenchymal Stem Cells Have a Modest Positive Effect on Angiogenesis in Hindlimb Ischemia

Kenon Chua[1], *Fui Ping Lim*[2,3]*, *Victor Kwan Min Lee*[4,5,6], *Toan Thang Phan*[2], *Bee Choo Tai*[7] *and Yih Kai Tan*[8,9]

[1] *Programme in Cancer and Stem Cell Biology, Duke-NUS Medical School, Singapore, Singapore,* [2] *Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore,* [3] *Alice Lee Centre for Nursing Studies, National University of Singapore, Singapore, Singapore,* [4] *Department of Pathology, National University Hospital, Singapore, Singapore,* [5] *National University Cancer Institute, Singapore, Singapore,* [6] *Department of Pathology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore,* [7] *Saw Swee Hock School of Public Health, National University of Singapore, Singapore, Singapore,* [8] *Novena Vascular and Varicose Vein Centre, Mount Elizabeth Novena Specialist Centre, Singapore, Singapore,* [9] *Department of Surgery (Vascular), Changi General Hospital, Singapore, Singapore*

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Mayasari Lim, Fujifilm Irvine Scientific, Inc., Santa Ana, United States

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Laura Iop, University of Padua, Italy
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***Correspondence:**
Fui Ping Lim
fuiping@nus.edu.sg

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Purpose: We investigated the use of human Cord Lining Mesenchymal Stem Cells (CL-MSCs) (US Patent number 9,737,568), in a rabbit hindlimb ischemia model, and evaluated their potential in stimulating neovascularization. Allogenic human CL- MSCs could potentially be used to treat patients with lower limb ischemia and non-healing wounds.

Methods: Twenty rabbits were divided into two separate groups. We created a hindlimb ischemia model surgically. At 21 and 49 days post-operatively, animals in the treatment group were injected with CL-MSCs (500,000 cells per 0.2 ml on each site) at 10 different sites (Quadriceps- 4 sites, Hamstrings- 4 sites and Calf-−2 sites) in the hindlimb muscles. The control group received only saline injection to the corresponding sites at the same time point as the treatment group. We then evaluated the effects of treatment on neovascularization by angiography, laser doppler perfusion imaging, as well as by histology. We evaluated the tissue samples for any signs of local immune reaction to the cell implantation. We also observed the rabbit clinically for any adverse effects after treatment.

Results: We found a higher number of CD31 positive cells in the treatment group, with a greater number of capillaries found in the treated muscles. The Rectus Femoris demonstrated a median vessel count/muscle fiber of 0.121 for the treatment group, compared to 0.076 in the control group (median difference 0.04; 95% CI 0.001–0.11; $p = 0.041$). The Gastrocnemius demonstrated a median vessel count/muscle fiber of 0.175 for the treatment group, compared to 0.089 in the control group (median difference 0.087; 95% CI −0.006 to 0.234; $p = 0.07$). Blood perfusion quantification through Laser Doppler Perfusion Imaging (LDPI) also demonstrated a non-statistically significant increase in perfusion in favor of the treatment group. CL-MSCs demonstrated no toxicity associated morbidity and minimal local immune reaction to implantation.

Conclusion: CL-MSCs have a positive effect on angiogenesis in a rabbit hindlimb ischemia model. This preliminary data is encouraging and paves the way for future large animal studies or for clinical trials.

Keywords: cell therapy, stem cell, mesenchymal stem cell, critical limb ischemia, peripheral arterial disease, hindlimb ischemia, angiogenesis

INTRODUCTION

Critical limb ischemia (CLI) is regarded as one of the most detrimental forms of peripheral artery disease with high rates of disability and mortality (Tu et al., 2015; Shishehbor et al., 2016; Teraa et al., 2016). Globally, CLI has affected more than 200 million people worldwide, with lower-middle income countries accounting for an increase in prevalence rate of 29%, and higher income countries reflecting a 13% increase (Fowkes et al., 2013; Sampson et al., 2014; Jelani et al., 2018). Significantly, this was closely associated with a 1-year major amputation rate of 40% (Ryu et al., 2012; Ponemone et al., 2017), while mortality rates increased by 20% within 6 months of diagnosis and 50% after 5 years of diagnosis (Norgren et al., 2007; Teraa et al., 2016). The current standard therapy aims to increase blood circulation to the affected limb either through surgery or endovascular revascularization (Tretinyak et al., 2001; Adam and Bradbury, 2007). However, an estimation of 20–50% of patients are high-risk surgical patients or with undesirable endovascular anatomy which limit their current interventional options (Dormandy et al., 1999; Idei et al., 2011). In addition, postoperative arterial re-occlusion is a rapid occurrence which further limits the intervention, and leaves CLI patients with no ideal alternatives for intervention (Lawall et al., 2011).

While pharmacological interventions such as antilipidemic, antiplatelet and antihypertensive therapies have been used to address the underlying state of atherosclerosis, none of these interventions have resulted in a decrease in amputation rates in patients with CLI (Conte et al., 2009; Gupta et al., 2014). Presently, the US Food and Drug Administration (FDA) has no authorized treatment for CLI. Often, the only choice that remains for no-option CLI patients will be treatment directed toward pain management, wound care, and eventually limb amputation. Taking into account the limiting circumstances of present interventions and its soaring mortality figure, the quality of life for CLI patients is not dissimilar to that of terminal cancer patients (Powell et al., 2011). These patients are left with no treatment options and constitute a population of patients with a detrimental and potentially fatal condition, as well as an unfulfilled medical need. Clearly, there is a pressing call to make headway for an alternative therapeutic approach to treat this intractable disease (Liew and O'Brien, 2012).

In the last two decades, there has been an exponential increase in the number of articles detailing the potential effects of stem cell therapy and featuring it as an up-and-coming alternate treatment for CLI (Lawall et al., 2010; Sprengers et al., 2010; Powell et al., 2011; Schiavetta et al., 2012; Wang et al., 2014). It has been reported in several studies that Mesenchymal Stem Cells (MSCs) express multiple proangiogenic growth factors; such as VEGF, VEGFR, ANG-1 and SDF-1, and can migrate to hypoxic areas, thereby enhancing angiogenesis and restoring the vasculature network in animal models of hindlimb ischemia (Liang et al., 2014, 2017). In clinical studies involving human volunteers, the administration of bone marrow cells has yielded favorable outcomes in patients with CLI; such outcomes include an improved Arterial Brachial Index and Transcutaneous Oxygen Pressure, an improved limb perfusion, increased walking distance, reduced pain and ultimately, reduced rate of major amputation, improved overall ischemic symptoms, and quality of life (Tateishi-Yuyama et al., 2002; Kajiguchi et al., 2007; Lawall et al., 2011; Powell et al., 2011). A recent systematic review that appraised several studies which had used cell-based therapy on patients with CLI reiterates a similar impression that cell-based treatment is effective in reducing amputation rate, improving perfusion, and prolonging the amputation-free survival (Rigato et al., 2017).

MSCs have been successfully harvested from several tissue types, namely, the bone marrow (BM), adipose tissue (AT), umbilical cord (UC) tissue, and umbilical cord blood (UCB) (Kern et al., 2006; Kim et al., 2007; Cho et al., 2009; Lv et al., 2012; Luo et al., 2017). However, the clinical adoption of BM, AT, UC, and UCB—derived MSCs is limited by certain drawbacks. Aged cells from older patients are less biologically active and have a limited capacity for proliferation and differentiation. Cell extraction involves invasive procedures with significant donor site morbidity (Chamberlain et al., 2007; Dimmeler and Leri, 2008). A significant delay is also involved during cell culture and expansion. To overcome the pre-existing difficulties in the translational use of stem cells, we will be using a novel source of MSCs derived from human cord lining (US Patent number 9,737,568). In our previous study, we demonstrated that these Cord Lining Mesenchymal Stem Cells (CL-MSCs), when compared with MSCs derived from other gestational tissues; namely umbilical cord blood (CB-MSCs), placenta (P-MSCs), and Wharton's jelly (WJ-MSCs); have showed the highest proliferation and migration rate as well as prolongation in survival, which is attributed to their ability to dampen TH1 and TH2 responses (Stubbendorff et al., 2013). Moreover, CL-MSCs, when compared to other gestational tissues, yielded the lowest immunogenicity, which is correlated with lower HLA 1 expression (Stubbendorff et al., 2013), as well as its ability to reduce the release of IFN-gamma in mixed lymphocyte reaction (Stubbendorff et al., 2013). Similarly, CL-MSCs when compared with bone marrow MSCs (bmMSCs) have significant

lower HLA 1 expression, higher production of tolerogenic TGH-beta and IL-10, and highest proliferation rate (Deuse et al., 2011). Altogether, the CL-MSCs are superior cells, with the most promising potential for cell based therapy because of their higher proliferative capacity, lower immunogeneity, and stronger immunosuppressive potential. This cGMP grade CL-MSCs or CorLiCyte has been approved by US-FDA for Phase-1 clinical trial for non-healing diabetic wounds and is believed to be a promising alternative source of stem cells. It offers a practical and affordable source of cells for cell-therapy for peripheral vascular disease. Besides the biological differences compared to other MSCs, they are processed from a rich source of cells (umbilical cord lining) which is routinely discarded after birth. This reduces the economic cost and logistic burden of therapy, facilitating greater adoption, and ability to penetrate into populations where financial resources may be scarce. This can potentially lower the incidence of major amputation in CLI patients.

To the best of our knowledge, the functionality of this novel MSCs on ischemic hindlimb has not been evaluated. Our study represents the first investigation of the therapeutic effect of CL-MSCs on hindlimb in a rabbit model. We investigated the use of CL- MSCs in hindlimb ischemia in a rabbit model and evaluated their potential in generating neovascularization. We predict that the rabbits in the treatment group (standard care with CL-MSCs) will demonstrate evidence of increased neovascularization compared to the control group (standard care with no CL-MSCs). We also predict that the rabbits in the treatment group will demonstrate evidence of increase functional collateral network formation compared to the control group.

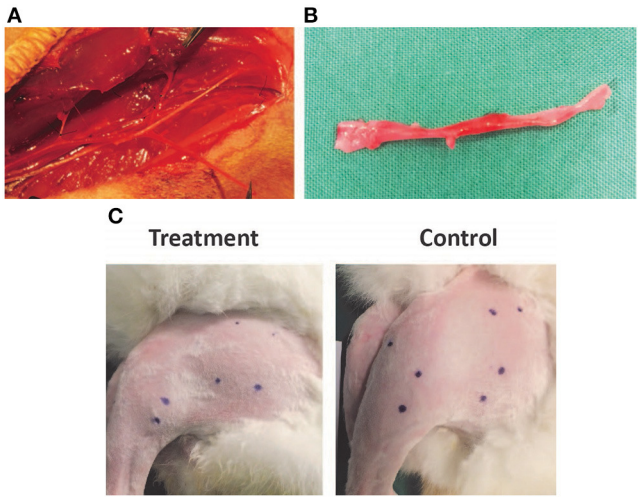

FIGURE 1 | (A,B) Dissection and ligation of the superficial femoral artery in the rabbit hindlimb. **(C)** Injection sites in the rabbit hindlimb muscle bellies marked prior to treatment to ensure consistent localization.

MATERIALS AND METHODS

Cord Lining Mesenchymal Stem Cells Isolation, Characterization and Culture

CL-MSCs isolation and cultivation were performed according to the patented protocol by CellResearch Corp Pte. Ltd. (US Patent number 9,737,568). Stem cell characterization and quality control were performed in compliance with cGMP processing protocol that has been submitted to US-FDA and is approved for clinical trials on human subjects.

Briefly, CL-MSCs were isolated from the outer amniotic lining of the umbilical cord and were cultured in PTT4 media (CellResearch). Cells were grown in their specific growth media [CMRL-1066 + antibiotics + L-glutamine] (Cell Research Corp). Cells were split at a confluency of 80–85% by mechanically lifting the cells from the tissue culture plastic surface using a cell lifter (Costar, Corning). Before transplantation, MSCs were dissociated by collagenous type 1 solution at a concentration of 0.025% for 5 min at 37°, and resuspended in sterile PBS at 1×10^6 cells per 50 μl. MSC viability was ∼95% as determined by trypan blue staining.

Preparation of Rabbit Hindlimb Ischemia Model

This study was approved by the Institutional Review Board and SingHealth Institutional Animal Care and Use Committee (#: 2013/SHS/ 836). Twenty male rabbits (New Zealand White strain) of weight 2.5–3 kg, ∼11–13 months in age, were used in this experiment. Hindlimb (left) ischemia in the rabbits was created by surgical ligation of the common femoral artery (CFA). The right hindlimb was kept intact. A longitudinal skin incision on the medial aspect of thigh, from the inguinal ligament to the knee, was made in the left hindlimb. The CFA, superficial femoral artery (SFA), and profunda femoral artery were dissected, ligated (with 6/0 prolene) and divided. SFA was then dissected down to popliteal and saphenous arteries. Popliteal and saphenous arteries as well as their associated branches were ligated (with 6/0 prolene) and divided at the knee level. The entire length of SFA and part of the popliteal artery and the saphenous artery were excised and discarded (**Figures 1A,B**).

Twenty rabbits were divided into two separate groups. Group 1 was the treatment arm. At 21 and 49 days post-operatively (after the establishment of hindlimb ischemia model), animals in Group 1 were injected with CL-MSCs (500,000 cells per 0.2 ml on each site) at 10 different sites (**Figure 1C**; Quadriceps- 4 sites, Hamstrings- 4 sites and Calf-−2 sites) of the ischemic left hindlimb muscles. Group 2 was the control group which received only saline injection to the corresponding sites in the ischemic left hindlimb muscles, at the same time point as group 1. The effect of CL-MSCs compared to control was monitored using laser doppler flowmeter (Imager) over 15 weeks (at week 7, 11, and 15) following the first treatment at week 3. The presence of constitutional symptoms such as fever, loss of appetite, loss of weight, movement, and rabbit grimace scale were documented throughout the experiment. At week 15 post-operation, all the

animals were euthanized. Peripheral angiography was performed at this time point. The left hindlimb Rectus Femoris muscle and Gastrocnemius muscle were harvested for histology analysis.

Angiography

Peripheral angiography by C-Arm machine was performed before animal euthanasia in a lead-lined section of the experimental operating theater. Each rabbit was anesthetized. Midline laparotomy was performed and small bowel was mobilized to expose the abdominal aorta. The aorta was cannulated with a 4-French Micro sheath (Angiodynamics) using the Seldinger technique. Digital Subtraction Angiography was carried out with 2.5 ml of contrast (Visipaque) being injected via the 4-French catheter. The distance between the image intensifier and the table was fixed at 25 cm for the angiography performed. Region of Interest (ROI) was drawn in the area of the medial thigh to include the newly developed collaterals. Same ROIs were used in all limbs in order to reproduce equal sampling areas. Quantification of neovascularization was done by standard grid overlay counting of collateral vessels in the region of interest. Analysis was done by 1 independent operator blinded to the treatment.

Laser Doppler Perfusion Imaging

The total local microcirculatory blood perfusion is measured using Perimed Laser Doppler Perfusion Imaging (LDPI) at week 7, 11, and 15 post-surgical ligation of the CFA. Each animal is sedated using Ketamine (50 mg/kg) and Xylazine (10 mg/kg) intramuscularly prior to the imaging procedure and maintained under inhalational anesthesia [Isoflurane (1–2%)] during the procedure. The hair from the hindlimb is removed using an electric shaver followed by hair removal cream as necessary. The measurements were expressed as perfusion units (PU).

Histology

The muscle tissues were dissected from the hindlimbs of euthanized rabbits, and consecutively fixed in formalin. Two muscle groups, the Rectus Femoris and Gastrocnemius, were analyzed using histopathologic procedure. Specimens were embedded in paraffin and sectioned. To locate and to mark up the endothelial cells and capillaries, the IHC staining for CD31 using UltraView DAB polymer kit (Dako-Immunoglobulins ApS, Produktionsvej, 42, 2600, Glostrup, Hovedstaden Denmark; Dilution of 1/100) was performed on Roche Ventana Ultra Automated machine (Retrieval: CC1-64 min; Incubation time: 32 min; Incubation temp: 37°C). The tissues were further stained with CD68 antibody (Boster Biological Technology Co., Ltd., Wuhan, China; Dilution of 0.4 ug/ml; Incubation time: 30 min; Incubation temp: 36.9°C) and evaluated by a clinical pathologist to look for evidence of inflammation and fibrosis. All samples were coded. Unbiased histological examination was performed by a pathologist, blinded to the treatment group. Five microscopic fields (400X magnification) with increased capillary density were randomly selected and the number of stained capillaries and fibers within each field were quantified using manual counting. The values were represented using median number of vessel count/muscle fiber (V/M) from two sections 200 μm apart.

Statistical Analysis

The difference in median (V/M) ratio between groups was compared using the Wilcoxon rank sum test. The effect of treatment on perfusion over time was evaluated using the linear mixed model with random intercept, to take into account possible intra-subject correlation in the serial measurements. Natural log transformation was carried out to normalize the perfusion data. All statistical analyses were generated using STATA V14, assuming a two-sided test at the 5% level of significance.

RESULTS

Cord Lining MSCs Injections Had No Increased Morbidity or Local Reaction

There was no report of adverse or serious condition occurring in both groups of rabbits. All surgical wounds healed with no complications and there were no soft tissue tumors noted at the injection site for both the treatment and control groups. The rabbits in the treatment arm experienced no adverse changes in temperature or mobility. There is no difference in the weight of rabbits between the control and the treatment groups (**Figure 2A**). Animals in the treatment arm had a 100% survival rate to the end of the experiment (**Figure 2B**). The rabbit grimace scores suggested minimal post implantation pain and were also comparable for both groups (**Figure 2C**). Macroscopic evaluation on the sites of CL-MSCs injection showed no tissue necrosis or foreign body reaction. There was a minimal increase (<5%) in inflammatory cells or fibrosis between the two groups (**Figures 3A–C**). This indicates no gross immune response on the injected tissue.

Cord Lining MSCs Injections Resulted in Increased Angiogenesis in the Rectus Femoris in the Ischemic Limb

Gross microscopic evaluation of the Rectus Femoris muscle and Gastrocnemius muscle from both the control and treatment groups showed no significant ischemic or fibrous scarring after ligation of the SFA and at termination of the experiment. This indicates that the rate of angiogenesis and existent collateral blood flow is adequate to prevent tissue necrosis after ligation, even in the non-treatment arm in our hindlimb ischemia model. As expected, both groups demonstrated no inhibition in mobility after ligation secondary to lower limb muscle necrosis.

Of interest is the increase in angiogenesis in the Rectus Femoris and Gastrocnemius in the treatment arm. The Rectus Femoris demonstrated a median vessel count/muscle fiber of 0.121 in the treatment group, compared to 0.076 for control group (median difference 0.04; 95% CI 0.001–0.11; $p = 0.041$; **Figures 4A–C**). The Gastrocnemius demonstrated a median vessel count/muscle fiber of 0.175 in the treatment group, compared to 0.089 for control group (median difference 0.087; 95% CI −0.006 to 0.234; $p = 0.07$; **Figures 4A–C**). The difference

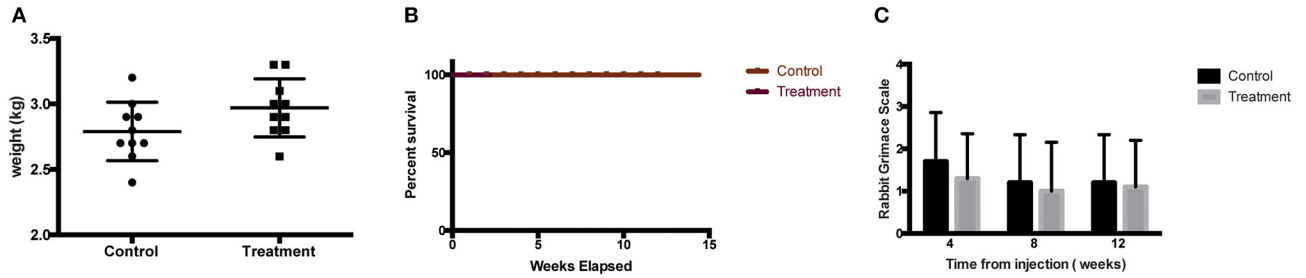

FIGURE 2 | (A) Weight of rabbit at final time point before sacrifice demonstrated no difference between control and treatment groups. **(B)** Kaplan Meier survival graph for treated rabbit demonstrated no difference between control and treatment groups. **(C)** Rabbit Grimace Scale demonstrated no difference between control and treatment groups.

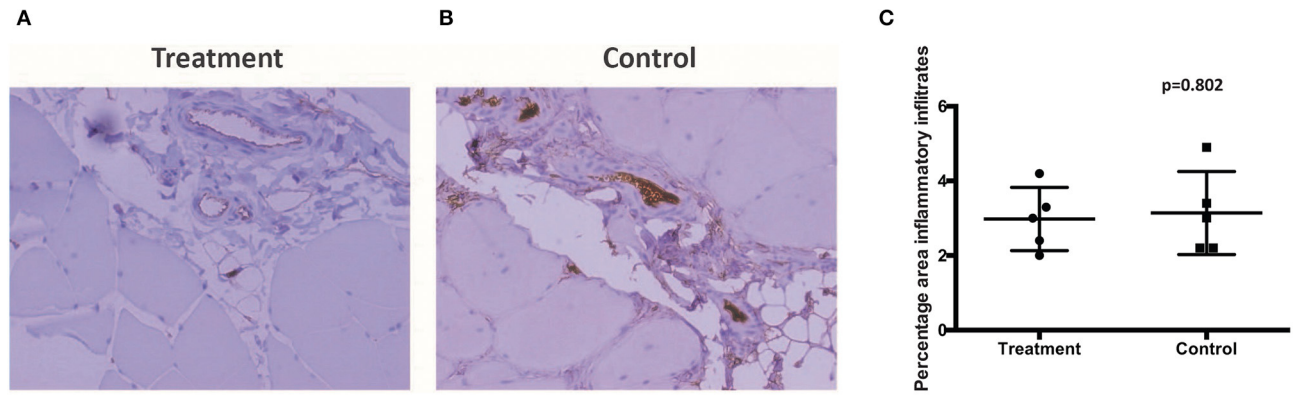

FIGURE 3 | (A,B) Muscle samples taken at the injection site demonstrated no difference in percentage inflammatory infiltrate, fibrosis, or local reaction between control and treatment. **(C)** Proportion of infiltrated inflammatory cells between control and treatment.

in Rectus Femoris but not the Gastrocnemius is significant as it indicates that the CL-MSCs injections had an effect on the rabbit angiogenesis after ligation of the SFA in the proximal muscles but not the distal muscles. This is not surprising, as the majority of the injections for implantations were done in the proximal muscles (8 injection sites) compared to the distal muscles (2 injection sites). This suggests that the therapeutic effect of the CL-MSCs on angiogenesis in ischemic muscles is likely to be dependent on cell quantity.

Cord Lining MSCs Injections Resulted in Marginal Increase in Perfusion in Hindlimb Ischemic Limbs

Peripheral angiography revealed a marginal increase in perfusion toward the treatment group. However, it did not demonstrate a statistically significant difference between groups (**Figures 5A,B**). Similarly, images acquired through LDPI revealed a slight increase in perfusion toward the treatment group (**Figure 5C**). There is no significant difference in the Log Perfusion Unit (PU) between treated and untreated groups. Both groups demonstrate an uptrend pattern in the LogPU over time, with the treated group projecting a slightly steeper slope (**Figure 5D**).

DISCUSSION

In this observational report, we have demonstrated that CL-MSCs have low morbidity associated with systemic toxicity. In our histology analysis, we have established that injection of CL-MSCs did not trigger adverse tissue response. This is an important first step in establishing the safety profile of this treatment modality in preparation for pre-clinical trials. Any cell-based therapy has the potential for immune rejection and inflammation. This is of even greater concern in the use of non-autologous cells, such as CL-MSCs. CL-MSCs have the practical advantage of being bankable from different donors in large numbers. This ensures that therapeutic cells are of low passage number. They are derived from the umbilical cord lining and hence have an inherently low immunogenicity.

We have also demonstrated that there is significant effect in the stimulation of angiogenesis in the hindlimb ischemia rabbit model in quantitative histological outcomes, which were the most sensitive and least subjective investigative technique we had. Due to the phase dependent nature of the clinical angiogram, we were not able to get a statistically significant result in this particular investigative modality. LDPI revealed a marginal increase in LogPU over weeks but did not demonstrate a significant difference between groups. LDPI is a crude measurement

A

Parameter	Treated	Untreated	p-value
Left Rectus Femoris Median Interquartile Range Difference (95% CI)	0.121 (0.078-0.175) 0.040 (0.001 to 0.110)	0.076 (0.065-0.096)	0.041
Left Gastronemius Median Interquartile Range Difference (95% CI)	0.175 (0.093-0.319) 0.087 (-0.006 to 0.234)	0.089 (0.073-0.156)	0.070

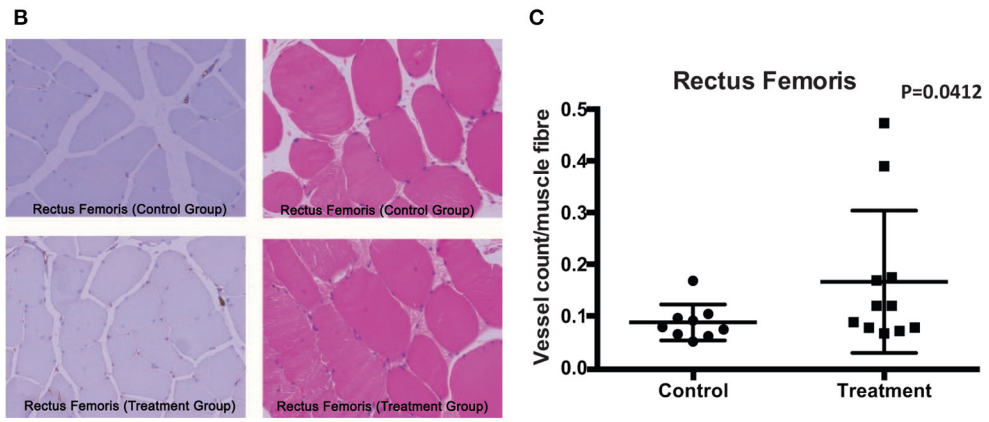

FIGURE 4 | (A) Comparison of median V/M ratio between treated and untreated groups. **(B,C)** CD31 and H&E stains of the Rectus Femoris muscles demonstrated a trend toward increased V/M ratio scores for the treatment group compared to the control group.

that is subject to multiple confounders. This includes varying degrees of fur covering the limb, environmental or animal core and peripheral temperature, as well as ambient light. It may not be adequately sensitive to determine small quantitative differences in perfusion but it does have the advantage of being non-invasive and repeatable over time. The fact that the Rectus Femoris (proximal) but not the Gastrocnemius muscle (distal) demonstrated a significant difference in the degree of angiogenesis between the treatment and control suggests that the cell therapy has a localized effect rather than a longer range or systemic effect. This is consistent with other reports on the mechanism of action of MSCs (Powell et al., 2011; Schiavetta et al., 2012; Liang et al., 2017). CL-MSCs should have a more consistent clinical efficacy compared to marrow or adipose derived stem cells as they are not taken from aging donor (peripheral vascular disease is typically a disease of the middle aged or older adults) and can be available in large numbers. There is also no waiting time for cell expansion in contrast to bone marrow derived cells which can take up 2–3 weeks for cell expansion. This gives CL-MSCs a huge advantage in the treatment of conditions that are more time sensitive and in avoiding a missed therapeutic window.

The characterizations of CL-MSCs were well-established and showed typical MSC characteristics in previous reports. Flow cytometry clearly showed that the isolated cells expressed CD44, CD73, CD90, and CD105 on the cell surface, confirming the qualifying criteria of MSCs (Kita et al., 2010; Deuse et al., 2011; Martinez et al., 2013; Stubbendorff et al., 2013). The prominent feature of CL-MSCs is that the cells also express several Stem Cells (SC) markers in addition to MSC markers, namely, noteworthy that 100% of the cells expressed Nanog, which is one of the key molecules necessary for the maintenance of self-renewal of SCs, and Oct-4, another well-accepted molecule to define SCs (Stubbendorff et al., 2013). Many of our cells expressed SSEA-4 (Kita et al., 2010; Deuse et al., 2011; Stubbendorff et al., 2013), which is shown to have superior propagative activity, consistent with the observation of the numerous formation of colonies (∼30 colonies, each larger than 2 mm in diameter), again indicating the CL-MSCs retain the capacity to propagate and actively migrate during proliferation. Another general defining criteria of CL-

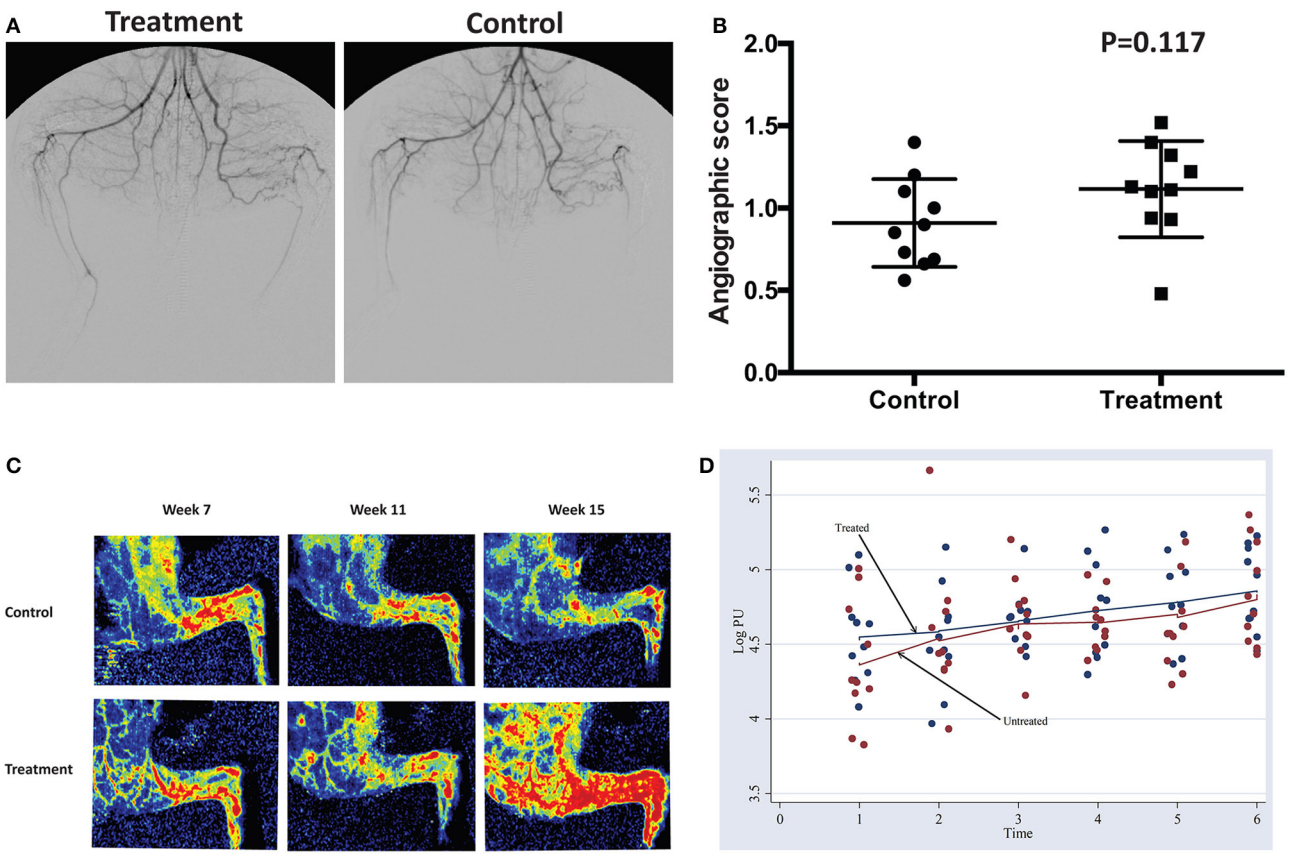

FIGURE 5 | (A,B) Peripheral angiography performed at week 15 before euthanasia demonstrated a weak trend toward a higher angiographic score in the treatment compared to the control group. **(C)** Laser Doppler perfusion Imaging of the hindlimb at week 7, week 11 and week 15 demonstrated a trend toward progressively more flow in the treatment group. **(D)** Pattern of change in logPU over time.

MSCs is the ability to differentiate into at least 3 lineages; osteogenic, adipogenic and chondrogenic (Martinez et al., 2013; Stubbendorff et al., 2013). Further, CL-MSCs did not show anchorage-independent growth, indicating that CL-MSCs did not show any tumor cell-specific features, an important aspect for future applications of CL-MSC in regenerative medicine.

There were certain limitations in the scope of our study. This study did not investigate the mechanism of action of the CL-MSCs. The mechanism of action of MSCs in angiogenesis has been well-elucidated and characterized in many other studies (Lawall et al., 2010; Sprengers et al., 2010; Powell et al., 2011; Schiavetta et al., 2012; Liang et al., 2014, 2017). We also did not investigate serum and organ specific changes during treatment. This would have been important in ensuring that besides the local immune reaction, there is no minor systemic toxicity, which may not have been exhibited in morbidity or symptoms from the rabbits. However, the physiological outcome measurements documented in our study are adequate for us to demonstrate that there was no major local or systemic reaction to the cell therapy.

Another limitation is that the microenvironment of the hindlimb ischemia model may not be identical to the clinical presentation. Even in the control arm, all the subjects showed negligible ischemic changes in the muscle after vascular ligation. This may be due to extensive collateral flow, or the rapidity of neovascularization in rabbits. However, since tissue hypoxia is known to be important in cell signaling and angiogenesis, this difference may account for the blunted therapeutic response in our treatment arm, compared to the control arm. Lastly, we did not label the implanted cells as there were concerns over how cell labels might markedly change the nature of our CL-MSCs and confound our findings. Further, the survival of CL-MSCs *in vivo* has been determined in earlier studies. FLuc positive CL-MSCs were detected up to 10.9 ± 1.2 days in immunocompetent Balb/c mice (Martinez et al., 2013), 11 days after injection in xenogeneic murine and allogeneic human ELISPOT assays (Lilyanna et al., 2013), and 17.2 ± days for immunodeficiency SCID-beige mice (Martinez et al., 2013). It has thus been established that injected cells do not survive beyond 2–3 weeks, and do not form part of the new capillaries.

CONCLUSION

CL-MSCs (US Patent number 9,737,568) implantation is safe and well-tolerated. The implantation of CL-MSCs demonstrates no toxicity associated morbidity and minimal local immune reaction to implantation. CL-MSCs have the capacity to stimulate angiogenesis in a rabbit hindlimb ischemia model. This early

safety and efficacy data is encouraging and paves the way for future large animal studies or for clinical trials. The cGMP grade CL-MSCs or CorLiCyte has been approved by US-FDA for Phase-1 clinical trial for human study, bringing it a step closer to clinical application. These cells can potentially be used for patients with ischemic limbs in the near future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Review Board and SingHealth Institutional Animal Care and Use Committee, Singapore.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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frontiers in Cell and Developmental Biology

REVIEW
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The Current Status of Mesenchymal Stromal Cells: Controversies, Unresolved Issues and Some Promising Solutions to Improve Their Therapeutic Efficacy

*David García-Bernal[1,2†], Mariano García-Arranz[2,3†], Rosa M. Yáñez[2,4,5†], Rosario Hervás-Salcedo[2,4,5], Alfonso Cortés[2,6], María Fernández-García[2,4,5], Miriam Hernando-Rodríguez[2,4,5], Óscar Quintana-Bustamante[2,4,5], Juan A. Bueren[2,4,5], Damián García-Olmo[2,3], Jose M. Moraleda[1,2], José C. Segovia[2,4,5] and Agustín G. Zapata[2,7]**

[1] Hematopoietic Transplant and Cellular Therapy Unit, Medicine Department, Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca, University of Murcia, Murcia, Spain, [2] Spanish Network of Cell Therapy (TerCel), Instituto de Salud Carlos III, Madrid, Spain, [3] Instituto de Investigación Sanitaria-Fundación Jiménez Díaz (IIS-FJD, Autonomous University of Madrid (UAM)), Madrid, Spain, [4] Advanced Therapies Mixed Unit, Instituto de Investigación Sanitaria-Fundación Jiménez Díaz (IIS-FJD, Autonomous University of Madrid (UAM)), Madrid, Spain, [5] Centre for Cytometry and Fluorescence Microscopy, Complutense University, Madrid, Spain, [6] Hematopoietic Innovative Therapies Division, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas and Centro de Investigación Biomédica en Red de Enfermedades Raras, Madrid, Spain, [7] Department of Cell Biology, Complutense University, Madrid, Spain

Mesenchymal stromal cells (MSCs) currently constitute the most frequently used cell type in advanced therapies with different purposes, most of which are related with inflammatory processes. Although the therapeutic efficacy of these cells has been clearly demonstrated in different disease animal models and in numerous human phase I/II clinical trials, only very few phase III trials using MSCs have demonstrated the expected potential therapeutic benefit. On the other hand, diverse controversial issues on the biology and clinical applications of MSCs, including their specific phenotype, the requirement of an inflammatory environment to induce immunosuppression, the relevance of the cell dose and their administration schedule, the cell delivery route (intravascular/systemic vs. local cell delivery), and the selected cell product (i.e., use of autologous vs. allogeneic MSCs, freshly cultured vs. frozen and thawed MSCs, MSCs vs. MSC-derived extracellular vesicles, etc.) persist. In the current review article, we have addressed these issues with special emphasis in the new approaches to improve the properties and functional capabilities of MSCs after distinct cell bioengineering strategies.

Keywords: MSC bioengineering, MSC homing, MSC immunomodulation, MSC preconditioning, MSC therapeutic efficacy

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Sina Naserian,
Hôpital Paul Brousse, France
Selim Kuci,
University Hospital Frankfurt, Germany

***Correspondence:**
Agustín G. Zapata
zapata@bio.ucm.es

†These authors have contributed equally to this work

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INTRODUCTION

Mesenchymal Stromal Cells: Lights and Shadows in the Knowledge of Their Mechanisms of Action

Numerous questions on the biology of mesenchymal stromal cells (MSCs), the most promising cell type for cell therapy strategies, remain unknown (Galipeau and Sensebe, 2018). This would explain the variability of both, the reported preclinical and clinical results and the difficulties to establish a general pattern of functioning for these cells. A current survey on the heterogeneity of MSCs, their immunogenicity, routes of delivery and migratory capacity, and principally on the mechanisms governing their immunomodulatory properties needs a substantial revision in order to design protocols for improving their therapeutic capacities, including MSC bioengineering.

MSCs were initially described as colony forming units-fibroblasts (CFU-Fb) capable of differentiating into distinct connective tissue lineages (i.e., osteoblasts, chondroblasts and adipocytes) (Friedenstein et al., 1970; Caplan, 1991; Pittenger et al., 1999). Multiple parameters can affect the therapeutic properties of MSCs including tissue origin (Ketterl et al., 2015), cryopreservation procedure (Oja et al., 2019), culture time and media supplementation with different growth factors (von Bahr et al., 2012; Moll et al., 2014b), optimal dosage (Golpanian et al., 2016) and *in vivo* cell delivery (Caplan et al., 2019; Moll et al., 2019) can affect substantially the cellular therapeutic properties of MSCs. Therefore, a better knowledge of these cell processes would improve the therapeutic outcomes of MSCs.

Mesenchymal Stromal Cell Immunophenotype and Immunomodulatory Properties

There are no specific markers to characterize the immunophenotype of the MSCs. In humans, MSCs express CD73, CD90, CD105, CD166, CD29, and CD44 that are also present in many other cell types (Pittenger et al., 1999). Negative markers include CD34, CD45, CD14, CD11b, CD79a, CD10, and HLA-DR, except in the presence of IFNγ (Alfaro et al., 2020). In addition, they express numerous cytokine and chemokine receptors as well as distinct Toll-like receptors (TLRs) that play distinct immunomodulatory functions including the inhibition of T cell responses, antigen-presenting cell maturation, cytotoxicity of resting NK cells and differentiation of monocytes to immature dendritic cells (DCs) (Beyth et al., 2005; Jiang et al., 2005; Spaggiari et al., 2006; de Castro et al., 2019). Indeed, MSCs exhibit high plasticity over time and probably related with their origin in different microenvironments (Wilson et al., 2019). This MSC heterogeneity is due, at least in part, to the occurrence of distinct expression profiles (i.e., surface markers, transcriptome and proteome), and functional properties (Phinney et al., 2006; James et al., 2015; Mattar and Bieback, 2015). Some authors have proposed, but not conclusively demonstrated, that induced pluripotent stem cells (iPSCs)-derived MSCs could constitute a more homogeneous cell population (Bloor et al., 2020).

Nevertheless, it is important to clarify more conclusively the relevance of an inflammatory environment for the MSC-mediated immunomodulation. Two recent publications by Naserian and colleagues (Beldi et al., 2020a,b) have provided new and relevant information on the role played by TNF-α signaling in these processes. TNF-α exerts its effects through interaction with two receptors, TNFR1 and TNFR2. Whereas TNFR1 is ubiquitously expressed, TNFR2 expression is restricted to some cell types, including MSCs (Salomon et al., 2018; Yang et al., 2018). Remarkably, TNFR2 signaling results in pro-angiogenic and survival effects, but activation of TNFR1 signaling pathway generally induces apoptosis (Faustman and Davis, 2013). Furthermore, MSCs isolated from TNFR2 KO mice are less efficient in governing immunosuppression, including reduced capability to induce T cell differentiation toward Treg cell lineage (Beldi et al., 2020b). More recently, extended analysis of these TNFR2 deficient MSCs demonstrated that impeded TNFR2 signaling courses with reduced MSC colony-forming units (CFUs), proliferative rate and expression of diverse MSC cell markers. In addition, these deficient TNFR2 MSC produce more pro-inflammatory molecules (i.e., TNF-α, IFNγ, IL-6), less IL-10, TGFβ and nitric oxide (NO), and show reduced regenerative capabilities for wound healing, vascular tube formation and neoangiogenesis (Beldi et al., 2020a).

It has been proposed that the therapeutic properties of MSCs depend on the crosstalk of these cells with the host tissues (Ankrum et al., 2014; Galleu et al., 2017; de Witte et al., 2018; Galipeau and Sensebe, 2018), as suggested by the mechanisms which control their immunoregulatory properties and the status of pre-sensitization of host (Avivar-Valderas et al., 2019). During acute inflammation, MSC activation is critical for the production of immunoregulatory factors, in contrast with non-activated MSCs, which do not exhibit a significant production of these molecules. In acute inflammatory conditions, activated T cells secrete pro-inflammatory cytokines (i.e., IFNγ, TNF-α, IL-1, or IL-17), that activate MSCs initiating the modulation of immune responses by releasing anti-inflammatory molecules, such as prostaglandin E_2 (PGE_2), IL-10, HLA-G, indoleamine-2,3-dioxygenase (IDO), hepatocyte growth factor (HGF), TGFβ, NO, galectins, semaphorin-3A or heme-oxigenase (HO) as well as multiple chemokines (i.e., CXCL10, CXCL11, CXCL12, and CXCL19) (Jimenez-Puerta et al., 2020).

In general terms, activated MSCs in an inflammatory microenvironment block or largely inhibit activation of the complement system, neutrophils, T cells, B cells and NK cells. MSCs stimulate functional maturation of anti-inflammatory type 2 macrophages, regulatory DCs and B and T regulatory cells as well (Saparov et al., 2016; Wang et al., 2018, 2019; Jimenez-Puerta et al., 2020). Therefore, TLRs and numerous immunomodulatory factors secreted or expressed by MSCs are orchestrated to function together. Indeed, it has not been possible to identify one single mechanism responsible for the immunomodulatory properties of MSCs and distinct factors seem to act, coordinately and/or sequentially in the blockade of the immune system (Ferreira et al., 2018). On the other hand, PGE_2 production largely depends on IL-10 signaling

and MSCs stimulated by kinurenin through aryl-hydrocarbon receptors (AhR) show an increased production of iNOS, IDO and PGE_2 (Jiang et al., 2005; Wang et al., 2018; de Castro et al., 2019). Moreover, low levels of TGFβ correlate with reduced IDO (Xu et al., 2014), and TNF-stimulated gene 6 (TSG6), that inhibit neutrophilia by blocking CXCL8-mediated chemotaxis, is regulated by AhR and IDO (Wang et al., 2018). Moreover, TLR2 activation induces galectin-3 production, increasing its capacity to suppress T cell activation (Sioud et al., 2010). However, effects mediated through TLR3 and TLR4 are controversial, although it is generally assumed that TLR3 signaling induces an anti-inflammatory MSC profile (MSC-2), while TLR4 signals promote pro-inflammatory MSCs (Shammaa et al., 2020). Importantly, and apart from their immunomodulatory properties, other studies have found that MSCs also possess robust anti-bacterial properties through secretion of a variety of anti-microbial peptides and/or proteins such as lipocalin-2, IL-37, hepcidin, keratinocyte growth factor and β-defensin-2 which has led to MSCs being considered as a therapeutic option for sepsis and septic shock (Krasnodembskaya et al., 2010; Gupta et al., 2012; Alcayaga-Miranda et al., 2015; Sung et al., 2016).

The key role played by Treg cells for governing MSC-mediated immunosuppression deserves further, more extensive analysis. MSCs induce Treg cell differentiation by increasing production of PGE_2, TGFβ and IL-10. In addition, they increase Treg cell proliferation via TLR2 and TLR3 signaling, thrombospondin, IL-2 and TNF-α through activation of Stat5 that increases CD39 and CD73 expression, both molecules involved in the adenosine production necessary for Treg cell function (de Castro et al., 2019). On the other hand, as previously indicated, a close relationship has been established between TNFR2 expression and Treg cell function (Salomon et al., 2018; Yang et al., 2018; Naserian et al., 2020). Remarkably, Treg lymphocytes express TNFR2 which is directly related to their immunosuppressive effects (Leclerc et al., 2016; Naserian et al., 2020).

On the other hand, some reports suggest that systemically injected MSCs have immunosuppressive properties because they are entrapped in the lung microvasculature, die by apoptosis and are engulfed by local macrophages that become type 2 macrophages which secrete IL-10 and arginase immunosuppressive factors (Anderson et al., 2013; Braza et al., 2016). Engulfed MSCs appear mainly in non-classical $Ly6C^{low}$ monocytes that polarize toward $CD14^+CD16^+CD296^+$ monocytes, an intermediate phenotype with anti-inflammatory properties that produces IL-10 and express PDL-1. In addition, these primed monocytes that engulfed MSCs induce $CD4^+CD25^{high}$ Treg cell formation (Weiss and Dahlke, 2019).

These results indirectly support that apoptotic, metabolically inert or even fragmented MSCs would have the same immunomodulatory properties as living MSCs (Chang et al., 2012; Galleu et al., 2017; Weiss and Dahlke, 2019). Therefore, the viability of MSCs would not be a pre-requisite for some of their exerted immunomodulatory effects (Weiss and Dahlke, 2019). In this respect, Thum and colleagues pointed out that the apoptosis of MSCs is caused by modulation of both innate and adaptive immunity (Thum et al., 2005) and further studies support this idea. Apoptotic MSCs exhibited an immunosuppressive behavior in a Th2-type inflammatory model, inducing IDO production in host phagocytic cells (Galleu et al., 2017), and supernatants of cultured macrophages that engulfed MSCs improved the survival of hypoxic cardiomyocytes (Lu et al., 2013). Remarkably, systemic administration of apoptotic adipose tissue-derived MSCs provide better therapeutic results than the treatment with living MSCs in a cecum ligation and puncture-induced sepsis model (Sung et al., 2013). On the other hand, MSCs heated for 30 min at 50°C that provokes an irreversible blockade of cell metabolism but maintains the cell integrity, were able to reduce the inflammatory response in mice receiving LPS by a significant reduction of the serum levels of IFNγ and increased production of IL-10 (Luk et al., 2016). Also, normal MSCs and metabolically inactive MSCs showed similar effects on monocyte function with a significant reduction of TNF-α production in response to LPS (Jiang et al., 2005). By contrast, the intrapulmonary administration of apoptotic MSCs did not increased survival or reduced the severity of endotoxin-induced acute lung injury (Gupta et al., 2007), in contrast with several studies demonstrating the significant positive effect of living MSCs in the reduction of sepsis in different experimental models (Gupta et al., 2007; Johnson et al., 2017).

MESENCHYMAL STROMAL CELL MANUFACTURING

MSC manufacturing for clinical use has been regulated worldwide for over a decade in an attempt of protecting potential users. Production of cell medicaments with protocols accepted by regulatory agencies under GMP conditions generates a cellular product with specific properties and a high level of safety. Autologous MSC manufacture was the first step and these cells are currently used in the majority of clinical trials and treatments; however, this procedure has disadvantages such as the time required to obtain an adequate number of cells from older or fragile patients, or the difficulty of growing MSCs *in vitro* from patients with different pathologies. For this reason, cryopreservation of cells has been frequently used to allow delayed treatment or for allogeneic donors; although cryopreservation is not an innocuous process for cells.

Cryopreservation has interesting benefits in clinical practices and is mandatory for MSC banking, but its effects on MSC biology are controversial. While some authors have discussed that the cryopreservation process reduces MSC potency, other studies have found no significant influence on their immunomodulatory properties (Cruz et al., 2015; Luetzkendorf et al., 2015). Two freezing steps with, at least, one preceding cell culture passage before freezing do not seem to affect the essential biological parameters of MSCs (i.e., cell yield, growth kinetics and population doubling number), but ≥4 freezing steps could accelerate the senescence of cultured MSCs. In addition, the immunosuppressive potential of frozen and thawed MSCs, independently of the number of freezing steps, is reduced by about 50% as compared to freshly cultured MSCs, but definitively

do not abolish the process, even after long periods (>10 months) of cryopreservation (Klinker et al., 2017; Oja et al., 2019; Giri and Galipeau, 2020). Moreover, a variety of methods for long-term storage of MSCs using different formulations of cryopreservation media or different procedures for MSC freezing and thawing may subsequently greatly affect the MSC potency (Ding et al., 2010; Liu et al., 2010; Moll et al., 2014a; Miyagi-Shiohira et al., 2015; Rogulska et al., 2019). Therefore, the improvement of the cryopreservation conditions to ensure the intrinsic biological properties of MSCs needs further investigation in order to extend the utility of MSC banking for subsequent cell therapy uses.

On the other hand, the production of a sufficient number of MSCs by *in vitro* expansion for obtaining a clinical dose may have some impact on the native properties of the MSCs. Although MSCs can be growth up to 20 passages, these long-term cultured MSCs have shown senescence genes up-regulation, morphologic changes, decreased differentiation potential, chemokine receptor down-regulation, telomere length shortening and decreased immunosuppressive properties compared to short-term cultured counterparts (Jiang et al., 2002; Rombouts and Ploemacher, 2003; Honczarenko et al., 2006; Izadpanah et al., 2008; Li et al., 2012; Lian et al., 2016). Accordingly, the establishment of universal protocols for maintenance, banking and culture of MSCs would be welcome.

ALLOGENEIC OR AUTOLOGOUS MESENCHYMAL STROMAL CELLS FOR THERAPEUTIC USAGE

A second controversial issue is whether allogeneic better than autologous MSCs would be used clinically. Indeed, both preclinical studies and clinical trials show an increasing use of allogeneic MSCs. Autologous MSC transplantation has some limitations. Firstly, the high cost of cell preparation just for a single recipient. Moreover, it is difficult to obtain a clinical dose of MSCs from some patients. For example, MSCs isolated from elder donors have decreased proliferation, less differentiation, and less regenerative potential, subsequently leading to ineffective treatments (Maredziak et al., 2016). By contrast, it is evident that the use of allogeneic vs. autologous MSCs for cell therapy has clear advantages (Hare et al., 2012; Zhang et al., 2015). Allogeneic MSCs from young healthy donors are an optimal choice to solve this problem. In addition, the expansion of autologous MSCs to obtain a clinical dose is time-dependent, making this therapeutic approach difficult for the early treatment of diseases in acute phase (e.g., COVID-19, brain stroke, septic shock or myocardial infarction). However, allogeneic MSCs, cryopreserved and stored once obtained, can be readily available, quickly thawed, and immediately administered to the patient who requires them. For all these reasons, cryopreserved allogeneic MSCs are a promising therapeutic alternative to autologous MSCs with multiple advantages in terms of time, cost of production and quality assurance. Importantly, allogeneic MSCs from pooled mononuclear cells of multiple third-party donors have been reported to exhibit decreased heterogeneity and to exert significantly higher immunosuppresive potential than those obtained from individual donors (Kuci et al., 2016).

However, this proposal leads to the unsolved question of the MSC immunogenicity. It is well known that there is immune activation of host cytotoxicity mediated by complement, NK cells and/or cytotoxic T cells (Noone et al., 2013; Ankrum et al., 2014; Berglund et al., 2017; Kot et al., 2019). In fact, syngeneic MSCs persist for more than 200 days, whereas allogeneic cells rapidly disappear (Eliopoulos et al., 2005). Although, low or null immunogenicity for allogeneic MSCs has been claimed (Le Blanc et al., 2003; Escacena et al., 2015), recent *in vivo* and *in vitro* evidence suggests that MSCs generate both innate and adaptive host immune responses (Caplan et al., 2019). However, anti-MSC responses are lower than those against other allogeneic cells are (Khan and Newsome, 2019), perhaps because MSCs do not express MHC class II antigens or co-stimulatory molecules. Therefore, the balance between their immunogenicity and the release of immunosuppressive factors, highly dependent on the local microenvironment, determines the MSC behavior (Khan and Newsome, 2019). Even more, this cytotoxic activity is important for MSC-mediated immunosuppression because it results in phagocytosis of apoptotic cells and then macrophage polarization (Galleu et al., 2017; de Witte et al., 2018). Thus, reduction of the activity of host immune system could diminish the efficiency of MSCs (Caplan et al., 2019).

Accordingly, the study of HLA matching between donor MSCs and recipient of these cells is being recently proposed (Avivar-Valderas et al., 2019). On a phase III clinical trial for the treatment of complex perianal fistulous pathology in patients with Crohn's disease, the authors carried out a study on the immunological responses and MSC efficacy taking into account the haplotypes of the donor cells and the recipient concluding that an HLA-screening to the donor MSCs would be performed to limit the humoral response between donor and recipient.

DELIVERY OF MESENCHYMAL STROMAL CELLS

There is no consensus on the best method for MSC delivery (Caplan et al., 2019). Intramuscular delivery is a safe and simple method, but its efficiency is frequently low (Jahromi et al., 2019). In some organs, *in situ* direct injection is almost mandatory but may impede interactions between MSCs and host cells, particularly in lungs and spleen, thus limiting their therapeutic activity. In addition, delivery of a high number of cells could induce important cell damage, including high cell mortality by trauma, hypoxia or NK cell-mediated MSC apoptosis. On the contrary, systemic infusion of MSCs allows interactions with host cells and tissues but needs an adequate biodistribution and homing to affected tissues, which is sometimes limited. Intra-arteriolar delivery would be the most efficient method but also can be potentially harmful because MSCs mechanically entrap in the microvasculature elsewhere (Toma et al., 2009). The most frequently used method is the systemic delivery by intravenous injection but, particularly in rodents and in lesser

extent in humans, results in a high number of entrapped MSCs in lung capillaries that limit the number of cells reaching target organs and increase the risk of thromboembolism (Scarfe et al., 2018; Coppin et al., 2019). Although there are only a few clinical trials reporting MSC-associated thrombotic events (Jung et al., 2013; Wu et al., 2017), MSC delivery triggers the activation of the complement system and the coagulation cascade inducing the so called "Instant Blood-Mediated Inflammatory Reaction" (IBMIR) (Moll et al., 2012, 2019). MSCs express the pro-coagulant tissue factor (CD142) (Drake et al., 1989), and MSC systemic injection significantly increases C3a and sC5b-9 levels and activation of the thrombin-anti-thrombin complex, inducing a drop in platelet numbers and increased values of D-dimer (Moll et al., 2015). These results remark the relevance of monitoring MSC pro-coagulant activity after their systemic infusion (Caplan et al., 2019). On the other hand, release of complement activation factors after exposure to MSCs could modulate their immunomodulatory and chemotactic activity (Schraufstatter et al., 2009; Moll et al., 2011), and protocols to avoid or attenuate complement-mediated cell damage would improve the efficiency of MSC-based therapies (Moll et al., 2016).

MESENCHYMAL STROMAL CELL HOMING

Similar to leukocytes and hematopoietic stem cells, MSCs must undergo a multistep process to extravasate from the circulating blood and migrate through the vessel walls to the damaged tissues. This process includes various sequential steps: (1) an initial decelerative tethering followed by direct rolling contacts with endothelial cells; (2) activation of integrins (mainly induced by chemokines); (3) integrin-dependent firm adhesion to endothelial cells; (4) transendothelial migration; and (5) interstitial migration toward the injured tissue (Nitzsche et al., 2017). However, MSC homing to the damaged organs is very inefficient, and only a small proportion of cells reach target tissues (Devine et al., 2003). A restricted repertoire of functional homing and chemokine receptors exhibited by MSCs could be reason for this inefficiency (Honczarenko et al., 2006; Chamberlain et al., 2008). Among them, MSCs express neither the sialofucosylated glycoforms of CD44 nor P-selectin glycoprotein ligand-1 (PSGL-1). These molecules, called hematopoietic cell E-/L-selectin ligand (HCELL) and cutaneous lymphocyte antigen (CLA), respectively, contain the sialyl Lewis X (sLeX) moiety that mediate migration to E-selectin-bearing endothelial beds in sites of inflammation (Sackstein et al., 2008; Sackstein, 2009). In addition, the response of MSCs to CXCL12 gradients is controversial because it has been reported that they do not express its receptor, CXCR4 (Ullah et al., 2019). By contrast, MSCs extravasation is mediated by the expression of FGF receptors that interact with bFGF on endothelial cells mediating galectin-1-dependent adhesion to P-selectin (Langer et al., 2009). Then, MSCs send out filopodia and cross the intraluminal space with the concourse of metalloproteinases and the development of a front cell pole through their intracellular adaptor FROUNT and the chemokine receptor CCR2 (Zachar et al., 2016). But this mechanism of extravasation is less effective.

On the other hand, it has been found that *in vitro* prolonged expansion of MSCs in culture produces a down-regulation of a variety of homing molecules including chemokine receptors, such as CCR1, CCR7, CCR9, CXCR5, and CXCR6, thus lacking the chemotactic response to these chemokines (Rombouts and Ploemacher, 2003; Honczarenko et al., 2006). Accordingly, attempts for improving MSC homing are complex and require further optimization. Some of them have focused on introducing modifications in the expression of different homing molecules on migrating MSCs through a wide variety of genetic, enzymatic or ligand conjugation approaches. Enzymatic treatment of MSCs by α(1,3)-exofucosylation of the CD44 receptor with either stereospecific fucosyltransferase VI or fucosyltransferase VII in presence of its substrate GDP-fucose, or by fucosyltransferase VI gene transfection, has been shown to engender the potent E-selectin ligand HCELL on the MSC surface. This transient modification increases efficiently the *in vivo* tethering and rolling contacts on E-selectin-expressing endothelial beds in bone marrow microvasculature and in inflamed tissues (Sackstein et al., 2008; Abdi et al., 2015; Dykstra et al., 2016; Chou et al., 2017). Remarkably, recent findings have shown that exofucosylated MSCs display an altered secretome characterized by an augmented expression of anti-inflammatory molecules, leading to higher MSC immunosuppressive properties, as well as increased migration ability toward some pro-inflammatory chemokines such as CCL5, CCL20 and CXCL16 (Garcia-Bernal et al., 2020). Other authors reported that covalent binding of sLeX to the MSC surface through a biotin-streptavidin bridge, by conjugation of E-selectin-targeting peptide on the MSC membrane or by mRNA transfection to overexpress PSGL-1 and sLeX on MSCs resulted in an augmented rolling behavior on P- and E-selectin-coated surfaces and on inflamed vascular endothelium *in vivo* (Sarkar et al., 2008; Cheng et al., 2012; Levy et al., 2013). Lo et al. fused the first 19 aminoacids of PSGL-1 to human IgG and, after overexpression of this construct on HEK293T cells (a cell line with endogenous fucosyltransferase VII expression), they coupled this fusion protein to the MSC surface using palmitated protein G (PPG), leading to an increased rolling on P- and E-selectin-coated surfaces under hydrodynamic flow (Lo et al., 2013). By contrast, Ko et al. coated MSCs with PPG and anti-ICAM-1 antibodies, thus improving its ability to adhere to this endothelial ligand (Ko et al., 2009).

Engineering approaches for improving other MSC functional capacities, that will be described below, have been used for increasing MSC homing. Genetic modification by mRNA transfection is highly efficient and non-toxic to MSC as well as compatible with ectopic co-expression of multiple mRNAs at the same time (Kormann et al., 2011; Hamann et al., 2019). Using these types of strategies, Liao et al. tested the therapeutic capacity of engineered MSCs expressing PSGL-1, sLeX, and IL-10 via mRNA transfection in a mouse model of experimental autoimmune encephalomyelitis observing a decreased infiltration of immunocompetent cells into the white matter of the spinal cord (Liao et al., 2016). More recently, Hervás-Salcedo et al. have shown the improved therapeutic

efficacy of human AdMSCs transfected with mRNAs encoding for specific migration and anti-inflammatory molecules. In particular, these data demonstrated that the transient co-expression of CXCR4 and IL-10 in human AdMSCs, using a single bicistronic mRNA, increases the migration of these cells to inflamed sites and enhances their anti-inflammatory properties in a local inflammation mouse model (Hervas-Salcedo et al., 2021).

Other experimental approaches were focused on the overexpression of the chemokine receptor CXCR4 for enhancing migration and mobilization of MSCs through activation of the CXCL12/CXCR4 signaling pathway. As above indicated, CXCR4 is usually absent on the surface of culture-expanded MSCs, but after the *in vitro* treatment of MSCs with diverse cytokines it is highly expressed (Rombouts and Ploemacher, 2003; Shi et al., 2007). Thus, pre-treated MSCs with insulin-like growth factor 1 (IGF-1) for 48 h markedly increased the CXCR4 expression *in vitro,* and a greater number of MSCs treated with IGF-1 engrafted and survived in the peri-infarcted area when the cells were transplanted in a rat model of myocardial infarction (Guo et al., 2008). IL-3-pre-conditioned human MSCs up-regulated the CXCR4 expression, enhancing their *in vitro* migration toward CXCL12 and their *in vivo* migration in immunocompromised mice (Barhanpurkar-Naik et al., 2017).

Another strategy to increase the CXCR4 expression in MSCs is by genetic modification. Zheng et al. (2019) transduced mouse bone marrow derived-MSCs with a lentiviral vector carrying the CXCR4 gene. Then, mice suffering colitis associated tumorigenesis, injected with MSCs-CXCR4 showed relieved weight loss, longer colons, lower tumor numbers and decreased tumor burden compared to mice receiving the unmodified MSCs. Kim et al. (2017) demonstrated in a mouse diabetic hindlimb ischemia model that CXCR4-overexpressing adipose derived-MSCs contributed more efficiently to the early homing and engraftment into ischemic areas than unmodified MSCs, also improving the long-term engraftment and muscle tissue regeneration.

Other strategies aimed to improve the homing capacity to target tissues include the employment of different scaffolds (i.e., hydrogels and chitosans) (Schantz et al., 2007; Shen et al., 2010; Thevenot et al., 2010), magnetic guidance after MSC labeling with iron oxide magnetic particles (Arbab et al., 2004; Yanai et al., 2012; Yun et al., 2018), coated MSCs with biotinylated lipid vesicles, and irradiation or pulsed-focused high intensity ultrasounds that frequently improve MSC engraftment by up-regulating CXCL12 release by activation of different mechanotransduction pathways (Ziadloo et al., 2012; Zang et al., 2017; Liu et al., 2020). Nevertheless, these are complex methods that require a rigorous optimization (Ullah et al., 2019).

ENGINEERING MESENCHYMAL STROMAL CELLS FOR ENHANCING THEIR THERAPEUTICAL PROPERTIES

The myriad of processes that governs the biology and function of MSCs makes difficult to manipulate them for improving their therapeutic possibilities. Different experimental approaches have engineered MSCs (i.e., MSCs-2.0) aimed to enhance their therapeutic efficacy compared to native MSCs and have been tested in several preclinical models of a variety of diseases. MSCs have been mainly modified to increase their survival, retention, migration capacities and growth factor production, principally through genetic modifications, usually achieved by means of viral vectors but also using non-viral methods. Standard protocols can reach high levels of transduction without affecting the lineage differentiation or the intrinsic properties of MSCs. Constitutive rather transient transformation provides the best therapeutic effects (Lin et al., 2011). The most common vectors used to modify MSCs are retrovirus, lentivirus, adenovirus and adeno-associated virus (AAV) (Sage et al., 2016). Among the non-viral approaches mRNA lipofectamine-mediated transfection, PEGylated DNA template nanocomposite system, biotinylated MSC, spermin pullulan, hyper-branched polyamidoamine and jetPEI-mediated transfection have been used (Pawitan et al., 2020).

On the other hand, several studies described that the incorporation of anti-inflammatory genes such as IL-10, HGF, IDO and FoxP3 could improve the therapeutic potential of MSCs. The overexpression of other factors including VEGF, BMP2, osteogenic molecules (i.e., TGFβ, Cbfa-1, and Osterix), or molecules involved in homing (CXCR4 and CXCL12), etc. have been shown to enhance the MSC capacities (Pawitan et al., 2020). Particularly, enhanced IL-10 production has been intensively tested. IL-10 is a strong anti-inflammatory cytokine produced by monocytes/macrophages, Th2 lymphocytes and regulatory T cells. IL-10 inhibits the production of pro-inflammatory cytokines by Th1 lymphocytes and improves survival, proliferation and antibody production of B-lymphocytes. Therefore, enhanced IL-10 expression could represent a promising therapeutic approach for diverse pathologies in which immunosuppression is needed (Grutz, 2005; Mosser and Zhang, 2008).

As previously indicated, triple-transfected PSGL-1/sLeX/IL-10 MSCs injected in an mouse model of local inflammation in the ear, induced a transient increase in the levels of IL-10 in the inflamed ear, and mediated a superior anti-inflammatory effect *in vivo* compared to *wild type* MSCs (Levy et al., 2013). These results are also supported by the cited above study in which the authors demonstrated the enhanced anti-inflammatory potential of human AdMSCs transfected with a single mRNA encoding for the receptor CXCR4 and IL-10 (Hervas-Salcedo et al., 2021).

The administration of IL-10-transduced bone marrow allogeneic MSCs attenuated the severity of acute graft-vs.-host disease in a murine model, while unmodified MSCs were not able to control the disease progression (Min et al., 2007). Different studies found that serum levels of IL-10 in rheumatoid arthritis-suffering patients was lower than that found in healthy people, but some pro-inflammatory factors, such as IL-17, IL-1β and TNF-α, were higher (Baek et al., 2012; Shoda et al., 2017). Using an adenovirus system to overexpress IL-10, Tian et al. analyzed the therapeutic effect of IL-10-overexpressing bone marrow-derived MSCs (IL10-BMMSCs) in a collagen-induced rheumatoid arthritis rat model. After 4 and 8 weeks of treatment

IL-10-BMMSCs receiving rats improved significantly their clinical condition. The repairing rate of osteoarticular cartilage and the inhibition of synovial proliferation were higher in the IL-10-BMMSCs group than in the unmodified counterparts. Accordingly, serum levels of the pro-inflammatory cytokines IL-17, IL-1β, and TNF-α were also lower (Tian et al., 2019). In a model of *Escherichia coli* pneumosepsis in rats, IL-10 overexpression in umbilical cord derived-MSCs (UC-MSCs) enhanced the capacity to attenuate lung injury compared to unmodified UC-MSCs, due to increased macrophage phagocytosis and killing of *E. coli* (Jerkic et al., 2019). Recently, Zhao et al. also found that MSCs transfected with a recombinant plasmid IL10-PEGFP-C1 were able to suppress the pancreatic cancer cell proliferation *in vitro* and to reduce the growth of tumor xenograft *in vivo,* prolonging the mouse survival, inhibiting tumor angiogenesis and reducing blood levels of TNF-α and IL-6 in mice with tumors (Zhao et al., 2020).

IL-10 has been also claimed to play a neuroprotective and vasculoprotective role in cerebrovascular disorders by attenuating pro-inflammatory signals and by upregulating anti-apoptotic proteins (Zhou et al., 2009). Nakajima et al. investigated the therapeutic benefit of adeno-associated virus (AAV)-mediated IL-10 overexpression in MSCs transplanted during the acute phase of ischemic stroke in Sprague-Dawley rats. MSC-IL10 grafting significantly inhibited microglial activation and pro-inflammatory cytokine expression. Moreover, overexpression of IL-10 suppressed neuronal degeneration and improved survival of engrafted MSCs in the ischemic hemisphere (Nakajima et al., 2017).

In a rat model of myocardial infarction, Meng et al. transduced bone marrow-derived MSCs using an adenoviral vector to secrete IL-10 (Ad.IL-10−MSCs). These modified MSCs were transplanted into injured hearts resulting in reduced myocardial infarcted area, cardiac impairment and cell apoptosis. Even, genome-editing technology using transcription activator-like effector nucleases (TALENs) has been used to generate functionally improved amniotic MSCs (Meng et al., 2019). The administration of these IL-10 gene-edited amniotic MSCs in an acute myocardial infarction mouse model showed higher anti-inflammatory properties and enhanced recovery of heart function, also providing a favorable environment for neovascularization.

On the other hand, FoxP3-expressing MSCs prevent rejection of allogeneic grafted liver, increasing the median survival time of treated mice by increasing the numbers of Treg cells and the PD-L1 expression on MSCs (Qi et al., 2015). In other studies, HGF-expressing MSCs exhibited enhanced regenerative and anti-apoptotic effects in murine models of radiation-induced toxicity (Zhang J. et al., 2014; Wang et al., 2015), and induction of early immunosuppression in mice undergoing rheumatoid arthritis (Dong et al., 2020). Bcl-2 is also a robust anti-apoptotic protein, which has been overexpressed in MSCs. These cells ameliorated myocardial infarction damage in mice by increasing cell engraftment and VEGF-mediated neovascular formation (Li et al., 2007). Other factors overproduced by MSCs (i.e., lipocalin-2) indirectly improved their therapeutic capacity inducing production of regenerative factors such as HGF, IGF, FGF, and VEGF (Roudkenar et al., 2018). In fact, MSCs can secrete both angiogenic and anti-angiogenic factors in response to signals from microenvironment. For example, MSCs respond to TGFα/EGF receptor by increasing VEGF production (De Luca et al., 2011). On the other hand, VEGF signaling pathway is defective in TNFR2 KO mice (Luo et al., 2006), and a correlation between TNFR2 expression by MSCs and NO production, that directly induces VEGF, has been recently established (Beldi et al., 2020a). Previously, it has been found that VEGF production by TNF-α-primed human bone marrow MSCs was TNFR2 dependent (Crisostomo et al., 2008; Zhang A. et al., 2010).

As above mentioned, CXCL12/CXCR4 signaling pathway is important for *in vivo* MSC homing to injured sites but also increase VEGF expression, thus contributing to neoangiogenesis. Accordingly, CXCL12-secreting MSCs have improved wound healing, dermal fibroblast migration and new blood vessel formation (Nakamura et al., 2013), whereas CXCR4-overexpressing MSCs improved the outcome of myocardial infarction by increasing cell engraftment and angiogenesis and reducing myocardial remodeling (Huang et al., 2010; Zhang D. et al., 2010). Obviously, effects mediated by VEGF-overexpressing MSCs are related to the potent pro-angiogenic capacity of this molecule that improves the blood flow and the heart function in preclinical assays of critical limb ischemia (Beegle et al., 2016) and myocardial infarction (Zhu et al., 2012), respectively.

MSC overexpressing genes involved in osteogenesis, particularly BMP2 have been repeatedly tested in several types of bone defects, improving the bone healing (Chang et al., 2003, 2004, 2010; Tsuchida et al., 2003; Jiang et al., 2009; Zhao et al., 2010). Sometimes, BMP2 and VEGF overexpression have been combined. In these cases, VEGF promotes blood vessel neoformation that favors BMP2-mediated osteogenesis (Lin et al., 2010, 2011, 2012, 2015; Fu et al., 2015).

Engineered MSCs have been also used as anti-tumor therapeutic agents alone or in conjunction with chemotherapeutic drugs (Pawitan et al., 2020). Three strategies have been used: (i) to insert suicide genes that transform non-toxic pro-drugs into cytotoxic molecules; (ii) to use MSCs as vehicles to transport cytokines for enhancing *in vivo* anti-tumoral immunity or (iii) as agents to kill directly the tumor cells. Several genes encoding for "suicide proteins" have been used in anti-tumoral therapies, including cytosine deaminase, thymidilate kinase from either herpex simple or SV40 viruses, and cytochrome P450 reductase. In general, MSCs are resistant to these agents, particularly to alkylating agents, although evidence on the effects mediated by nucleoside analogs is scarce. IL-12 (Gao et al., 2010), IFNγ (Seo et al., 2011), and TNF-α (Tyciakova et al., 2015) have been overexpressed in engineered MSCs to enhance anti-tumoral immune responses. Another tested strategy is based on the use of MSCs to deliver pro-apoptotic agents to tumor cells. The most frequently used is the overexpression of TNF-related apoptosis inducing ligand (TRAIL), a transmembrane protein that binds to death domain-containing receptors that selectively trigger apoptotic of cancer cells (Luetzkendorf et al., 2010). Other approaches include engineered MSCs to release different anti-angiogenic factors

(Zheng et al., 2012) or oncolytic viruses (Yong et al., 2009). In general, most studies using these strategies work quite well in preclinical models but their efficiency in human patients has been very limited.

Modifications of the culture protocols also change the MSC functionality and would be considered in this section. MSCs in three-dimensional culture conditions (3D) have shown an upregulated expression of TSG6, SCT1, LIF, IL24, TRAIL, and CXCR4 (Potapova et al., 2008). MSC spheroids generate reduced levels of some pro-inflammatory molecules such as TNF-α, IL-1β, CXCL12, MIP-2, and PGE_2, and stimulate their pro-angiogenic activity, increasing their anti-fibrotic properties (Xu et al., 2016). It is important to remark that 3D spheroid cultures create a microenvironment where inner cell layers are exposed to lower levels of oxygen and nutrients generating a hypoxic environment (Cesarz and Tamama, 2016) that importantly affects the MSC biology (see below).

Furthermore, the nanoparticle use is being analyzed to enhance cell therapeutic efficacy. Engineering strategies that associate nanoparticles with MSC membranes have improved their homing ability, tumor tropism and attraction to inflammatory tissues (Wang et al., 2020). MSC-derived cell membrane coated nanoparticles have proven to be a useful biomimetic strategy to design therapeutic devices that have shown great potential in diagnostic and therapeutic applications. Among them, we can highlight the administration of drugs, immune modulation, vaccination and detoxification (Narain et al., 2017).

Hypoxia controls the MSC biology as well and it has been a target for improving their capabilities. In general, MSCs are cultured in normoxia, around 21% oxygen, but the optimal oxygen concentration can vary among tissues *in vivo*. The MSC niche is hypoxic, around 5% oxygen, compared to highly perfused organs. A low level of oxygen during the *in vitro* culture would make available a positive environment for MSCs to simulate their physiologic conditions. Thus, hypoxia could contribute to maintain the stemness and the proliferative capacities of MSCs during the *in vitro* culture. Choi J. R. et al. (2014) reported that adipose tissue-derived MSCs cultured in 2% oxygen tension maintained their stemness capacity, increased proliferation rate and enhanced their chondrogenic differentiation compared to MSCs cultured in normoxia with 21% oxygen. Indeed, numerous studies confirm hypoxia as a preconditioning factor of MSCs that induces increased production of pro-angiogenic factor (Liu et al., 2015), as well as anti-oxidative and anti-apoptotic effects in healthy and pathological conditions (Zhang W. et al., 2014).

These results were related with an increase in the expression of hypoxia-induced factor-1α (HIF-1α) under hypoxia (Choi J. R. et al., 2014). HIF-1α activation in MSCs cultured in hypoxia conditions induces increased expression of neovascularization promoters such as VEGF and angiotensin (Imtiyaz and Simon, 2010; Ahluwalia and Tarnawski, 2012). Roemeling-van Rhijn et al. showed that immunosuppressive properties of adipose tissue derived-MSCs were maintained under hypoxic conditions. The oxygen level had no effect on the proliferation of adipose tissue derived-MSCs and colony forming unit efficiency was similar under 1 and 20% oxygen. Also, they did not observe cell toxicity neither changes in the immunophenotype, except a downregulation in the expression of CD105 (Roemeling-van Rhijn et al., 2013). Martinez et al. transduced human dental pulp MSCs with a lentiviral vector codifying for HIF-1α. Compared to unmodified MSCs, HIF-1α-MSCs showed the same capacity to inhibit T cell activation, but HIF-1α-MSCs were able to impair DC differentiation more efficiently. As well, HIF-1α-MSCs induced higher attraction of monocytes, exhibited greater resistance to NK cell-mediated lysis and also exhibited a pro-angiogenic profile due to an increased expression of the chemokines CXCL12 and CCL5 and a complete loss of CXCL10 transcription (Martinez et al., 2017). Schive et al. investigated the *in vivo* therapeutic potential of hypoxic-cultured MSCs in a mouse model of streptozotocin-induced insulitis and hyperglycemia compared to MSCs cultured in normoxic conditions. Either hypoxic-cultured or normoxic-cultured MSCs were injected into this mouse model. Both groups of animals had higher pancreas insulin content compared to untreated control group, but the hypoxic-cultured MSC group had lower fasting blood glucose and improved oral glucose tolerance compared to untreated mice. The authors concluded that hypoxic preconditioning potentiates MSCs ability to protect against hyperglycemia *in vivo* (Schive et al., 2017).

Alternatively, 3D MSC cultures based on the different conditions of cultures vs. those of two-dimensional (2D) cultures constitute another way for improving the biological and therapeutic properties of MSCs. In fact, 3D MSC cultures reflect better the natural physiological environment than the 2D cultures. Thus, the use of 3D MSC cultures could mimic better the physiologic state of MSCs in their specific resident tissues and influence their paracrine mechanisms. The 3D MSC cultures are based on MSC spheroids encapsulated with various types of scaffolds such as hydrogels, polymers, hydrophilic glass fibers and electrospun silk fibroin meshes. Other approaches that are not based on the use of scaffolds include magnetic levitation, hanging drop microplates or ultralow attachment spheroid microplates (Langhans, 2018; Millan-Rivero et al., 2019; Sankar et al., 2019). The spheroid 3D cultures create a microenvironment in which inner layers are exposed to lower levels of oxygen and nutrients, resembling to a hypoxic environment that affects notably the MSC behavior. Compared to 2D cultures, 3D MSC cultures have shown an augmented secretion of molecules with paracrine function (i.e., cytokines, chemokines, and growth factors), better anti-oxidative and anti-apoptotic functions and higher production of extracellular matrix components (Cushing and Anseth, 2007; Sun et al., 2018; Mukherjee et al., 2020), as well as improved therapeutic effects in some preclinical models such as corneal or skin wound healing (Carter et al., 2019; Millan-Rivero et al., 2019).

This accumulating preclinical evidence on the promising potential of MSC-based cell therapy in the treatment of multiple diseases has allowed its translation to the clinical practice, having been launched to date more than 1,000 clinical trials. However, clinical trials based on the use of engineered MSCs are still very scarce, and only a few studies, mainly phase I and phase I/II, have

TABLE 1 | Clinical trials involving engineered MSCs in Clinicaltrials.gov website.

Study title	MSC source	Modification	Pathology	Phase	Identifier
MV-NIS infected MSCs for treating patients with recurrent ovarian, primary peritoneal or fallopian tube cancer	Adipose tissue	MSCs transduced with Edmonston's strain measles virus (MV) genetically engineered to produce sodium iodine symporter (NIS)	Recurrent ovarian, primary peritoneal or fallopian tube carcinoma or adenocarcinoma	Phase I/II	NCT02068794
Genetically Modified MSC Therapy Against Head and Neck Cancer (Gx-051)	N/A (Gx-051)	MSCs expressing modified interleukin-12 (MSCs/IL-12M)	Head and neck neoplasm	Phase I	NCT02079324
Osteogenic effects in human MSCs enhanced by Wnt signaling	Bone marrow	Viral administration of Wnt3a-transduced MSCs with hydroxyapatite nanoparticles	Osteoarthritis	Observational	NCT01323894
Efficacy and safety of allogeneic MSCs of bone marrow, cultured under hypoxia in the treatment of patients with severe pulmonary emphysema	Bone marrow	MSCs cultured under hypoxic conditions	Severe pulmonary emphysema	Phase I/II	NCT01849159
A single dose of BRTX-100 for patients with chronic lumbar disc disease	Bone marrow (BRTX-100)	Hypoxic-cultured bone marrow mononuclear cells highly enriched in MSCs from autologous bone marrow with autologous platelet lysate	Chronic lumbar disc disease	Phase II	NCT04042844
Intravenous infusion of fucosylated bone marrow MSCs in patients with osteoporosis	Bone marrow	Enzymatic exofucosylation by fucosyltransferase VIII and GDP-fucose treatment	Osteoporosis with low impact bone fractures	Phase I	NCT02566655

N/A, data not available. Fast track designation of commercial cell therapy products is also indicated.

been implemented to evaluate "MSCs-2.0" safety and efficacy in a variety of pathologic conditions summarized in **Table 1**, and their results are eagerly awaited.

On the other hand, in the last years, several studies have emphasized the presumptive relevance of the MSC secretome as a better way of treatment than the own cells for their clinical application (Poltavtseva et al., 2019), although reported results are frequently contradictory and the clinical assays using total secretome or extracellular vesicles (EVs) are limited. On the other hand, despite the difficulties for a conclusive definition of their phenotype, content and physiological function, EVs present some benefits, such as low immunogenicity, stability during extended storage and protection of their content (Kusuma et al., 2017). The term "secretome" includes diverse soluble molecules, such as growth factors, cytokines, immunomodulatory molecules and the named EVs (Kusuma et al., 2017). EVs are a heterogeneous population of lipid-bilayer vesicles that contain biologically active biomolecules such as lipids, proteins, single-stranded DNA and different types of RNAs (Bister et al., 2020; Watanabe et al., 2021). They include small exosomes (40–120 nm) originated from multivesicular bodies of the endosomal compartment that are secreted by exocytosis, and larger microvesicles (200–1,000 nm) that bud directly from the plasma membrane (Kusuma et al., 2017).

Moreover, MSCs derived from different sources produce some specific factors: adipose tissue-derived MSCs secrete more IGF-1, VEGF, and IL-8 than those from the bone marrow, whereas MSCs from the umbilical cord Wharton's jelly secrete the highest amounts of immunomodulatory molecules such as IL-6, IL-7, IL-10, PDGF-A, and TGFβ2. On the contrary, adipose tissue-derived MSCs produce more extracellular matrix components such as collagen-1 and -2, and metalloproteinases (Amable et al., 2014), and a common group of secreted molecules including chemokines (CCL2 and CCL5), growth factors (bFGF and IGF-1), cytokines (IL-6 and TGFβ) and others (TNFR-I) (Wang et al., 2019). However, not only soluble factors can be secreted by MSCs. Remarkably, mitochondria can be transferred between cells via tunneling nanotubes, cell fusion or contained into secreted EVs (Torralba et al., 2016; Morrison et al., 2017). Therefore, the secretome recapitulates many of the properties described for MSC themselves (Ferreira et al., 2018), including immunomodulation (Teng et al., 2015), inhibition of both apoptosis and fibrosis (Li et al., 2013; Teixeira et al., 2015), induction of vascularization (Teng et al., 2015) and promotion of tissue remodeling and cell recruitment (Chen et al., 2014). Furthermore, EVs derived from MSCs activated or not with IFNγ exhibit distinct capacities. Both EVs reduce the frequency of $CD14^{+}CD16^{+}$ inflammatory monocytes, but those derived from IFNγ-treated MSCs also promote anti-inflammatory PD-L1 expressing monocytes (Goncalves et al., 2017).

Although the number of clinical trials using MSC-derived EVs for the treatment of different pathologies is still very limited (see **Table 2**) and their conclusions unpublished, numerous preclinical studies support their functional capabilities. Different lung injuries improve after treatment with MSC-derived EVs.

TABLE 2 | Clinical trials involving MSC-derived EVs in Clinicaltrials.gov website.

Study title	MSC-ECVs source	Pathology	Phase	Identifier
Exosomes of MSCs for multiple organ dysfunction syndrome after surgical repair of acute type A aortic dissection	N/A	Surgical repair of acute type A aortic dissection	Not applicable	NCT04356300
MSC-exos promote healing of macular holes	Umbilical cord	Large and refractory macular holes	Phase I	NCT03437759
Effect of UMSCs derived exosomes on dry eye in patients with cGvHD	Umbilical cord	Dry eye symptoms in chronic GvHD	Phase I/II	NCT04213248
Safety and efficacy evaluation of allogeneic adipose MSC-exos in patients with Alzheimer's disease	Adipose tissue	Mild/moderate dementia associated to Alzheimer's disease	Phase I/II	NCT04388982
Effect of microvesicles and exosomes therapy on B-cell mass in type I diabetes mellitus	Umbilical cord blood	Type 1 diabetes mellitus	Phase II/III	NCT02138331
MSC-EVs in dystrophic epidermolysis bullosa	Bone marrow (AGLE-102)	Dystrophic epidermolysis bullosa	Phase I/II	NCT04173650
Expanded access protocol on bone marrow MSCs derived extracellular vesicle infusion treatment for patients with COVID-19 associated ARDS	Bone marrow (ExoFlo[1]")	COVID-19 associated acute respiratory distress syndrome	Phase II	NCT04657458 NCT04493242
Clinical study of mesenchymal stem cell exosomes nebulizer for the treatment of ARDS	N/A	COVID-19 associated acute respiratory distress syndrome	Phase I/II	NCT04602104
Pilot clinical study on inhalation of MSC exosomes treating severe novel coronavirus pneumonia	Adipose tissue	COVID-19 pneumonia	Phase I	NCT04276987
Effects of ASC secretome on human osteochondral explants	Adipose tissue	Osteoarthritis and/or articular regeneration	Observational	NCT04223622
iExosomes in treating participants with metastatic pancreas cancer with KrasG12D mutation	N/A	Metastatic pancreatic ductal adenocarcinoma	Phase I	NCT03608631
Allogeneic MSC derived exosome in patients with acute ischemic stroke	N/A	Acute ischemic stroke	Phase I/II	NCT03384433

N/A, data not available. Fast track designation of commercial cell therapy products is also indicated.

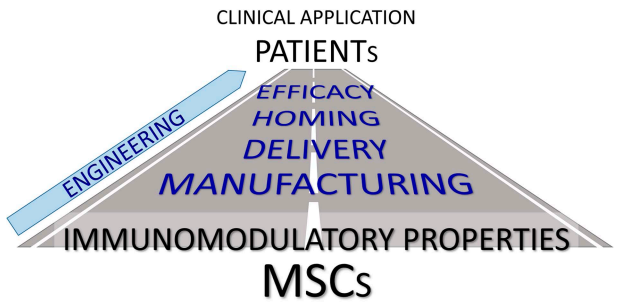

FIGURE 1 | Next steps to improve the immunomodulatory properties of MSCs to treat patients efficiently.

In acute respiratory distress syndrome (ARDS) models, the administration of MSC-derived $CD44^{+}$ EVs reduced the lung injury (Morrison et al., 2017). Remarkably, EV-mediated mitochondrial transfer induces a highly phagocytic and an anti-inflammatory macrophage phenotype (Morrison et al., 2017). In addition, MSC-derived exosomes remodel vascular network and diminish the hypoxia pulmonary hypertension in rodent models (Weiss et al., 2019). Systemically injected EVs have been shown to reduce both the collagen deposits and the inflammatory infiltrates in a murine model of silica-induced lung fibrosis (Choi M. et al., 2014).

The effects of MSC-derived EVs have been tested in other models of tissue fibrosis: treatment with EVs enriched in miRNA-let7c, a model of renal fibrosis, induced a downregulated expression of collagen IV, metalloproteinase-9, TGFβ1 and its receptor (Wang et al., 2016). Also, ECV enriched in miRNA-125b, that target Shh signaling activated in liver fibrosis, rescues liver progenitor cell expansion and stellate cell activation (Hyun et al., 2015). MSC-EVs containing miRNA22 improve cardiomyocyte survival in a murine model of myocardial ischemia-reperfusion (Arslan et al., 2013; Feng et al., 2014).

Interestingly, $CD69^{-/-}$ mice, which produce less exosomes, have shown significant reduced bone junctions. This problem can be recovered after injection of EVs isolated from MSC conditioned media, a process presumably mediated by RNAs (Furuta et al., 2016). EVs from embryonic MSCs promote cartilage regeneration in a rat osteochondral defect model by increasing both neoformation of tissue and extracellular matrix components (Zhang et al., 2016), whereas exposure of MSC-derived EVs increases stem cell engraftment in irradiated bone marrow (Wen et al., 2016). Effects of MSC-derived EVs on the immune system reflect their origins and tend to show immunosuppressive properties. MSC-derived EVs containing miRNA inhibit macrophage activation by controlling NF-κB activation and induce changes in the profile of expression of several immune molecules, including IL-1β, COX-2, IL-10, TNF-α, MyD88, TLR-1, -4, -5, -7-9, IRAK1, and TRAF6 (Phinney et al., 2015). On the other hand, EVs from MSCs obtained from bone

marrow increase the IL-10 production and the proliferation of Treg cells in PBMNC cultures stimulated through CD3/CD28 (Del Fattore et al., 2015; Dal Collo et al., 2020).

COMMENTS AND CONCLUSION

We have reviewed the current state of the art of the biology of MSCs with special emphasis to the advances that might improve their therapeutic efficiency. A summary of the topics discussed is shown in **Figure 1**. Firstly, we reported data on the phenotypical and functional characteristics of MSCs highlighting the difficulties to get specific markers that would allow to isolate enriched, homogeneous MSC subpopulations and to identify one or a few molecules masters for governing their properties.

MSC were used for the first time as cellular pharmaceutical agents in humans in 1995 (Lazarus et al., 1995). After several years utilizing MSCs as therapeutical agents, numerous questions on their behavior remain unsolved, including the heterogeneity of the MSC populations in the final product, the adequate conditions to activate *in vivo* their immunomodulatory capabilities, the consequences of the banking procedures, the best route for their delivery, the use of allogeneic vs. autologous cells, the problems to reach the target host tissues, their response to stressful conditions, specially hypoxia, or the real therapeutic relevance of products secreted by MSCs, such as the soluble fraction of their secretome or EVs.

Accordingly, we have summarized recent published results on these issues in an attempt to provide new approaches for a better clinical application of the named "MSCs-2.0." On the other hand, it is urgent a universal standardization of the protocols for manufacturing MSCs such as MSC culture and banking conditions, and also the route of cell delivery, the optimal dosage and the best way to use allogeneic vs. autologous MSCs. The available results on the effects of cryopreservation on MSC biology are contradictory and, although the use of allogeneic MSCs exhibit evident advantages in cell therapy, it is important to recognize their unquestionable immunogenicity, although immune responses elicited by allogeneic MSCs appear to be lower or less aggressive than autologous ones, presumably because a balance between immunogenicity and release of immunosuppressive factors is established in these circumstances.

Differences between MSC homing in preclinical models and humans must be conclusively clarified as well as the mechanisms governing the MSC homing into the target tissues by their relevance for a definitive establishment of the best route for MSC delivery, according to the disease to be treated. On the other hand, because MSC-mediated thromboembolism limits the MSC migration to the target tissues, this physiological condition, would be carefully evaluated before the systemic infusion of MSCs. Some simple, although transient, chemical manipulations of MSCs for improving their homing are highly promising but require technical optimization and a better knowledge on their consequences for the MSC biology. Also, usage of MSCs on scaffolds of diverse origin is complex and needs further research and improvement.

As indicated, to manipulate the whole factors known to affect MSC behavior is highly improbable since the therapeutic application of MSCs in a concrete disease requires the strengthening of the action of one or few discrete molecules. As summarized herein, genetic procedures have been attempted in the last years. However, this approach has the same problems than those reported in gene therapies applying other cell types. On the other hand, it is obvious that MSC engineering is a robust technology extensively tested in numerous experimental models but the translation of these results to the clinical practice need more time and research to be successful.

Apart from gene overexpression procedures, MSCs can be engineered changing the culture conditions by using 3D cultures or hypoxic conditions, or adhering distinct types of nanoparticles to the MSC membrane. Hypoxia has been frequently used as a preconditioning factor that favors MSC stemness and proliferation, and exhibit pro-angiogenic, anti-oxidant and anti-apoptotic effects.

EVs obtained from MSC secretome have provided in the last years numerous although frequently contradictory results but few effective clinical trials. Unfortunately, their heterogeneous condition, the lack of specific markers for establishing their true nature and, in general, the absence of information on the mechanisms controlling their effects, make difficult their therapeutic use, although increased numbers of clinical trials are being currently reported. It is therefore important to establish conclusively their real clinical value.

In summary, many of these research fields that try to improve MSC efficiency are ongoing with promising preclinical results, although the translation of their findings to the clinical practice seems to be yet remote.

AUTHOR CONTRIBUTIONS

DG-B, JS, MG-A, RY, RH-S, AC, MF-G, MH-R, ÓQ-B, JB, DG-O, JM, and AZ contributed to intellectual discussion of the findings. DG-B, JS, MG-A, RY, and AZ supervised the writing and drafting the manuscript. All the authors revised and approved the final version of the manuscript.

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PERSPECTIVE
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Therapeutic Effects of Mesenchymal Stromal Cell-Derived Small Extracellular Vesicles in Oxygen-Induced Multi-Organ Disease: A Developmental Perspective

*Angeles Fernandez-Gonzalez[1,2], Gareth R. Willis[1,2], Vincent Yeung[1,2], Monica Reis[1,2], Xianlan Liu[1,2], S. Alex Mitsialis[1,2] and Stella Kourembanas[1,2]**

[1] Division of Newborn Medicine, Department of Pediatrics, Boston Children's Hospital, Boston, MA, United States, [2] Department of Pediatrics, Harvard Medical School, Boston, MA, United States

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***Correspondence:**
Stella Kourembanas
stella.kourembanas@childrens.harvard.edu

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Despite major advances in neonatal intensive care, infants born at extremely low birth weight still face an increased risk for chronic illness that may persist into adulthood. Pulmonary, retinal, and neurocognitive morbidities associated with preterm birth remain widespread despite interventions designed to minimize organ dysfunction. The design of therapeutic applications for preterm pathologies sharing common underlying triggers, such as fluctuations in oxygen supply or in the inflammatory state, requires alternative strategies that promote anti-inflammatory, pro-angiogenic, and trophic activities—ideally as a unitary treatment. Mesenchymal stem/stromal cell-derived extracellular vesicles (MEx) possess such inherent advantages, and they represent a most promising treatment candidate, as they have been shown to contribute to immunomodulation, homeostasis, and tissue regeneration. Current pre-clinical studies into the MEx mechanism of action are focusing on their restorative capability in the context of preterm birth-related pathologies, albeit not always with a multisystemic focus. This perspective will discuss the pathogenic mechanisms underlying the multisystemic lesions resulting from early-life disruption of normal physiology triggered by high oxygen exposures and pro-inflammatory conditions and introduce the application of MEx as immunomodulators and growth-promoting mediators for multisystem therapy.

Keywords: development, BPD, preterm, oxygen, inflammation, multiorgan, MEx

INTRODUCTION

The survival of premature infants born at extremely low birth weight (ELBW) has improved dramatically due to recent advances in neonatal care (Matthews et al., 2015). However, 40% of infants born before 28 weeks of gestational age (GA < 28) are at risk of one or more long-term complications, as they often require prolonged mechanical ventilation and exposure to high and variable oxygen tension (Younge et al., 2017). Preterm ELBW infants, even those who do not

require supplemental oxygen, are exposed to "perceived" hyperoxia that often causes direct tissue damage and/or triggers maladaptive physiological responses. Such disruption not only impacts lung growth but can also lead to multiorgan dysfunction including cardiac, retinal, and neurological deficits as well as gastrointestinal and renal abnormalities (Weinberger et al., 2002; Johnson et al., 2011). Bronchopulmonary dysplasia (BPD) and other morbidities associated with prematurity, such as retinopathy of prematurity (ROP) and neurodevelopmental disabilities, share a common pathogenesis (i.e., inflammation and oxygen toxicity) not adequately addressed with current established therapies. Therapeutic advances based on new concepts and less invasive techniques are being currently tested to prevent BPD injury and the consequences of post-delivery respiratory care but do not target the underpinnings of perinatal diseases that accompany BPD. Moreover, the contribution of placental, microbial, and immune factors to the extrapulmonary sequelae of prematurity is just being recognized, and it is prompting the search for new therapeutic options (Collins et al., 2017).

Mesenchymal stem/stromal cell (MSC)-derived extracellular vesicles (MEx) have shown considerable anti-inflammatory and regenerative capacity in preclinical animal models, (Lee et al., 2012; Sdrimas and Kourembanas, 2014; Willis et al., 2018b), and a safety study of stem cell-derived extracellular vesicles in preterm neonates at high risk for BPD is currently ongoing (NCT03857841). The MEx advantage lies in their potential to modify immune, vascular, and parenchymal processes precipitated by preterm birth and exacerbated by oxygen fluctuations. This article will incorporate existing knowledge of the outcomes of prematurity and BPD as relating to MEx treatments in preclinical models of neonatal diseases, provide new information and perspective on the topic, and conclude by advancing the postulation that MEx can serve as a unitary therapeutic vector against systemic multi-organ pathologies of prematurity with common underlying triggers.

PREMATURITY AND ASSOCIATED ORGAN PATHOLOGY

BPD is a major cause of morbidity and mortality in surviving extremely preterm infants. Its incidence has increased between 2009 and 2012 (Stoll et al., 2015). BPD pathogenesis is triggered by prenatal factors, postnatal mechanical trauma, and hyperoxia during mechanical ventilation and is exacerbated by other stressors, including infection, inflammation, and pulmonary volume overload. The pathology of BPD has evolved during the past 50 years, thanks to the introduction of surfactant therapy (Jobe, 1999) and improvements in prenatal and neonatal care. Infants born during the late canalicular and early saccular stage of normal lung development are likely to develop BPD pathology that includes a reduced number of alveoli and secondary septa. The large decrease in surface area is associated with reduced and dysmorphic pulmonary microvasculature, leading to increased vascular resistance and pulmonary hypertension (Mourani et al., 2015). Survivors with severe BPD also manifest a deterioration of lung function and a higher risk of developing chronic obstructive pulmonary disease later in life (Broström et al., 2010).

The pathologic processes driving abnormal lung growth affect the development of other systems as well. It is estimated that about 25–50% of surviving preterm infants suffer neurodevelopmental deficits such as cognitive deterioration (Larroque et al., 2008; Linsell et al., 2018), functional disability (Johnson et al., 2009), behavioral and psychological abnormalities that manifest later in life (Olsen et al., 2018), and, in a small proportion of cases, development of cerebral palsy and motor impairment (Jarjour, 2015).

An additional outcome of extreme preterm survival and supplemental oxygen is the occurrence of ROP (Silverman, 2004). Advanced ROP may lead to retinal detachment and significant visual impairment (Tin et al., 2001; Sommer et al., 2014). Although, clinically, ROP is considered a vascular disease precipitated by the premature exposure to high oxygen tension, there is evidence that neurons within the retina are also affected, causing a reduction in visual acuity, perception, and performance with increasing age (Leung et al., 2018).

Current therapies designed to treat and prevent BPD and other prematurity-related complications attempt to ameliorate acute stress reactions and rapidly improve organ function. An update on the current pharmacological therapies for BPD has been reported by Michael and colleagues (Michael et al., 2018). For example, the use of postnatal corticosteroids reduces inflammation and facilitates extubation in ventilator-dependent preterm infants (Doyle et al., 2006). Milder ventilation approaches, targeted oxygen saturation levels, growth factors, antioxidants, and other drug therapies are also being considered as less invasive alternatives for the prevention and treatment of BPD (Collins et al., 2017). Anti-vascular endothelial growth factor (VEGF) and erythropoietin therapies are used in the treatment of ROP and preterm brain injury, respectively (Patel et al., 2016; Younge et al., 2017). However, these interventions have limited efficacy as they only target isolated organs and stages of the disease. More importantly, they may contribute to the development of a "new" and "evolving" pathophysiology of prematurity in which the long-term outcomes are difficult to predict. Preterm birth is associated with arrested structural or functional development of key organs, causing impairment that will likely persist into adulthood. Thus, the development of new protective strategies requires an understanding of the molecular and cellular vulnerabilities of developing organs during increased oxygenation and concomitant inflammation with the goal of finding common pathogenic mechanisms amenable to therapeutic intervention.

ORGAN DEVELOPMENT AND OXYGEN VULNERABILITY

The transition from *in utero* environment to extrauterine life in the preterm born neonate triggers a sudden rise of lung and systemic oxygen tension, and supplemental oxygen application intensifies their vulnerability to injury. Postnatal development of several organ systems including the lung,

brain, gastrointestinal tract, and lymphoid organs occurs during defined and particularly vulnerable stages in humans and rodents (Picut and Parker, 2017). Oxygen fluctuations during this stage fuel an inflammatory response and cause the arrested growth of parenchyma as well as dysregulated angiogenic and immune responses which can be regarded as having a common mechanistic origin.

Lung

Contrary to what occurs in full-term human neonates that are born during the alveolar stage, rodents are born at a term during the saccular stage of lung development. From approximately postnatal (P) day 4–P21, the sacculae undergo thinning and reconstruction in a process termed alveolarization. Capillaries also grow superimposed to the process of alveolarization to create a network for blood supply and air gas exchange. The lungs of human preterm newborns experience an increase in arterial oxygen tension, even without supplemental oxygen, which leads to arrested alveolarization and vascularization. Preclinical studies have demonstrated that low antioxidant levels underlie the activation of transcription factors and pathways leading to endothelial and alveolar type II cell dysfunction and survival and the inactivation of surfactant (Stenmark and Abman, 2005). Exposure to hyperoxia also results in pulmonary accumulation of inflammatory cells and increased cytokine production (Ambalavanan et al., 2009).

Brain

In humans, the period of rapid brain growth and therefore greater vulnerability occurs during the last 3 months of pregnancy and continues postnatally with outgrowth and reorganization of the central nervous system. Similarly, although rodent brain development is generally completed at birth, cell apoptosis, neuronal pruning, and cell migration are still occurring by P7 and not completed until P14, while myelination progresses beyond P21, the equivalent of a 2-year-old human (Semple et al., 2013). Moreover, blood vessels in the rodent brain develop in parallel with the cortical parenchyma, due in part to their association with microglia during the first week of postnatal development (Mondo et al., 2020). A major pathological feature of preterm brain injury and early postnatal exposure to toxic levels of oxygen is the presence of white matter lesions. Oligodendrocyte precursors are highly sensitive to oxygen fluctuations (Back and Miller, 2014), and hyperoxia induces their apoptosis and reduces their proliferation, causing white matter deficiencies that persist well beyond the juvenile age (Schmitz et al., 2011). In addition, other cellular changes including abnormal glial maturation and phenotype (Schmitz et al., 2011; Leaw et al., 2017), cell death (Truttmann et al., 2020), and disrupted cortical development and neuronal maturation have been described in preclinical and preterm brain injury (Fletcher et al., 2017; Fleiss et al., 2020).

Retina

Hyperoxia also plays an initiating role in the pathogenesis of ROP in preterm infants and in experimental models of oxygen induced-retinopathy (OIR) (Vessey et al., 2011; Zin and Gole, 2013). Retinal vascularization in humans is completed at 36–40 weeks post-conceptual age. Preterm birth suddenly exposes the incompletely vascularized retina to hyperoxia, leading to vascular structural and functional compromise. Glial cells have been implicated in the genesis of retinal pathology in animal models of OIR, as they support the development of the vasculature and regulate cell apoptosis and neuronal metabolism and activation (Vessey et al., 2011; Shen et al., 2012). Müller cells express angiogenic growth factors such as VEGF and up-regulate the expression of glial fibrillary acidic protein (GFAP) as a hallmark of reactive gliosis in response to pathological oxygen tension. Microglia, like those in the brain, are generally considered immune cells, but they also contribute to the vascular and neuronal pathologic effects of oxygen-induced toxicity.

It is clear that perinatal oxidant stress, inflammation, and other toxic stimuli adversely impact the premature neonate cared for in modern neonatal intensive care units, and these result in acute and long-term injuries. The lack of regenerative therapies addressing the multiple organ pathologies occurring during this critical developmental stage highlights the need to consider a new, for the lack of a better term, "holistic" approach that can redirect normal development and enhance early organ restoration.

MEx AS A REGENERATIVE AGENT FOR PERINATAL PATHOLOGIES

Therapies based on the MSC secretome have gained increased attention as promising candidates for a multifaceted approach, impacting multiple pathological aspects of premature infant disease. MSCs, as most cells, secrete a plethora of heterogenous extracellular vesicles (EVs) of diverse biogenetic origins. The molecular composition and associated bioactive cargo of each EV class reflect both their biogenesis as well as the specific stimulus triggering their formation. EV biogenesis and nomenclature have been extensively reviewed by Yeung et al. (2019). On a gross level, EVs are categorized into three subclasses: small EVs (sEVs) (∼30–150 nm in diameter) represent the class that includes the exosomes, i.e., EVs generated through the endosomal pathway; microvesicles (∼100 nm–1 μm in diameter) are generated through budding from the plasma membrane, and apoptotic bodies (>1 μm) are generated through decomposition of apoptotic blebs. These subclasses of vesicles, defined through their biogenesis and biophysical properties (such as size, density, and predominant protein markers), remain vaguely defined, especially in terms of functionality, due to limitations in EV isolation and the absence of universally accepted characterization methodologies. The International Society of Extracellular Vesicles spearheads the efforts to establish standardization in nomenclature, definitions, and methodology in this field (Lener et al., 2015; Théry et al., 2018; Witwer et al., 2019).

Although the mechanisms by which MEx induce their therapeutic effect are only partially understood, they appear to promote tissue restoration through the modulation of immune cell phenotype in the inflamed tissue microenvironment. Our

group has demonstrated that MEx suppress the levels of proinflammatory cytokines (IL6, TNF-α) and modulate the expression of anti-inflammatory markers (Cd206, Arginase-1) in proinflammatory (M1-like, LPS + IFNγ) and pro-remodeling (M2-like, IL4 + IL13) macrophages, respectively (Willis et al., 2018a). In the neonatal lung, a polarized "M2-like" macrophage phenotype is critical for the process of alveolarization and normal lung development (Jones et al., 2013). Immune system homeostasis, disrupted by high oxygen levels in murine models of BPD, appears to be restored by MEx treatment.

Paramount in the pursuance of MEx-based therapy for premature infant disease is the uniformity in MEx preparations. This emphasizes the need of reproducibility in the parental MSC clone characteristics, and factors such as age, gender, and health status should be taken into consideration when selecting MSC donors (Willis et al., 2017). MEx produced by either human umbilical cord Wharton's jelly or bone marrow MSCs have been used in preclinical studies, and they have been shown to be equally efficacious (Willis et al., 2018a).

Our group has extensively explored the role of MEx as vectors for lung-targeted therapy. MEx delivery suppressed pulmonary inflammation and the development of pulmonary hypertension and vascular remodeling in a model of hypoxia-induced lung injury (Lee et al., 2012) while preventing and reverting core features of pulmonary fibrosis, lung inflammation, and aberrant pulmonary morphology induced by bleomycin administration (Mansouri et al., 2019). Importantly, we have demonstrated a therapeutic effect of MEx in experimental BPD, ameliorating disrupted alveolarization and angiogenesis and restoring pulmonary function in adults. These actions were mostly mediated through the anti-inflammatory action of MEx on pulmonary macrophage number and phenotype (Willis et al., 2018a). Supporting results were also reported by other investigators in the field (Ahn et al., 2018; Braun et al., 2018; Chaubey et al., 2018).

MEx have been reported to restore myelination and functional outcome in experimental adult rodent models of demyelinating disease (Laso-García et al., 2018). Furthermore, MEx ameliorate white matter injury (Ophelders et al., 2016), inflammation, and gliosis (Drommelschmidt et al., 2017) and protect against impaired growth and altered cortical development from ischemic insults in models of neonatal preterm brain injury (Sisa et al., 2019). In addition, the therapeutic effect of MEx administration in ROP has been demonstrated in preclinical models. In OIR, MEx protect against retinal ischemia by preserving vascular flow and retinal thinning and enhancing functional recovery while decreasing inflammation and apoptosis. Importantly, these studies indicated that EVs are taken up by retinal neurons, retinal ganglion cells, and microglia (Moisseiev et al., 2017). Other investigators, using human placental amniotic membrane-derived MSCs, have also shown reduced pathological neovascularization in OIR (Kim et al., 2016). Collectively, MEx hold a great promise to potentially enhance the recovery of multiple organs and tissues. Their pleiotropic beneficial effect warrants their translation into clinical applications for ameliorating multifactorial pathologies, underlying not only BPD but also other prematurity-associated diseases.

Considering that the pathobiology of the preterm newborn involves multiple organ systems that share common mechanisms (i.e., inflammation precipitated by oxygen toxicity), we considered it essential to explore the impact of systemic administration of MEx on postnatal lung, brain, and eyes using a model of multisystemic injury induced by hyperoxia exposure (see **Figure 1A** legend for section "Materials and Methods"). As demonstrated previously by our group (Willis et al., 2018a), hyperoxia (Hyrx) exposure to postnatal day 1 (P1) mice caused disrupted lung alveolarization and vascularization that were prevented with a single dose of MEx (**Figure 1B**). We now report that analysis of the retina in the same injured animals showed restoration of the retinal structure, decreased gliosis, and re-establishment of microglial homeostasis (**Figure 1C**) produced by Hyrx exposure, emphasizing the synergistic action of systemic MEx on immune and glial cell activation. In addition, white matter in the brain (**Figure 1D**) was preserved while astrogliosis, microglial response, and aberrant neuronal cortical pattern caused by Hyrx were reverted to normoxic (Nrmx) levels by MEx treatment, indicating a beneficial effect on myelination, gliosis, and neuronal density as the brain repairs and matures.

THE REGENERATIVE CAPACITY OF MEx TREATMENT FOR POSTNATAL OXYGEN-INDUCED MULTIPLE ORGAN SYSTEM INJURY: PERSPECTIVES FROM NEWBORN MEDICINE

These and previously reported results indicate that, in addition to lung, the retina and brain, known to be also vulnerable to the detrimental effects of hyperoxia exposure, can benefit from an early systemic administration of MEx. Some pathologic mechanisms involve the activation of immune cells and supportive glial cells that are critical for the maintenance of the vasculature and tissue homeostasis and suggest common pathways and processes amenable for therapeutic intervention (**Figure 2**). Although the detailed molecular mechanisms of MEx action are still the focus of intensive investigation, the beneficial effects MEx bestow on perinatal pathologies seem to principally rely on their immunomodulatory actions on tissue-resident immune cells. Alveolar macrophages in the lung and microglia in the brain revert to their anti-inflammatory polarization state in response to MEx, favoring remodeling and tissue repair. In addition, MEx effects on brain and retina seem to suggest that, directly or indirectly through parenchymal glial cells, MEx promote normal vascular function and neuronal integrity. Although the major systemic action of MEx appears to be the modulation of inflammatory states precipitated by oxygen toxicity, we should note that their impact on myelination has been reported, in certain studies, to be associated with their ability to

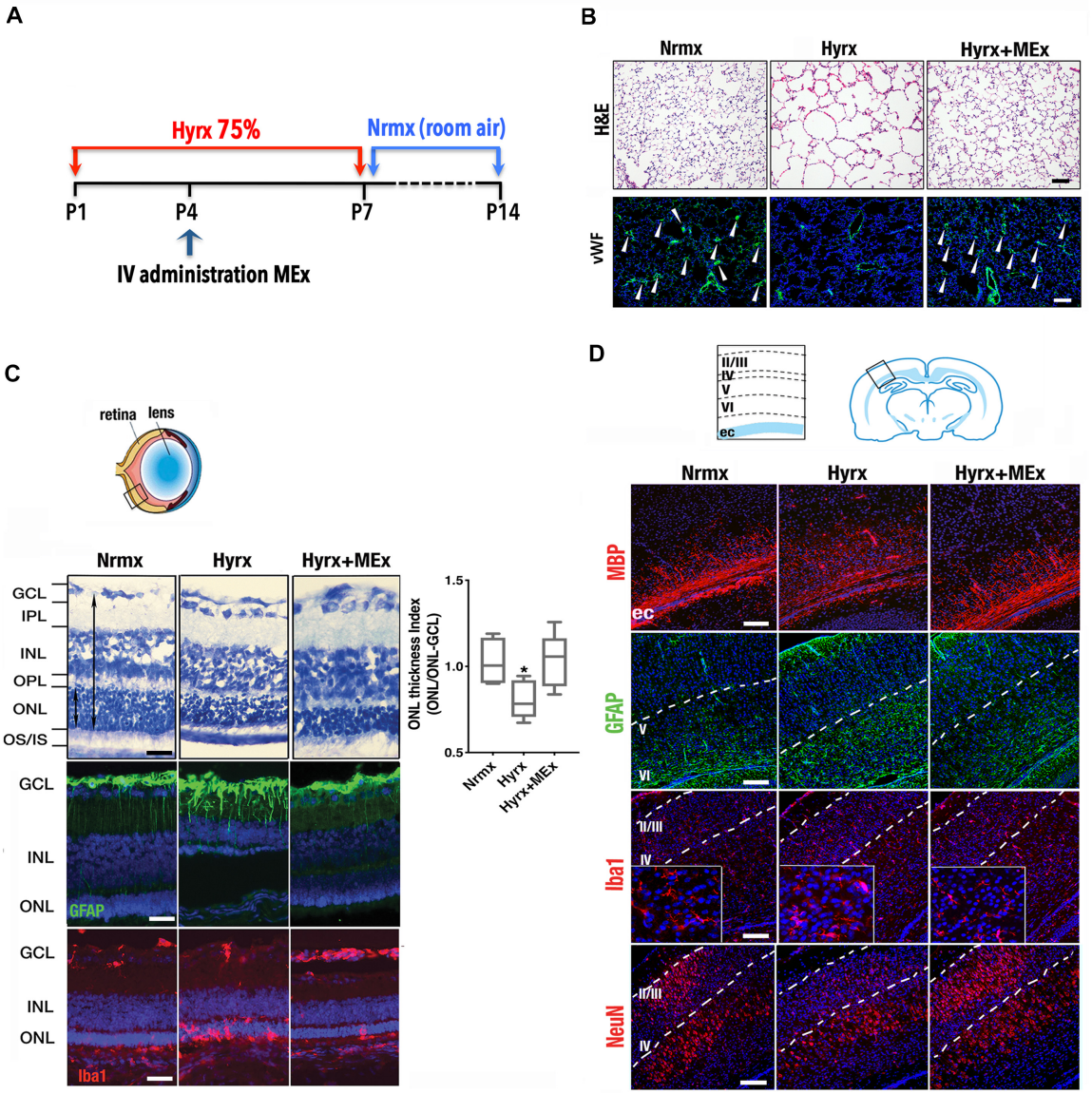

FIGURE 1 | Mesenchymal stromal cell (MSC)-extracellular vesicle (MEx) treatment reduces systemic cellular and inflammatory responses. **(A)** Schematic showing the experimental design. Neonatal mice were exposed to hyperoxia (Hyrx; 75% O_2) from postnatal day 1 (P1) to P7 and returned to room air (Nrmx) from P7 to P14 (this exposure modality impacts both alveolarization and myelination, as these processes progress postnatally). Hyrx mice were compared with age-matched control mice that remain in Nrmx conditions. The treated mice (Hyrx + MEx) received a single IV dose of MEx (50 μl corresponding to the product generated by 0.5×10^6 MSCs injected *via* the superficial temporal vein) at P4 and were sacrificed immediately after Hyrx exposure (P7, period of maximal vulnerability for microglial activation and cortical migration) or after an additional 7 days in Nrmx (P14). MEx belong to a subset of "small" EVs that includes exosomes and were derived from either human umbilical cord Wharton's jelly or bone marrow MSCs and purified in accordance with the 2018 Minimal Information for Studies of Extracellular Vesicles (Théry et al., 2018). Lungs, brains, and retinas were harvested, fixed, and processed for immunohistochemical and immunofluorescence analyses. **(B)** Disrupted alveolarization and vascularization in P14 Hyrx lungs was prevented with a single dose of MEx as shown in hematoxylin and eosin and von Willebrand factor-stained sections, respectively. Scale bar = 100 μm. **(C)** Hyperoxic injury of P14 retinal layers was prevented by MEx. Hyrx decreased retinal thickness as assessed with toluidine blue staining as a ratio of outer nuclear layer (ONL) thickness/ONL-ganglion cell layer (GCL) distance and shown to be restored with a single dose of MEx at P4 (Hyrx + MEx). Data are shown as mean ± SEM; $n = 4$ per group. $^*P < 0.05$ vs. Nrmx (ANOVA followed by Tukey's comparison test). Hyrx-induced gliosis at P14 is shown by GFAP immunofluorescence labeling of Müller cell bodies that are close to the inner nuclear layer with projections into the outer retina. MEx administration reverted Müller cell activation to Nrmx levels. Microglia activation and invasion into the ONL in P14 Hyrx retinas are depicted by increased ionized calcium binding adaptor molecule (Iba-1) immunofluorescence. MEx treatment restores microglial morphology and prevented invasion from injury. Scale bar = 25 μm. **(D)** Schematic depicting the cortical layers from which images were obtained. Myelin basic protein immunofluorescence showing marked staining decrease in the external capsule (ec) from P14 Hyrx mice and amelioration of white matter loss after a single dose of MEx at P4. Astrogliosis, as denoted by GFAP immunofluorescence staining at P14, was increased in early-exposed Hyrx mice but attenuated in the brains of MEx-treated littermates, particularly in areas in close association with white matter corresponding to the ec. Large Iba-1 positive cell bodies and thickened processes in P7 Hyrx cortex appear activated in comparison to Nrmx microglial cells. MEx normalized microglial morphological appearance in treated-Hyrx brain. Neuronal nuclear protein immunofluorescence staining of cortical P7 Hyrx brains, showing a less defined neuronal labeling pattern. MEx treatment restored cortical lamination after injury to Nrmx levels. Scale bar = 50 μm. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IV, intravenous; ONL, outer nuclear layer; OS/IS, photoreceptor outer and inner segments junction; OPL, outer plexiform layer.

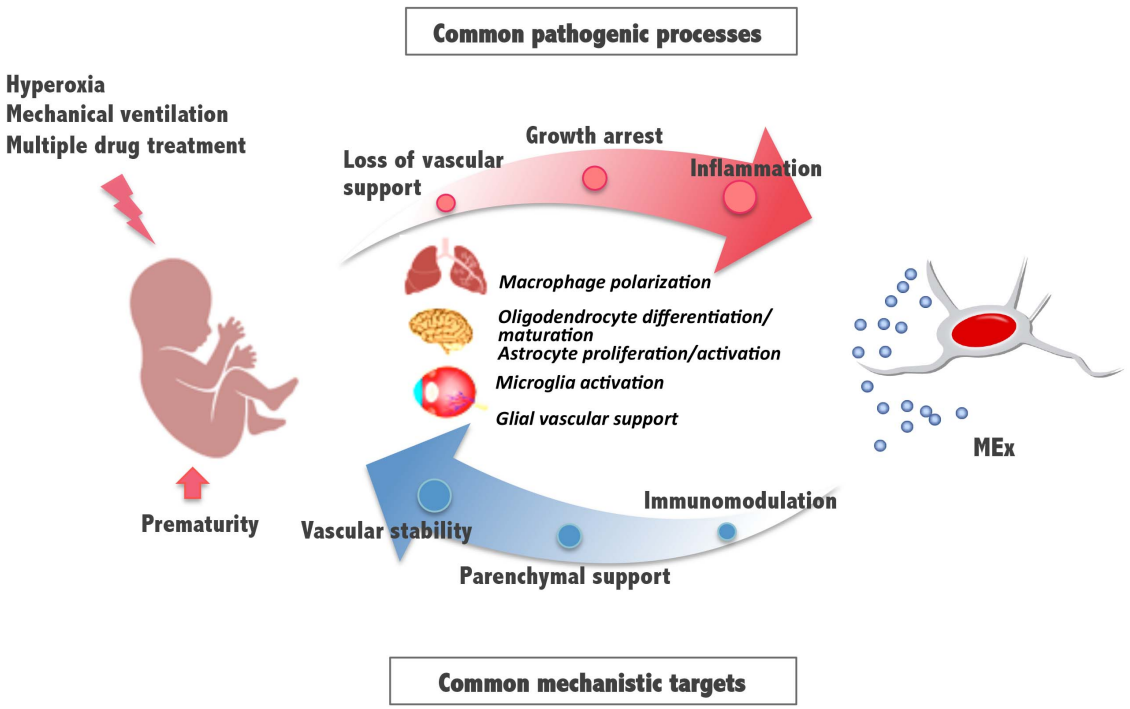

FIGURE 2 | Schematic diagram illustrating mesenchymal stromal cell-extracellular vesicle (MEx) as an alternative systemic therapy for neonatal pathologies with common cellular targets. Prematurity and postnatal stressors can trigger pathological processes such as inflammation, growth arrest, and loss of vascular support, all of them essential to the function of the developing lung, brain, and retina. Multiple actions of MEx include modulation of macrophage and microglial cell activation and polarization, cell differentiation and maturation, and glial maintenance, all common mechanistic targets that support immunomodulation, preservation of parenchymal integrity, and vascular stability.

promote oligodendrocyte differentiation and maturation (Otero-Ortega et al., 2018).

Following early systemic administration, MEx can be delivered to injured developing tissues and improve organ development through anti-inflammatory, angiogenic, and pro-survival mechanisms. This early intervention would require the development of reliable biomarkers of disease and better brain and lung imaging tools, thus allowing the identification of patients likely to develop BPD and associated illnesses as early as possible. Most current investigations of live cell MSC-based or MEx-based therapeutic approaches are focused on evaluating the safety and efficacy of these treatments after preterm birth. However, efficient MEx therapies should also consider the effects on the host immune system. As preterm neonates are more skewed toward a tolerogenic development and are more susceptible to infections, the immunomodulatory impact of MEx treatment has to be considered when applying those therapies in compromised newborns with risk of infection. Efforts must be made to minimize contamination and aid consistency in MEx preparations. Finally, given the systemic effect of MEx, the advancement of *in vivo* tracing methods will help elucidate their interaction with target organs and help decipher the contribution of circulating EVs on systemic signaling and immunomodulation during the critical period surrounding preterm birth and the development of lung and multiorgan complications.

CONCLUSION

This perspective advances the postulation that common triggers and pathways underlying dysregulated angiogenesis, growth, and immune system development converge in precipitating systemic neonatal injury in susceptible organs, including but not limited to the lung, brain, and retina. This demands new approaches to simultaneously address the potential pathogenic mechanisms of BPD and associated pathologies of prematurity. Numerous reports of preclinical studies, including those presented here, highlight the protective potential of MEx treatment against such early-life injuries. A phase I clinical study on the effects of MEx treatment in BPD is currently in progress, and basic research studies defining the molecular and cellular mechanisms responsible for the protective effects of MEx are actively underway. We are confident that future research in MEx therapies based on these strategies will lead to a more holistic approach for effectively preventing or treating complications of prematurity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Boston Children's Hospital Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AF-G and GW participated in study design and execution, data collection, and data analysis. VY and MR participated in study execution and data analysis. XL participated in study execution and technical assistance. SAM and SK contributed to study design, supervision of study execution, manuscript writing, data analysis, and final article editing and approval. All authors contributed to the article and approved the submitted version.

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REVIEW
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Translational Animal Models Provide Insight Into Mesenchymal Stromal Cell (MSC) Secretome Therapy

*Rebecca M. Harman†, Charlotte Marx† and Gerlinde R. Van de Walle**

Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY, United States

The therapeutic potential of the mesenchymal stromal cell (MSC) secretome, consisting of all molecules secreted by MSCs, is intensively studied. MSCs can be readily isolated, expanded, and manipulated in culture, and few people argue with the ethics of their collection. Despite promising pre-clinical studies, most MSC secretome-based therapies have not been implemented in human medicine, in part because the complexity of bioactive factors secreted by MSCs is not completely understood. In addition, the MSC secretome is variable, influenced by individual donor, tissue source of origin, culture conditions, and passage. An increased understanding of the factors that make up the secretome and the ability to manipulate MSCs to consistently secrete factors of biologic importance will improve MSC therapy. To aid in this goal, we can draw from the wealth of information available on secreted factors from MSC isolated from veterinary species. These translational animal models will inspire efforts to move human MSC secretome therapy from bench to bedside.

Keywords: mesenchymal stromal cells, stem cells, secretome, human, veterinary, translational models

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Anastasia Efimenko,
Lomonosov Moscow State University, Russia
Borhane Guezguez,
German Cancer Consortium, German Cancer Research Center (DKFZ), Germany

****Correspondence:***
Gerlinde R. Van de Walle
grv23@cornell.edu

† These authors have contributed equally to this work

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are adult multipotent progenitor cells found in many organs and tissue types. Due to their relative ease of isolation and expansion in culture, combined with the lack of ethical constraints associated with the collection and manipulation of embryonic stem cells, MSCs hold great promise as a multi-faceted cell-based therapy (Pittenger et al., 2019). Originally considered as whole-cell therapy, whereby injected MSCs migrate to the site of tissue damage and differentiate into cells needed for repair or regeneration, it is now accepted that transplanted MSCs do not survive for long and that the effects of MSC-based therapies are due to a broad array of secreted bioactive factors, collectively referred to as the secretome (Maguire, 2013; Moll et al., 2020; Wu et al., 2020). The recognition that MSC secreted factors are responsible for the positive effects of MSCs on tissue repair is significant, as it spurs the design of MSC-based therapies that do not require administration of the cells themselves, thus avoiding negative immune reactions or unwanted tumor growth (Sun et al., 2019).

The secretome of cells in general, is a commixture of soluble factors as well as molecules associated with extracellular vesicles (EV); lipid bilayer delimited particles of various sizes and complexities containing proteins and nucleic acids released from cells into the extracellular space. Soluble factors, such as nucleic acids, proteins, and lipids, can all be detected in the cellular secretome, at various concentrations and activity levels determined by cell type and environment (Daneshmandi et al., 2020). The human MSC secretome is no exception and has been characterized as containing EV (Gowen et al., 2020), a multitude of regulatory non-coding RNAs (Harrell et al., 2019), as well as an abundance of proteins including growth factors, cytokines, peptides,

and hormones (Abbasi-Malati et al., 2018). Lipid mediators are less well documented but have been described as active factors released by human MSC (Vasandan et al., 2016) (**Figure 1**). A solid understanding of the individual bioactive factors secreted by MSCs that affect injured target cells or tissues is indispensable to refine MSC secretome-based therapies and is not contradictory to evidence that cell free-based treatments greatly benefit from the administration of the complete secretome (Eleuteri and Fierabracci, 2019; Daneshmandi et al., 2020).

The MSC secretome has been actively explored for over 25 years (Haynesworth et al., 1996) but despite promising preliminary studies, no MSC secretome-based therapies are approved by the United States Food and Drug administration (FDA) for use in human medicine. This is in part because the secretome is not definitively characterized and typically varies significantly between MSC cultures dependent on individual donor, tissue source of origin, culture conditions, and passage (Rizk et al., 2016). To (i) help define the MSC secretome, (ii) understand which molecules secreted by MSCs are therapeutically valuable, and (iii) learn how to manipulate MSC to preferentially secrete these molecules, we can take advantage of information obtained from studies of MSCs isolated from veterinary species, many of which are relevant translational models for human conditions. The wealth of reports describing the activity of specific factors secreted by MSCs from veterinary species, as well the manipulation of these MSC to secrete factors of biological interest, will benefit human medicine by expanding the general knowledge of MSCs. And, by virtue of the fact that many veterinary species suffer from diseases that are physiologically analogous to human conditions that could be treated with MSC-secreted factors, they serve as relevant translational models.

This review starts with a brief overview of secreted factors from naïve human MSCs and laboratory rodent MSCs, which historically have been considered well-accepted animal models for human research. This is followed by a review of studies in which human or laboratory rodent MSCs were manipulated to improve inherent properties to optimize the therapeutic benefits of MSC secreted factors. Next, we provide an extensive overview of studies on the secretome of naïve, as well as manipulated, MSCs isolated from veterinary species, which function as physiologically relevant translational models for human MSC secretome-based therapies but are less well accepted.

THE SECRETOME OF MESENCHYMAL STROMAL CELLS (MSCs) FROM HUMANS AND LABORATORY RODENTS

Naïve Human MSCs

The secretome of human MSCs regulates a wide variety of physiological processes. The pioneering studies of the effects of these secreted factors on specific target cells or in experimental rodent models have been extensively reviewed elsewhere (Vizoso et al., 2017; Abbasi-Malati et al., 2018; Eljarrah et al., 2019; Harrell et al., 2019). To provide an overview of the research conducted on human MSC secreted factors without being redundant, **Table 1** summarizes human MSC secretome studies that have been published in more recent years (2018–2020). The majority of these studies evaluates either the effects of the complete secretome or exosomes (EXOs), a class of EV. An advantage to delivering MSC-secreted factors as EXOs is that EXOs can cross physiological barriers *in vivo*, which makes them attractive as treatments for diseases in tissues with restricted drug access such as the retina or the central nervous system (CNS) (Alvarez-Erviti et al., 2011; Zhang et al., 2015). Many of the studies do not identify the specific MSC secreted factors that mediate the observed effects on target cells or tissues, which illustrates the difficulty of determining precisely which factors in the complex secretome are responsible for biological responses.

Naïve Rodent MSCs

As relatively inexpensive, well-accepted models for human disease, rats and mice have been used for evaluating MSC-based therapies for human diseases. Of the many published rodent MSC studies, **Table 2** gives an overview of recent work (2013–2018) focused specifically on MSC secretome components. As with the human studies, the results tend to promote the utility of the entire secretome and/or or EXOs, as opposed to confirming the specific molecules responsible for the observed biological effects.

Manipulated MSCs

Although it is clear that MSCs have great therapeutic potential and are being explored in multiple medical fields, results are often inconsistent and (pre-)clinical studies show only minor effects or do not lead to the desired outcomes at all (Lukomska et al., 2019; Wilson et al., 2019). A potential reason for the observed inconsistencies is the heterogeneity of cultured MSCs, which is influenced by the individual donor and tissue of origin, isolation technique, culture environment, and cell passage number (Wilson et al., 2019; Harman et al., 2020). Purposefully manipulating MSCs to improve therapeutic benefits, which by default could result in a more standardized and/or customized secretome, is not a novel idea, but one that has not yet been maximally explored (Liesveld et al., 2020; Ocansey et al., 2020).

The methods by which MSCs are manipulated to control secretome components broadly fall into two categories: (i) priming and (ii) genetic modification (**Figure 2**). Priming MSCs to improve their immunomodulatory properties, migratory potential, and/or hypo-immunogenicity, has become a field of intense research. The most popular strategies for priming include treatment with pharmacological or chemical agents, stimulation with cytokines, alterations of culture conditions via use of bio-scaffolds and/or 3D cultures, and the use of hypoxic culture conditions (**Figure 2**). The various strategies to influence human MSC behavior via priming have been reviewed in detail recently (Noronha et al., 2019). Genetic modification of MSCs to silence or overexpress genes of interest via transfection and/or transduction is gaining increased attention (**Figure 2**). Transfection approaches include microinjection, electroporation, and nanocarriers (including polymers, lipids, polysaccharides, peptides, and inorganic materials) (Hamann et al., 2019). Transduction, using viral vectors such as lentivirus

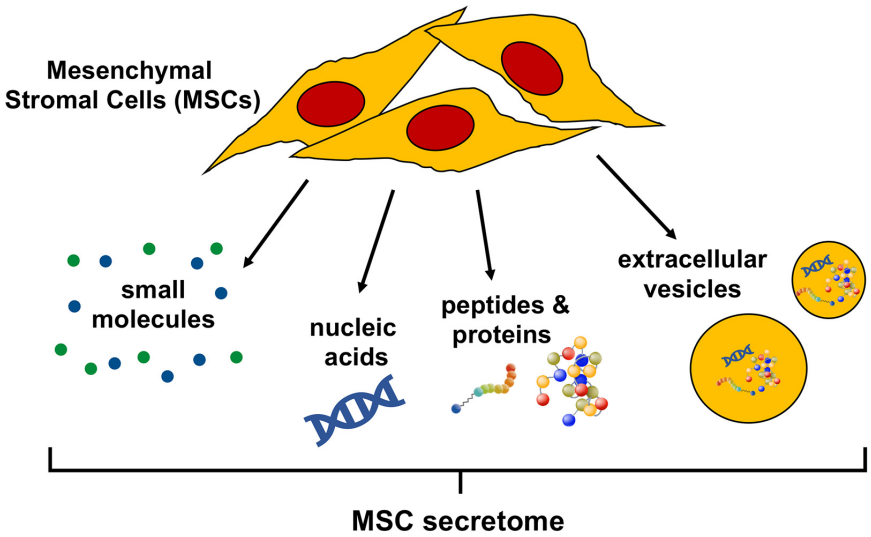

FIGURE 1 | Bioactive components of the mesenchymal stromal cell (MSC) secretome. The MSC secretome is comprised of all factors secreted by MSCs. The bioactive components of the secretome include a wide range of small molecules, nucleic acids (importantly, regulatory RNAs), peptides, and proteins. These molecules can either be released freely or packaged in extracellular vesicles, which are lipid bilayer-delimited particles of various size and composition.

and adenovirus, has the advantage of being more efficient than transfection, however, safety concerns related to potential immunogenicity and mutagenicity of the viral vectors are disadvantages of this technique for clinical use. MSCs secrete miRNAs, which are short oligonucleotides with critical post-transcriptional regulatory functions that are either released within EV or protein-associated, where miRNAs are vesicle-free but associated with high-density lipoproteins or Argonaute-2/nucleophosmin-1 (Chen et al., 2012). Overexpressing or inhibiting miRNAs in MSCs is considered a valuable approach to improve clinical outcome (He and Hannon, 2004; Yeo et al., 2013) (**Figure 2**).

A potential, albeit minimally explored at present, method of manipulating the MSC secretome is via the exogenous bioengineering of isolated EVs. Approaches to bioengineer EVs secreted from various cell types and their therapeutic applications are the topic of a recent review (Wiklander et al., 2019). While these methods have not been widely used for MSC-derived EVs, they could become an interesting approach to control the composition of bioactive factors in the MSC secretome.

Manipulated Human MSCs

The manipulation of human MSCs to enhance secretion of desired factors, to increase homing abilities, and/or to decrease their immunogenicity, has been reviewed in depth by other authors (Najar et al., 2019; Costa et al., 2020; Ullah et al., 2019). Here, we will give a short overview of recent studies in which human MSCs have been manipulated either by priming or genetic engineering (**Table 3**). Of note is that studies of human MSCs are usually conducted *in vitro*. In order to investigate the effects of manipulated MSC *in vivo*, researchers typically fall back on laboratory rodent models, as outlined below, raising the question of direct translation from mice to men. As an exception, pilot studies and clinical trials have been conducted in humans using MSC for gene-directed enzyme/prodrug therapy (GDEPT) (von Einem et al., 2017, 2019). With the GDEPT approach, MSCs are solely used as vehicles to carry cargo, which consists of enzymes capable to activate the prodrug form of chemotherapeutics, into tumors. The fact that the administered inactive prodrug gets converted by the enzymes at the tumor site offers the advantage that the complete anti-tumor effect of the chemotherapeutic only unfolds locally without causing severe side effects systemically (Matuskova et al., 2015).

Manipulated Rodent MSCs

Laboratory rodent models are the gold standard for investigating the effects of manipulated MSCs *in vivo*. Advantages of these models are the relatively low cost, the availability of genetically modified animals designed to model human diseases, and the availability of commercially customized reagents for the use of research in rodents. Since it is common to use rodents for *in vivo* research, experimental work *in vitro* with optimized human MSCs is often followed up by related *in vivo* studies using rodent MSCs in experimentally induced disease models. **Table 4** gives an overview of recent studies testing the efficacy of manipulated rodent MSC.

THE SECRETOME OF MESENCHYMAL STROMAL CELLS (MSCs) FROM VETERINARY SPECIES

Translational Potential of MSC Research in Veterinary Species

Mesenchymal stromal cells derived from veterinary species have been isolated, characterized and extensively studied *in vitro*, with

TABLE 1 | Human MSC secretome components, targets, effects, and potential therapeutic uses.

MSC source	Secretome components	Targets: effects	Therapeutic use	References
Bone marrow	Complete secretome including VEGFC, TGF-β1, TGF-β2, GDF6	Secretome not tested with targets in a model system	Hematological malignancies	Baberg et al., 2019
Adipose	EV derived alpha-1-antitrypsin	*S. aureus, K. pneumoniae, P. aeruginosa*: microbicidal effect on gram negative bacteria	Pulmonary disease	Bari et al., 2019
Umbilical cord, dental pulp	Complete secretome	HUVEC: decreased apoptosis and senescence, increased migration, tube formation, *in vitro* vascularization	General MSC based therapies	Caseiro et al., 2019
Umbilical cord	TSG-6 in complete CM, EXO	Newborn mouse model of BPD: improvement of lung, cardiac, and brain pathology	Bronchopulmonary dysplasia	Chaubey et al., 2018
Adipose	Complete secretome	Arsenic injured human neurons: prevent arsenic induced damage	Prevention of arsenic induced toxicity	Curtis et al., 2018
Hoffa's fat pad, synovial membrane, umbilical cord, cartilage	Complete secretome including MMPs, IL-17, complement factors, TGF-β1 and PGE2	-Human PBMC: inhibition of proliferation, migration and cytokine secretion -Human chondrocytes: increased aggrecan gene expression	Articular cartilage repair	Islam et al., 2019
Adipose, bone marrow, Wharton's jelly	Complete secretome	-Human monocyte: increased migration -Human macrophage: increased differentiation -Human endothelial cells: induced pro-angiogenic phenotype -Murine vasculature: increased vascularization in Matrigel plug assay	Ischemic diseases	Kehl et al., 2019
Bone marrow	Complete secretome including IL-5, IL-6, IL-8, IL-9, IP-10 MCP-1, FGF-2 and VEGF	Human keratinocytes in hypoxic, low serum culture: increased migration and proliferation, cell spreading and F-actin expression	Chronic wounds	Kosol et al., 2020
Umbilical cord	Complete secretome	-Rat bone marrow MSC from aged animals: increased cell growth, differentiation, potential, decreased senescence -Aged rats: improved bone formation capacity	Age-related osteoporosis	Liang et al., 2019
Bone marrow	miR-21-5p from EXO	Human engineered cardiac tissue: increased contractility, calcium handling	Cardiac therapies	Mayourian et al., 2018
Bone marrow	Complete secretome including EXO-related proteins related to the ubiquitin-proteosome and histone systems	Human neural progenitors: induced neural differentiation Rat model of Parkinson's disease: rescued dopamine neurons, increased behavioral performance in staircase test	Parkinson's disease	Mendes-Pinheiro et al., 2019
Adipose	Complete secretome, soluble factors and EV cargo including proteins involved in RNA metabolism and miRNAs targeting processes involved in regeneration, regulation of inflammation	-Human and rat cell lines: increased proliferation and differentiation, protection against senescence -Mouse model of skeletal muscle injury: enhanced rate of regeneration after acute damage	Muscle regeneration	Mitchell et al., 2019
Adipose	Complete secretome including TIMPs and cartilage protecting factors	TNFα-stimulated primary articular chondrocytes: blunted hypertrophy, reduced levels of osteocalcin and collagen X and MMP13 activity	Osteoarthritis	Niada et al., 2019
Adipose	Innate and IFNγ preconditioned/complete secretome including > 60 secreted cytokines/chemokines and >240 EV-miRNAs	-Macrophages: increased anti-inflammatory phenotype marker CD163 -Chondrocytes: reduced inflammation marker VCAM1	Joint disease	Ragni et al., 2020
Cornea	Complete secretome	*Ex vivo* porcine cornea injury model: enhanced survival of corneal endothelial cells	Corneal endothelial cell injury	Rouhbakhshzaeri et al., 2019
Adipose	Concentrated secretome including GDNF and FGF2	Rat model of bilateral abdominal cryptorchidism: restored seminiferous tubules, increased *GATA4* expression	Non-obstructive spermatogenesis disorders	Sagaradze et al., 2019
Cornea	EXO	-Cultured corneal epithelial cells: increased migration -Murine epithelial debridement wounds: increased wound healing	Ocular surface injuries	Samaeekia et al., 2018

(Continued)

TABLE 1 | Continued

MSC source	Secretome components	Targets: effects	Therapeutic use	References
Bone marrow, adipose	Complete secretome	Hypoxic primary rat alveolar epithelial cells: increased viability, reduced secretion of inflammatory mediators, enhanced IL-10 production, increased active MMPs	Pulmonary syndromes	Shologu et al., 2018
Wharton's jelly, bone marrow	EXO	Mouse model of bronchopulmonary dysplasia: ameliorated alveolar simplification, fibrosis and pulmonary vascular remodeling due to hyperoxia	Pulmonary disease	Willis et al., 2018

BPD, bronchopulmonary dysplasia; EV, extracellular vesicle; EXO, exosomes; FGF2, fibroblast growth factor 2; GATA4: GDF6, growth differentiation factor 6; HUVEC, human umbilical vein endothelial cells; IFNγ, interferon γ; IL, interleukin; IP-10, interferon γ -induced protein 10; MCP-1, monocyte chemoattractant protein-1; MMPs, matrix metalloproteases; PBMC, peripheral blood mononuclear cells; PGs, prostaglandins; PGE2, prostaglandin E2; TIMPs, tissue inhibitors of MMPs; TGF, transforming growth factor; TSG-6, tumor necrosis factor α-stimulated gene-6; VEGF, vascular endothelial growth factor; VECAM1, vascular cell adhesion protein 1; VEGFC, vascular endothelial growth factor C.

TABLE 2 | Rodent MSC secretome components, targets, effects, and potential therapeutic uses.

Rodents	MSC source	Secretome components	Targets: effects	Therapeutic use	References
Mouse 	Bone marrow	MV	LPS stimulated microglia: prevented production of pro-inflammatory molecules and upregulation of proteins associated with activation, decreased phosphorylation of MAPK in activation pathway	Neuroinflammatory disease	Jaimes et al., 2017
	Bone marrow	IGFBP7	Mouse experimental colitis model: ameliorated the clinical and histological severity of inflammation, restored gastrointestinal mucosal tissues	Crohn's disease	Liao et al., 2016
Rat 	Bone marrow	EXO-rich secretome	-Injured liver cells in culture: reduced cytotoxicity, improved cell recovery *-In vivo* rat liver failure models: improved liver regeneration	Injured liver disease	Damania et al., 2018
	Bone marrow	Complete secreted factors including Wnt4	Injured islet microvascular endothelial cells: prevented apoptosis, inhibited eNOS and VCAM-1 elevation, increased β-catenin	Diabetes	Wang et al., 2017
	Bone marrow	EXO	*In vivo* rat stroke model: improved functional recovery, enhanced neurite remodeling, neurogenesis, and angiogenesis	Stroke	Xin et al., 2013
	Bone marrow	EXO including argonaute 2	Primary cortical neurons: promoted axonal growth	Traumatic brain injury	Zhang et al., 2017

eNOS, endothelial nitric oxide synthase; EXO, exosome; IGFBP7, insulin-like growth factor binding protein 7; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MV, microvesicles; VCAM-1, vascular cell adhesion protein 1.

the goal of determining the potential of these cells as therapies for a variety of diseases, many of which also affect humans (De Schauwer et al., 2013; Calloni et al., 2014; Devireddy et al., 2017; Sultana et al., 2018; Dias et al., 2019; Hill et al., 2019). In addition, case studies and clinical trials of MSCs isolated from companion animals have provided *in vivo* data further supporting the efficacy of MSC-based therapies (Caniglia et al., 2012; Carvalho et al., 2013; Renzi et al., 2013; Arzi et al., 2016; Hoffman and Dow, 2016; Geburek et al., 2017; Quimby and Borjesson, 2018). This section provides an overview of data from studies specifically designed to look at the effects of secreted factors of MSC isolated from veterinary species for diseases that are relevant to human medicine.

Veterinary species as physiologically relevant translational models for human diseases have several advantages over rodent models. When working with veterinary species MSC, results from *in vitro* experiments can be easily tested in the same species *in vivo*. In addition, most veterinary species (i) are made up of individuals with genetic variation that reflects the diversity found in human populations, (ii) have larger body sizes and longer lifespans compared to rodents, and (iii) are often exposed to the same environmental insults as humans, causing them to be susceptible to similar naturally occurring diseases, such as musculoskeletal disorders, immune-modulatory diseases, respiratory diseases, and certain cancers. As such, they serve as valuable "real world" models (Hoffman and Dow, 2016).

Small companion animals, most often dogs and cats, are domesticated animals whose physical, emotional, behavioral and social needs are met by close daily relationships with humans. Human bonds to companion animals create a demand for new and optimal pet therapies, including state-of-the-art cell-based therapies. Consequently, the growing interest in MSC-based therapies has resulted in MSCs from dogs and cats to be isolated, characterized, and studied in both the laboratory setting and clinical trials (Hoffman and Dow, 2016). Generally, the dog is considered an excellent model for human disease. In addition to sharing similar environments with humans, dogs naturally develop diseases that resemble pathologic conditions

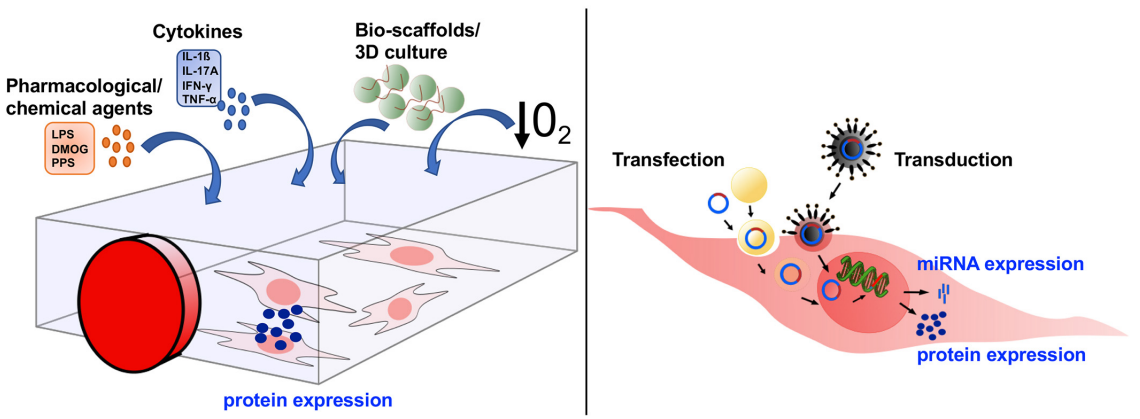

FIGURE 2 | Altering the mesenchymal stromal cell (MSC) secretome through *in vitro* manipulation. In order to increase the secretion of desired molecules, such as proteins or miRNA, MSCs are manipulated in culture through either priming or genetic engineering. The four main approaches of priming are (i) addition of pharmacological/chemical agents, (ii) treatment with cytokines, (iii) culture in 3D cultures/bio-scaffolds, and (iv) culture under induced hypoxic conditions. Genetic engineering is used to express/overexpress specific proteins or miRNA by a targeted RNA or DNA transfer into the MSCs via transduction, transfer by virus or viral vector, or transfection, transfer through various biological/chemical/physical approaches. DMOG, dimethyloxalylglycine; LPS, lipopolysaccharides; PPS, pentosan polysulfate; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; 3D, 3-dimensional; O_2, oxygen.

of humans (Hoffman and Dow, 2016). In this regard, MSC-based therapies have been widely investigated in diseases in dogs, including osteoarthritis (OA), spinal cord injury, bone regeneration, pulmonary and cardiac disorders, cancer, intervertebral disk degeneration, atopic dermatitis, inflammatory bowel disease (IBD), non-ischemic cardiac disease, Alzheimer's disease, amyotrophic lateral sclerosis, and epilepsy (Hoffman and Dow, 2016). Cats have proven to be good models for immune-mediated diseases such as IBD as well as non-ischemic cardiac disease, and chronic kidney disease (Hoffman and Dow, 2016) (**Figure 3**).

Mesenchymal stromal cells have been isolated and characterized from large animals including cows, pigs, sheep, goats, and horses (Calloni et al., 2014). Of these, the majority of MSC studies have been carried out with MSCs isolated from horses, driven by the high demand of horse owners for innovative regenerative therapies, primarily geared toward the treatment of musculoskeletal injuries. Horses are well-characterized as models for specific human diseases, most notably orthopedic injuries such as OA (McCoy, 2015), but also skin wounds (Harman et al., 2019), and respiratory diseases (Klier et al., 2019), all of which have the potential to be managed by treatment with MSC secreted factors (De Schauwer et al., 2013) (**Figure 3**). Pigs have long been considered valuable preclinical models for a variety of human therapies, as exemplified by the use of pig organs that are quite similar to those of humans in terms of size, morphology, and function (Roth and Tuggle, 2015). Assessing stem cell-based therapeutics in pig models for skin wounds, acute liver failure, neurodegenerative disorders, general wound healing and tissue repair, diabetes mellitus, and influenza A infections, have all been proposed, and are mainly performed to fine-tune preclinical testing (Sullivan et al., 2001; Rajao and Vincent, 2015; Seaton et al., 2015; Bharti et al., 2016) (**Figure 3**). Moreover, there is evidence that pig MSCs can function cross-species *in vivo* (Li et al., 2014). Lastly, Sheep and goats are primarily used to model human OA (McCoy, 2015), and sheep also serve as models for human respiratory diseases (Meeusen et al., 2009). The sheep model has also been used to examine the therapeutic effects of EVs derived from human BM-MSCs in a preclinical model of hypoxic-ischemic brain injury in preterm neonates (Ophelders et al., 2016). In this study, ovine fetuses were subjected to global hypoxia-ischemia followed by *in utero* intravenous treatment with EVs. As compared to controls, brain function in fetuses treated with EVs exhibited improved brain function as determined by total number and duration of seizures and preserved baroreceptor reflex sensitivity. Although cerebral inflammation remained unaffected by this treatment, the authors proposed that MSC EVs might be a novel approach to reduce neurological consequences of hypoxic-ischemic injury of the fetal brain in humans (Ophelders et al., 2016).

It is important to point out that many MSC secreted factors are similar across species, making studies on the MSC secretome from animals relevant to human medicine. For example, immunomodulatory molecules, growth factors, anti-tumoral, and anti-microbial molecules, have all been documented to be secreted by MSCs isolated from humans, laboratory rodents, and veterinary species (Harman et al., 2017b; Vizoso et al., 2017; Cassano et al., 2018; Mancuso et al., 2019; Villatoro et al., 2019). Although a quantitative analysis of MSC secreted factors across species has not been carried out to our knowledge, such studies could further strengthen the translational aspect of MSC secretome studies in animals.

Naïve Small Companion Animal MSCs

The primary source of MSC isolated from dogs and studied for therapeutic use is adipose tissue (AT). The secretome of

TABLE 3 | Manipulation of human MSCs to optimize the therapeutic effects of the MSC secretome.

Modification (MSC source)	Manipulation	Outcome	Therapeutic use	References
Priming				
Protein profile in EXO (BMMSC)	Retinal cell CM (TNF-α)	MSC CM and EXO had neuro-protective effects on retinal ganglion cells, increased PEDF and VEGF-A in primed EXO	Optic nerve injury	Mead et al., 2020
Immune-modulatory properties (Gingival MSC)	IL-1β	Overexpression of TGF-β and MMP pathway agonists (MMP-1, MMP-9), Primed MSC CM promoted cell migration, epidermal-dermal junction formation, inflammation reduction *in vitro* and improved epidermal engraftment *in vivo* (mice)	Wound healing	Magne et al., 2020
Protein profile in EXO (BMMSC)	Hypoxia	Exosomes from primed MSC are enriched with specific subclassifications of proteins, including secretory and ECM associated proteins, EXO enhances secretion of growth factors of neuroblast-like cells	CNS related diseases	Yuan et al., 2019
Immune-modulatory properties (UCMSC)	3D culture	CM had increased anti-inflammatory profile (IL-10, LIF) and trophic factors (PDGF-BB, FGF-2, I-309, GM-CSF, increased therapeutic effect *in vivo* (rats)	Rheumatoid arthritis	Miranda et al., 2019
Senescence and immune-modulatory properties (BMMSC)	Substance P	Increased secretion of PDGF-BB in primed MSC. Primed MSC CM increased viability of retinal pigmented epithelium	Age related macular degeneration	Jung et al., 2019
Metabolic pathways (BMMSC)	INF-γ/TNF-α	Primed MSC show increased glycolysis and fatty acid oxidation, glycolysis is linked to MSC-mediated T cell suppression through the JAK/STAT1/IDO axis by posttranslational modification (glycosylation) of STAT1	General MSC therapy: immune-modulatory properties	Jitschin et al., 2019
Induction of quiescent state (BMMSC)	Hypoxia, SF-media	Increased survival, adaptive response mechanism after transplantation, primed MSC maintained their stemness by reaching a quiescent state.	General MSC therapy	Ferro et al., 2019
Senescence and immune-modulatory properties (BMMSC)	3D culture in FBS-containing medium and xeno-free medium	MSC in 3D culture contained their immune-suppressive profile over multiple passages. Upregulation of COX-2, TNF alpha induced protein 6, SCT-1. Secretion of PGE_2, TSG-6, STC-1	General MSC therapy: immune-modulatory properties	Bartosh and Ylostalo, 2019
Genetic modification: miRNA overexpression				
miRNA-26a-5p (BMMSC)	Lentivirus	Alleviation of damages on synovial fibroblasts by targeting PTGS2 *in vitro* and retardation of damage in OA *in vivo* (rats)	Osteoarthritis	Jin et al., 2020
miRNA-181a (UCMSC)	Lentivirus	Reduced inflammatory response and promoted Treg polarization *in vitro* and an *in vivo* (mice) ischemic damage model	Myocardial infarction	Wei et al., 2019
miRNA-122 (ADMSC)	Lipofection	Reduced collagen, inhibition of pro-inflammatory cytokines, reduction of liver enzymes, elevated expression of antifibrotic proteins *in vitro* (human HSC) and *in vivo* (mice)	Liver fibrosis	Kim K.H. et al., 2019
miRNA-126 (UCMSC)	Lipofection	Alleviated effects of hypoglycemia induced inflammation *in vivo* (rats), suppressing of HMGB-1 signaling pathway and inflammation *in vitro* (human retina cells)	Retinal inflammation in diabetes	Zhang et al., 2019
Genetic modification: protein overexpression via transfection				
VEGF (BMMSC)	Microporation	Improved angiogenic potential *in vitro*	Peripheral artery disease	Serra et al., 2019
CXCR4 (BMMSC, ADMSC)	Microporation in combination with minicircle transfection	Increased homing in a skin wound mouse model	General MSC therapy: homing	Mun et al., 2016
Genetic modification: Transduction of viral immune evasion proteins				
Herpesviral immunoevasion protein US11 (BMMSC)	Lentivirus	Downregulation of MHCI proteins, increased persistence of MSC in immune-competent mice with depleted NK	General MSC therapy/increase of immune evasiveness	de la Garza-Rodea et al., 2011
Cytomegaloviral immunoevasion proteins US6/US11 (not specified)	Retrovirus	Downregulation of HLA-I, protection against NK in vitro, increased liver engraftment in pre-immune fetal sheep	General MSC therapy/increase of immune evasiveness	Soland et al., 2012

(Continued)

TABLE 3 | Continued

Modification (MSC source)	Manipulation	Outcome	Therapeutic use	References
Genetic modification: Gene-directed enzyme/prodrug therapy				
HSVtk (BMMSC)	Retrovirus	Clinical trial, administration of transduced MSC in combination with prodrug ganciclovir: 4 of 6 patients reached stable disease, safe and feasible	Gastrointestinal adenocarcinoma	von Einem et al., 2017
HSVtk (BMMSC)	Retrovirus	Clinical trial, administration of transduced MSC in combination with prodrug ganciclovir: 50% of patients reached stable disease, safe and feasible	Gastrointestinal adenocarcinoma	von Einem et al., 2019
CD::UPRT or HSVtk (ADMSC)		Systemic administration of human CD::UPRT-MSC or HSVtk-MSC in combination with 5-FC and ganciclovir inhibited growth of lung metastases in mice	Gastrointestinal adenocarcinoma	Matuskova et al., 2015

AD, adipose tissue derived; BM, bone marrow derived; BNDF, brain-derived neurotrophic factor; CD, cluster of differentiation; CM, conditioned medium; CNS, central nervous system; COX, cyclooxygenase; EXO, exosomes; FBS, fetal bovine serum; FGF, fibroblast growth factor; GM-CSF, granulocyte-macrophage colony stimulating factor; HGF, hepatocyte growth factor; HLA, human leukocyte antigen; HSC, hepatic stellate cells; IDO, indoleamine-pyrrole 2,3-dioxygenase; IL, interleukin; INF, interferon; JAK, janus kinase; LIF, leukemia inhibitory factor; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; MSC, mesenchymal stromal cells; NK, natural killer cells; OA, osteoarthritis; PEDF, pigment epithelial derived factor; PGE_2, prostaglandin E_2; PSGL, P-selectin Glycoprotein Ligand; PTGS2, prostaglandin-endoperoxide synthase 2; STC-1, stanniocalcin 1; SF, serum free; STAT, signal transducer and activator of transcription; TGF, transforming growth factor; TNF, tumor necrosis factor; TSG, tumor necrosis factor-stimulated gene; UC, umbilical cord derived; VEGF, vascular endothelial growth factor; HSVtk, herpes simplex thymidine kinase; CD::UPRT, fusion yeast cytosine deaminase::uracil phosphoribosyltransferase, 5-FC: 5-fluorocytosine.

AT-derived MSCs from dogs has the potential to influence neurologic diseases, immune-related diseases and cancer. *In vitro* experiments designed to examine the paracrine action of dog AT-derived MSCs on neuronal and endothelial cells showed that treating a neuronal cell line with conditioned medium (CM) from MSC cultures significantly increased cell proliferation, neurite outgrowth and expression of the neuronal marker βIII-tubulin (Al Delfi et al., 2016). Exposure of an endothelial cell line to this dog MSC CM increased cell proliferation and migration, as well as inducing tubule formation in a soluble basement membrane matrix, suggesting that the MSC secretome contains pro-angiogenic factors (Al Delfi et al., 2016). The authors concluded that these data support the hypothesis that transplanted MSC can promote increased neural function in dogs with CNS damage, due to paracrine activity on nerves and blood vessels (Al Delfi et al., 2016). Of note is that the neuronal and endothelial cell lines used in this study for the experiments were of human origin. The fact that dog-derived secretome components stimulated human cells suggests common inter-species mechanisms that endorse the use of veterinary models for human medicine. Another *in vitro* study aimed to compare immunomodulatory properties of dog AT- and bone marrow (BM)-derived MSCs. In these experiments, proliferation of stimulated peripheral blood mononuclear cells (PBMCs) from dogs co-cultured with AT- or BM-derived MSCs was inhibited when compared to non-co-cultured controls (Russell et al., 2016). The authors concluded that the *in vitro* immunomodulatory effects were mediated by MSC secreted factors and proposed future *in vivo* experiments to determine the efficacy of MSCs to modulate the immune system during inflammation-based conditions in dogs (Russell et al., 2016). Experiments testing the effects of dog AT-derived MSC CM on tumor cell growth showed that the MSC CM enhanced proliferation and invasion of a dog hepatocellular carcinoma cell (HCC) line *in vitro* and altered mRNA expression levels of genes related to tumor progression in HCC cells (Teshima et al., 2018). The results of this study seemingly contradicted multiple earlier studies on the effects of human MSC CM on human HCC cell lines, where inhibition of proliferation and invasion were observed (Li et al., 2010; Zhao et al., 2012). This indicates that more research is needed in both species to determine if and how the MSC secretome influences cancer cell growth and tumor progression (Teshima et al., 2018).

The secretome of AT-derived MSC from cats has been studied as well, particularly in the context of immune modulation. IBD is an autoimmune disease common in both cats and humans. *In vitro* experiments showed that prostaglandin E_2 (PGE_2) secreted by cat AT-MSC induced elevation of Forkhead box P3 (*FOXP3*) mRNA and altered the expression of inflammatory cytokines in concanavalin A (Con A)-stimulated PBMCs (An et al., 2018). Complementary studies in a Dextran sulfate sodium -treated mouse model of colitis demonstrated that intraperitoneal infusion of cat AT-MSCs reduced the clinical and histopathologic severity of colitis, and FOXP3+ T cells were significantly increased in the inflamed colon of MSC-treated mice as compared to controls (An et al., 2018). The authors concluded that PGE_2 secreted by cat AT-MSC likely reduced inflammation by increasing FOXP3+ regulatory T cells in the mouse model and proposed that MSC-derived PGE_2 may improve IBD and other immune-mediated inflammatory diseases in cats (An et al., 2018). Cat AT-MSC secreted factors have been shown to decrease proliferation of Con A-stimulated PBMCs, suggesting an additional anti-inflammatory mechanism (Parys et al., 2017). Inhibition of cat AT-MSC secreted PGE_2 by indomethacin or NS-398 was shown to reduce the anti-proliferative effects of AT-MSC CM on cat PBMCs, confirming that PGE_2 is involved in the immunomodulatory effects exerted by the MSC secretome (Chae et al., 2017; Taechangam et al., 2019). One of these studies also showed that cat AT-MSC secreted factors alter cytokine expression in cat PBMCs as well as a murine macrophage cell line, providing more evidence that inter-species studies of the MSC secretome can provide data that is relevant to human medicine (Chae et al., 2017). A more detailed comparison of the cat and human AT-MSC secretome using ELISAs and enzyme activity assays, revealed that AT-MSC from both species secrete PGE_2, indoleamine 2,3 dioxygenase, transforming growth factor beta (TGFβ) and

TABLE 4 | Manipulation of laboratory rodent MSCs to optimize the therapeutic effects of the MSC secretome.

Modification (MSC source)		Manipulation	Outcome	Therapeutic use	References
Priming					
Rat 	Angiogenesis, immune modulatory properties (ADMSC)	Biomaterial: PAMAM-IKVAV/HA	Production of pro-angiogenic cytokines, anti-inflammatory miRNAs, increased proliferation, improved cell retention and tissue angiogenesis in rat MI model	Myocardial infarction	Su et al., 2019
	Antibacterial and immune modulatory properties (BMMSC)	LPS	Improved inhibition of bacterial growth *in vitro* Reduced inflammation and better bacterial clearance in septic mice. Improved survival of mice	Sepsis	Saeedi et al., 2019
	Immune-modulatory properties (BMMSC)	TNF-α/IL-1β	Increase inhibitory potential on lymphocyte proliferation mediated by NO, prolonged corneal allograft survival *in vivo*	Corneal allografts	Murphy et al., 2019
Genetic modification: miRNA overexpression					
Mouse 	miRNA-30b-3p (BMMSC)	Lentivirus	Promotion of proliferation and inhibition of apoptosis in AEC (*in vitro*), protection against LPS-induced ALI in mice	Acute lung injury	Yi et al., 2019
Rat 	miRNA-17-92 (BMMSC)	Electroporation	-Increased functional recovery, neurite remodeling and brain EXO content in an *in vivo* stroke model -Increased EXO release in cultured astrocytes deprived of oxygen and glucose	Recovery after stroke	Xin et al., 2017
	miRNA-133 (BMMSC)	Lentivirus	Improved cardiac function in an *in vivo* MI model, decreased inflammatory levels and infarct size	Myocardial infarction	Chen et al., 2017
	miRNA-9-5p (BMMSC)	Lipofection	Promotion of MSC migration *in vitro* by activation of β-catenin signaling pathway	General MSC therapy	Li et al., 2017
	miR-17-92 cluster (BMMSC)	Electroporation	Enhanced axonal growth of primary cortical neurons	Traumatic brain injury	Zhang et al., 2017
Genetic modification: protein overexpression					
Mouse 	CCL19 (BMMSC)	Lentivirus	T-cell dependent anti-tumor effect *in vitro* and *in vivo*	Colon carcinoma	Iida et al., 2020
	Fucosyltransferase VI (BMMSC)	Lipofection	Increased homing into kidneys after fucosylation of CD44. Alleviation of renal injury, partly through enhanced polarization of macrophages in a mouse model	General MSC therapy (homing)	Chou et al., 2017
Rat 	HMGB-1 (BM MSC (F344)	Lentivirus	Vascular protective effect, increased motility and potential to differentiated toward EC	Chemotherapy side effects	Tao et al., 2019
	CXCR4 (BMMSC)	Adenovirus	Increased homing towards SDF-1 *in vitro*	General MSC therapy (homing)	Sanghani-Kerai et al., 2017
	CXCR4 (BMMSC)	Adenovirus	Homing of MSC into bones in an *in vivo* osteoporosis model, increased mineral bone density	General MSC therapy (homing)	Sanghani et al., 2018

AD, adipose tissue derived; AEC, alveolar epithelial cells; ALI, acute lung injury; BM, bone marrow derived; CD, cluster of differentiation; CM, conditioned medium; EC, endothelial cell; EXO, exosomes; HMGB1, high-mobility group box 1; IL, interleukin; INF, interferon; LPS, lipopolysaccharide; MI, myocardial infarct; MSC, mesenchymal stromal cells; NO, nitric oxide; PAMAM-IKVAV/HA, polyamidoamine-IKVAV/hyaluronic acid; PGE_2, prostaglandin E_2; SDF-1, stromal cell-derived factor 1; TNF, tumor necrosis factor; UC, umbilical cord derived.

interleukin (IL)- 6, and that secretion of these proteins was increased when MSCs were co-cultured with stimulated PBMCs (Clark et al., 2017). Clinical trials using cats with naturally occurring inflammatory and immune-mediated diseases could, therefore, be used as surrogate models for human clinical trials (Clark et al., 2017). Supernatants from cat BM- and AT-derived MSC cultures have also been shown to modulate immune cells by inhibiting the reactive oxygen species (ROS) production by cat neutrophils *in vitro*. Although the authors of this study did not attempt to identify specific factors in the supernatants that were responsible for exerting this effect, it was determined to be dose dependent, with ROS production decreasing when neutrophils were cultured in medium made up of increasing percentages of MSC supernatants (Mumaw et al., 2015). Moreover, cat MSCs from both sources displayed similar effects on neutrophil ROS production, and the authors further concluded that supernatants from cat BM- and AT-derived MSC cultures could be clinically useful in diseases in which neutrophilic inflammation plays a significant role (Mumaw et al., 2015).

A concise overview of these studies of dog and cat MSC secreted factors is presented in **Table 5**.

Naïve Large Animal MSCs

Horse MSC isolation and therapeutic application for orthopedic injuries was first described in 2003 (Smith et al., 2003). The technique was declared "rational and feasible," but conclusions could not be made based on a single case report without appropriate controls. More comprehensive studies, carried out

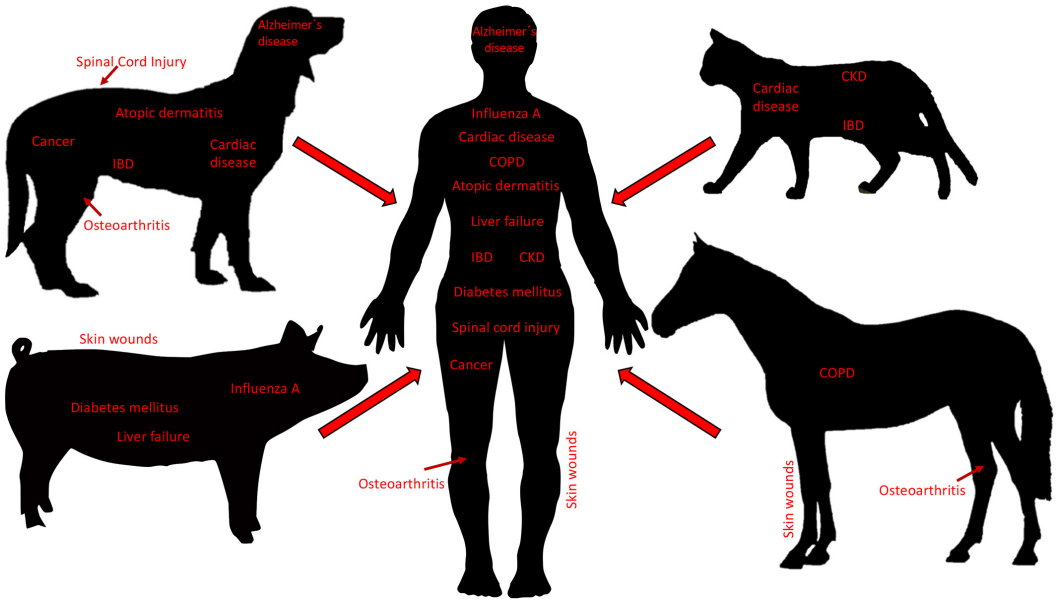

FIGURE 3 | Diseases in veterinary species as translational models for human disease. Small companion animals (i.e., dogs and cats), as well as large animals (i.e., pigs and horses), develop pathologies that are similar to diseases in humans and thus, are used as translational animal models for neurological, cardiac, pulmonary, musculoskeletal, nephrological, gastroenterological, dermatological, infectious, and cancerous, diseases. Body sizes are not to scale. IBD, inflammatory bowel disease; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease.

TABLE 5 | Small companion animal MSC secretome components, targets, effects, and potential therapeutic uses.

Animal	MSC source	Secretome components	Targets: effects	Therapeutic use	References
Dog 	Adipose	Complete secretome	-Human neuronal cells: increased proliferation, neural outgrowth, immunopositivity for neural marker βIII-tubulin -Human endothelial cells: increased migration, proliferation, tube formation	Neurological disorders	Al Delfi et al., 2016
	Adipose, bone marrow	Complete secretome	Canine lymphocytes: suppressed proliferation	Immune-related diseases	Russell et al., 2016
	Adipose	Complete secretome/growth factors, MMP2	Canine hepatocellular carcinoma cells: increased proliferation and invasion	Cancer	Teshima et al., 2018
Cat 	Adipose	PGE2	-Feline regulatory T cells: increased *FOXP3* gene expression, altered cytokine gene expression -Mouse colitis model: reduced severity of disease	Inflammatory bowel disease treatment	An et al., 2018
	Adipose	Complete secretome	Feline PBMC: reduced proliferation	Inflammatory disorders	Parys et al., 2017
	Adipose	Complete secretome	-Feline PBMCs: decreased *TNF-α, INFγ, IL-6* gene expression, increased *IL-10* gene expression -Human macrophages: decreased *TNF-α, iNOS, IL-1β* gene expression	Immune-related diseases	Chae et al., 2017
	Adipose	Complete secretome/PGE2	Feline T lymphocyte: reduced proliferation	CD4+ and CD8+ mediated alloreactive diseases	Taechangam et al., 2019
	Adipose	Complete secretome	Feline PBMCs: reduced activated T-cell proliferation	Regenerative medicine	Clark et al., 2017
	Bone marrow	Complete secretome	Feline neutrophils: reduced production of ROS	Diseases dependent on neutrophilic inflammation	Mumaw et al., 2015

IFN-γ, interferon-γ; IL-6: IL-10, interleukin-10; FOXP3, forkhead box P3; MMP2, matrix-metallopeptidase 2; PBMCs, peripheral blood mononuclear cells; PGE2, prostaglandin E2; ROS, reactive oxygen species; TNFα, tumor necrosis factor α.

in the past 15 years, have established that autologous MSC therapy is not detrimental to horses and the use of MSCs to treat orthopedic injuries has been accepted as a valuable therapeutic approach (Bogers, 2018; Al Naem et al., 2020). There are still many unanswered questions about the immunogenicity of allogenic horse MSC. Although allogenic MSC hold promise as

treatments for numerous diseases of horses, such as endotoxemia, IBD, asthma and recurrent uveitis, more data are needed before allogenic cells can be used clinically (Berglund et al., 2017b; MacDonald and Barrett, 2020).

To the best of our knowledge, neither the cell-free complete secretome nor specific secretome components of horse MSCs have been delivered to horses *in vivo*. However, we and others have worked extensively *in vitro*, and to a lesser extent *in vivo* in rodent models, to characterize the horse MSC secretome and its effects on target cells (**Table 6**). Specifically, our research focuses on the horse peripheral blood (PB) derived-MSC secretome in the context of cutaneous skin wounds, which is not only of significant importance in equine medicine but can also greatly benefit human medicine by taking advantage of horse cutaneous wounds as translational models for the evaluation of human MSC-based therapies (Harman et al., 2019). For example, we found that *in vitro* (i) endothelin 1 (ET1), IL-8, platelet derived growth factor-AA (PDGF-AA), and insulin-like growth factor binding protein 2 (IGFBP2), present in the horse PB-MSC secretome promotes angiogenesis and (ii) plasminogen activator inhibitor 1 (PAI-1) and tenascin-C (TNC) secreted by PB-MSC increases fibroblast migration (Bussche and Van de Walle, 2014; Harman et al., 2018). Importantly, we confirmed the active roles of PAI-1 and TNC in fibroblast migration by repeating the experiments with secretome in which these factors were silenced using RNA interference and followed up by testing the contribution of these MSC-secreted proteins to wound healing *in vivo* in a mouse full-thickness skin injury model. Although this work confirmed the bioactive roles of these factors, our results showed that these two proteins did not account for the full wound healing effect of the complete secretome (Harman et al., 2018). Additional studies from our group identified anti-microbial peptides in the horse PB-MSC secretome that inhibit the growth of contaminating bacteria commonly found in skin wounds, as well as cysteine proteases that destabilize bacterial biofilms *in vitro* (Harman et al., 2017b; Marx et al., 2020). Moreover, we confirmed that the chemokine C-X-C motif ligand 6 (CXCL6) secreted by horse PB-MSC acts as a chemoattractant for neutrophils *in vitro* (Harman et al., 2020). Collectively, our research has identified specific factors secreted by PB-MSC that promote various aspects of skin wound healing, supporting the notion that the complete MSC secretome provides therapeutic benefits by targeting various aspects of specific disease processes.

Early studies of MSCs from cows, pigs, sheep, and goats, primarily focused on MSC characterization based on phenotypic marker expression and the potential to differentiate into adipocytes, chondrocytes, and osteocytes *in vitro* (Bosch et al., 2006; Cardoso et al., 2012; Heidari et al., 2013; Mohamad-Fauzi et al., 2015). More recently, global proteomic analysis of the secretome of cow endometrial MSCs identified 302 unique proteins, including those with anti-inflammatory or antibacterial properties and proteins related to tissue remodeling. After stimulating these MSCs with lipopolysaccharide (LPS), an increased 397 proteins were detected in the secretome, particularly those proteins involved in immunomodulation and tissue repair, leading the authors to conclude that these cow MSCs could be useful to treat reproductive diseases of cattle (de Moraes et al., 2017). Additional *in vitro* studies showed that the secretome from fetal cow MSC reduced the growth of *S. aureus* (Cahuascanco et al., 2019) and promoted endothelial cells to form tubules, an *in vitro* proxy for angiogenic potential (Jervis et al., 2019). Based on currently available data, the cow MSC secretome has been proposed as a treatment for mastitis, wound healing, nerve injuries, degenerative joint diseases and other diseases of the skeletal system, as well as diabetes mellitus (Gugjoo et al., 2019; Hill et al., 2019).

The immunomodulatory functions of soluble factors secreted by pig MSC have been studied *in vitro,* demonstrating that PGE_2 suppresses the functionality of dendritic cells and T-cells (Khatri et al., 2015). Applying the secretome of pig corneal MSCs to injured corneal endothelial cells *ex vivo,* significantly reduced endothelial cell loss when compared to control conditions (Rouhbakhshzaeri et al., 2019). Moreover, it was found that LPS-damaged pig enteric ganglia were protected upon treatment with the secretome of pig MSC using an *in vitro* model of IBD (Dothel et al., 2019). The activity of pig MSC-derived EVs has also been studied in depth. For example, a study comparing the miRNA, RNA, and protein, expression profiles in the complete secretome of pig AT-derived-MSC to those profiles found in the EV fraction from these cells, showed that 4 miRNAs and 255 mRNAs were specifically enriched in EVs (Eirin et al., 2017). Another study evaluated the anti-influenza activity of EVs isolated from pig BM-MSC in lung epithelial cells *in vitro* and in a pig model of influenza infection *in vivo* (Khatri et al., 2018). The *in vitro* experiments showed that EVs were incorporated into epithelial cells, inhibited the hemagglutination activity and replication of influenza virus, and reduced virus-induced apoptosis of lung epithelial cells. *In vivo,* treatment with EVs significantly reduced influenza virus shedding in the nasal epithelium, viral replication in the lungs, and virus-induced proinflammatory cytokines in the lungs of infected pigs (Khatri et al., 2018).

Sheep MSCs have primarily been studied in terms of their potential to contribute to joint and cartilage repair (Music et al., 2018). For example, the administration of chondrogenically predifferentiated MSCs, embedded in hydrogels at the site of induced osteochondral injury in the medial femoral condyle of sheep, resulted in significantly improved histological scores at 6 months and 1-year post-administration when compared to controls (Zscharnack et al., 2010; Marquass et al., 2011). Since it is known that implanted MSC do not persist long term, these encouraging findings could be a result of factors secreted by the MSCs. To the best of our knowledge, however, specific effects of the sheep MSC secretome on osteochondral defects has not been examined *in vitro* nor *in vivo.*

Manipulated Small Companion Animal MSCs (Dog)

Many strategies to optimize therapies with dog MSCs focus on enhancing the differentiation potential of these cells, primarily into chondrocytes and osteocytes. A main goal of establishing a stable chondrocyte phenotype from dog

TABLE 6 | Horse MSC secretome components, targets, effects, and potential therapeutic uses.

MSC Source	Secretome components	Targets: effects	Therapeutic use	References
Peripheral blood	ET1, IL-8, PDGF-AA, IGFBP2	Endothelial cells: increased angiogenesis	Tissue regeneration	Bussche and Van de Walle, 2014
Bone marrow	Glycosaminoglycan	BM-MSC: decreased PDL	Maintaining stemness	Sasao et al., 2015
Peripheral blood	Complete secretome	Dermal fibroblasts: increased migration, altered gene expression	Cutaneous wound healing	Bussche et al., 2015
Amnion	Complete secretome, EV	LPS stimulated and unstimulated alveolar macrophages: cytokine secretion	Inflammatory lung diseases	Zucca et al., 2016
Peripheral blood	Antimicrobial peptides	*E. coli, S. aureus*: inhibition of growth, biofilm formation	Cutaneous wound healing	Harman et al., 2017b
Peripheral blood	Complete secretome	Dermal fibroblasts, healthy and dysregulated: alterations in morphology, proliferation, gene expression, contractile capacity and susceptibility to senescence	Fibroproliferative disorders	Harman et al., 2017a
Bone marrow	Complete secretome	Corneal stromal cells: increased migration	Corneal wound healing	Sherman et al., 2017
Bone marrow	Galectin-1/3	BMMSC: increased motility	Osteoarthritis	Reesink et al., 2017
Peripheral blood	PAI-1, tenascin-C	Dermal fibroblasts, mouse skin wounds: increased migration, wound closure	Cutaneous wound healing	Harman et al., 2018
Amnion	MicroRNAs	Secretome not tested with targets in a model system	Regenerative medicine	Lange-Consiglio et al., 2018
Adipose	EV derived small RNAs	Secretome not tested with targets in a model system	Regenerative medicine	Capomaccio et al., 2019
Bone marrow	Inflammatory, angiogenic proteins	Secretome not tested with targets in a model system	Osteoarthritis	Bundgaard et al., 2020
Peripheral blood	Complete secretome, cysteine proteases	*P. aeruginosa, S. aureus, S. epidermidis*: inhibition and destabilization of biofilms	Bacterial skin infections	Marx et al., 2020
Peripheral blood	CXCL6	Neutrophils: chemotaxis	Tissue repair	Harman et al., 2020

BM-MSC, bone marrow-derived MSC; CXCL6, chemokine (C-X-C motif) ligand 6; ET1, endothelin 1; EV, extracellular vesicles; IGFBP2, insulin growth factor binding protein 2; IL-8, interleukin-8; LPS, lipopolysaccharide; NA, not applicable; PAI-1, plasminogen activator inhibitor 1; PDGF-AA, platelet derived growth factor-AA; PDL, population doubling level.

MSCs is to increase their deposition of articular cartilage proteins, so that these cells can become an effective treatment option for chronic OA. For example, it was found that exposing canine AT-MSCs to hypoxic conditions resulted in increased proliferation, a downregulation of genes associated with senescence like histone acetylase 1 (HDAC 1) and DNA-cytosine-5-methyltransferase (DNMT1), and an upregulation of genes that are associated with the potential to differentiate into chondrocytes like collagen type II alpha 1 (COL2A1) (Lee et al., 2016). Another study showed that culturing dog AT-MSCs with dimethyloxalylglycine (DMOG), which mimics hypoxic conditions by stabilizing hypoxia-inducible factor-1alpha (HIF1a), led to an increased expression of the signal protein vascular endothelial growth factor (VEGF), important in angiogenesis and thus, beneficial in diseases with ischemic conditions. However, high concentrations of DMOG did inhibit MSC proliferation (Kim S.M. et al., 2019). Importantly, it was found that the serum used in MSC cultures can alter the immunomodulatory properties of dog AT-MSC, since MSCs cultured in serum-free medium secreted lower levels of PGE_2 and were less efficient in inhibiting interferon (INF)-γ secretion by activated T-cells (Clark et al., 2016). Culturing dog BM-MSC with pentonsan polysulfate (PPS) in a micromass culture system, successfully enhanced chondrogenesis and proteoglycan deposition. However, repeating these experiments in an alginate culture system did not result in a chondrocyte phenotype, pointing out the importance of the culture conditions for obtaining the desired MSC phenotype (Bwalya et al., 2017). Increased chondrogenesis and glycosaminoglycan (GAG) deposition was also observed when dog BM-derived peri-adipocytes (BMPAGs) were stimulated with fibroblast growth factor 2 (FGF2) in serum-free medium. BMPAGs are MSCs derived from cells adhering to adipocytes in the BM, and the authors proposed that this special site of isolation explained a lower donor variability in their results when compared to earlier studies that used BM-MSC (Endo et al., 2019). To increase clinical effectiveness of dog BM-MSC, Steffen et al. (2019) attached BM-MSC on a collagen microcarrier scaffold, in the presence or absence of immobilized TGF-ß1 and found an increased chondrogenic phenotype *in vitro*. Following up on this finding in a clinically study with canine patients suffering from intervertebral disc (IVD) degeneration, however, did not find any improvement that could be associated with the MSC treatment (Steffen et al., 2019).

While the majority of dog MSC studies focus on increasing differentiation potential, as outlined above, some studies did investigate the effects on paracrine signaling by priming MSCs. Dog umbilical cord blood (UC)-derived MSCs were primed with β-tricalcium phosphate, a combination previously found to produce promising osteogenic material (Jang et al., 2008), and then evaluated in an ectopic implantation model. On day 1 after implantation, tissue collected from UC-MSC-β-calcium implants showed an increase of *IL-1*, *IL-6*, and *VEGF* RNA expression as well as increased protein levels of IL-6 and VEGF when compared

to controls, and this cytokine release was proposed to mediate the enhanced bone formation observed (Byeon et al., 2010). In addition to the potential of dog MSCs to modulate chondro- and/or osteogenesis, their benefits in immune-mediated diseases, such as inflammatory bowel syndrome that affects both dogs and humans, have also been explored (Hoffman and Dow, 2016). In this context, it was investigated how gastrointestinal microbes interact with dog AT-MSCs in order to understand if an altered gastrointestinal microbiome affects MSC therapy outcome in IBD (Kol et al., 2014). Based on the knowledge that (i) MSCs are known to express pattern recognition receptors (PRRs) and (ii) activation of MSCs through PRR ligands alters the MSC secretome (DelaRosa and Lombardo, 2010), it was explored whether co-culture of dog AT-MSCs with gastrointestinal commensal (*Lactobacillus acidophilus*) and pathogenic (*Salmonella typhimurium*) bacteria affected their phenotype. Although no increased cell death or upregulation of surface proteins major histocompatibility complex (MHC)-II, cluster of differentiation (CD)80/CD86, or CD1 was detected, the canine MSCs (i) did express higher RNA levels of *COX2, IL6* and *IL8*, (ii) secreted more PGE_2, IL-6 and IL-8, and (iii) showed a higher ability to inhibit mitogen induced T-cell proliferation (Kol et al., 2014). The authors concluded that microbe-MSC interaction alters MSC functionality, and thus, that this needs to be taken into consideration when MSCs are explored as therapy in diseases associated with bacterial colonization (Kol et al., 2014). Another study evaluated the value of PRR expression for priming of AT-MSC in both mouse and dog models (Johnson et al., 2017). First, they showed that activation of mouse MSCs with poly I:C through the PRR Toll-like receptor 3 (TLR3) resulted in an altered secretome profile including increased secretion of the monocyte chemoattractant CC-chemokine ligand 2 (CCL2). CM from these primed MSCs (i) led to increased murine monocyte recruitment in an *in vitro* migration assay and (ii) stimulated neutrophils to increase their phagocytosis of bacteria *in vitro*. A follow up *in vivo* experiment in mice showed increased homing of activated MSCs to infected wounds (Johnson et al., 2017). When the effects of intravenously injected allogenic poly I:C-activated dog AT-MSCs were evaluated in canine patients suffering from chronic multi-drug resistant bacterial infections, the authors found that the MSC infusions were well tolerated, with no notable side effects, and that the conditions in all enrolled dogs either improved of resolved by the end of observation period. This prompted the authors to conclude that their pre-clinical study provides strong rationale to establish primed MSCs as a therapy for chronic bacterial infections (Johnson et al., 2017).

Due to their low immunogenicity and their homing ability, dog MSCs have also been explored as "trojan horses." For example, the canine adenovirus ICOCAV17 has anti-tumor effects, but this virus is readily neutralized by the host immune system. To allow ICOCAV17 to reach the tumor site, dog AT-MSCs were infected with this virus and used to treat 27 dogs suffering from various cancerous diseases (Cejalvo et al., 2018). Of those, 74% benefited from the therapy and 14% even showed total remission. Interestingly, the study found increased immune cell infiltrations into the tumors after treatment, and this immune-related response to the infected MSCs was deemed to play an important role in the observed clinical benefits (Cejalvo et al., 2018). Similar studies in humans, using the human oncolytic adenovirus ICOVIR-5 to infect human MSCs, however, were less promising (Garcia-Castro et al., 2010; Melen et al., 2016; Ruano et al., 2020). When the cellular responses of human and dog MSCs to ICOVIR-5 and ICOCAV17, respectively, were compared, it was found that ICOVIR-5, but not ICOCAV17, intrinsically induces a strong phosphorylation of AKT and c-JUN (Rodríguez-Milla et al., 2020). Activation of the AKT pathway is associated with (i) virus latency by suppressing apoptosis of the host cell, (ii) host cell survival in chronic viral infections, and (iii) short-term cellular survival in acute viral infections depending on the virus and type of infection (Cooray, 2004). The authors concluded that an impaired cellular signaling in dog MSCs after ICOCAV17 infection, due to the lack of AKT activation, might lead to a more restricted host immune response after injection of dogs with these virus-infected MSCs, which could explain the better clinical outcome (Rodríguez-Milla et al., 2020). This study is a nice example of how comparative studies between species can lead to a better understanding of the underlying mechanisms responsible for certain clinical outcomes.

In addition to infecting dog MSCs with oncolytic viruses, several studies have been conducted to evaluate the use of genetically modified MSCs to treat cancer in dogs. For example, the effects of INF-β overexpression in dog AT-MSCs against canine melanoma has been studied intensively (Seo et al., 2011; Ahn et al., 2013; Han et al., 2015b). Transfected MSCs performed slightly better than naïve cells *in vitro*, with their secretome showing improved pro-apoptotic and cell growth inhibitory effects on canine melanoma cells (Han et al., 2015b). *In vivo*, INF-β overexpressing dog AT-MSCs did migrate to the tumor site after subcutaneous injection in a mouse model, and these mice showed increased survival time when the MSC treatment was combined with the chemotherapeutic cisplatin (Seo et al., 2011). These findings were corroborated a few years later when INF-β overexpressing dog AT-MSCs were used *in vitro* and in a xenograft *in vivo* mouse model of canine melanoma. Importantly, this study found that melanoma cell proliferation was inhibited using an indirect co-culture system, indicating that the anti-tumor effects rely on factors in the MSC secretome (Ahn et al., 2013). Genetically modified dog AT-MSCs overexpressing cytotoxic T-lymphocyte antigen 4 (CTLA4), with the goal to increase immune suppressive properties, have also been explored. Specifically, T-lymphocyte infiltration into thyroid glands was found to be decreased, as well as thyroglobulin antibodies in the serum, upon treatment with CTLA4-AT-MSCs in an induced thyroiditis model in beagles (Choi et al., 2015). These cells have also been used as treatment in a case study of a dog with therapy-resistant pemphigus foliaceus, an immune-mediated disease that leads to severe skin lesion and reduces quality of life immensely (Olivry, 2006). In this case study, the dog received 21 injections with CTLA4-AT-MSCs over a period of 20 months. The lesions improved and the patient reached a stable state of disease

that could be controlled with low-dose prednisolone for a year (Han et al., 2015a). Lastly, dog AT-MSCs transduced with a tyrosine mutant adeno-associated virus 2 vector to overexpress stromal-derived factor-1 (SDF-1), with the goal to promote MSC homing and survival, have been evaluated in dogs with dilated cardiomyopathy (Pogue et al., 2013). Although the SDF-1-AT-MSCs were successfully administered via retrograde coronary venous delivery and no adverse effects were observed, the treatment failed to improve clinical outcome in the enrolled dogs (Pogue et al., 2013).

Manipulated Large Animal MSCs (Horse)

As previously mentioned, the horse is a widely used and well-accepted model for OA. Early on, the hypothesis was that MSCs would engraft in pathological joints and differentiate into chondrocytes, and as such, mitigate joint trauma. In order to increase clinical outcome, horse BM-MSCs were primed *in vitro* with TGF-β1 and insulin-like growth factor-I (IGF-I), which increased the chondrogenic potential of these horse MSCs (Worster et al., 2001). However, it is now generally accepted that the observed therapeutically effects of MSCs are not due to their engraftment, but due to immunomodulation via paracrine signaling. Although it is known that the expression of immunogenic and immunomodulation-related genes and molecules in MSCs change in a proinflammatory environment, potentially activating the immunomodulatory properties of these cells, it was found that the equine synovial fluid of inflamed joints alone was not sufficient to enhance the immunoregulatory profile of horse BM-MSCs (Barrachina et al., 2016). Consequently, several studies focused on increasing the immunomodulatory properties by priming horse MSCs *in vitro* in culture before administration *in vivo*. For example, a dose-dependent stimulation of horse BM-MSCs with the cytokines tumor necrosis factor α (TNF-α) and INF-γ led to an upregulation of immunoregulatory genes without affecting viability and differentiation potential (Barrachina et al., 2017a, 2018). Although INF-γ priming increased the chondroprotective effect of horse BM-MSCs, the expression of MHC-I and MHC-II was also upregulated, implicating an increased immunogenicity (Barrachina et al., 2017b; Hill et al., 2017; Cassano et al., 2018). In line with these *in vitro* results, an *in vivo* study in an equine OA model showed only slightly improved clinical signs, as well as synovial inflammatory signs, when horses were treated with allogeneic naïve or TNF-α/INF-γ primed horse BM-MSCs. Moreover, injection of these primed MSCs led to a transient local inflammation reaction after the second injection, most likely due to the production of allo-antibodies that recognized these primed MHC-mismatched MSCs with high expression levels of MHC-class I and II molecules (Berglund and Schnabel, 2017; Barrachina et al., 2018, 2020). More recently, priming horse BM-MSCs with TGF-β2 has been identified as a promising strategy to inhibit INFγ-induced MHC I and II surface expression *in vitro*, thus, potentially improving MSC survival and therapeutic efficacy (Berglund et al., 2017a).

Genetically modified horse MSCs have also been explored and one of the first *in vivo* studies used horse BM-MSCs that were successfully transduced with an adenoviral vector to overexpress IGF-I in a model of equine tendinitis (Schnabel et al., 2009). Tendon histological scores improved after treatments with both naïve MSCs and IGF-I-MSCs, leading to the conclusion that horse MSCs might be beneficial for the treatment of tendinitis, but without a superior effect from transfected MSC (Schnabel et al., 2009). In equine OA, the nuclear factor κB (NFκB) signaling pathway, which can be activated by the cytokines IL-ß1 and TNF-α that are naturally present in inflamed joints, has been determined to be a key signaling pathway contributing to disease pathology. IL1-β and TNF-α not only activate NFκB but become in turn also upregulated by this activated pathway, thus creating a positive autoregulatory loop that can amplify inflammation (Marcu et al., 2010). Equine MSCs have been engineered with the goal to interrupt this inflammatory response. For example, the usefulness of a tunable gene expression vector under the control of an NFκB-responsive enhancer/promoter that can be regulated by the pro-inflammatory cytokines IL-1β and TNF-α has been explored (Gabner et al., 2016). As proof of concept, the reporter gene luciferase was used to show that stimulation of transduced MSCs with IL-1β and TNF-α indeed led to the expression of the reporter gene in a dose dependent manner (Gabner et al., 2016). In a follow-up study, the authors then replaced the reporter gene with the gene encoding for interleukin-1 receptor antagonist (IL-1Ra) and found that its expression could be modulated by repeated cycles of induction with TNF-α. Importantly, they could demonstrate that IL-1Ra present in the secretome of these transduced MSCs effectively blocked OA onset in an *in vitro* model using horse chondrocytes (Gabner et al., 2018). Based on these findings, the authors suggested that transduced MSCs that are administered to inflamed joints and express tunable IL-1Ra in response to the pro-inflammatory cytokines present in these inflamed joints, are a promising strategy to promote joint homeostasis.

DISCUSSION

Understanding the biologically active factors that make up the human MSC secretome and manipulating these cells to consistently secrete factors of therapeutic importance, will improve MSC secretome-based therapies. Emerging single-cell technologies will undoubtedly help decipher the heterogeneity of MSCs and allow for the selection of MSC subsets that secrete therapeutically desirable factors. To date, single-cell transcriptomic analyses of human MSCs resulted in varied outcomes. For example, umbilical cord-derived MSCs were found to exhibit limited heterogeneity, whereas Wharton's jelly derived MSCs were found to be functionally heterogeneous in terms of proliferative capacity and wound healing potential (Huang et al., 2019; Sun et al., 2020). Single-cell RNA sequencing of mouse BM-derived MSCs revealed multiple profiles as well, some associated with distinct differentiation potential (Freeman et al., 2015). Our group used single-cell transcriptomics to analyze donor-matched equine MSCs isolated from three different tissue sources and we found inter- and intra-source genetic heterogeneity that resulted in functional

heterogeneity in immune function and cell motility (Harman et al., 2020). The emerging technology of high-resolution precision proteomics is currently only being used to evaluate cancer cellular heterogeneity (Waas and Kislinger, 2020), but will certainly be transferrable to MSCs, where this technique can provide additional insights into the heterogeneity of MSC populations to allow for the purification of MSC subpopulations with high secretory potential.

In addition to identifying the molecules produced by MSCs that have the functional characteristics to lead to desired clinical outcomes, there are further aspects to consider when moving MSC secretome therapy from bench to bedside. Here, we discuss two of those aspects by asking the following questions. First, is the use of a rich compilation of bioactive MSC secreted factors required for maximal therapeutic benefit, and second, what options are available for delivery of the MSC secretome to target tissues?

Advantages and Disadvantages of Using a Compilation of Bioactive MSC Secreted Factors

The use of a compilation of bioactive factors secreted by MSCs that have either been primed to overproduce therapeutically valuable molecules or genetically engineered to produce and secrete these molecules, may be more effective than solely administering the individual factors of interest. As discussed earlier, studies from our group indicated that although specific proteins in the horse PB-MSC secretome contribute to cellular functions associated with wound healing, they did not account for the full effectiveness of the complete secretome as observed in both *in vitro* and *in vivo* wound healing assays (Harman et al., 2018). In general, most studies documenting the efficacy of the MSC secretome to promote tissue repair and/or modulate the immune system do not indicate precisely which factors are responsible for the beneficial effects. The most obvious reason for using a compilation of bioactive MSC secreted factors over discrete individual factors, is the fact that the secretome is comprised of a myriad of bioactive nucleic acids, proteins, and lipids, that all have the potential to interact with target cells and tissues on different levels (Harrell et al., 2019). Consequently, the use of a compilation of MSC secreted bioactive factors provides numerous molecules that may function together in networks in order to obtain the maximal effect. This is illustrated by previously discussed data from our own group. We have identified proteins with regenerative properties and proteins with antimicrobial properties in the secretome of horse PB-MSCs (Bussche and Van de Walle, 2014; Bussche et al., 2015; Harman et al., 2017a,b, 2018, 2020, Marx et al., 2020). Using these identified factors individually could be therapeutically useful to promote wound healing or to fight bacterial infections. However, using the secretome as a whole may capitalize on a treatment that reduces bacteria in infected wounds, while simultaneously restoring the tissue damage caused by acute injury and pathogens.

This benefit is also evident by the recognition that MSC-derived EVs, widely studied as a form of cell-free MSC therapy, are made up of a compilation of factors, including nucleic acids, particularly small regulatory RNAs, proteins and lipids. Secreted factors contained in EVs are more stable than secreted factors that are free in solution and they are more likely to be taken up by target cells via interactions of surface ligands/receptors, adhesion of membrane integrins, or endocytosis of the EVs (Sarko and McKinney, 2017; Eleuteri and Fierabracci, 2019). EVs from many cell types are known to be involved in cell-cell signaling, as well as tissue regeneration, and it has been demonstrated that EVs are comprised of RNAs, proteins, and lipids, that are distinct from those secreted freely from their cells of origin (Barreca et al., 2020). For example, comparative analyses of miRNAs detected in EXOs and the EXO-cells-of-origin have clearly demonstrated that miRNA composition of EXOs and the cells they were secreted from differ widely, suggesting active packaging of miRNAs into this class of EV (Zhang et al., 2015). Among miRNAs known to be upregulated in EVs, are those involved in the regulation of angiogenesis (Liang et al., 2016; Salinas-Vera et al., 2018). Moreover, EVs transport various cytosolic proteins involved in cell proliferation and migration, such as FGF2 that lacks the exocytosis signals needed to be secreted through the endoplasmic reticulum-Golgi pathway (Candela et al., 2010). In general, EVs are enriched in sphingosine-1-phosphate, a signaling lipid that in itself induces cell proliferation and migration (Xiang et al., 2018).

In addition to the important therapeutic benefit of administering a compilation of bioactive factors secreted by MSCs, there are certainly some disadvantages to this approach. Most notably, and as discussed throughout this review, is the inconsistency in the effectiveness of secretome therapy due to variability of the MSC secretome based on individual donor, tissue source of origin, culture method, and duration of MSCs in culture. As reviewed, these inconsistencies can be addressed by priming and/or genetically modifying MSCs to generate a more consistent secretome. Other strategies to produce a more consistent MSC secretome include (i) consideration of the age, sex and health status of MSC donors, (ii) purposefully using MSC from specific tissue sources, (iii) carefully documenting MSC culture conditions and (iv) limiting the length of time MSCs are maintained in culture (Sagaradze et al., 2018). Indeed, experts in the field of MSC secretome-based drug development state that generating a consistent MSC secreted product with testable potency is the first step needed to move this cell-free therapy from laboratory testing to clinical use (Sagaradze et al., 2018).

Delivery of the MSC Secretome

As described in this review, the secretomes of MSCs isolated from various species and/or different tissue sources contain bioactive factors that have the potential to be used as cell-free therapies. In order to be effective, MSC secretome components must be delivered to target tissues and interact with target cells. On the one hand, administering the MSC secretome therapeutically avoids some of the hurdles associated with MSC cell-based therapies such as the risk of triggering the innate or adaptive immune responses and the possibility of donor cell engraftment and tumorigenicity (Caplan et al., 2019). On the other hand, MSC

secretome-based therapies presents challenges such as retaining secreted factors at the appropriate sites and protecting them from degradation. Select strategies for the delivery of the stem cell secretome have been recently reviewed (Daneshmandi et al., 2020), so here, we will primarily discuss additional strategies that have been described for the delivery of the secretome from other cell types that may also be appropriate for administering MSC secreted factors. Modes of secretome administration can be roughly divided into two categories: direct and associated with a delivery vehicle.

Direct administration includes the injection or application of CM from cultured MSCs at the site of injury, e.g., cutaneous wounds, as well as the injection of exosomes (EXOs) systemically into the blood stream (Daneshmandi et al., 2020). The MSC secretome may also be delivered directly by inhalation for certain diseases (Khan et al., 2017; Grinblat et al., 2018; Dane et al., 2020). For example, treatments with CM from human induced pluripotent stem cells (iPSCs) were delivered by inhalation every 5 days following unilateral pneumonectomy (PNX) in a dog model of destructive lung disease. This study revealed that repetitive inhalation of the iPSC secretome increased alveolar angiogenesis and enhanced septal remodeling associated with improved gas exchange compensation in the lungs (Dane et al., 2020). Moreover, intranasal delivery of the MSC secretome may also have neuroprotective effects, as shown in a study evaluating the efficacy of the secretome of the human amnion-derived multipotent progenitor cells, named ST266 (Grinblat et al., 2018). Specifically, five daily intranasal treatments with ST266 of mice with surgically induced optic nerve crush injuries, resulted in increased retinal ganglion cell (RGC) survival and showed a trend toward reduced RGC axon and myelin damage (Grinblat et al., 2018). In another study, intranasally administered ST266 showed potent neuroprotective and anti-inflammatory effects on the optic nerve in a mouse experimental autoimmune model of multiple sclerosis (Khan et al., 2017).

Historically, MSCs themselves have been used as "delivery vehicles" for their secretome which evolved from the practice of injecting MSCs, locally or systemically, with the goal of having them differentiate and expand at the site of injury to replace damaged tissue (Galipeau and Sensébé, 2018; Krueger et al., 2018; Martin et al., 2019). As it became clear that MSCs do not survive long after administration and that the beneficial effects they exert are due to the factors they secrete that influence recipient target cells, MSCs have served as delivery vehicles in their own right (Spees et al., 2016; Krueger et al., 2018). An active field of research is the engineering of delivery vehicles that allow for the prolonged release of therapeutic molecules at sites of injured tissue. Cells that secrete bioactive factors or bioactive factors in solution can be carried in the vehicles. Delivery vehicles include (i) synthetic polymer-based scaffolds, which increase hydrophilicity and improve cell/secretome immobilization, (ii) hydrogels that retain cells or secreted factors and allow for controlled release, and (iii) fabricated secretome-loaded microparticles that can reside in damaged tissues releasing bioactive factors for days without being rejected by the recipient immune system (Ryu et al., 2019; Daneshmandi et al., 2020). Our group conducted an *in vitro* proof-of-concept study aimed to determine if equine PB-MSCs survived when encapsulated in core shell hydrogel microcapsules, and if MSC secreted factors could diffuse through the capsules and affect target cells (Bussche et al., 2015). We found that MSCs survived for over 3 weeks in the capsules and that CM collected from these encapsulated MSCs promoted dermal fibroblast migration and changes in fibroblast gene expression, suggesting that MSCs encapsulated in this way may be appropriate for therapy (Bussche et al., 2015). Commercially available, implantable cell devices may also serve as delivery vehicles for the MSC secretome. In a rat model of myocardial infarction, animals were subjected to permanent ligation of the left anterior descending coronary artery and then treated with a subcutaneous implantation of human cardiac stem cells enclosed in a TheraCyte device (Kompa et al., 2020). This device retained the cells and protected them from the recipient immune system, while allowing cellular secreted factors to exit. Treated rats showed preserved cardiac function, reduced fibrotic scar tissue, interstitial fibrosis and cardiomyocyte hypertrophy, as well as increased myocardial vascular density when compared to controls (Kompa et al., 2020).

Taken together, both the direct and indirect methods shown to be effective at delivering the secretomes of other cells types are highly likely translatable to the MSC secretome as well. Such methodologies can then be tested and refined in translation animal models, as discussed above, to further improve and optimize MSC secretome therapy in humans.

CONCLUSION

To optimize the human MSC secretome as a therapy, MSC-secreted factors must be better characterized and MSC cultures should be consistent in terms of reliably producing adequate quantities of biologically active molecules of interest. Methods to minimize variation between MSC cultures and to promote enhanced or selective secretion of specific bioactive molecules include priming and genetic engineering of MSCs. Once secretome components are optimized for maximum therapeutic benefits, targeted delivery methods are needed to direct them to injured tissues. Studies in veterinary species have already provided a wealth of information on which molecules are present in the MSC secretome and are therapeutically valuable, and on how to manipulate MSCs to preferentially secrete molecules of interest. Importantly, many of these studies have been carried out *in vivo* in these translational animal models with similar diseases as seen in humans, and they will continue to serve as valuable models to evaluate effective MSC-secreted factor delivery methods in a preclinical setting, that will not only benefit the model species, but human medicine as well.

AUTHOR CONTRIBUTIONS

RH and CM performed the bibliographic research, drafted the manuscript, and created the figures and tables. GV supervised the process, revised the manuscript, and finalized the manuscript.

All the authors contributed to the article and approved the submitted version.

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REVIEW
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Acceleration of Translational Mesenchymal Stromal Cell Therapy Through Consistent Quality GMP Manufacturing

*Premkumar Jayaraman[1], Ryan Lim[1], Jacqueline Ng[1] and Mohan C. Vemuri[2]**

[1] Thermo Fisher Scientific, Singapore, Singapore, [2] Thermo Fisher Scientific, Frederick, MD, United States

Human mesenchymal stromal cell (hMSC) therapy has been gaining immense interest in regenerative medicine and quite recently for its immunomodulatory properties in COVID-19 treatment. Currently, the use of hMSCs for various diseases is being investigated in >900 clinical trials. Despite the huge effort, setting up consistent and robust scalable manufacturing to meet regulatory compliance across various global regions remains a nagging challenge. This is in part due to a lack of definitive consensus for quality control checkpoint assays starting from cell isolation to expansion and final release criterion of clinical grade hMSCs. In this review, we highlight the bottlenecks associated with hMSC-based therapies and propose solutions for consistent GMP manufacturing of hMSCs starting from raw materials selection, closed and modular systems of manufacturing, characterization, functional testing, quality control, and safety testing for release criteria. We also discuss the standard regulatory compliances adopted by current clinical trials to broaden our view on the expectations across different jurisdictions worldwide.

Keywords: mesenchymal stromal cells, cell therapy, GMP manufacturing, closed and automation systems, characterization and potency, regulatory compliance

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Marius Alexander Möbius,
Technische Universität Dresden, Germany

****Correspondence:***
Mohan C. Vemuri
mohan.vemuri@thermofisher.com

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INTRODUCTION

The immense potential of human mesenchymal stromal cells (hMSCs) for regenerative capacity and immunosuppression has been increasingly explored for treating a diverse group of diseases such as neurodegenerative, cardiovascular, autoimmune, bone, cartilage, kidney, liver, cancer, and other disorders (Galipeau and Sensébé, 2018; Pittenger et al., 2019; Saeedi et al., 2019; Kabat et al., 2020). It is well documented now that hMSCs, after transplantation, exert the therapeutic effects through two mechanisms: (i) differentiate into functional cells and facilitate tissue repair by homing into the injured sites (Mastrolia et al., 2019) and (ii) secrete growth factors and cytokines to stimulate immunosuppressive effects by modulating the immune cells (T-cells, dendritic cells, NK cells, and B-cells), angiogenesis, and extracellular matrix remodeling (Pittenger et al., 2019). In addition, hMSCs have low immunogenicity and thus they have the potential to be used for both autologous and allogeneic therapy (Hassouna et al., 2019).

With over 900 hMSC clinical trials listed on ClinicalTrials.gov, the field has expanded its understanding and application of hMSCs and seems poised for success (Levy et al., 2020). Initial successes include the 2018 European approval of TiGenix/Takeda, Alofisel®, for complex perianal fistulas in Crohn's disease. However, only around 300 trials were completed as of 2020, and the

total number of approved hMSCs therapy stands at just 10 (Levy et al., 2020). Earlier this year, one of the approved hMSC products' remestemcel-L (Ryoncil™, Mesoblast) phase 3 clinical trial showed significant improvement in pediatric patients who failed to respond to steroid treatment for acute graft-versus-host disease (aGVHD) (Kurtzberg et al., 2020).

Given the backdrop of increasing interest in using hMSC therapies to fulfill unmet patient needs, there are still inherent industry challenges that would pose barriers to market access, especially within the manufacturing process. Some of the main challenges are product consistency in terms of quality and efficacy, raw material qualification to ensure that the clinical product meets regulatory compliance, cost of cell processing as manufacturing is scaled up or scaled out, and lack of advanced check-point analytical tools to carry out the process and product quality assessment. If the clinical product is not consistent, batch failures are imminent, leading to loss of productivity and compromised sustainability. Protocol amendments can impact the timely progress of all functions including R&D, process development, quality control, manufacturing, regulatory, and clinical testing. To implement a substantial clinical protocol amendment, the median costs can be $141,000 for phase II and $535,000 for phase III protocols (Getz et al., 2016). That is why choosing the right starting raw materials is very important as early as the process development stage. When moving toward clinical trials, developers need more safety and regulatory features to foresee and meet regulatory requirements. It is the responsibility of the hMSC manufacturer to qualify the performance of the raw materials, assess the lot-to-lot variability, test residuals on the final cell product, and determine the need for any additional safety testing. Furthermore, the manufacturer needs to qualify whether the intended suppliers can provide raw material traceability, characterization, and regulatory filing support documentation.

Concomitantly, both autologous and allogeneic cell therapy manufacturing workflow comprises of many different unit operations and, thus, is very complex and labor-intensive due to open processing. Open manipulations are prone to errors and contamination leading to a risk of failed production runs. Besides, manual methods to synchronize different steps in scale-out or scale-up processes and proper workflow documentation to satisfy GMP compliance adds another layer of complexity. In contrast, closing the process and automating the entire manufacturing workflow through digital integration would reduce the risks of open operations and improves product consistency, which is a critical necessity for a GMP setting (Moutsatsou et al., 2019).

Furthermore, identification and assessment of critical quality attributes (purity, potency, and safety) for release criteria as early as the process development stage would ensure product consistency during commercial manufacturing (National Academies of Sciences Engineering, Medicine, Health and Medicine Division, Board on Health Sciences Policy, and Forum on Regenerative Medicine, 2017). In 2006, the International Society of Cell and Gene Therapy (ISCT) came up with a "minimal criteria" for defining hMSC (Dominici et al., 2006). While it is useful in defining the identity and some functionality of hMSCs, it stopped short of defining other critical attributes such as its immunomodulatory capability of cells and other novel biomarkers (Samsonraj et al., 2017). In 2019, the criteria were updated by the ISCT to include the tissue-source origin of the cells and a matrix of functional assays such as secretion of trophic factors ensuring more meaningful information is collected to properly assess the therapeutic potential of hMSCs (Viswanathan et al., 2019). Yet, there remains a lack of a minimum set of standard guide release criteria that hMSC manufacturers targeting different diseases can adopt for regulatory approvals. Additionally, if the manufacturers are targeting their hMSC products for multiple regions, it is important to align with each region's regulatory guidance as it is imperative to note that each region has unique raw material regulatory guidance documentation.

In this review, we discuss the raw materials considerations for hMSC manufacturing with a particular focus on QC/safety testing expectations from global regulatory guidelines. We propose a rationale of why closed, automated, and modular systems are integral to GMP manufacturing and discuss possible workflow solutions for both scale-out and scale-up processes. We focus on hMSC product characterization tools and provide insights to improve existing assays throughout the development and manufacturing process. We also shed light on the regulatory perspectives of current hMSC products in the market and potential future guidelines for regulatory approvals in different global regions.

CONSIDERATION IN THE SELECTION AND QUALIFICATION OF CULTURE SYSTEMS USED IN HMSC MANUFACTURING

hMSC culture systems have evolved over the last 40 years. **Table 1** summarizes common hMSC culture systems and critical raw materials used following regulatory expectations of quality and safety testing. There are many do-it-yourself and commercially available serum-free and xeno-free (XF) culture systems currently; however, there is no harmonization in the way culture systems are being classified. To avoid ambiguity, we attempt to define the classification of culture media in **Table 1** (Jayme and Smith, 2000). ISO/TS 20399 also lists definitions of ancillary materials that the field could aim to adopt. Today, there are over 30 hMSC expansion media marketed as "XF" by commercial suppliers (Gottipamula et al., 2013). We will focus on discussing XF culture systems supplemented with human platelet lysate (hPL) or recombinant human proteins and growth factors as these are current trends in the field (Guiotto et al., 2020; Yan et al., 2020).

Friedenstein et al. (1970) were the first to report the culture of fibroblast-like colonies from guinea pig bone marrow in media supplemented with fetal bovine serum (FBS). Media supplemented with 10–20% FBS has since been recognized as a conventional method to expand hMSCs from various tissue sources (Haynesworth et al., 1992) and has been used as an ancillary reagent in clinical trials since the early 1990s.

Safety concerns using undefined animal serum include risks of introducing pathogens, exposing patients to xenogenic infections, and unintended immunological reactions to bovine proteins (Macy et al., 1989; Heiskanen et al., 2007). Twenty to fifty percent of commercial FBS is tested positive for viruses and not all lots are suitable for MSC isolation and expansion (Wessman and Levings, 1999; Gottipamula et al., 2013). Although regulatory authorities allow the use of FBS as a raw material for clinical production, cell therapy manufacturers would have to ensure that FBS is adequately controlled and that viral testing/inactivation processes (gamma-irradiation/mycoplasma/sterility, 9CFR virus testing) (TSE/BSE sourcing) and specific risk assessments are thoroughly performed in conformity to the relevant regulatory guidance (**Supplementary Table 1**).

Given the lot-to-lot variability of FBS, significant investment in time and costs have to be made in rigorous screening, selection, and validation of suitable lots to ensure consistency and reproducibility in culture performance expansion (van der Valk et al., 2004). The presence of FBS during the hMSC expansion could also influence cell quality attributes—hMSC cultures could undergo early senescence with progressive loss of differentiation capacity (Bieback et al., 2012). Overall, FBS is viewed as a high-risk material (USP < 1043 > ancillary material risk tier 4) and regulatory agencies have recommended manufacturers to use non-animal, non-ruminant materials if the option exists. The field has thus shifted toward adopting xeno- and serum-free culture systems for hMSC manufacturing.

Today, hPL has been suggested as an XF substitute of FBS (Doucet et al., 2005), and it has been increasingly used in trials. A survey of bone marrow transplantation centers in Europe reported 77% of centers use hPL-supplemented media for trials utilizing hMSCs. Initially described by Doucet et al. (2005), hPL is derived from platelet-rich plasma of whole blood donations or apheresis collections (Schallmoser and Strunk, 2013). Platelets are subjected to lysis through repeated freeze/thaw cycles resulting in the release of bioactive molecules and growth factors involved in stimulating mitogenesis and promoting cell adherence (Guiotto et al., 2020; Yan et al., 2020). Research has shown that hMSCs expanded in hPL-supplemented media retain their *in vitro* and *in vivo* characteristics and generally achieve superior proliferation rates over hMSCs expanded in FBS-based systems (Schallmoser et al., 2007; Bieback et al., 2009; Ben Azouna et al., 2012; Griffiths et al., 2013). As such, hPL culture systems are viewed as a desirable option to enable large-scale commercial manufacturing of hMSCs in both 2D and 3D suspension-based platforms. There are, however, ongoing challenges with using hPL in hMSC manufacturing. hPL is undefined and its composition is inherently heterogeneous. Many factors such as donor differences (i.e., gender, age, blood group, metabolites) and production processes influence batch-to-batch variation (Lohmann et al., 2012; Pierce et al., 2017).

A key gap lies in the lack of standardized methods used in sourcing, producing, and quality/safety testing of hPL. Usually, hPL is prepared from a large allogenic pool of blood donation to balance out variation in growth factor concentrations across donors and manufactured lots. However, the size of the donor pool has recently come under regulatory scrutiny due to concerns over risks of transmitting bloodborne pathogens. European Pharmacopeia general chapter 5.2.12 recommends that pooled donations must be limited otherwise pathogen reduction treatment (PRT) needs to be applied during hPL production. Pathogens can be reduced or inactivated by several methods such as gamma-irradiation and treatment with amotosalen + ultraviolet (UV)A light, riboflavin + UVB light, UVC light, or solvent/detergent (S/D). While global regulatory agencies and pharmacopeia recommend limiting the size of the donor pool, no specific guidelines have been given aside from German regulations that restrict the size to 16 donors without the need for pathogen reduction.

hMSC therapy developers using hPL-based culture systems will face some limitations around batch consistency, safety, costs associated with outsourcing PRT, and additional performance testing to ensure that release criteria are met using hPL subjected to PRT. It is no surprise that the industry is in favor of using defined serum-free and XF formulations containing only human proteins and growth factors, but there are still several barriers to regulatory acceptance and commercialization to consider. Firstly, suppliers of hMSC XF systems do not often state that their formulation fully utilizes human recombinant proteins and growth factors. Such systems could still contain proteins purified directly from human plasma (i.e., human serum albumin or human transferrin). Cell therapy developers will have to ensure that the human plasma-derived proteins are sourced from low-risk origins and accredited blood banks and have the necessary adventitious agent testing and inactivation performed. Secondly, human recombinant proteins used in hMSC XF culture systems could be expressed in mammalian cell lines such as CHO or HEK. Regulatory guidance suggests following the principles of ICHQ5A (viral testing evaluation) and ICHQ5D (characterization/lineage history) in establishing master cell banks (MCB) for products derived from cell lines of human or animal origin. hMSC developers should consult with their suppliers on the quality and documentation available for proteins derived from mammalian MCBs. As a safer alternative,

TABLE 1 | Classification of hMSC culture systems.

Classification	Definition
Serum-containing media	Contains animal or human serum (i.e., FBS or Human serum)
Serum-free media	Does not contain animal or human serum or plasma as direct/primary ingredients. Media may still contain proteins purified from the blood (i.e., BSA and HSA)
Xeno-Free media	Contains human-derived blood components as direct ingredients (i.e., hPL, human serum) and may contain human proteins purified from human blood (i.e., HSA) and human recombinant growth factors.
Animal origin-free	Does not contain any human or animal components at the product and process level. Does not contain human recombinant proteins and growth factors. Could contain biological proteins expressed in plant and rice (i.e., soy hydrolyzate)
Chemically defined media	Media formulation with known chemical components and structures. Does not contain any proteins or complex raw materials.

hMSC developers and suppliers could aim to use recombinant proteins expressed only in non-mammalian and non-animal cell lines. Thirdly, unlike serum and undefined hPL, hMSC XF culture systems containing purified and/or recombinant proteins lack extracellular matrix proteins to support cell adhesion. Pre-coating surfaces with common cell adhesion proteins such as human collagen and human fibronectin are required. To ease the expansion process, hMSC developers have been exploring coating-free methods by simply supplementing cell adhesion proteins directly to the culture media. However, obtaining a consistent and affordable supply of GMP-grade collagen or fibronectin and their respective animal-origin free recombinant alternatives remains a current industry challenge. Recent studies have reported the use of recombinant vitronectin for hMSC expansion as an alternative substrate to fibronectin and collagen. Recombinant vitronectin protein fragments are widely used in the expansion of pluripotent stem cells with GMP-grade, animal-origin free versions available by several commercial suppliers.

With this background, developers need to identify and evaluate regulatory compliant hMSC raw materials and optimize expansion and characteristics in small scale during the early process development stage. This exercise would serve as a prerequisite for the next stage of large-scale GMP manufacturing in a closed and automated manner.

GMP BASED AUTOMATED, CLOSED SYSTEM MANUFACTURING

In this section, we will discuss the benefits of closing the process, automation, and single-use technologies (SUTs) followed by a review of existing and proposed closed automation solutions for each of the hMSC manufacturing unit operations.

MSC-based therapies require large-scale manufacturing, conserving both the phenotypic characteristics and functional potency of the donor-derived MSCs. Typically, both allogeneic and autologous hMSC manufacturing processes include cell isolation, followed by *ex vivo* cell expansion, harvesting the expanded hMSCs, wash and concentrate cells, and final fill and finish cell doses (formulation) either for direct infusion or for cryopreservation. Currently, most of these unit operations in the hMSC manufacturing are manual or semi-automated involving largely open processes (Timmins et al., 2012; Nguyen, 2016). Consequently, these are laborious, labor-intensive, prone to cross-contamination resulting in production loss, batch-to-batch inconsistency, high manufacturing costs due to the requirement of a large footprint of the facility with dedicated Class B processing areas, and increased environmental monitoring (Moutsatsou et al., 2019). This creates a major challenge for hMSC manufacturing under a stringent GMP regulatory framework, which is critical for commercial-scale GMP manufacturing. Closing and automating the entire processes produce consistent product quality and reduce the risk of contamination during each step of the workflow, enabling a significant cost reduction and ensuring regulatory compliance through standardized manufacturing and process reproducibility (James, 2017; Moutsatsou et al., 2019).

Closing the process is often achieved by using SUTs, which protect against contaminants outside of a cleanroom environment or biosafety cabinet. SUTs include disposable tubings, connectors, bags for cryopreservation, bioreactors and product transfer, vials, mixers, and filters. Closed system connectivity for sterile fluid transfer is accomplished through the use of tube welding/sealing or aseptic connectors. Tubing used in cell therapy manufacturing comes in different sizes (i.e., 1/8″ ID, 1/4″ ID, and 3/8″ ID) and materials (i.e., PVC and C-Flex®). Tube welding/sealing is a widely adopted method for sterile connections in biopharma industries because of its ease of use. The most commonly used tube welders are Terumo TSCD® II, Terumo SCB® IIB, and BioWelder® Total Containment from Sartorius. However, some inherent challenges remain such as particulate generation during the welding process, inability to join tubing of different sizes, different welders required for different tube sizes, and different types of thermoplastic tubings cannot be welded together (Clarke et al., 2016). To address these challenges, aseptic genderless connectors would offer the flexibility to work with any tubing size or material. Genderless connectors utilize three simple steps to enable sterile connection, "flip-click-pull" (CPC AseptiQuik®) or "click-pull-twist" (Pall Kleenpak® Presto). These aseptic connectors are however not pre-fitted with tubing. Cell therapy manufacturers would have to work with their suppliers to customize single-use bags with compatible tubing dimensions for sterile connections or welding to other SUTs.

Automation is critical for large-scale commercial GMP manufacturing and most importantly enables closed system processing (James, 2017). There are two types of closed automation platforms for cell therapy manufacturing: (1) Closed automated system with integrated incubation and (2) Closed automated system with centralized incubation (James, 2017; Ball et al., 2018). In option 1, all of the unit operation processes are combined into a single automation system (e.g., Miltenyi's Prodigy and Lonza's Cocoon) and are specifically designed for autologous cell therapy manufacturing. These devices can do parallel processing in Class C processing area with minimal labor, "but" the processing equipment is poorly utilized due to the lengthy incubation periods, e.g., one machine processes one patient at a time and it is locked for use for 1–2 weeks depending on the number of cells that are expanded. This would result in the need for more machines and increases the cost-of-goods (COGS) for processing more patients for a given duration (James, 2017; Ball et al., 2018). Also, scalability for different MSC batch sizes due to the limited incubator space is one of the major challenges (Nguyen, 2016) since multiple dosing might be required for MSC therapy due to limited engraftment and survival rate of transplanted cells (Wysoczynski et al., 2018; Pittenger et al., 2019). In contrast, option 2 provides end-to-end manufacturing by integrating different modular automated systems and is highly suited for both autologous and allogenic manufacturing. This modular approach allows parallel processing in Class C area with high equipment and facility utilization achieved by separating incubation. It provides process flexibility for optimizing different conditions and the ability to incorporate new technologies that are critical for the early stage translational therapy developers

(Ball et al., 2018). However, the modular approach requires careful selection of automation systems for physical and digital integration of different unit operations.

Existing and Proposed Closed Automation Solutions for Each of the Unit Operations

Cell Isolation

Cell isolation is the first unit operation in hMSC manufacturing. The most common sources of hMSCs are bone marrow (BM), adipose tissue (AT), placenta (P), and umbilical cord (UC). In current clinical trials, bone marrow is the most widely used source of hMSCs followed by umbilical cord, adipose, and placenta (Pittenger et al., 2019). Currently, most of the cell isolation methods are manual or semi-automated followed by plating in either multiple T75/T175 cell culture flasks or cell stacks/cell factories depending on the starting cell numbers. Subsequently, the attached cells are harvested and cryopreserved as MCBs following standard critical quality attributes (CQA) testing. Furthermore, MCBs undergo a series of seed trains where post-expanded cells at the right passage are harvested and cryopreserved as working cell banks after CQA evaluation. Based on the CQAs, identifying the maximum passage limit with the same clinical efficacy as the earlier passages for each cell line is very important. This would identify the number of passage expansions and working cell banks required to achieve the maximal number of doses from a single vial of MCB. Depending on the starting hMSC source, there are potential gaps in the isolation methods that need to be addressed before adopting the entire workflow in a GMP facility.

Typically for BM-MSCs isolation, manual Ficoll-based density gradient centrifugation of bone marrow aspirates is carried out to separate the mononuclear cell (MNC) fraction. Compared to manual MNC separation, automated Ficoll-based density gradient centrifugation devices such as Sepax C-Pro (Cytiva) can be used to generate clinical-grade MSCs from human bone marrow or cord blood with high recovery and less processing time (Aktas et al., 2008, 2010; Hanley et al., 2013).

Conventionally, UC-MSCs are isolated from sections of perivascular tissue by two manual methods: (1) Direct explant culture technique. Although the procedure is straightforward, there are many challenging steps and contamination risks involved in handling the samples, and it is difficult to translate into an automation platform (Beeravolu et al., 2017). (2) Using a combination of mechanical dissociation with enzymatic degradation followed by filtration of undigested particles and direct culture of separated cell suspension (Smith et al., 2016). In line with the second method, a semi-automated approach of placental MSC isolation was carried out using sterile paddle blender bags containing pieces of intact placenta along with a cocktail of digestive enzymes. The semi-automated process yields were comparable while more importantly the processing time was significantly reduced to 1.5 h against 4–5 h using the manual method (Timmins et al., 2012).

To isolate AD-MSCs, lipoaspirates or adipose tissue are mixed with an equal volume of saline; subsequent manual centrifugation of the lipid phase aspirates is enzymatically digested using collagenase followed by manual centrifugation to isolate stromal vascular fraction (SVF). Alternatively, without the need for enzymatic digestion, rapid isolation of AD-MSCs using the blood-saline portion of lipoaspirate was demonstrated through a simple five-step process (Francis et al., 2010). Güven et al. (2012) previously reported an automated procedure to isolate AD-MSCs from adult human lipoaspirates using Sepax C-Pro, and compared to manual separation, automation resulted in a 62% higher isolation yield and a 24% higher frequency of clonogenic progenitors. More recently, Rodriguez et al. (2017) reported that SVF isolated from adipose tissue using three semi-automated medical devices (GID SVF-1^{TM}, Puregraft^{TM}, and Stem.pras®) are equivalent to the reference manual method in terms of SVF yield, characteristics, and clonogenic potential.

Alternatively, other automation devices such as spinning membrane-based filtration device (LOVO^{TM}, Fresenius) (Wegener, 2014) and cell separation based on size using a counterflow centrifugation system (Gibco^{TM} CTS^{TM} Rotea^{TM}, Thermo Fisher Scientific) (Li et al., 2019; Dargitz et al., 2020) could be explored for non-Ficoll-based MNC isolation (BM-MSC), SVF wash and isolation (AD-MSC), and cord-tissue processing (UC-MSC) using the cited protocols. One other gap that needs to be highlighted here is the requirement and cost of different GMP-grade enzymes for digesting tissues from different sources. Interestingly, these alternatives or new-to-the-market bench-top closed automated cell processing systems could open more innovative ways to improve or simplify the existing protocols to maximize the isolation efficiency. Overall, we envision that the cell isolation process can be closed and automated as illustrated in **Figure 1**.

Cell Expansion, Processing, and Formulation

Based on the ongoing clinical trial data, hMSCs are transfused intravenously at typical doses of 1–2 million cells/kg and in few cases not exceeding more than 12 million cells/kg (Galipeau and Sensébé, 2018), which is approximately 100–150 million cells/patient (Kabat et al., 2020). In addition, depending on the disease indications, the estimated hMSC dosage per patient might be from 15 million to 6 billion cells (Chen et al., 2013). Choosing a suitable scale-out or scale-up strategy for autologous and allogenic hMSC manufacturing (**Figure 2**) is critical to plan right at the beginning stage of small-scale process optimization. Depending on the scale needed, the manufacturer must identify a suitable closed and automated cell processing system that can connect directly to multi-layered flasks and bioreactors to perform volume reduction, wash, medium exchange, and formulation (**Table 2**). Most of the academic developers and advanced cell therapy companies fail to address this aspect, causing profound risks such as increased time and costs to repeat clinical trials shadowed by re-optimization of entire process workflow (Tania et al., 2016; James, 2017). Specifically, MSCs expanded under different culture conditions such as 2D monolayer or 3D microcarrier-based suspension system have an impact on their biological properties and functions, making process and technology changes difficult after clinical trials begin (Cherian et al., 2020; Levy et al., 2020).

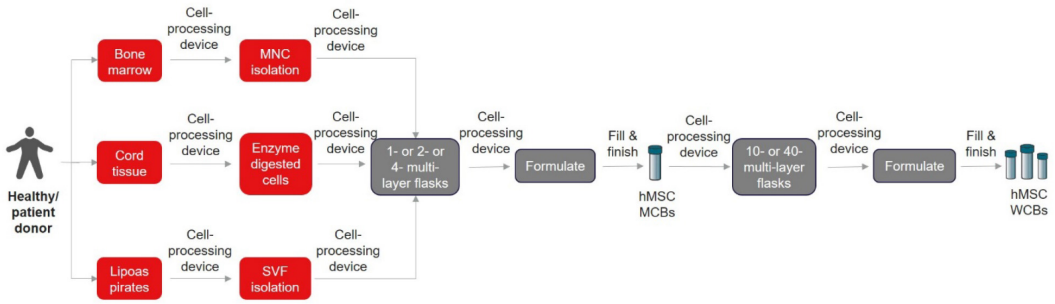

FIGURE 1 | Closed and automated cell isolation workflow. The list of cell processing devices and fill and finish instruments are shown in **Tables 2**, **3**, respectively.

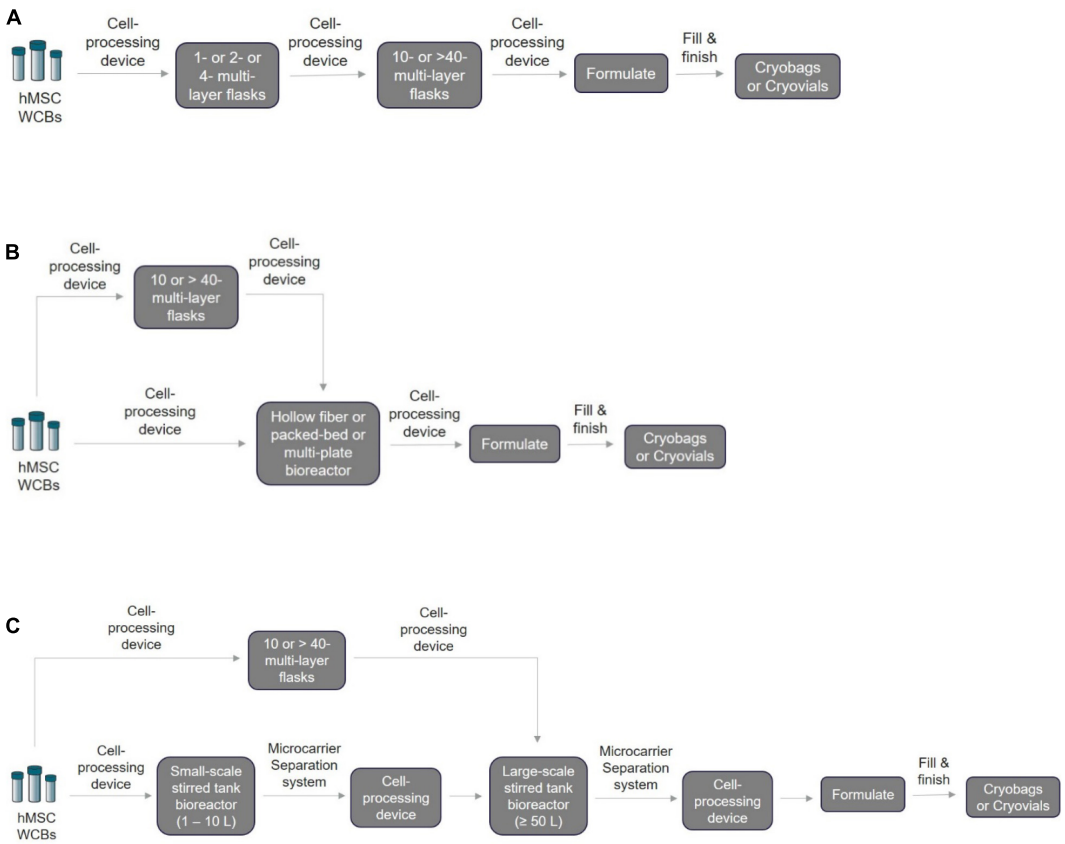

FIGURE 2 | Schematic of the closed and automated hMSC manufacturing workflow. For the three methods listed below, the first step is to thaw the cryopreserved WCBs based on the required seeding density using a cell processing device to wash away cryopreserved media and exchange it with fresh expansion media. Depending on the technology chosen, it may be possible for the same cell processing device to be used for volume reduction, harvesting, passaging, and final formulation into bags for fill and finish. **(A)** 2D monolayer scale-out expansion using multi-layer systems. Processed cells can be transferred directly to a multi-layered flask for expansion. Next, the expanded cells after harvesting and volume reduction can be passaged directly in larger flasks until final harvest and formulation. **(B)** Scale-out expansion using hollow fiber membranes/multi-plate/packed-bed bioreactors. Typically, for hMSC manufacturing using the HF, MP, or PB bioreactors, two approaches can be adopted depending on the passage limit: (1) direct cell seeding into these bioreactors for cell expansion or (2) cell seeding into a 2D monolayer multi-layer device first before seeding into bioreactors. **(C)** Scale-up using microcarrier-based suspension culture in stirred-tank bioreactors. For this technology, two different closed and automated scale-up approaches can be used after cryopreserved WBCs are processed: (1) 2D–3D expansion: cells can be seeded and expanded first into 2D monolayer multi-layered flasks followed by seeding and expansion in 3D microcarrier-based suspension bioreactors. (2) 3D–3D expansion: cells can be seeded directly on 3D microcarriers for expansion in small-scale stirred tank bioreactor followed by seeding and expansion in large-scale stirred tank bioreactors. For in-line separation of microcarriers and cells, closed single-use systems such as the Harvestainer microcarrier separation system can be used for both smaller-scale and larger-scale applications.

2D monolayer-based scale-out culture system:

Currently, for large-scale manufacturing of hMSCs in 2D monolayer cultures, "scale-out" expansion is carried out using multi-layered cell stackers (Corning® CellSTACK and Nunc™ Cell Factory™) through 1 layer to 40 layers. Mostly, these systems are equipped with dual ports closed by caps for filling and venting, respectively. Thus, for aseptic processing, these systems require a laminar hood for manual handling, which is not ideal for large-scale manufacturing. However, it can be completely closed by having a 0.2-μm pore, hydrophobic

TABLE 2 | Closed automation cell-processing instruments that are commercially available in the market.

Supplier	**Cytiva**	**Cytiva**	**Sartorius**	**Fresenius**	**Thermo Fisher Scientific**
Product specifications	**Sefia S-2000**	**Sepax C-Pro**	**kSep400**	**Lovo**	**Rotea**
Output volume (ml)	15–400	8–500 (Optimal output recommended is 70 ml)	>50	50–50000	≥5
Technology	Syringe chamber centrifugation	Electric centrifugation motor and pneumatic circuitry for piston drive	Counterflow centrifugation system	Spinning membrane filtration	Counterflow centrifugation system
Scalability	<10 L	20–1200 ml	0.05–500 L, cell capacity per cycle (1–80 × 10^9 cells)	Up to 22 L	0.03–20 L (no maximum volume, continuous processing possible), cell capacity per cycle (5 × 10^7–5 × 10^9 cells)
Versatility (applications)	Cell isolation by density gradient separation, harvest and formulation	Cell isolation by density gradient separation, spinoculation, harvest wash, and formulation	Wash, concentrate, and harvest	Fresh, cryo-preserved, and culture-expanded white blood cells, including, but not limited to, leukapheresis CD34 + cells, CAR T-cells, TILs, NK cells, and MSCs	Cell isolation and separation based on size, RBC depletion/lysis, fresh/cryopreserved/culture-expanded immune cells (CAR T cells, NK cells), MSCs, HEK, iPSC spheroids wash and concentrate, media exchange, harvest, and formulation
Key features	Ultrasound sensors for bubble detection, pressure and bag weight sensors, centrifugation up to 1600 *g*, Thermal mixer for temperature control between + 4°C and + 40°C and Separation chamber temperature monitor and control	Pressure monitoring and optical line sensors, centrifugation 100–800 *g*	3/8″ × 1/4″ C-Flex connections. Max g-force 1000 × *g*, max flow rates (1900 ml/min)	Membranes have 4-μm pores, using the Lovo Software 3.0. Multiple Source container processing, Administrator ability to pre-fill and lock operator entry fields and options	Instrument (Kit Barcode Reader, Bubble Sensors, pinch valves, camera, moisture sensor, Chamber Detector, OD and Pressure sensors), max g-force 3000 × *g*, max flow rates (165 ml/min) and Single-use kit (Bubble Trap, flexible 1/8-inch tube input (7) and output (1) lines, CFC Chamber) and Software (protocol builder with a simulator, process model and GUI). Able to connect to 2D and 3D expansion vessels. Visually monitor the cells in real time using Gibco™ CellCam™ video technology
Customization (consumables and protocol)	Two different protocol software's and two different kits	Seven different protocol software's and three different kits	One single-use class VI product. One software for all systems	Up to 10 protocols can be saved on the device and each wash cycle may be customized even further	One standard single-use kit (standard/high-flow version). More than 10 standard protocols for different applications. Protocols are highly customizable during process optimization. During GMP manufacturing software allows lockdown of protocol and restricts user access.
Dimensions (L × W × H); weight	51 × 74 × 91; 40 kg	40 × 27 × 46 cm; 16.3 kg	107.5 × 72 × 140 cm; 350 kg	45.7 × 50.8 × 67.3; 34 kg	29 × 50.8 × 76.2 cm; 20 kg
Translate to GMP	Yes (traceability using Barcode reader Data management with PDF reports)	Yes (traceability using Barcode reader Data management with PDF reports)	Yes	Yes (Exportable from DXT to Excel or LIMS)	Yes (OPC-UA interface to connect to a DCS, MES or 21 CFR Part 11–compliant system, digital integration using Delta V platform)

membrane filter in one of the ports to allow gas exchange without the risk of contamination, while the second port can be internally sealed using an aseptic connector that can be connected to media bags or cell processing instruments to allow direct fluid transfer via pumping or gravity. **Figure 2A** shows the schematic for a closed and automated 2D monolayer-based two passage, scale-out hMSC manufacturing. Although this method (**Figure 2A**) is the most cost-effective (Mizukami et al., 2018) and a preferred option for expanding hMSCs for clinical trials (Rowley et al., 2012), many bottlenecks still exist. It is not a scalable system as it requires a larger footprint for handling due to its restrictive surface-to-volume ratio, its non-homogenous expansion due to non-uniform surface coating resulting in batch-to-batch variability, it being laborious, and the fact that media exchange and cell harvesting can be impacted by the handling of multiple stackers at the same time (Cherian et al., 2020). Alternatively, GMP-compliant, closed automated and single-use systems may be suitable for hMSC manufacturing including hollow fiber-based (HF) continuous perfusion device, the Quantum cell expansion system from Terumo BCT (Hanley et al., 2014; Barckhausen et al., 2016; Khan et al., 2017; Frank et al., 2019; Vymetalova et al., 2020), 2D multiplate-based (MP) Xpansion® bioreactor system from Pall corporation (Rouard et al., 2020), and packed-bed (PB) iCeLLis® bioreactor from Pall corporation (Mizukami et al., 2018) (**Figure 2B**). Nonetheless, all of these systems (HF, MP, and PB) are better suited for autologous therapy or scale-out allogeneic therapy as they are limited by scalability, poor harvesting efficiency (especially PB), and the least cost-effective technology (Mizukami et al., 2018).

3D microcarrier-based scale-up suspension system:

On the contrary, microcarrier-based suspension culture using stirred tank bioreactors provides a high surface-to-volume ratio, enabling high-density cultures for large-scale allogenic hMSC manufacturing (Schnitzler et al., 2016). More importantly, microcarrier cultures are the most cost-effective in terms of COG/dose closely following 2D monolayer cultures or even surpassing if we optimize the harvesting efficiency (Mizukami et al., 2018). Many GMP-grade commercial microcarriers are available: cross-linked dextran-based Cytodex® 1 and 3 (Cytiva), SoloHill® polystyrene (Sartorius Stedim), and untreated or Synthemax II or CellBind-coated polystyrene (Corning®). On the single-use stirred-tank bioreactors, there are a variety of commercially available options (Mobius® from EMD Millipore, CelliGen® from Eppendorf, BIOSTAT® from Sartorius, HyPerforma™ from Thermo Fisher Scientific, Xcellerex® from Cytiva, and Allegro® from Pall) for different scales starting from bench-top (1–5 L) and large pilot scale (10–300 L) bioreactors (Schnitzler et al., 2016; Jossen et al., 2018). This microcarrier-based technology also has limitations, such as non-uniform binding during cell attachment, poor harvesting efficiency (∼60%) (Mizukami et al., 2018), and the need for an additional step to separate microcarriers and cells post-harvest. Thus, small-scale process optimization by pre-screening different microcarriers with different substrate coatings in the media of choice using shake flask or spinner flask is important to identify the top-performing microcarrier and further optimize attachment, expansion, and harvest parameters (aeration, impeller speed, and feeding regime) for the large-scale transition. To date, many reports have shown successful scale-up of hMSCs in small-scale and large-scale bioreactors (Timmins et al., 2012; Schirmaier et al., 2014; Cunha et al., 2017; Lawson et al., 2017; Bayne et al., 2019; Koh et al., 2020). In **Figure 2C**, we have shown the schematic of closed and automated hMSC manufacturing using both 2D to 3D and 3D to 3D expansion methods.

Fill and Finish

As a last step in the entire manufacturing workflow, closed automated fill and finish is one of the most important cell therapy manufacturing processes primarily to ensure product safety, consistency, and integrity for longer-term storage (Brandon Fletcher et al., 2020). As such, cell therapy manufacturers need to be aware of the choice of equipment and the aseptic containers used for filling their products in a sterile and scalable manner. The most commonly used forms of containers are closed cryovials or cryobags. Some commercially available cryobags are multiple-chamber CryoStore™ freezing bags (Origen), CryoMACS® Freezing Bags (Miltenyi Biotec), and Freeze-Pak™ Bio-Containers (CharterMedical) with different fill volumes. Gao et al. (2019) reported that human AD-MSCs cryopreserved at -150°C for 24 months in cryobags post-thaw had a viability of >90% with minimal cell clumps with functionality profiles similar to fresh cells. In the case of cryovials, it has been reported that the seal integrity could be compromised for glass vials with rubber stoppers at cryogenic temperatures thus presenting problems of losing product integrity during the critical cryopreservation stage (Zuleger et al., 2012; Hunt, 2019). Alternatively, CellSeal® cryovial (Sexton Biotechnologies) made from USP Class VI material was reported to be stable and durable after 12 weeks of storing cryopreserved regulatory T cells with high cell recovery post-thaw (Fearnot et al., 2014). Aseptic technologies' ready-to-fill AT-Closed Vial® is made up of a polymer body with a thermoplastic septum, and the filling process is simple and scalable (Hunt, 2019). Pharmaceutical-grade Daiko-Crystal Zenith plastic vial was found to be suitable for hMSC cryopreservation at either -85 or -196°C for 6 months, with post-thaw viability of >95% with comparable growth and differentiation profiles of fresh controls (Woods et al., 2010). Ideally, cryobags are used for large-volume and take a longer time for thawing, while the cryovials can be thawed rapidly because of lower fill volumes. For both cryobags and cryovials, slow freezing and rapid thawing are critical to protect the structural and functional integrity of the cells (Hunt, 2019). Overall, examples of commercially available fill and finish instruments are shown in **Table 3**. As listed, very few systems are available that have been designed to suit the specific needs of cell therapy. The challenge for manufacturers is to choose an optimal system, cryopreservation media, and containers that will suit their current needs but be scalable as they progress through the clinical trials and into commercial production.

Digital Integration of Different Unit Operations

While we can physically integrate modular closed and automation systems for each of the unit operations, enabling

digital integration using software platforms provides true automation across the entire autologous or allogenic hMSC end-to-end manufacturing workflow. In this complex environment, data logging the information through enterprise resource management (ERP) starting from sourcing of the raw materials, manufacturing process controls, quality management through to product storage and delivery to the patient is critical. This will ensure a proper flow of information ensuring traceability, which is a requirement for a GMP manufacturing. Building this foundation of cell therapy digital integration and data management enables the interaction of production (hardware and controllers) and control layers such as supervisory control and data acquisition (SCADA) and manufacturing execution systems (MES). Moreover, the enterprise layer will facilitate interlinking the process and plant control for managing all aspects of clinical manufacturing. Data mining tools allow for the acquisition of upstream and downstream process batch record data and using this; real-time data analysis can be performed for different batches for rapid process optimization and troubleshooting. There are few automation transformation platforms that hMSC clinical manufacturers could leverage for integrating all bioprocess unit operations in a GMP biomanufacturing capacity (**Supplementary Table 2**).

Taken together, it is extremely important to understand the existing process, COGS, and choose the best closed automation technologies suitable for unit operations that will seamlessly scale and transition to GMP manufacturing. For example, the hMSC manufacturers, depending on the scale, could choose cell stackers, or stirred tank bioreactors, etc. for incubation. Cell processing devices (**Table 2**), can be utilized for cell isolation, concentrating,washing cells, formulation, and then fill and finish into vials or bags using appropriate instrumentation (**Table 3**) that suits the preferred product format and number of doses. Note that cell processing systems will be used for multiple steps in the entire manufacturing workflow (**Figures 1**, **2**) and the manufacturers need to evaluate each system individually to identify the best fit that works for their process. During this early stage evaluation, it is also important to qualify tubing compatibility and connector options to integrate instruments from several suppliers. Overall, hMSC manufacturers need to embrace the idea to design a GMP facility capable of

TABLE 3 | Closed automated fill and finish instruments.

Specifications	Sexton CellSeal AF-500™	Sexton Signata™ CT-5	Terumo Finia	Flexicon FPC-50	Invetech's 3rd gen	Aseptic technologies L1 robot
Containers	Vials	Bags and vials	Bags	Vials	Bags	AT-Closed Vials
Fill accuracy	99%	N/A	±2 ml or ±10% of the target volume, whichever is greater	±0.5% > 1 ml and ± 1% > 0.2 ml	N/A	N/A
Fill volume	0.8–5 ml	Up to 1500 ml	20–174 ml	<0.2–100 ml	0.25–5 L	0.1–50 ml
Fill capacity	400 vials/h	1 ml to 400 ml/min	N/A	1500 vials/h	N/A	600 vials/h
Batch size	N/A	N/A	N/A	N/A	N/A	≥100–5000 vials per shift
Sterilization	Vapor Hydrogen Peroxide (VHP)	Single-use kits	Single-use gamma-sterilized functionally closed tubing sets	Single-use fluid path	Single-use kit	Vapor Hydrogen Peroxide (VHP)
GMP compatibility	Yes (traceability of process parameters that is automatically generated in batch records and audit trials)	Yes (lockdown GMP compliant routines)	Yes (data management capability tools allow monitoring the processing run data and tracking accessibility)	Yes (able to generate batch reports after each production run and it comes up with optional software to support 21CFR Part 11)	Yes (21CFR11 compliant, eBR integrated)	Yes
Temperature control	External	External	Yes	N/A	N/A	N/A
Dimensions (L′′ × W′′ × H′′)	19.7 × 46.3 × 20	N/A	19.6 × 35 × 30.9	53.8 × 21.7 × 27.2	N/A	47 × 30 × 37
Additional features	The machine is designed for a controlled environment in both Class B and C cleanrooms that includes a benchtop, biosafety cabinet, or isolators.	The system is flexible with ready-to-use consumable kits that can be connected to run custom routines or optimized protocols. Able to perform, cell wash/media exchange and media preparation.	The system can maintain final product temperature to within 3°C, cell viability of more than 95%, uniformity of cell concentrations to within 5% for all bags	The equipment is designed for use under a biosafety cabinet or in a restricted access barrier system or customized for integration into an isolator.	Can perform bulk media formulation of 50–250 L	No need for Class B room

flexible manufacturing using closed modular systems compatible with a digital connectivity platform to enable fully automated manufacturing.

CHARACTERIZATION AND SAFETY TESTING TO ENSURE PRODUCT CONSISTENCY THROUGHOUT THE MANUFACTURING WORKFLOW

During each step of the unit operations starting from cell isolation through to final product cryopreservation and ensuing patient delivery, it is imperative that the cell product undergoes QC and monitoring, such as critical process parameters and critical material attributes, to ensure that CQAs are met for the end product, thereby maintaining product consistency in terms of quality and performance.

Since the late 1960s, the US FDA has been establishing GMP guidelines for pharmaceuticals and has been periodically updating them (Immel, 2001). While those are useful for small-molecule drugs, it is almost impossible to apply those rules directly to our "living" drugs as we do not have a complete understanding of (1) the cells we work with and (2) the intricacy of the manufacturing process. Hence, regulators have developed new guidance with regular periodical updates for cell therapies.

The mandatory CQAs for characterization include identity, purity, potency, and safety. **Table 4** summarizes the main framework and requirements necessary for characterization testing. Of note, a review in 2017 observed that a substantial proportion of hMSCs trials did not result in publications; in particular, early stage trials were unsuccessful (Fung et al., 2017). Of those that did report their findings, a sizable number reported a lack of potency as a reason for not advancing their candidates (Fung et al., 2017). More importantly, manufacturing and non-manufacturing variables have been put forward as the possible cause for the discrepancy between previously observed efficacy in both *In vivo* and *In vitro* settings that differed from the clinical settings (Galipeau and Sensébé, 2018). An underlying reason for this is the inability to accurately predict critical hMSCs functions, such as its immunomodulatory activity properly define CQAs.

Expectedly, much focus has been given to identity, purity, and potency, particularly during process development. The aspect of safety has been thought to be a step considered only at the final stage of manufacturing. However, to ensure that the final hMSC product is free of any adventitious agents, safety should be considered at each stage of the manufacturing process, starting from the selection of raw material as previously mentioned. Additionally, it is important to note that safety testing has three separate components, namely, sterility, mycoplasma, and adventitious virus testing that must be completed (Guadix et al., 2019).

In this section, we describe what are the best practices as per literature in terms of hMSC characterization testing, and what should possibly be considered to overcome the current challenges in product quality assessment. Moreover, we suggest additional considerations that are being considered by the field that would improve hMSC product efficacy with regard to different clinical indications.

Established Technology Parameters

While certain methods have been well established, it does not mean that they can be used directly across multiple utilities due to key differences between them. For example, flow cytometry assay for cellular identity is well accepted and multiple parameters still should be considered. These include but are not limited to the need for a reference control for specific cell surface markers and to overcome the subjective nature of the instrument type and operator gating strategies. Interestingly, standardized automated gating has been shown to improve the precision of the assay, which can be included as an established method (Suni et al., 2003).

Quantification of Multiple Parameters in a Single Assay

The immunomodulatory functions of hMSCs via the release of cytokines have been demonstrated to be a key mechanism in which they are utilized for the treatment of various clinical indications. This point has been highlighted in multiple reviews, which summarize the plethora of cytokines that have been demonstrated to be released by hMSCs, as well as their downstream regulatory functions (Kyurkchiev et al., 2014; Gao et al., 2016; Han et al., 2019). As such, it is critical to characterize the hMSC cytokine release profile and expression levels, to demonstrate potency and consistency in manufacturing.

The testing of multiple cytokines individually for hMSCs would be costly, time-consuming, and ultimately impractical. However, the need for comprehensive characterization testing should not be a trade- off given its importance. As such, assays such as the multiplexed ELISA to test cytokine and growth factors residue for potency and purity are being considered (Ellington et al., 2010; Tighe et al., 2015). The attractiveness of this platform is that it allows for the concurrent quantification of multiple cytokines, at a fraction of the cost, relative to testing cytokines individually.

Of note, while this platform has yet to be fully adopted in approved hMSCs therapy, its feasibility, however, has been shown in other approved therapeutics. Recent examples include the vaccine development for the Sars-Cov-2 pandemic. As part of BioNTech-Pfizer COVID-19 RNA vaccine, a Luminex assay was used (the ProcartaPlex) to test 11 different T_H1 and T_H2 cytokines concurrently (Sahin et al., 2020). Another example was the development of Axicabtagene Ciloleucel. This CAR T-Cell Therapy for Refractory Large B-Cell Lymphoma employed a multiplex Luminex assay to measure seven different cytokines during their process development stage (Turtle et al., 2016). Hence, this is a platform that could be adopted for hMSCs as well. While some operational challenges are present, the benefits of such a platform warrant further looking into (Ellington et al., 2010; Tighe et al., 2015).

TABLE 4 | Characterization/CQA for MSC therapy.

	Identity	Potency	Purity	Safety
Definition and Purpose	21 CFR 610.14: Specific testing that will adequately identify the designated product and distinguish it from any other product being processed in the same site. Also, to ensure that the final product given to the patient is as intended and that the manufacturing process did not significantly alter the starting hMSCs (FDA, 1998a).	21 CFR 610.10: Potency assays are necessary to quantify specific hMSCs biological functions for the intended purpose (FDA, 2011).	21 CFR 600.13: To define that the final product is relatively free from any extraneous material (FDA, 2008).	21 CFR 610. 12: To ensure that the product is free from any adventitious agents and other contaminants (FDA, 2008).
Key consideration	Multiple factors such as culture duration and scaling up could result in changes to the final hMSCs product. The US FDA notes four major parameters that can affect MSCs characteristics (Mendicino et al., 2014): Working and Master cell bank Fetal bovine serum (FBS) Oxygen concentration Cryopreservation	A matrix of relevant assays that likely demonstrates the mechanism of action (MOA) of hMSCs for the intended purpose rather than a single assay should be considered. Given that hMSCs have multiple clinical indications, there is no established gold standard potency assay. Guidance from the US FDA recommends how to establish potency assays, and that they be performed during the early product development phase due to the large number of advantages it affords (FDA, 2011).	Broadly classified as two groups, pyrogenicity/endotoxin, and residual contaminants	Required testing includes adventitious viruses and sterility testing of bacterial and fungal and mycoplasma (FDA, 2008). US FDA recommends reading of two guidance document ["Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals" (FDA, 1993) and ICH guidance Q5A: "Guidance on Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin (FDA, 1998b)]
Requirements	No specific assay is stated; however, it is a requirement to confirm the identity of the cells via quantitative testing through phenotypic and/or biochemical assays such that it can be adequately identified and distinguished from other products (FDA, 1998a).	No specific assay is stated, however, to attain a biologics license, the hMSC product would have to meet the requirements of potency as stated in (21 CFR 601.2), which requires the validation of a potency assay "accuracy, sensitivity, specificity, and reproducibility" [21 CFR 211.165(e)]. Additionally, data from all tests, with the necessary standards and specifications, must be well documented (21 CFR 211.194) (FDA, 2011).	*Pyrogenicity/endotoxin:* (FDA, 2008) 21 CFR 610.13: Requires the rabbit pyrogen test method. If this test cannot be carried out, the Limulus Amebocyte Lysate test method (LAL) is an alternative, only if acceptable conditions as set by the FDA guidelines are met. US FDA recommends an upper limit for endotoxin at 5 EU/kg body weight/h, provided it is not administered intrathecally (upper limit would be reduced to 0.2 EU/kg body weight/h if so) *Residual contaminants:* (FDA, 1998a) Parameters include: Residual peptides and proteins used during production and purification Manufacturing reagents, such as cytokines, growth factors, antibodies, beads, and serum. Quantification of cell debris or contaminating cell type	*Adventitious agents:* (FDA, 1993; FDA, 1998a) As per 21 CFR 610.12 *In Vitro* and *In Vivo* viral testing is necessary. Key information required includes what are the tests performed and at which stage of the manufacturing process was it done. Species-specific testing for adventitious viruses is also important. With human cells, it is recommended that human pathogens such as CMV, HIV-1 and 2, HTLV-1 and 2, EBV, HBV, HCV, and B19 be tested. *Sterility testing:* (FDA, 1998a) Specific microbiological tests are described in 21 CFR 610.12 culture and non-culture-based methods. *Mycoplasma testing:* (FDA, 1993; FDA, 1998a) Both culture-based assays and polymerase chain reaction-based assays can be used.

Clinical Indication-Specific Assay

A best practice is to develop a matrix of relevant assays that likely demonstrates the mechanism of action (MOA) of the hMSCs for the disease indication for which the MSCs are intended. This is appropriate, given that hMSCs could have a specific effect on certain clinical indications. For example, in the treatment of graft-versus-host disease, hMSCs' immunomodulatory capacity via anti-inflammatory cytokines should be part of the matrix of the potency assays (Caplan, 2009). However, should the same cell product be used for neural regeneration, whereby the hMSCs can stimulate angiogenesis in nerves and motor function recovery, other potency assays would be more relevant (Masgutov et al.,

2019). As such, the selection of specific clinical indication potency assays would be more relevant in not only demonstrating the MOA of the cells but also reducing the need to perform other irrelevant potency assays and thus save the effort and time driving lower COGS in product development.

When and Where Should Characterization Assay Be Done

The necessity of performing characterization assay is not solely because it is a requirement, rather, it is to help the developer ensure that the development and manufacturing process is consistent, and the final product will function as intended. The US FDA further recommends that characterization testing be done during the early product development phase due to a large number of advantages it affords such as the ability to "Demonstrate product activity, quality and consistency throughout product development; evaluating product stability; provide a basis for assessing manufacturing changes," etc. (FDA, 2011). Hence, characterization testing should be performed as a part of in-process testing instead of just being part of the release criteria.

In addition to performing characterization assays early, it is also a good practice that such in-process testing should be done at key points of the manufacturing process, as well as critical risk areas for certain parameters. For example, when using frozen products, if it undergoes manipulation such as washing or culturing after thawing, it may be necessary to repeat the sterility test. This is more so if it is performed in open systems (FDA, 1998b). Moreover, concerning mycoplasma, two major sources in which contamination can occur are the culture facility: in particular open culture systems and the use of animal serum products (FDA, 1998a). It is recommended that mycoplasma testing be done at stages where cell pooling is involved to harvest cells or when there is an extended culture procedure (FDA, 1993).

Other Consideration to Enhance the Development of MSC Therapy

Development of More Representative Characterization Assays: Direct Function Assays

One key challenge in the development of successful therapy is due to hMSC therapy being "living" drugs. As such, they are fundamentally a heterogeneous population, whereby their gene and protein expression profiles can be entirely different from each other. Several studies have demonstrated that this heterogeneity is due to multiple parameters, such as hMSC origin source (Hass et al., 2011), the method of extraction (Juneja et al., 2016), as well as *in vitro* culture conditions and methods (Yin et al., 2019), just to name a few.

As multiple parameters can affect hMSC functions, it stands to reason that more meaningful characterization assays should be employed (in addition to what is minimally needed) to certify that the final hMSC product has the necessary therapeutic activity (Chinnadurai et al., 2018). Indeed, more groups are looking to engineer more predictive characterization assays to ensure a greater correlation between the therapeutic activity and final product (Zhukareva et al., 2010; Chinnadurai et al., 2018; Russell et al., 2018). One such assay is the inhibition of activated CD4 + T-cells proliferation to measure hMSC immunomodulatory function. Given that this method allows for a phenotypic change to be measured, relative to surrogate measurements of cytokines such as TNF-α receptor expression, it is believed to be a more accurate representation (Bloom et al., 2015).

New Characterization Methods: Utilizing Genetic Data to Improve Clinical Indication Prediction

Given the decades of hMSC research and clinical trials, we have an enormous database of gene expression data that thoroughly investigates almost every aspect of the hMSC transcriptome throughout every stage of the manufacturing process. Hence, studying this readily available treasure trove of data is extremely useful to properly elucidate the nature of hMSCs, their role in multiple diseases, and potential clinical indication via its mechanism of action (Pittenger et al., 2019).

Indeed, one interesting transcription factor that was demonstrated to regulate hMSC effector function was Twist1 (Boregowda et al., 2016). Boregowda et al. (2016) observed that altering expression levels of Twist1 resulted in a corresponding change in hMSC potency in both *in vitro* and *in vivo* settings. Their team subsequently developed a CLinical Indications Prediction (CLIP) scale that could be used to prognosticate hMSC heterogeneity against hMSC effector function for multiple clinical indications. While this has the potential to be a useful tool for screening populations, more work should be done to establish it further.

Improving on Pre-existing Assays: Using New Assays to Speed Up Critical Characterization Parameters

There is a need to accelerate the development of hMSCs therapy; hence, there is much focus on improving existing protocols of characterization of CQAs (Samsonraj et al., 2017).

Concerning potency, as previously mentioned, the implementation of the multiplex ELISA to perform multiple cytokine measurements instead of single-target ELISA is being considered (Ellington et al., 2010; Tighe et al., 2015; Turtle et al., 2016; Sahin et al., 2020). For identity characterization, using gene expression assays such as Taqman^{TM} to replace standard Tri-lineage differentiation staining assay is another example. While the current Tri-lineage differentiation assay requires 2–3 weeks for the cells to differentiate followed by staining and imaging (Owston et al., 2019; Sanjurjo-Rodriguez et al., 2019; Rajpar and Barrett, 2020), newer methods such as the measurement of hMSC Tri-lineage gene expression following 1–2 weeks of differentiation reduces the time needed while providing quantitative information relative to the current method (Szepesi et al., 2016; Hwang et al., 2017; Ling et al., 2020).

Once properly validated, these new assays would be used as an alternative to current methods. One such example is the characterization of safety, in particular, mycoplasma detection. Traditionally, the detection of mycoplasma was done via culture methods that typically require around 28 days. However, it is now accepted by regulators that mycoplasma detection can be done via

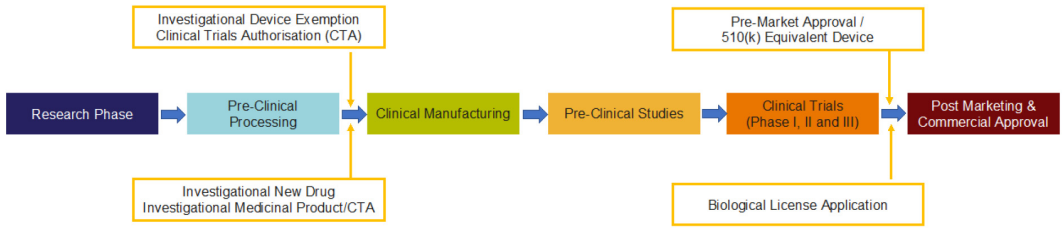

FIGURE 3 | Process for the generation of MSC-based clinical product with compliance to regulatory requirements by FDA (Mendicino et al., 2019) in the cell and gene therapy category.

PCR-based assay, which is cheaper and less time-consuming than the traditional method (FDA, 2010).

Improving Manufacturing Processes

While developing better potency assays would allow the detection and subsequent removal of suboptimal hMSCs, it would be far better to develop techniques that indicate hMSCs' therapeutic potential. This concept is not lost on several companies that had improved on the current manufacturing process.

One stage that was targeted was cryopreservation. A study showed that freshly thawed and washed hMSC had a large range in its viability (36–85%) (Matthay et al., 2019). This could be a result of cellular damage due to the cryopreservation stage in which a study observed that the process of cryopreservation affects the cytoskeleton (Chinnadurai et al., 2014). Of note, these detrimental changes to the hMSC physiology could lead to an increase in complement-mediated clearance (Moll et al., 2014), thereby significantly reducing its overall efficacy for the patient due to a decline in hMSC persistence in the system. In addition to viability and persistence, cryopreservation was also observed to reduce hMSC immunosuppressive function (Francois et al., 2012). Taken together, improving the cryopreservation process and characterizing the hMSC product before and after this stage is vital.

While this has not been fully validated, there is some evidence that provides credence to this strategy in improving MSC quality. Take the most recent approved hMSC therapy Alofisel®. During its phase 3 clinical trial, they took into consideration the pitfalls of cryopreservation by including an additional process. Before administration, they thaw the cells and formulated 120 million cells in 24 ml of culture medium before shipping to the respective hospital as a formulated product that can be stored for 48 h (Panes et al., 2016). This allowed "recovery" of freshly thawed product and selection of viable cells to occur, addressing the issue observed above. While this is promising, more studies would have to be done to further validate this strategy.

REGULATORY PERSPECTIVES OF HMSC PRODUCTS

Despite decades of research showing hMSC clinical potential and a vast number of hMSC clinical trials covering an array of indications, only a handful of hMSC products (Ancans, 2012) have been approved for market authorization globally compared to the large number of ongoing clinical trials that meet the safety requirements (Jahani et al., 2020). For country-specific information, the readers are directed to http://www.aabb.org/advocacy/regulatorygovernment/ct/international/Pages/default.aspx.

Regulations for Mesenchymal Stromal Cell-Based Medicinal Products in the United States and European Union

The process of approval for MSC-based clinical product is developed following a rigorous procedure designed in a sequential and stepwise manner as shown in **Figure 3** in accordance with the global regulatory agencies (**Table 5**).

TABLE 5 | Regulatory agencies in different countries.

Country	Regulatory agency	Website link
United States	Food and Drug Administration (FDA)	https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products
Canada	Health Canada	https://www.canada.ca/en/health-canada/services/drugs-health-products/biologics-radiopharmaceuticals-genetic-therapies/applications-submissions.html
Europe	European Medicines Agency (EMA)	https://www.ema.europa.eu/en
China	National Medical Products Administration (NMPA)	https://www.emergobyul.com/resources/china/china-food-drug-administration
Japan	Pharmaceuticals and Medical Devices Agency PMDA	https://www.pmda.go.jp/english/
Korea	Pharmaceutical Affairs Act (PAA) Ministry of Food and Drug Safety (MFDS)	https://www.mfds.go.kr/eng/index.do
India	Central Drugs Standard Control Organization (CDSCO)	Central Drugs Standard Control Organization (CDSCO)
Australia	Therapeutic Goods Administration (TGA)	https://www.tga.gov.au
Germany	The Paul Ehrlich Institute	http://www.pei.de (not a regulatory site; but informative)

Research Phase

The most critical activity that happens is tissue procurement/donor qualification. The tissue procurement is done following Good tissue practices (GTP), using 21 CFR 1271[1]. It is intended to help manufacture human cells, tissues, and cellular and tissue-based products (HCT/Ps) and to comply with the comprehensive regulatory framework for HCT/Ps, outlined in Title 21 of the Code of Federal Regulations, Part 1271 (21 CFR Part 1271).

In Europe, the research phase mainly involves tissue procurement, and it is mainly done through EU Tissue and Cell Directives (EUTCD) following the Directive 2004/23/EC[2].

Preclinical Processing

In this phase, in addition to 21 CFR 1271, additional rules of 361 PHS Act are applied. Under section 361 of the Public Health Service Act (42 U.S. Code §264), the U.S. Secretary of Health and Human Services is authorized to take measures to prevent the entry and spread of communicable diseases from foreign countries into the United States and between states.

In Europe, preclinical processing is carried out under the authority of EUTCD using the following directives: (i) Directive 2004/23/EC, (ii) Directive 2006/17/EC, and (iii) Directive 2006/86/EC.

Clinical Manufacturing

Once preclinical processing is approved, clinical manufacturing of cell doses is manufactured in good manufacturing practice (GMP) facility following 21 CFR 210 and 21 CFR211.

(a) 21 CFR Part 210 refers to Current Good Manufacturing Practice in Manufacturing Processing, Packing, or Holding of Drugs.
(b) 21 CFR Part 211 refers to Current Good Manufacturing Practice for Finished Pharmaceuticals.

In Europe, clinical manufacturing is also done in GMP facilities following the Directive 2003/94/EC.

Preclinical Studies

This is usually conducted in a good laboratory practice (GLP) facility using specifically approved animal models for a specific disease. Specific regulations governing this process are 21 CFR 58 and 21 CFR 610.

(a) 21 CFR Part 58 refers to GOOD LABORATORY PRACTICE FOR NONCLINICAL LABORATORY STUDIES.
(b) 21 CFR 610 refers to general biological products standards[3].

In Europe, preclinical studies are done in GLP under (i) Directive 2004/9/EC and (ii) Directive 2004/10/EC.

Clinical Trials Phase I, Phase II, and Phase III

Once the data from preclinical studies are finalized, an investigational new drug (IND) application should be filed with the FDA before commencing a clinical trial. The IND application should include the clinical protocol and detailed descriptions of previous clinical experience, preclinical studies, manufacturing, and testing. These are done again in a sequential manner of Phase I (safety), Phase II (Efficacy), and Phase III (larger cohort).

(a) For IND filing, 21 CFR 312 is used. 21 CFR 312 refers to procedures and requirements governing the use of investigational new drugs, including procedures and requirements for the submission to, and review by, the Food and Drug Administration of investigational new drug applications (INDs). An investigational new drug for which an IND is in effect in accordance with this part is exempt from the premarketing approval requirements that are otherwise applicable and may be shipped lawfully to conduct clinical investigations of that drug.
(b) Besides, throughout all the phases of clinical trials, the following FDA regulations are applied:

(i) 21 CFR 50—refers to the protection of human subjects
(ii) 21 CFR 54—refers to the financial disclosure by clinical investigators
(iii) 21 CFR 56—refers to the institutional review boards
(iv) 21 CFR 11—refers to the electronic records and electronic signatures

In Europe, all clinical trials are done in IMP with the Directive 2001/20/EC and GCP with the Directives 2005/28/EC and Directive 95/46/EEC.

Post-marketing and Commercial Approval (Biological License Application, BLA)

Ensuring safety, efficacy, and success in a large cohort, the study moves to a final phase of commercial approval for use as standard care of therapy. For this to move into the final stage of therapy, the following regulations are applied.

(a) 21 CFR 600—refers to the regulations for biological products in general
(b) 21 CFR 601—refers to the applications for biological licenses; procedures for filing
(c) 451 PHS Act—refers to the natural resources and environmental protection act of public health safety

In Europe, Market authorization is done through (i) Directive 2001/83/EC, (ii) Directive 2009/120/EC, and (iii) Regulation EC 1394/2007 and Regulation EC 726/2004.

What is new with FDA regulations in 2020:

The FDA regulates the commercialization of cell therapy products through the Public Health Service Act (PHSA) of 1944 and the Food, Drug, and Cosmetic Act of 1938. FDA uses Sections 351 and 361 of PHSA to establish regulatory requirements for commercialization and safety for the human

[1]https://www.fda.gov/regulatory-information/search-fda-guidance-documents/regulation-human-cells-tissues-and-cellular-and-tissue-based-products-hctps-small-entity-compliance

[2]https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:102:0048:0058:en:PDF

[3]https://www.gmp-compliance.org/guidemgr/files/CFR_2019/CFR-2019-title21-vol7-part610.pdf

cells, tissues, and cellular and tissue-based products (HCT/Ps). For details of FDA administration of Section 361 versus Section 351 products, please refer to recent governmental regulations (Fang and Vangsness, 2020).

Regulations for Mesenchymal Stromal Cell-Based Medicinal Products in Korea

Korea tops the list among the globe for the highest number of cell therapy- and gene therapy-approved products. The Korean Ministry of Food and Drug Safety (MFDS) is the regulatory body and uses their Pharmaceutical Affairs Act (PAA) for the regulations to govern the release or commercial launch of cell and gene therapy products[4].

The process goes through just like the regulatory activities outlined in the figure for the United States, with minor modifications (Galli and Serabian, 2015). The process consists of three steps:

(a) Pre-IND meeting: this should have a prototype of the product developed in GLP in preclinical development.
(b) Application of IND: Consists and Phase I and Phase II clinical trials using a product manufactured in GMP.
(c) Submission of NDA: Following Phase III clinical trial, MFDS reviews (115 days) the data and enables NDA approval, following which the product is released into the market.

MFDS notifications include Regulations on Review and Authorization of Biological Products (RRABP) (Qiu et al., 2020). In the RRABP,

(a) Article 25: Safety and Efficacy Review Criteria[5]
    (i) Annex 2: types of information needed for cell therapy products
    (ii) Annex 3: information needed for gene therapy products
(b) Article 30: provides the specifications and test methods for cell therapy[6]
(c) Article 31: review criteria for gene therapy products[7]

CONCLUSION

Although there are more than 900 hMSC clinical trials that are currently ongoing around the world, we have seen only limited success in product approvals for clinical therapy. A host of reasons go into the factor of why we see this trend, such as regulatory burdens due to high-risk raw material selection, failure to show product consistency and efficacy, cost of manufacturing, and open system processing. To overcome this, manufacturers need to start with the end goal in mind. First, choosing high-quality, low-risk raw materials with all the necessary QC/safety testing and regulatory support documentation is an utmost priority. Qualification of whether these raw materials can be supplied in a secure and scalable manner is essential to sustainability. Second, the evaluation of scalable closed and automated solutions must be done upfront during the early process development stage. Changes in scale and automation solutions are unavoidable because novel technologies will be developed in the future to address the current challenges. This process flexibility can only be adopted if the manufacturing workflow is modular in design. Third, it is empirical to test CQAs at every step of the manufacturing unit operations starting from the isolation step of hMSC, creation of MCBs and WCBs, to final product scale-up/scale-out expansion, before fill and finish and post-thawing of the cryopreserved product. Lastly and most importantly, there is no "one size fits all" approach for hMSC therapy regulatory guidance. Early and constant engagement with regulatory agencies to understand the relevant documentation required for multiple regions is requisite for smooth regulatory approvals. Taken together, planning ahead of time for a GMP regulatory-compliant manufacturing is key for successful hMSC-based therapies in the future.

[4]https://www.mfds.go.kr/eng/index.do
[5]https://www.mfds.go.kr/eng/brd/m_61/view.do?seq=46
[6]https://www.mfds.go.kr/eng/brd/m_27/view.do?seq=70469
[7]https://www.mfds.go.kr/eng/brd/m_61/view.do?seq=10

AUTHOR CONTRIBUTIONS

PJ wrote the GMP-based closed and automated manufacturing section. RL contributed to the product characterization for MSC therapy. JN wrote the critical raw material quality assessment. MV covered the regulatory perspectives for MSC-based cell therapy products. All the authors, in addition to writing specific sections, reviewed and commented on the other sections. PJ and MV conceived the design, assembly of data, analysis, and interpretation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.648472/full#supplementary-material

Supplementary Table 1 | Common hMSC culture systems, raw materials, and QC/Safety testing expectations.

Supplementary Table 2 | List of different digital integration platforms.

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frontiers in Cell and Developmental Biology

REVIEW
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The Lack of a Representative Tendinopathy Model Hampers Fundamental Mesenchymal Stem Cell Research

Marguerite Meeremans[1], Gerlinde R. Van de Walle[2], Sandra Van Vlierberghe[3] and Catharina De Schauwer[1]*

[1] Comparative Physiology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, [2] Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY, United States, [3] Polymer Chemistry and Biomaterials Group, Centre of Macromolecular Chemistry, Faculty of Sciences, Ghent University, Ghent, Belgium

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Janina Burk, University of Giessen, Germany

***Correspondence:**
Marguerite Meeremans
Marguerite.Meeremans@ugent.be

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Overuse tendon injuries are a major cause of musculoskeletal morbidity in both human and equine athletes, due to the cumulative degenerative damage. These injuries present significant challenges as the healing process often results in the formation of inferior scar tissue. The poor success with conventional therapy supports the need to search for novel treatments to restore functionality and regenerate tissue as close to native tendon as possible. Mesenchymal stem cell (MSC)-based strategies represent promising therapeutic tools for tendon repair in both human and veterinary medicine. The translation of tissue engineering strategies from basic research findings, however, into clinical use has been hampered by the limited understanding of the multifaceted MSC mechanisms of action. *In vitro* models serve as important biological tools to study cell behavior, bypassing the confounding factors associated with *in vivo* experiments. Controllable and reproducible *in vitro* conditions should be provided to study the MSC healing mechanisms in tendon injuries. Unfortunately, no physiologically representative tendinopathy models exist to date. A major shortcoming of most currently available *in vitro* tendon models is the lack of extracellular tendon matrix and vascular supply. These models often make use of synthetic biomaterials, which do not reflect the natural tendon composition. Alternatively, decellularized tendon has been applied, but it is challenging to obtain reproducible results due to its variable composition, less efficient cell seeding approaches and lack of cell encapsulation and vascularization. The current review will overview pros and cons associated with the use of different biomaterials and technologies enabling scaffold production. In addition, the characteristics of the ideal, state-of-the-art tendinopathy model will be discussed. Briefly, a representative *in vitro* tendinopathy model should be vascularized and mimic the hierarchical structure of the tendon matrix with elongated cells being organized in a parallel fashion and subjected to uniaxial stretching. Incorporation of mechanical stimulation, preferably uniaxial stretching may be a key

element in order to obtain appropriate matrix alignment and create a pathophysiological model. Together, a thorough discussion on the current status and future directions for tendon models will enhance fundamental MSC research, accelerating translation of MSC therapies for tendon injuries from bench to bedside.

Keywords: tendinopathy, biomaterials, tendon, *in vitro* tendon models, bioreactors

INTRODUCTION

Tendon overuse injuries are one of the most common sports-related injuries both in humans and horses (Carpenter and Hankenson, 2004). The Achilles tendon in human patients and the superficial digital flexor tendon (SDFT) in equine patients are frequently injured structures due to their capacity to store energy during high-speed locomotion. The cumulative degenerative damage to tendons caused by high-intensity exercise and age-related microdamage might result in chronic problems of tendinopathy. Currently, injuries to the equine SDFT is the most appropriate animal model for human Achilles tendon injuries (Patterson-Kane et al., 2012; Burk et al., 2013a). Besides the chronic pain and early retirement in equine and human athletes, tendinopathy also causes economic losses and animal welfare concerns (Patterson-Kane et al., 2012). Tendons are hierarchically organized based on a triple-helix of cross-linked tropocollagen, forming insoluble collagen molecules which aggregate progressively into microfibrils, fibrils, and fibers (**Figure 1**). Different fibers are combined into a bundle, called "fascicles", surrounded by endotenon. In their turn, different fascicles are grouped and surrounded by epitenon. Both endo- and epitenon supply the tendon with blood vessels, nerves and lymphatics (Wang, 2006; Docheva et al., 2015; Tan et al., 2015; Schneider et al., 2018). Tendon extracellular matrix (ECM) consists physiologically mainly of collagen I (95%), while collagen III is present in the endotenon (1–3%) (Spaas et al., 2012; Tan et al., 2015). In addition to collagen, elastin renders the tendon tissue flexible and extensible, while the ground substance in the ECM is essential for proper metabolism, shock absorption, viscoelasticity, and support (Schneider et al., 2018). Important components of the latter include proteoglycans (e.g., decorin and lumican) and glycoproteins (e.g., tenascin-C, tenomodulin, and cartilage oligomeric matrix protein). Scleraxis and Mohawk are major tenocyte-specific transcription factors which support matrix production, tenocyte proliferation, and differentiation (Liu et al., 2015). The cellular compartment of tendons consists of specialized fibroblasts, i.e., tenocytes and tenoblasts, and recently identified tendon stem/progenitor cells (Bi et al., 2007; Wang et al., 2017; Costa-Almeida et al., 2018b; Sensini and Cristofolini, 2018; Zhang et al., 2020).

Immediately following acute tendon damage, an inflammatory phase is observed, in which various inflammatory and immune cells are attracted to the injury site. Subsequently, the proliferation phase starts, characterized by fibroplasia, angiogenesis and new ECM synthesis. Finally, during the remodeling or maturation phase, tendon fibers are realigned and scar tissue is replaced by tissue-specific cells and matrix to restore native tissue properties (Spaas et al., 2012). In adult tendons, however, the healing process results in the presence of inferior scar tissue lacking the structural integrity and elasticity of the original tendon (Evans, 2012; Spaas et al., 2012; Docheva et al., 2015; Adekanmbi et al., 2017; Schneider et al., 2018; Sensini and Cristofolini, 2018; Costa-Almeida et al., 2019; Khatibzadeh et al., 2019). The limited functionality of healed tendon tissue represents a high risk of reduced performance and/or reinjury (Dyson, 2004; Spaas et al., 2012). To date, we lack knowledge on the molecular and cellular basis of tendon physiology and fail to capture essential aspects of its pathology (Nichols et al., 2019; Wunderli et al., 2020), particularly during the early stages of injury (Dakin et al., 2012). Hypocellularity and hypovascularization of the tendon may affect its ability to respond to inflammation and reduce its efficacy to repair injured tissue (Dakin et al., 2012; Nichols et al., 2019). Indeed, neovascularization occurs in response to hypoxia-associated vascular endothelial growth factor (VEGF) secretion. However, these neovessels are not completely functional and fail to deliver properly nutrients and oxygen. Consequently, the persistent hypoxia aggravates inflammation and MMP secretion, which results in further disruption of the tendon (Chung and Shum-Tim, 2012; Costa et al., 2007). Current tendinopathy treatments in humans and horses include physical therapies (cold, pressure, support, shock wave therapy, and rehabilitation programs), drug treatments (systemic or intra-lesional anti-inflammatory medication) and surgery (tenoscopy and tendon splitting), but all these therapies fail to provide tendon regeneration and restore the functionality of the original tendon tissue (Smith, 2008; Docheva et al., 2015; Ramos et al., 2019). The poor success of conventional therapy supported the need to search for novel treatments to regenerate a tissue mimicking the tendon to the greatest extent as possible (Richardson et al., 2007). Despite the great interest in MSCs due to their ability to repair tissue and reduce inflammation (Sevivas et al., 2018), common clinical applications have been hampered by several limitations. First, many experimental and pre-clinical studies have been evaluating the regenerative potential of MSCs for tendon healing and although clinical translation appears temptingly close (Pacini et al., 2007; Nixon et al., 2008; Schnabel et al., 2009; Godwin et al., 2012; Ricco et al., 2013; Smith et al., 2013; Van Loon et al., 2014), convincing evidence based on randomized, controlled,

Abbreviations: 2D, two-dimensional; 3D, three-dimensional; AT-MSC(s), adipose tissue-derived mesenchymal stem cell(s); BM-MSC(s), bone marrow-derived mesenchymal stem cell(s); dECM, decellularized extracellular matrix; ECM, extracellular matrix; ELAC, electrochemically aligned collagen; HUVEC(s), human umbilical vein endothelial cell(s); MSC(s), mesenchymal stem cell(s); PCL, poly-ε-caprolactone; PDMS, polydimethylsiloxane; PGA, polyglycolic acid; PLA, polylactic acid; PLGA, polylactide-co-glycolide; SDFT, superficial digital flexor tendon; UTS, ultimate tensile strength; VEGF, vascular endothelial growth factor.

clinical studies in equine or human patients, is still lacking (Pas et al., 2017; Phelps et al., 2018; Khatibzadeh et al., 2019) and outcomes of long-term follow-up studies do not meet expectations (Geburek et al., 2017; Ahrberg et al., 2018). Second, various practical considerations regarding MSC source, dosage, administration technique, and timing, remain unanswered (Costa-Almeida et al., 2019; Shojaee and Parham, 2019). It is known that MSCs isolated from different sources display significantly diverse properties indicating potential advantages and disadvantages for the use of each MSC type in particular clinical applications (Burk et al., 2013b; Harman et al., 2020). Bone marrow, adipose tissue, and peripheral blood are the most commonly used MSC sources in equine regenerative medicine. Although adipose tissue-derived MSCs (AT-MSCs) are more easily accessible and have a higher yield after harvesting compared to bone marrow-derived MSCs (BM-MSCs), better results in treating tendon injuries are obtained with the latter (Dai et al., 2015; Zarychta-Wisniewska et al., 2019). MSCs isolated from neonatal sources, however, are reported to have a longer lifespan than MSCs isolated from adult tissues, secrete more extracellular vesicles, and show broader differentiation capacity (Burk et al., 2013b; Iacono et al., 2017; Gugjoo et al., 2019). Moreover, it has been reported that the regenerative capacity of aged cells can be restored when exposed to a young environment. These findings suggest opportunities to reverse the aging process of tissues by targeting their niche (Lui and Wong, 2020). As such, MSC-based strategies isolated from neonatal sources might represent promising therapeutic tools for tendon repair and regeneration (Sevivas et al., 2018). Third, most *in vitro* studies investigate tenogenic differentiation of MSCs while some studies explored the interaction between MSCs and tenocytes, MSCs and tendon ECM or the effects of their secretome products, but their underlying mechanisms of action have been rarely studied (Liu et al., 2017; Burk, 2019). A decade ago, Dirks and Warden reviewed the models available to study tendinopathy and concluded that a wide range of models (*in vitro, ex vivo* and *in vivo* models) is mandatory to completely understand the pathogenesis of tendinopathy. To gain insight in the underlying molecular pathways, however, *in vitro* models serve as important biological tools to study cell behavior under controlled conditions, bypassing the confounding factors associated with *in vivo* clinical trials (Dirks and Warden, 2011). Nowadays, a wide diversity of *in vitro* tendon models are used to improve our fundamental understanding of tendon mechanobiology and to study tissue replacement processes, cell-based treatments, and drug screening applications, but no generally accepted *in vitro* model exists (Butler et al., 2008; Patterson-Kane et al., 2012; Patel et al., 2017; Wang et al., 2017; Laternser et al., 2018). To the best of our knowledge, both in humans and (laboratory) animals, a generally accepted *in vitro* tendinopathy model is not available yet.

Initially, the use of MSCs for primary tissue regeneration was advocated based on their ability to migrate to and engraft in the injury site, where they would differentiate into various appropriate cell types. Nowadays, however, MSCs are considered "medicinal cell factories" secreting a variety of bioactive molecules, either in soluble form or via extracellular vesicles, with immunomodulatory, ECM modeling, trophic and anti-apoptotic activities, collectively identified as the secretome (da Silva Meirelles et al., 2009; Manning et al., 2015; Presen et al., 2019; Zhang et al., 2020). Indeed, MSC-conditioned medium (MSC-CM), which includes all their secretome products, has similar regenerative effects as MSCs, which illustrates the impact of these secretome products (Phelps et al., 2018). It has also been demonstrated *in vitro* that cell proliferation and migration of injured tenocytes were promoted after MSC-CM administration (Chen et al., 2018; Li et al., 2020). Nevertheless, the regenerative capacities of MSCs are multifaceted and strongly cell- and tissue context-dependent, and the insights into their trophic and protective mechanisms in the context of tendon therapies remain scarce (Pas et al., 2017; Vizoso et al., 2017; Bogatcheva and Coleman, 2019; Burk, 2019; Harrell et al., 2019; Presen et al., 2019; Al Naem et al., 2020). As there is an urgent need to (i) unravel the MSCs' mechanisms of action, (ii) find a therapy that consistently yields positive results, (iii) investigate the most optimal treatment protocol, (iv) unravel the wide potential of the MSCs' bioactive factors, and (v) identify the most appropriate MSC source, an optimal *in vitro* tendon model might provide a lot of answers. Moreover, by establishing a state-of-the-art physiologically representative *in vitro* tendinopathy model, the use of experimental animals will be drastically reduced. The effect of potential therapies for tendinopathy, with great emphasis on MSCs and their secretome, can be studied *in vitro*, and answers to questions relevant for clinical applications (like timing of treatment, dosage, immunomodulatory activities, etc.) can be provided, further reducing the number of *in vivo* experiments considerably.

An overview of the models available to mimic tendon tissue *in vitro* with increasing complexity is given throughout the review (**Figure 2**). Elements to establish a representative *in vitro* tendinopathy model are suggested, including techniques which might be promising but are not yet optimized to incorporate in a tendon model. Rather than citing all available literature on tendon tissue engineering, models are discussed with an emphasis on their strengths and shortcomings regarding fundamental research on the regenerative capacities of MSCs. Therefore, the evaluation criteria incorporated in this review are i) representative cellular phenotype (of tenocytes, tenogenic-differentiated MSCs, fibroblasts, or tendon stem/progenitor cells) as demonstrated by the spindle-shape morphology and tenocyte marker expression, (ii) production of ECM (evaluated by gene expression and immunohistochemistry) and allowing cell–matrix interactions, (iii) supporting nanometric and axially aligned structure (anisotropy), (iv) responsive to physiological levels of uniaxial strain, (v) neuro-vascular supply, and (vi) mimicking micro-damage, like acute injuries and chronic overuse.

BASICS: MODELS WITHOUT SCAFFOLDS

Two-Dimensional (2D) Models

2D cell cultures are commonly used to study cell behavior. In such a simplified environment, basic morphology, gene expression,

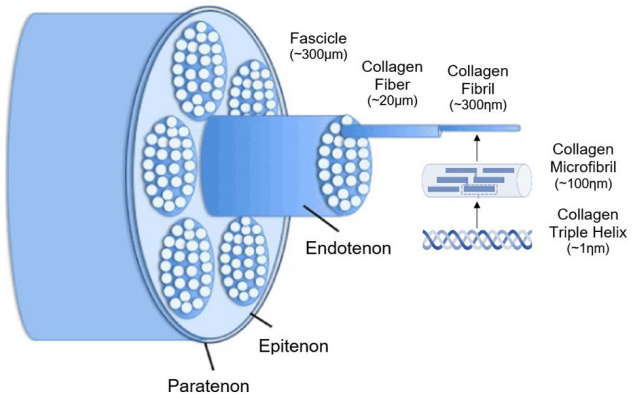


FIGURE 1 | Schematic illustration of the hierarchical structure of tendons. Adjusted from Durgam and Stewart (2016) and ChemBAM (n.d.).

and differentiation are easily studied without confounding factors (Laternser et al., 2018). However, mimicking the biomechanical and -chemical environment of native tendon is crucial when studying the behavior and mechanisms of action of MSCs (Grier et al., 2017; Al Naem et al., 2020). A common issue in 2D tenocyte cultures is dedifferentiation. With increasing cell passage, tenocytes lose their characteristic spindle-like morphology and consequently, their functionality (Yao et al., 2006). Their changing morphology is accompanied by a significant decrease in collagen I and tenomodulin mRNA expression, as demonstrated in the study of Zhu et al. (Zhu et al., 2010). Unlike *in vitro* cultured tenocytes, tenocytes *in vivo* are not organized in confluent sheets and are able to actively interact with the ECM (Patterson-Kane et al., 2012; Laternser et al., 2018). Therefore, 2D cultures are no longer used for tissue functionality or regeneration studies. However, these models are still useful for setting up preliminary experiments, implementation as control condition or, for example, investigating cytotoxic effects (Occhetta et al., 2013; Fessel et al., 2014). Because of their simplicity, 2D models are more cost-effective than the sophisticated techniques explained below (**Figure 3**) (Wunderli et al., 2020).

2D Models Combined With Mechanical Stimulation and/or Surface Topography

Contact-guidance might offer a solution to maintain tenocyte differentiation in 2D cell cultures. Mechanical stimulation and surface topography of cell culture surfaces influence cell density, cellular alignment, and the organization of newly deposited matrix (Nikolovski et al., 2003; Park et al., 2006; Zhang et al., 2021; Kuo et al., 2010). *In vivo*, it is known that mechanical stimulation is important to maintain tendon homeostasis (Screen et al., 2005). However, cultured cells *in vitro* also respond to mechanical strain by displaying a more spindle-shaped morphology and adjusting their DNA synthesis toward the production of collagen I (**Figure 3**) (Wang et al., 2003; Matheson et al., 2006). As such, Riboh et al. (2008) induced the tenogenic phenotype in epitenon tenocytes, sheath fibroblasts, bone BM-MSCs and AT-MSCs by exposing the cells to intermittent cyclic strains (4%, 0.1 Hz, 1 h on/2 h off). To study the impact of surface topography on cellular alignment and tenocyte characteristics, Kapoor et al. (2010) used grooved substrates with different diameters (50–250 μm) to verify the influence of physical parameters on tenocytes. Both cell density and cellular alignment were affected by the microtopography of the substrates, with 50 μm grooves having the most pronounced impact, as demonstrated by denser and more longitudinally oriented collagen fibers. No significant impact was observed on matrix gene expression or cell phenotype (Kapoor et al., 2010). However, when micro-grooved silicone surfaces were combined with cyclic uniaxial stretching, human tenocytes showed a phenotype comparable to the *in vivo* situation and an increased cellular production of collagen type I in a stretching-magnitude-dependent manner (4 and 8% stretch) (Wang et al., 2003; Yang et al., 2004). Despite the improved knowledge on cell proliferation and cellular alignment, these modified 2D cell cultures still lack structural complexity to mimic tendinopathy *in vitro*.

Three-Dimensional (3D) Models

Apart from contact-guidance, tenocyte dedifferentiation can also be countered by spheroid formation. As such, a 3D set-up was developed using hanging drop cultures, in which tenocytes are exposed to microgravity (Theiss et al., 2015). Theiss et al. (2015) and Kraus et al. (2017) generated equine tenocyte spheroids when specific growth factors were supplemented to the culture medium. They observed that tenocytes within spheroids better preserved their spindle-like morphology and showed enhanced expression of tenogenic genes like collagen I, collagen III, and scleraxis and expression of the chondrogenic transcription factor *SOX9* (Theiss et al., 2015; Kraus et al., 2017). Spheroids provide the functionality which is lacking in 2D cultures, and as such, are more suitable to study pathological conditions *in vitro* (**Figure 3**) (Laternser et al., 2018). Calve et al. (2004) were the first to engineer viable tendon tissue constructs *in vitro* without using artificial scaffolds. They created these constructs by allowing self-assembly of isolated rat Achilles tendon tenocytes into a cylinder, which resembled embryonic tendon consisting of collagen fibrils, many tenocytes and a non-collagenous ECM. Moistening of the constructs was provided by bathing each sample individually in culture medium. Both for spheroids and the self-assembled tenocyte cylinders, cell–cell and cell–matrix interactions can be studied as the cultured tenocytes produce ECM. Therefore, to extrapolate *in vitro* results to *in vivo* clinical trials, it would be of great benefit to study MSCs in 3D environments (Burk, 2019), as for example the immunomodulatory potential of the MSCs is shown to be altered in 3D vs. 2D (Follin et al., 2016; Li et al., 2018). The disadvantages of these techniques are the small model size and the low mechanical properties of the constructs when compared to mature tendons due to the rather immature morphology of the tenocytes and the lack of mechanical stimulation during the culture period (Calve et al., 2004).

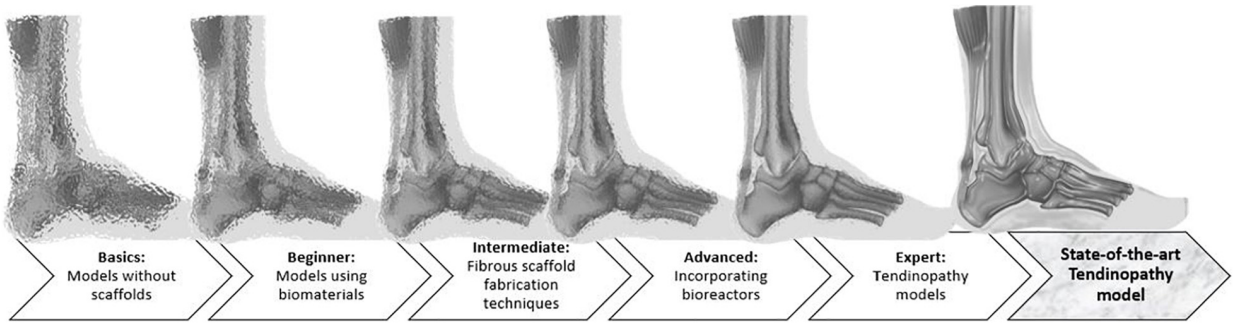


FIGURE 2 | Illustration of the review' structure, starting from very basic but very unclear, blurred models to increasingly complex models, which are more appropriate to represent an injured Achilles tendon and to clarify the MSC mechanisms of action.

Explant Models

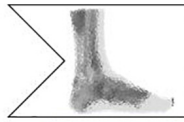
Another approach to study tendon tissue is by using tendon explant models. Intact native tissue samples can be dissected and cultured *ex vivo*. The main advantage of this method is the intact tissue architecture, which allows studying cell-ECM interactions in a near-physiological environment. These models have been used for structure and function characterization of tendon tissue, to study cell-mediated processes and to investigate crosstalk mechanisms (Wunderli et al., 2020). "Clamp and stretch" models of these explants are often implemented to define mechanical characteristics of tendon tissue (Goldstein et al., 1987). This technique is characterized by the application of mechanical load in a longitudinal manner on a tissue sample clamped at both ends, while the deformation and applied forces are monitored (Dyment et al., 2020). Many different bioreactors have been developed for mechanical stimulation of cell and tissue cultures, which are described in more detail in the section "Advanced: Incorporating bioreactors". The most frequently described problems of longitudinal stretch systems are the rather heterogeneously transmitted strain, along with grip slippage due to the high mechanical forces used (**Figure 3**) (Brown, 2000). Basic "clamp and stretch" studies often cover a small-time interval because of the lack of nutrient supplementation, resulting in dry, non-physiological circumstances. Devkota and Weinhold (2005) developed an advanced tissue explant system to create and monitor mechanical changes occurring with tendon overuse. The machine can be placed in a standard incubator and is equipped with video strain analysis capabilities for monitoring. Another improvement over previous models is the use of load-controlled operation, preventing grip slippage (Devkota and Weinhold, 2005). The incorporation of mechanical stimulation mimics the *in vivo* load-bearing function. Explant models as such can provide useful insight into tendon (patho-)physiology and have also previously been used to study MSC characteristics. Costa-Almeida et al. (2018a) studied the communication between AT-MSCs and native tendon ECM in a *trans*-well tendon explant model. Although the AT-MSCs were not directly in contact with the tendon explant, significant changes in MMP secretion, collagen III and tenascin-C deposition were monitored when AT-MSC were co-cultured compared to single tenocyte cultures, suggesting ECM remodeling. The authors proposed explant co-cultures as a tool to unravel cellular communication and tendon healing (Costa-Almeida et al., 2018a). Wunderli et al. (2020) excessively reviewed tendon explant models for physiologically relevant *in vitro* studies and confirmed their suitability for investigating cellular cross-talk. Furthermore, Youngstrom et al. (2015) reseeded decellularized tendon scaffolds with BM-MSCs to evaluate the effects of different strain protocols on ECM composition, gene expression and mechanical properties of the scaffolds. The goal of these experiments, however, was to validate the custom-designed bioreactor and not specifically to characterize MSCs (Youngstrom et al., 2015). Nevertheless, explant models are subjected to variable conditions which are not controllable enough to obtain reproducible results when aiming to elucidate exact MSCs' mechanisms of action (Wunderli et al., 2020).

BEGINNER: MODELS USING BIOMATERIALS

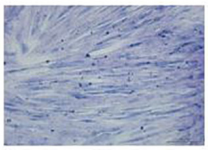
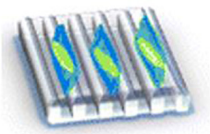
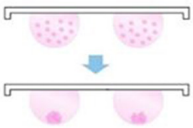
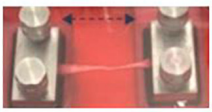
Biomaterials are defined as any material that is able to interact with biological systems and can consist of natural and/or synthetic materials (National Institute of Biomedical Imaging and Bioengineering, n.d.). While standard cell culture materials do not resemble physiological circumstances, e.g., native ECM, biomaterials are specifically designed to deliver mechanical, structural, and compositional stimuli to the cells (Caliari and Burdick, 2016). Most requirements for biomaterials are based on demands for tissue engineering. In relation to an *in vitro* tendon model these include (i) correct biochemical composition and structure, (ii) biocompatibility toward appropriate cell population, (iii) appropriate mechanical strength and elasticity to mimic cell-microenvironment interactions of *in vivo* tendon tissue, and (iv) an easily processable material (Ma, 2004; Kuo et al., 2010; Rodrigues et al., 2013). An overview of the advantages and disadvantages of the discussed materials is given in **Figure 4**.

Natural Materials

Currently used natural materials for tendon applications are hyaluronic acid, silk fibroin, collagen and gelatin. The general advantage of using natural materials for tissue engineering



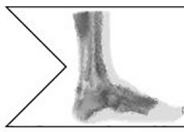
Basics:
Models without scaffolds

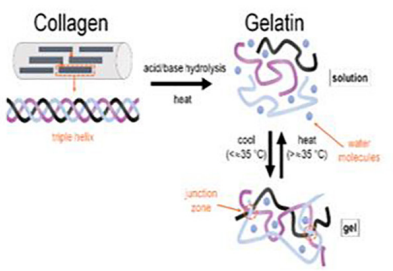
	1. 2D models	2. 2D models + mechanical stimulation and/or surface topography	3. 3D models	4. Explant models
Advantages	Straightforward	Straightforward	Maintenance of tenogenic phenotype	Intact tissue structure
	Suitable for preliminary experiments	Suitable for preliminary experiments	Cell-ECM interactions	Cell-ECM interactions
	Cost-effective	Maintenance of tenogenic phenotype	Cell environment mimics *in vivo* conditions	Mechanical strength
		Mechanical stimulation		Mechanical stimulation
		Cost-effective		
Limitations	Tenocyte dedifferentiation	No ECM incorporation	Lack of structural complexity	Lack of control
	No ECM incorporation	Lack of structural complexity	Small model size	Difficult standardization
	Lack of structural complexity	No (neuro-)vascular supply	No mechanical stimulation	No (neuro-)vascular supply
	No mechanical stimulation		Mechanical weakness	
	No (neuro-)vascular supply		No (neuro-)vascular supply	

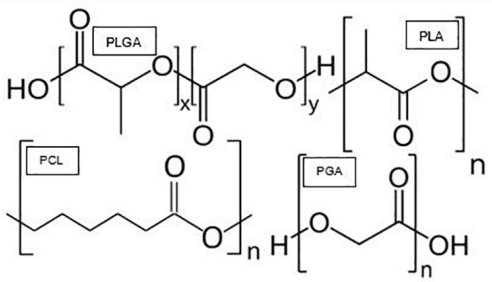
2D: Two-dimensional; 3D: Three-dimensional; ECM: Extracellular matrix.

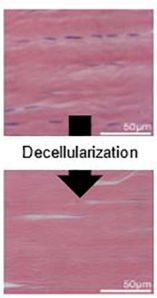
FIGURE 3 | Advantages and disadvantages of basic tendinopathy models without scaffolds. Adjusted from Krishna et al. (2016); Ryu et al. (2019) and Tohidnezhad et al. (2020).



Beginner:
Models using biomaterials







	1. Natural materials	2. Synthetic materials	3. Hybrids	4. Decellularized materials
e.g.	Hyaluronic acid, silk fibroin, collagen and gelatin	PCL, PLA, PGA and PLGA	PCL/silk fibroin and PCL/gelatin	Decellularized tissue sample and dECM
Advantages	Good cytocompatibility	Mechanical strength	Good cytocompatibility	Good cytocompatibility
	ECM mimicking	Cost-effective	Mechanical strength	Cell-ECM interactions
	Cost-effective	Easy processable	Many different combinations	Intact tissue structure
	Wide availability			Mechanical strength
Limitations	Mechanical weakness	Poor cytocompatibility	Higher costs	Lack of control
	No (neuro-)vascular supply	Need surface-modification for cell adhesion	More processing steps needed	Difficult standardization
	Batch-to-batch variability	No (neuro-)vascular supply	No (neuro-)vascular supply	Expensive processing
			Lack of consistency in use	No (neuro-)vascular supply

dECM: decellularized extracellular matrix; ECM: Extracellular matrix; PCL: poly-ε-caprolactone; PGA: polyglycolic acid; PLA: polylactic acid; PLGA: polylactide-co-glycolide.

FIGURE 4 | Advantages and disadvantages of tendinopathy models incorporating biomaterials. Adjusted from Barrett et al. (2013) and ChemBAM (n.d.).

is the good cytocompatibility due to the functional chemical groups available for cellular binding. For example, the tripeptide arginine-glycine-aspartic acid (RGD) sequence functions as integrin-binding sites, which are of critical importance for cell adhesion (Ruoslahti, 1996). For tissue engineering, it is important that cells delivered through the scaffold remain in place, but also for an *in vitro* model, it is of great importance that cultured cells can interact with the biomaterial.

Collagen I is the most extensively used natural material because of its low cost and high physiological prevalence in tendon tissue (Kuo et al., 2010; Caliari and Burdick, 2016; Wu et al., 2018). Although collagen gels, sponges and extruded fibers are being used for tendon tissue engineering, their main drawback is mechanical weakness (Qiu et al., 2016; Lake et al., 2020). Cheng et al. (2008) were able to upregulate the mechanical strength of collagen (30-fold) by producing electrochemically aligned collagen (ELAC) bundles. When these bundles were seeded with MSCs, the expression of tendon-specific genes (scleraxis and tenomodulin) was upregulated when compared to randomly oriented collagen threads, which illustrates that collagen might be used to replace tendon (Kishore et al., 2012; Shimada et al., 2014). As with all natural materials, another disadvantage of extracted collagen is batch-to-batch variability, which can be circumvented by using recombinant collagen (Slaughter et al., 2009; Tytgat et al., 2019). Gelatin is denatured collagen which can be used as an ECM mimic after chemical modification and crosslinking to provide stability at elevated temperatures and which is less immunogenic when compared to collagen (Laternser et al., 2018; Van Hoorick et al., 2019). Other advantages are low cost and wide availability, especially when considering large-scale *in vitro* studies (Van Hoorick et al., 2019). Furthermore, we recently demonstrated the excellent biocompatibility of cross-linked gelatin (gelatin-methacrylamide and gelatin-norbornene) to support equine tenocyte cultures (Meeremans et al., 2021). Silk fibroin is a worthy alternative to collagen and gelatin for tendon and ligament tissue engineering and is collected from silkworms, mostly *Bombyx mori*. The advantages of silk are its strong mechanical characteristics, good cytocompatibility, easy processability, and its potential to be changed into many different forms (Minoura et al., 1995; Rockwood et al., 2011; Yao et al., 2016). In the study of Chen et al. (2010), knitted silk-collagen scaffolds were used in which seeded MSCs showed good adherence to the scaffold, proliferated well, and showed tendon biocompatibility after mechanical stimulation. Tenogenic differentiation of MSCs, characterized by adopting a tenocyte-like shape and expression of tendon-related genes, illustrates the suitability of silk for supporting tenocyte cultures and tendon tissue engineering. Hyaluronic acid belongs to the group of glycosaminoglycans and is often implemented in tendon tissue engineering to increase the mechanical strength (Liu Y. et al., 2008). Funakoshi et al. (2005) fabricated a 3D chitosan/hyaluronic acid scaffold to repair tendon defects in an *in vivo* rabbit model. This newly designed scaffold had previously shown potential as a biomaterial for cartilaginous tissue scaffolds and was, therefore, hypothesized to enhance collagen I production when implanted in *in vivo* tendon defects. This study found that in addition to the enhanced collagen production, the mechanical strength of the regenerated tendons also increased when seeded with fibroblasts, displaying potential for an *in vitro* tendon culture (Funakoshi et al., 2005; Yamane et al., 2005).

Synthetic Materials

Synthetic materials are also widely used in tissue engineering as they provide excellent mechanical support, are easily processable, and are cost-effective. Synthetic polymers applied in tendon tissue engineering are e.g., polylactic acid (PLA), polyglycolic acid (PGA), and their copolymers such as polylactide-co-glycolide (PLGA) (Ouyang et al., 2003; Cao et al., 2006; Chen et al., 2010; Yang et al., 2016; Aldana and Abraham, 2017; Wu et al., 2018). All belong to the group of polyesters and are attractive for *in vivo* use due to the formation of natural metabolites upon degradation. However, because of the hydrophobic nature of polyesters, cell adhesion is far from optimal and their *in vitro* application is less attractive (Liu Y. et al., 2008). Cao et al. (2006) were able to generate tendon tissue *in vitro* by culturing tenocytes on PGA fibers arranged into a cord-like construct, both with and without application of constant strain (two groups). They found that the generated tissue resembled natural tendon tissue histologically in both groups, as opposed to the cell-free control group. The application of constant strain improved mechanical characteristics but was detrimental for scaffold thickness and collagen fiber alignment, and thus, considered suboptimal. The authors suggested that aligned fibers instead of non-woven fibers should be used preferably and that the strain regime for mechanical load should be intermittent instead of constant (Cao et al., 2006). The fact that polymer fibers should be aligned to mimic the highly organized collagen fibers, was further corroborated by a study of Lee et al. (2005), where they developed tissue-engineered ligaments of polyurethane. Tendons and ligaments indeed have some features in common such as the hierarchical structure and the non-linear mechanical properties (Sensini and Cristofolini, 2018). Moreover, when aligned PLA scaffolds were used, Yin et al. (2010) showed that tendon stem cells displayed a spindle-shape morphology and tendon-like tissue was formed. Ouyang et al. (2003) and Sahoo et al. (2006) compared different PLGA production technologies to create a tendon/ligament biodegradable scaffold. Both *in vivo* and *in vitro* studies described favorable BM-MSC morphology (spindle-shape) and alignment (Ouyang et al., 2003; Sahoo et al., 2006). Synthetic polymers are often implemented for their superior mechanical strength but they lack functional chemical groups for cellular binding and often need surface modification (Kuo et al., 2010). Locke et al. (2020) recently assessed the use of synthetic polyethylene glycol (PEG) hydrogels. To create a material suitable for tendon regeneration, a degradable linker peptide, a multifunctional collagen mimetic peptide and integrin-binding peptide sequences were incorporated into the hydrogel (Locke et al., 2020). Although the authors highlighted the potential of such multifunctional and synthetic hydrogels for tissue regeneration, especially when considering their mechanical properties, the approach is rather complex to (repeatedly) implement in an *in vitro* model.

Hybrids

An *in vitro* tendon model needs comparable mechanical strength as natural tendon to mimic the physiological stiffness the cells experience *in vivo*, but also specific surface stimuli for cell proliferation, hybrids are preferred for tendon tissue engineering. Hybrid scaffolds consist of various synergistically combined natural and synthetic polymers (Kuo et al., 2010). As previously mentioned, a knitted collagen-silk scaffold was suitable for tenogenic differentiation of human MSCs, but no comparison with other scaffolds was made in that particular study (Chen et al., 2010). Liu H. et al. (2008) seeded a combined scaffold of knitted silk and microporous silk sponge with BM-MSCs for (anterior cruciate) ligament tissue engineering and successfully overcame limitations of individual designs. Poly-ε-caprolactone (PCL), a synthetic biomaterial is often used in tissue engineering because of its biocompatibility, low cost and slow degradation. However, its hydrophobic nature prevents efficient cell attachment and the use as mono-material for tendon tissue engineering or for *in vitro* model design is, therefore, not recommended (Yang et al., 2016; Chen et al., 2017). Chen et al. (2017) combined PCL and silk fibroin in aligned scaffolds for supporting dermal fibroblast attachment and guidance of cell proliferation along the orientation of the nanofibers. By combining both materials, a new superior nanofiber scaffold for tendon tissue engineering was produced (Chen et al., 2017). Since the dermal fibroblasts differentiated into tenocytes (Chen et al., 2017), a similar response is in our opinion to be expected after tenocyte seeding and thus this strategy is suitable for *in vitro* tendon design. A multi-layered PCL/gelatin scaffold for tendon tissue engineering was designed by the group of Yang. The scaffolds seeded with AT-MSCs were found to mimic the native tendon tissue structure, mechanical properties and cell phenotype (Yang et al., 2016).

Decellularized Constructs

Instead of using polymers and complicated chemical production technologies, decellularized tendons are also widely used for tissue engineering (Lake et al., 2020). Decellularization protocols often use detergents to solubilize cell debris of tendon tissue samples aiming to remove all immunological signals. These constructs provide the same mechanical properties and integrin binding sites as natural tendon tissue, allowing implementation as a biological graft material *in vivo* and creating new opportunities for fundamental research models (Kuo et al., 2010). However, the amount of removed cells, DNA, immunological signs, and mechanical characteristics are depending on the used decellularization process (Kuo et al., 2010; Youngstrom and Barrett, 2016). When decellularization is combined with chemical oxidation, mechanical characteristics of the tendon extracts are preserved and all DNA is withdrawn (Whitlock et al., 2007). In a study of Youngstrom and Barrett (2016) decellularized equine SDFT was stated as ideal for tendon tissue engineering because of the preservation of the biochemical composition, structure and mechanics of native tendon (Barrett et al., 2013). Tissue sample decellularization can also be combined with enzymatic digestion to create soluble decellularized ECM (dECM), which can be analogously processed like natural and synthetic materials, or even combined with them (Santschi et al., 2019). When developing an *in vitro* model, however, the exact dECM composition should be determined every time in order to obtain reproducible results, which results in an undesirably expensive and cumbersome production process.

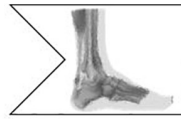
INTERMEDIATE: FIBROUS SCAFFOLD FABRICATION TECHNIQUES

It is important to emphasize different production processes, as scaffold properties are strongly influenced by the used processing techniques and applied parameters (Grier et al., 2017). For example, collagen gels are mechanically very weak, but their strength can be influenced by different crosslinking methods. Maximal collagen strength could be reached by physical crosslinking using dehydrothermal and ultraviolet light, but this came at the cost of decreased migration of dermal fibroblasts (Cornwell et al., 2007). Hydrogels, a water-swollen network of polymers, have emerged as the most promising out of the different biomaterial systems (Caliari and Burdick, 2016). The main advantage of hydrogels is their large water content, mimicking the hydrophilic nature of (tendon) tissue ECM (Yang et al., 2016; Schneider et al., 2018). A disadvantage is the typically weak mechanics, which need improvement by various chemical modifications required for crosslinking.

Important requirements for 3D constructs are spatiotemporal control of the 3D cellular microarchitecture and ECM distribution (Lu et al., 2013). Available literature underlines the advantage of aligned nanofibers over randomly oriented fibers to mimic tendon tissue. However, ideal fiber characteristics, such as diameter, pore size, spacing and angle are still under debate. The oldest production techniques use a "top-down" approach, in which cells are seeded onto a designed scaffold. After proliferation, the seeded cells need to produce new ECM. Complex functional tissues are hard to design top-down and size is confined by diffusion limitations (e.g., oxygen diffusion, 100–200 μm) (Radisic et al., 2006; Loh and Choong, 2013; Lu et al., 2013; Richards et al., 2016). By "bottom-up" engineering, microscale tissue building blocks with specific micro-architecture are carefully assembled together to build larger constructs. Different building blocks can consist of different cell populations and biomaterials, creating micro-organs (Lu et al., 2013). Another classification can be made regarding conventional methods vs. additive manufacturing technologies. A more detailed overview of the techniques discussed is shown in **Figure 5**.

Self-Assembly

In this bottom-up method, very small nanofibers (<100 nm to a few nm) are produced through weak interactions. The productivity of this technique is rather low and the process is only under limited control (Alghoraibi and Alomari, 2018). Cornwell et al. (2007) improved the mechanical strength of self-assembled collagen threads, formed by extrusion into a bath of fiber formation buffer, by dehydrothermal treatment and



Intermediate:
Fibrous scaffold fabrication techniques

	1. Self-assembly	2. Phase separation	3. Freeze-drying	
Advantages	Straightforward Modifiable strength		More physiological, no organic solvents Straightforward Modifiable structure	
Limitations	Small nanofibers Mechanical weakness Random fiber orientation Low productivity Limited control Expensive	Heterogeneous pore structure Limited number of suitable materials Short fibers Random fiber orientation	Difficult to create organised scaffolds Random fiber orientation	
	4. Microfluidic alignment	**5. Electrochemically alignment**	**6. Electrospinning**	**7. 3D Bioprinting**
Advantages	Straightforward Encapsulation possible Aligned fibers Mechanical stimulation Cost-effective	Aligned fibers Different geometry formation Improved controllability Cost-effective	Facilitate cell attachment and metabolic activity ECM mimicking Mimicking tissue structure Aligned & random fibers Mechanical strength Production flexibility	Encapsulation possible Structural mimicking Controlled geometrical properties Facilitate cell attachment and metabolic activity Production flexibility
Limitations	Lack of structural complexity No (neuro-)vascular supply	Lack of structural complexity Fibers diameter too big (50-400µm) Mechanical weakness	No cell encapsulation Heterogeneous scaffold seeding No (neuro-)vascular supply Limited control over pore geometry	Low achievable sizes Limited number of suitable materials No completely aligned fibers

ECM: Extracellular matrix.

FIGURE 5 | Advantages and disadvantages of the available fibrous scaffold fabrication techniques for tendon tissue engineering.

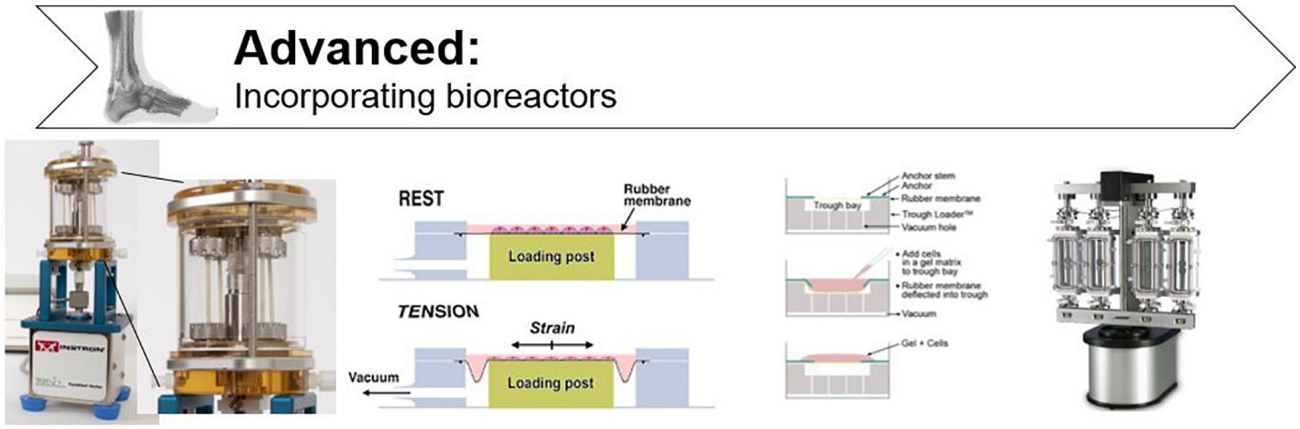
ultraviolet crosslinking. Another way to improve strength is by administering strain prior to fiber drying. Through this way, the ultimate tensile strength (UTS) in pre-stretched fibers is reported to be five times higher (Kew et al., 2011). The research group of Attenburrow compared different co-agents to produce extruded collagen fibers. They aimed to preserve the advantages of extruded collagen fibers and, at the same time, upregulate production and stability. Both polyethylene glycol and NaCl are considered highly suitable to reconstruct collagen fibers (Zeugolis et al., 2008a,b). Due to the poor mechanics of collagen, the formation of stable 3D constructs is not yet achieved, and the high costs associated with this technique hamper research and practical applications (Lu et al., 2013). Another disadvantage of collagen extrusion techniques is their limited efficiency as the production process is very time consuming (Kew et al., 2011).

Phase Separation

Phase separation is generated by introducing a non-solvent. Physical incompatibility between polymer and solvent results in separation and formation of nanofibers after gelation. However, these scaffolds have a heterogeneous pore structure, which makes them unsuitable for tissue engineering (Lu et al., 2013). Moreover, the porosity needs to be interconnected to allow cell infiltration and diffusion of culture media (Freyman et al., 2001). An alternative is thermally induced phase separation, but only few (synthetic) polymers are suitable for this process and short fibers are obtained, limiting the *in vitro* model capability (Alghoraibi and Alomari, 2018).

Freeze-Drying

Using this technique, the polymer is frozen (−80°C) to obtain a porous structure, after which the formed ice crystals are sublimated (De France et al., 2018). This process is more appropriate for biomedical applications due to the lack of organic solvents and the more simple method, yet it is difficult to obtain hierarchically organized scaffolds with this method (Alghoraibi and Alomari, 2018). Collagen freeze-drying is regularly used to create skin regeneration scaffolds, but too little structure is present to consider this approach for tendon applications (Freyman et al., 2001). Caliari and Harley (2011) provided alignment by adding a collagen-glycosaminoglycan melt to a polytetrafluoroethylene-copper mold before freeze-drying. Equine tendon cells showed increased attachment to the surface, metabolic activity, and cell alignment, when compared to an isotropic (non-aligned) scaffold. By including directional solidification, contact guidance cues were implemented and a more physiologically relevant model was created (Caliari and Harley, 2011). This model was also utilized by Grier et al. (2017) to study the influence of pore size and stiffness on tenocyte metabolic activity and gene expression. Increased tenogenic gene expression and cell activity was seen for higher crosslinking densities and smaller pore sizes (Grier et al., 2017).



	1. *Ex vivo* loading	2. 2D loading models	3. 3D loading models	4. Stretch & Perfusion models
Advantages	Intact tissue structure	Straightforward	Cell-ECM interactions	Cell-ECM interactions
	Cell-ECM interactions	Suitable for preliminary experiments	Loading of various materials	Loading of various materials
	Biomechanical characteristics similar as *in vivo*	Cost-effective	Custom-made set-up possibilities	Custom-made set-up possibilities
				Mimicking vascular supply
				Inter-sample repeatability
Limitations	Variation (strain protocol, sample size and decellularization process)	No ECM incorporation	Low inter-sample repeatability	No neurological supply
	Lack of control	Lack of structural complexity	No (neuro-)vascular supply	Expensive
	Difficult standardization	No (neuro-)vascular supply	Expensive	

2D: Two-dimensional; 3D: Three-dimensional; ECM: Extracellular matrix.

FIGURE 6 | Advantages and disadvantages of bioreactors used in tendon tissue engineering. Adjusted from Ligagen® L30–4C (DynaGen systems; Dartmouth, NS, Canada), BioFlex® culture plates and TissueTrain® (Flexcell International; Hillsborough, NC, United States), and BOSE BioDynamic® 5,200 multi-chamber (TA Instruments; New Castle, United Kingdom).

Microfluidic Alignment

While contact guidance in 2D cultures is provided by culture plastic surface modifications (see section "Two-Dimensional (2D) Models" above), microfluidic alignment is a popular method in 3D cultures to create scaffolds with well-defined geometry. By collagen polymerization inside channels (often in polydimethylsiloxane, PDMS) fiber alignment is realized. Lee et al. (2006) examined the effect of different channel widths on alignment. They considered this technique suitable for cell studies on movement, signaling, growth and differentiation pathways, although it must be mentioned that they did observe a reduced alignment efficiency of threads with increasing channel width, resulting in a greater angle between fibers (Lee et al., 2006; Uquillas et al., 2012). Occhetta et al. (2013) combined micro-molding within PDMS stamps and hydrogel photopolymerization to establish an *in vitro* model. Instead of seeding, they encapsulated BM-MSCs and endothelial cells in the hydrogel, which is much more efficient, more physiologically relevant and more compatible with vascular supply (Occhetta et al., 2013; Waheed et al., 2019).

Electrochemical Alignment

An electrical current can be applied to the collagen solution to produce densely packed, aligned fibers, the so-called ELAC threads, as already mentioned earlier. Advantages of this technique are low cost, different geometry formation by changing electrode geometry and improved controllability (Uquillas et al., 2012). Cheng et al. (2008) were the first to produce ELAC threads. Collagen bundles were formed with an average diameter of 50–400 μm, which is much larger than the fiber diameter achieved with self-assembly. Tenocytes seeded on ELAC threads were able to survive and proliferate on the bundles. However, only half the strength of native tendon was reached (Cheng et al., 2008). The group of Uquillas was able to increase the mechanical strength by crosslinking ELAC with 2% genipin (Uquillas et al., 2012). However, to increase biomimetic fibril structure, a smaller diameter is desirable (±100 nm range) (Smith, 2008; Kew et al., 2011Xie et al., 2020).

Electrospinning

Non-modified Electrospinning Structures

Using electrospinning, ultrafine fibers within nanometer range are produced which enables tendon tissue engineering applications. The micro- and nanostructure can be precisely designed by altering process parameters and polymer solution (De France et al., 2018). Fibers are produced through subjecting natural or synthetic materials in a syringe pump to a high-power electrical field, surface tension is overcome by electrostatic forces, and a jet of fluid is ejected onto a grounded collector (Zhong, 2016). Electrospun nanofibers have a high surface

area to volume ratio, mimic the tendon ECM and, therefore, facilitate cell attachment and metabolic activity (Sahoo et al., 2006). Many derivative methods of traditional electrospinning have been developed in which highly anisotropic structures are created, thus, applicable for tendon applications, such as wet spinning, melt electrospinning, or coaxial electrospinning. In wet electrospinning, the usual metal collector is replaced by a liquid bath. If the polymer substance is heated during extraction, it is called melt electrospinning. A cylindrical collector is typically used, resulting in more aligned fibers when compared to a traditional setup (Alghoraibi and Alomari, 2018; Sensini and Cristofolini, 2018). Coaxial electrospinning combines two polymer solutions in a core-shell setup. For a more comprehensive technical description of the various techniques, we refer to other reviews (Alghoraibi and Alomari, 2018; Sensini and Cristofolini, 2018).

In various electrospinning studies, scaffolds are evaluated for tendon tissue engineering by the (un)successful tenogenic MSCs differentiation. For example, James et al. (2011) used PLGA to compare an electrospun matrix with a 2D film. Enhanced collagen I expression was observed after growth factor supplementation in both matrices, but scleraxis expression was only upregulated when AT-MSCs were cultured on the electrospun matrix. The tensile strength of the scaffold was within the range measured for regular human flexor tendons though not sufficient for those perceived by Achilles tendons (Sensini and Cristofolini, 2018). Full-thickness cell infiltration was evaluated in multi-layered aligned electrospun PCL matrices by Orr et al. (2015). In that study, it was demonstrated that fiber alignment has no positive effect on ECM production and cell infiltration after 28 days of culture, but tenomodulin expression by AT-MSCs and mechanical properties were increased (Orr et al., 2015). Yang et al. (2016) combined PCL and methacrylated gelatin onto a rotating vessel. Using these scaffolds, ECM structure and mechanical anisotropy were mimicked, and tenocytic differentiation of AT-MSC was induced. In the study of Ramos et al. (2019), in which PCL was combined with cellulose acetate, decreased fiber diameter and tensile strength were observed with increasing cellulose acetate concentrations. The latter illustrates that the balance between the hydrophobic nature of PCL and the hydrophilic nature of cellulose acetate is difficult to realize in order to promote cell interactions and, at the same time, provide sufficient strength.

Natural materials are less frequently used as a mono-material in electrospinning. Ghiasi et al. (2014) designed nano-coated textured silk yarns for tendon and ligament scaffold application. A better surface roughness was achieved, resulting in a more porous surface to support cell migration to the inner part of the scaffolds. Unfortunately, the mechanical strength of the constructs was insufficient (Ghiasi et al., 2014). Tu et al. (2013) combined a PLA core with a collagen sheath to create aligned fibers with high mechanical strength and adequate biocompatibility. PLA/PCL/collagen scaffolds were fabricated by Xu et al. (2014), by using a dynamic water flow system for electrospinning. When tendon stem cells were seeded onto the scaffolds, they showed good proliferation and increased gene expression in response to mechanical stimulation (Xu et al., 2014).

Textile Manufacturing Techniques

To reconstitute filamentous collagen structures and improve the mechanical strength, electrospinning configurations have been modified to produce bundles and yarns and have been combined with different textile production methods such as braiding, knitting, and weaving. Sahoo et al. (2006) designed a nano-microfibrous polymer tendon scaffold by electrospinning PLGA onto a knitted PLGA scaffold, as such bypassing the poor cell seeding results associated with braided fabrics. Although favorable cell properties were obtained, mechanical properties were far from comparable with native tendon (Sahoo et al., 2006). Barber et al. (2013) braided three, four, or five, aligned bundles of electrospun PLA nanofibers. The three-bundle braided scaffold showed superior mechanical characteristics compared to the other groups. Tenogenic differentiation of MSCs on the three-bundle scaffolds was observed after supplementation of tenogenic growth factors and upon applying cyclic tensile strain, highlighting the *in vitro* potential (Barber et al., 2013). Braiding, however, results in tightly packed biomaterials, having a negative impact on cell proliferation and infiltration (Laranjeira et al., 2017). Czaplewski et al. (2014) investigated the effect of fiber chemistry and braiding angle on the scaffold's mechanical properties and tenogenic differentiation using human induced pluripotent stem cells. They showed that large angles, thus less dense packaging, better supported tenogenic differentiation (Czaplewski et al., 2014). Vuornos et al. (2016) compared different medium compositions, biomaterials and scaffold structures in order to identify the most efficient set-up to produce tendon-like matrix *in vitro*. When compared to foamed PLA/PCL scaffolds, braided PLA scaffolds were superior both for cell characteristics and tenogenic differentiation of AT-MSCs. Moreover, the PLA scaffolds expressed a similar elastic modulus as native Achilles tendons (Vuornos et al., 2016).

Hybrids

Both production technologies and biomaterials mentioned above can be extensively combined to establish the ideal *in vitro* tendon model. However, it is mandatory that the structure does not become too complex to be generally accepted and stays controllable. Each single component should be evaluated for necessity and added value. Wang et al. (2018) designed a novel scaffold for tendon regeneration by combining a PCL shell with an electrospun PCL/polyethylene oxide core. The scaffold had tendon-like mechanical properties and the cultured tenocytes expressed a higher amount of phenotypic markers when compared to isotropic control scaffolds (Wang et al., 2018). Despite the achieved mechanical strength, they were not able to mimic native ECM. As the synthetic polymers used in this model did not contain the functional chemical groups available for cellular binding, this setting is deemed not suitable as a good *in vitro* tendon model. The group of Rinoldi et al. (2019) combined coaxial extrusion printing and wet spinning of a bioink composed of alginate and methacrylated gelatin, which allows cell encapsulation. Tenogenic differentiation of

BM-MSCs and aligned cell/fiber orientation was successfully achieved (Rinoldi et al., 2019). Laranjeira et al. (2017) designed an artificial tendon construct consisting of continuously aligned nanofiber threads (PCL/chitosan) reinforced with cellulose nanocrystals. Biocompatibility, cell elongation, and anisotropic organization was assessed after seeding tenocytes and AT-MSCs. Tenogenic differentiation of AT-MSCs was reached and tenocyte dedifferentiation prevented (Laranjeira et al., 2017). Unfortunately, vascularization was not provided which hampers extensive use of this model. Wu et al. (2017) produced a nanofibrous, woven biotextile, made of electrospun PCL yarns interlaced with PLA multifilaments. The woven scaffolds had a significantly larger pore size and showed better mechanics than the non-woven controls. Tenocyte and AT-MSCs proliferation and gene expression were upregulated in the woven scaffolds. Subsequently, after supplemental seeding of human umbilical vein endothelial cells (HUVECs) on the woven scaffolds with AT-MSCs and tenocytes, tenogenic gene expression further increased (Wu et al., 2017). Although vascularization building-blocks are included in this model, the exact function of the HUVECs was not evaluated in this study.

More than one hundred scientific papers and several reviews have described different electrospun nanofibers which can be used for tendon tissue engineering because of their ability to mimic ECM structure and its production flexibility (Sensini and Cristofolini, 2018). However, current models still require some optimization before a standalone model can be established. Most electrospinning set-ups are not suitable for cell encapsulation, which is more physiologically relevant and significantly more compatible with the incorporation of vascular supply than cell seeding (Waheed et al., 2019). In addition, effective vascularization has not yet been achieved in electrospun scaffolds and homogeneous seeding is often difficult (Merceron et al., 2015). The search for the ideal material (or combination of materials) is still ongoing. A combination of PCL and chemically modified collagen to combine a certain mechanical strength with biocompatibility, could be promising for a tendon *in vitro* model (Zeugolis et al., 2008a; Uquillas et al., 2012).

3D Bioprinting

In additive manufacturing, different processes are used to replicate 3D objects layer-by-layer by computer-aided design with controlled geometrical properties (Sculpteo, n.d.; De France et al., 2018; Vaezi et al., 2018). The conventional chemical engineering methods (as discussed above) fail to control exact pore size, pore geometry, and spatial distribution of the pores (Vaezi et al., 2018). Therefore, advanced additive manufacturing techniques are gaining popularity in recent tissue engineering strategies.

A popular technique is 3D bioprinting, in which a bioink is used to mimic the ECM and encapsulate the desired cells (Laternser et al., 2018). The most promising techniques are direct printing techniques, in which a 3D gel is directly printed in a single processing step (De France et al., 2018). The most common methods are micro-extrusion, inkjet and/or light−induced methods, which include laser−assisted bioprinting and stereolithography (Mandrycky et al., 2016). Bioprinting must be carefully prepared by collecting accurate tissue information, transferring information to a suitable computer-aided design model and, finally, by creating a stable structure (He et al., 2018). Specific demands for material and cell source have been listed: (i) the material should maintain cell viability and promote specific activity after printing, (ii) a great number of encapsulated cells needs to be available and (iii) cells should be able to survive the (post-)printing process (Zhang et al., 2017).

Extrusion-based 3D printing is a direct printing method which is widely researched and identified as an appropriate method for bone, cartilage and adipose tissue engineering. In contrast, this technique is still in its infancy for tendon tissue engineering, (Costantini et al., 2016; Sun et al., 2016; Naghieh et al., 2017; De Mori et al., 2018; Fernandez, 2019; Colle et al., 2020; Tytgat et al., 2020; Van Damme et al., 2020). For example, Sun et al. (2016) have demonstrated the overall superior qualities of 3D printed scaffolds over freeze-dried scaffolds for cartilage tissue engineering. Different extrusion-based bioprinters are designed to deposit various biopolymers, hydrogels and many different cell types to produce 3D bio-constructs (Vaezi et al., 2018). Merceron et al. (2015) developed a muscle–tendon unit construct, suitable as *in vitro* model by applying pneumatic pressure and heat to extrude filaments. The tendon part consisted of PCL co-printed with fibroblasts (NIH/3T3). The polymers were printed separately from the cell-laden bioinks by interspersing rows, and this process was repeated layer-by-layer with the whole construct being crosslinked at the end. After 7 days of culture, good cell viability was observed as well as the characteristic cell morphology. In addition, the construct was repeatedly produced with precise dimensional accuracy (Merceron et al., 2015). Laternser et al. (2018) described a novel drug screening platform for tendon and muscle applications. A tendon tissue model was designed by alternating layers of bioink and rat tenocytes in a dumbbell shape around postholder inserts in a microplate. The printed tenocytes showed high viability and maintained good differentiation, suggesting a good but rather basic approach for tendon drug screening (Laternser et al., 2018). Stanco et al. (2020) incorporated AT-MSCs in a nanofibrillar cellulose and alginate bioink for 3D printing, to evaluate tenogenic differentiation and suitability for tissue engineered constructs. The AT-MSCs survived the printing process and displayed the favorable tenogenic-like phenotype without an inflammatory response to the bioink. The research group reported a first approach for upscaling the clinical use of 3D printed tendon constructs, opening up possibilities for model design (Stanco et al., 2020). Park et al. (2018) designed a 3D bioprinted scaffold sleeve composed of PCL, PLGA and β tricalcium phosphate to reconstruct an anterior cruciate ligament. The scaffold was seeded with MSCs and evaluated in bone-tunnels of an *in vivo* rabbit model to assess bone-tendon regeneration. In the treatment groups, improved bone-tendon healing was observed at all time points and thus the authors concluded that their scaffold has the potential to accelerate bone-tendon healing in anterior cruciate ligament reconstruction (Park et al., 2018). Unfortunately, MSCs were still seeded afterward in this model instead of being encapsulated.

Recently, incorporating dECM in bioinks has attracted attention in the field of tissue engineering (Zhang et al., 2017; Santschi et al., 2019). Toprakhisar et al. (2018) developed a bioink from tendon dECM and evaluated murine fibroblast (NIH/3T3) viability and morphology. Vascularization, however, was not included in this model and cultures were only followed for 3 days. Furthermore, fibers were not properly aligned in contrast to fibers obtained with electrospinning. Regardless, the use of dECM in an *in vitro* model is limited due to the fact that its composition is variable and needs to be analyzed before every procedure.

Current drawbacks of direct bioprinting for tissue engineering applications include the low achievable sizes and the limited number of suitable materials (De France et al., 2018). When considering *in vitro* studies, the small scaffold size is not a constraint, but better fiber alignment has to be achieved and the impact of mechanical stimulation on 3D printed tendon constructs still needs to be evaluated.

The use of computer-aided design in bioprinting enables the combination with other fabrication techniques such as electrospinning. Jordahl et al. (2018) used 3D jet writing (a combination of electrospinning and 3D computer-aided design) to create bone constructs. Stability was obtained with PLGA and MSCs were seeded (Jordahl et al., 2018). In this approach, the benefits of both bioprinting and electrospinning were combined. When vascularization would be integrated in this model, this approach could be suitable for *in vitro* tendon models as well, providing a breakthrough in this research field.

ADVANCED: INCORPORATING BIOREACTORS

Bioreactors are used to sustain the life of cells and tissues *in vitro*, while under the influence of dynamic, but controllable, physiological conditions (Youngstrom and Barrett, 2016; Ozbolat, 2017). Many different bioreactors have been used in tendon tissue engineering, as chronologically listed by Dyment et al. (2020). As already mentioned above, mechanical loading is essential to mimic tendon physiology, as cell signaling systems are modified through mechano-transduction pathways (Chiquet et al., 2009; Buxboim et al., 2010; Riehl et al., 2012; Govoni et al., 2017; Wang et al., 2017). Therefore, only bioreactors applying mechanical stimulation are discussed in this literature review, with the focus on parameters like mechanical stimulation, stability, and repeatability for fundamental research. A main drawback associated with bioreactors is that all researchers have used different protocols and it is challenging to compare studies. An overview of the cited bioreactors and corresponding stimulation protocols is given in **Figure 6** and **Table 1**. Tension protocols ranged from 0 to 10% (magnitude) and a frequency between 0.0167 and 1 Hz.

To study tendon homeostasis under mechanical load, biomechanical parameters such as strength, toughness and viscoelasticity are evaluated (Murata, 2012). Other tensile properties for characterization of biomaterials are UTS, yield or failure load/stress/strain, and elasticity. While UTS displays the maximum stress before physical deformation disrupts the material irrevocably, the yield load/stress/strain displays the stress a material can tolerate before physical deformation occurs and the failure load/stress/strain illustrate the properties at the moment of failure (Malkin and Isayev, 2012b). Stress is the applied force over cross-sectional area. Strain, however, is a measure for the deformation in response to the applied stress. The ratio of stress over strain is called elasticity (or Young's modulus) and illustrates how force results in deformation of the tissue (Malkin and Isayev, 2012a).

To allow cells to respond in an *in vitro* model similarly as *in vivo*, they should experience the physiological stiffness of the scaffold and undergo physiological levels of strain (Sensini and Cristofolini, 2018). The tendon response to mechanical load is varying depending on the location in the body, the age of the animal, and the differentiation level of the cells (Patterson-Kane et al., 2012). As different results are observed *in vivo* compared to *in vitro*, measurements cannot simply be extrapolated and should be evaluated 'relatively' (Smith and Goodship, 2008). For example, *in vivo* the strain of the SDFT in galop is measured 12-16%, while *in vitro* 15–17% is measured as ultimate tensile strain (Gerard et al., 2005; Thorpe et al., 2010). The effects of mechanical forces on MSC differentiation have been widely studied (Huang et al., 2009; Youngstrom et al., 2015; Youngstrom et al., 2016; Fahy et al., 2017). Depending on the mechanical stimulation protocol and MSC source used, differentiation into an osteogenic, chondrogenic, adipogenic, or tenogenic phenotype is observed (Delaine-Smith and Reilly, 2012). Therefore, the appropriate strain/stretch protocols for MSCs in a tendon environment should be identified in order to support tenogenic differentiation of the MSCs and, more importantly, the secretion of tenogenic trophic factors. In a study of Youngstrom et al. (2015) equine BM-MSCs were seeded on decellularized SDFT, subjected to 3 and 5% strain, 0.33 Hz for up to 1h daily and compared to static controls (0%). Upregulation of scleraxis expression was seen both in the 3 and 5% group compared to the control group, albeit only significant in the 3% group. Other evaluation criteria such as gene expression, elastic modulus, and UTS, were also superior in the 3% group. The 5% strain approach gave results in between the 0 and 3% strain group, leaning more toward the 0%, suggesting that 3% strain is more suitable for SDFT studies (Youngstrom et al., 2015). Human tenocytes cultured on rat tail collagen gels, on the other hand, expressed anabolic changes in matrix metalloproteinases and tenogenic genes when exposed to 5% strain, 1 Hz (Jones et al., 2013). In a study of Wang et al. (2013) in which rabbit Achilles tendons were subjected to 0–9% strain, 0.25 Hz for 8 h daily, 6% cyclic tensile strain was identified as optimal to maintain structural integrity and cellular functions (Wang et al., 2013). An electrospun PLA/PCL/collagen scaffold, seeded with rat tendon stem cells was subjected to cyclic tensile strain with different magnitudes and frequencies by Xu et al. (2015). The optimal protocol for enhancing tenogenic gene expression was determined as 4% and 0.5 Hz (Xu et al., 2015). Burk et al. (2016) seeded equine AT-MSCs on a decellularized tendon scaffold. When the scaffolds were exposed to 2% strain and 1 Hz, the viability decreased while tenogenic gene expression (tenascin-C and scleraxis) increased. Cell alignment was present on all scaffolds (static vs. cyclic stretching) and was more

TABLE 1 | Overview of bioreactors used in tendon tissue engineering which all apply mechanical stimulation.

	Study	Biomaterial + Cells	Bioreactor	Stimulation	Results
Ex vivo	Angelidis et al., 2010	Decellularized rabbit hind paw Flexor tendon + AT-MSCs, fibroblasts	Ligagen L30–4C (DynaGen systems), clamped	Uniaxial strain, 1.25 N over 5 days. 1 cycle/minute in alternating 1h periods of mechanical loading and rest.	UTS and E comparable with fresh tendons. Cells reoriented parallel to the direction of the strain.
	Saber et al., 2010	Decellularized rabbit hind paw Flexor tendon + tenocytes	Ligagen L30–4C (DynaGen systems), clamped	Uniaxial strain, 1.25 N over 5 days. 1 cycle/minute in alternating 1 h periods of mechanical loading and rest.	UTS and E of loaded construct superior to non-loaded controls.
	Wang et al., 2013	Rabbit AT	Clamp grips, in medium	8 h/day, 0–9%, 0.25 Hz. 6 days	Loss of structure integrity and increased collagen III expression in unloaded tendons. 6% cyclic strain optimal for structure integrity and cellular function.
	Lee et al., 2013	Decellularized porcine anterior tibialis tendon	Vertically, in culture medium	10% tension, 1 Hz, 90° torsion. 7 days	20% lower UTS in decellularized grafts vs. normal tissue but doubled UTS after 7 days incubation
	Youngstrom et al., 2015	Decellularized equine SDFT + BM-MSCs	Horizontally clamp gripped, in medium	0%, 3%, 5% strain, 0.33 Hz, up to 1 h/day, 11 days.	Gene expression, elastic modulus and UTS favorable with 3%.
	Burk et al., 2016	Decellularized equine SDFT + AT-MSCs	Clamp grips, in medium	2% strain, 1 Hz, short (2 stretches/cycle) and long (3 stretches/cycle) protocol.	Short mechanical stimulation best cell alignment, successful tenogenic differentiation.
2D loading	Riboh et al., 2008	Rabbit tenocytes, sheath fibroblasts, BM-MSCs, AT-MSCs	UniFlex culture plate + Flexcell Tension System (Flexcell International)	Continuous strain (8%, 1 Hz). Intermittent strain (1 h on/5 h off, 4% 0.1 Hz).	Cell proliferation, collagen I production and tenocyte morphology increased with intermittent strain.
	Zhang and Wang, 2013	Mice tenocytes or TSPCs of AT or patellar tendon	Silicone dishes connected to stretching apparatus	12 h, 4% or 8%	Tenogenic gene expression increased in TSPCs with 4% mechanical stretching. Tenocyte and non-tenocyte related gene expression increased in TSPCs with 8% mechanical stretching. Tenocytes no strain-dependent response in non-tenocyte related gene expression.
	Gaspar et al., 2016	Human dermal fibroblasts, tenocytes, BM-MSCs + macromolecular crowding	MechanoCulture FX (CellScale Biomaterials Testing), clamp grips	12 h/day, 10%, 1 Hz	Cell/ECM alignment superior, increased ECM deposition and similar metabolic activity with mechanical loading.
	Gaspar et al., 2019	Human tenocytes, BM-MSCs, neonatal/adult dermal fibroblasts + macromolecular crowding	MechanoCulture FX (CellScale Biomaterials Testing), clamp grips	12 h/day, 10%, 1 Hz	Tenogenic phenotype maintained by tenocytes. No (*trans*)differentiation of BM-MSCs or fibroblasts.
3D loading	Altman et al., 2002	Collagen type I gel + bovine ligament fibroblasts, human BM-MSCs	Vertically oriented ligament growth between 2 anchors	Translational (10%, 2 mm) and rotational strain (25%, 90°). 0.0167 Hz (1 cycle of stress/relaxation per minute), 21 days.	Ligament markers upregulated, cell alignment/density increased and oriented collagen fibers.
	Garvin et al., 2003	Collagen type I gel + avian tenocytes	Tissue Train 3D Culture System (Flexcell International), culture plate with 2 anchors	1 h/day, 1% elongation, 1 Hz, 11 days	Tenogenic gene expression and linear morphology. Stronger loaded constructs vs. non-exercised controls.

(Continued)

TABLE 1 | Continued

	Study	Biomaterial + Cells	Bioreactor	Stimulation	Results
	Scott et al., 2011	Collagen type I gel + mouse multi-potent mesenchymal cell line (C3H10T1/2)	Tissue Train 3D Culture System (Flexcell International), culture plate with 2 anchors	Static vs. cyclic load, 2 h/day, 5%, 0.1 Hz for 1, 2 or 3 weeks. 2 h/day, 0, 2.5, 5, 7.5, or 10%, 0.1 Hz for 2 weeks. 2 h/day, 10%, 0.1 Hz, 10,100, or 1,000 cycles/day, 10s rest	Tenogenic gene expression increased with cyclic loading. Gene expression increased with increasing magnitude, with 10s rest and increased repetitions.
	Jones et al., 2013	Collagen type I gel + human AT tenocytes	Tissue Train 3D Culture System (Flexcell International), culture plate with 2 anchors	5% cyclic uniaxial strain, 1 Hz, 48 h	Matrix metalloproteinases and tenogenic genes anabolically influenced.
	Bosworth et al., 2014	PCL + human BM-MSCs	BOSE BioDynamic chamber 5110 (TA Instruments), clamp grips	1 h/day, 5%, 1 Hz (3,600 cycles/day), 225 N, 7 and 21 days	Cell orientation more uniaxial, tendon gene upregulation due to dynamic loading.
	Wu et al., 2017	PCL/PLA scaffold + human tenocytes, AT-MSCs and HUVECs	MechanoCulture T6 Mechanical Stimulation System (CellScale Biomaterials Testing), clamp grips	2 h/day, 4%, 0.5 Hz, 12 days	Total collagen secretion upregulated, enhanced tenogenic differentiation with dynamic stretching.
	Atkinson et al., 2020	Collagen type I + equine tenocytes	Custom-designed bioreactor with clamps	20 min/day, 10%, 0.67 Hz, 14 days	Mechanical properties improved, more gel contraction by the tenocytes with loading.
Stretch and perfusion	Barber et al., 2013	Decellularized equine SDFT + rabbit BM-MSCs	Oscillating stretch-perfusion bioreactor, 6 separate chambers	3× 15–30–60 min of activity alternated with 15–30–60 min off, 2×/day. 3%, 0.33 Hz, 7 days. Perfusion: 100 μm/s	Collagen production and alignment superior in cyclic load vs. static culture.
	Hohlrieder et al., 2013	PLA nanofibers in yarns + human BM-MSCs	BOSE BioDynamic 5200 multi-chamber (TA Instruments), clamp grips	2 h/day, 10%, 1 Hz, 10 days Perfusion: 20 ml/min	Cytoskeleton realignment in fiber/applied strain direction, BM-MSCs adherence to fibers, tenogenic differentiation when differentiation medium + cyclic tensile strain.
	Xu et al., 2015	Braided silk fibroin + human ACL fibroblasts	Custom-made bioreactor, 10 independent reactor vessels, vertical movement	45° rotational and 3.5 mm translational deformations, 0.0667 Hz	Exact control of environmental conditions possible, load and stiffness of silk scaffolds matches native ACLs.
	Talò et al., 2020	PLA-PCL/Collagen scaffold + rat TSPCs	Custom-designed, in culture medium, loading plates	Cyclic tensile strain, 3 h/day, 2, 4, and 8 and 0.3, 0.5, and 1.0 Hz, 7 days	No difference in cell viability. Tenogenic gene expression highest with 4%, 0.5 Hz.

ACL, anterior cruciate ligaments; AT, achilles tendon; AT-MSCs, adipose tissue-derived mesenchymal stem cells; BM-MSCs, bone marrow-derived mesenchymal stem cells; E, elastic modulus; HUVECs, human umbilical vein endothelial cells; PCL, poly-ε-caprolactone; PLA, polylactic acid; SDFT, superficial digital flexor tendon; TSPCs, tendon stem/progenitor cells; UTS, ultimate tensile strength.

pronounced compared to monolayer cell cultures, indicating the importance of ECM mimicry (Burk et al., 2016).

Not only the amount of strain applied is significant, the regimen used is also of great importance. Continuous static loading not only affects tendon phenotype negatively, it is also not representative for the *in vivo* situation. Optimal for physiological mimicking is, therefore, the application of intermittent cyclic loading (Cao et al., 2006; Scott et al., 2011). Different studies show favorable cell characteristics (elongation, metabolic activity, gene levels and protein production) administering intermittent cyclic strain, while a negative influence of continuous cyclic strain is frequently reported (Cao et al., 2006; Riboh et al., 2008; Bosworth et al., 2014; Youngstrom et al., 2015; Atkinson et al., 2020). For example, Riboh et al. (2008) compared the application of constant versus intermittent cyclic strain in a 2D, Flexcell Strain Unit (Flexcell International; Hillsborough, NC, United States). Continuous cyclic strain inhibited cell proliferation and collagen production in tenocytes, sheath fibroblasts, BM-MSCs and AT-MSCs. Intermittent cyclic strain, on the other hand, increased cellular proliferation and total collagen production per cell (Riboh et al., 2008).

Ex vivo Loading

To define mechanical characteristics of tendons, explants can be loaded onto bioreactors immediately after dissection or after decellularization. For defining biomechanical characteristics of *in vivo* tendons, *ex vivo* loading is the most appropriate method. However, mechanical properties vary due to the applied strain

protocol, the size of the dissected tissue, and the decellularization process if applicable (Kuo et al., 2010; Youngstrom and Barrett, 2016). A custom bioreactor system (Ligagen L30-4C, DynaGen systems; Tissue Growth Technologies, Minnetonka, MN, United States) was used by two research groups to test the influence of oscillatory, uniaxial tensile stimulation on decellularized rabbit flexor tendons. Both reseeded the constructs with different cell types (AT-MSCs combined with fibroblasts, and tenocytes, respectively) and compared either seeded constructs to fresh tendons and unloaded constructs (Angelidis et al., 2010) or to unseeded tendons (Saber et al., 2010). Both studies found that the seeded tendon constructs showed mechanical characteristics (UTS and elastic modulus) comparable to those of freshly extracted tendon (Angelidis et al., 2010; Saber et al., 2010). Lee et al. (2013) evaluated porcine anterior tibial tendons for human ligament reconstruction in the bioreactor where the ligament was fixated vertically at both ends and tension up to 120% was applied while rotation was simulated also (0–90°). Decellularized grafts initially showed 20% lower UTS than normal tendon, but after 7 days of physical stimulation, the UTS was doubled, indicating that these constructs do have potential for human ligament reconstruction (Lee et al., 2013). Based on research with these *ex vivo* models, our knowledge about tendon physiological mechanics has substantially improved. Nevertheless, these models are too variable, and therefore, inadequate to study cellular responses *in vitro* (Wang et al., 2017).

2D Loading Models

As 2D models are still used to examine cell behavior [as discussed in section "Two-Dimensional (2D) Models"], implementing mechanical load is achievable in 2D loading bioreactors. Cells are cultured in modified cell-plates, allowing execution of a specified stretch protocol after cell adhesion to the bottom of the well. Both custom-made and commercial models are available and are usually placed within a standard incubator. The disadvantage of these 2D bioreactors is that they can only provide insights in cellular responses but not cell–matrix interactions (Wang et al., 2017; Yu et al., 2020). Zhang and Wang investigated tendon mechano-biological responses through different *in vivo* and *in vitro* experiments. For the *in vitro* experiment, mice AT/patellar tendon tenocytes and tendon stem cells were extracted, plated in silicone dishes and mounted on a custom-made stretching device (Wang et al., 2003). Their main finding was that a cell-dependent response occurred on the applied strain regimes. Tendon stem cells responded to moderate mechanical stretching (4%) with increased tenocyte-related gene expression (collagen I, tenomodulin). Exaggerated mechanical loading (8%) resulted in both increased tenocyte and non-tenocyte-related gene expression, suggesting differentiation into other cell types. Tenocytes, on the other hand, did not express this strain-dependent response. The researchers concluded that moderate mechanical loading could be beneficial in tendon homeostasis (Zhang and Wang, 2013). The combined effects of macromolecular crowding [where an attempt is made to create an optimal intracellular environment by the administration of various macromolecules, including different proteins and nucleic acids (Hata et al., 2018)] and mechanical loading in a commercially available MechanoCulture FX (CellScale, Biomaterials Testing; Waterloo, Canada) were evaluated by Gaspar et al. (2016) Increased alignment was seen together with increased ECM deposition, but with similar cell metabolic activity and viability. The same group later conducted a similar study using other cell types (tenocytes, BM-MSCs and dermal fibroblasts). While they successfully achieved maintenance of proper tenogenic phenotype by combining mechanical loading and macromolecular crowding, they failed to induce tenogenic differentiation of BM-MSCs and *trans*-differentiation of fibroblasts (Gaspar et al., 2019).

3D Loading Models

2D bioreactors are only suitable for loading monolayer cell cultures, therefore, a more sophisticated approach for 3D biomaterial scaffolds is needed (Yu et al., 2020). Various (bio-)materials can be loaded, and many alternative mount methods are available. For example, the end of the samples can be fixed with grips, but fixation between anchors is also possible. These reactors, however, lack vascularization, as the static presence of culture medium is not representative for *in vivo* blood flow. Additionally, culture medium is often individually provided per sample, limiting the inter-sample repeatability (Jaiswal et al., 2020). In the bioreactor of Altman et al., BM-MSCs seeded onto collagen I gel matrices were grown in 12 individual tubes in between two anchors (2 cm apart) for ligament tissue engineering. The complete system was designed to fit in a standard incubator. After application of translational and rotational strain, gene expression, cell alignment, and collagen fiber orientation suggested the BM-MSCs differentiated into ligament cells (Altman et al., 2002). Unlike some ligaments, tendons mostly experienced unidirectional stretching parallel to their orientation (Wang et al., 2017). The implementation of rotational strains is, therefore, unnecessary for tendon bioreactors. Garvin et al. (2003) inserted tendon constructs, made of a mixture of collagen I gel and tenocytes, in a commercially available Tissue Train 3D Culture System consisting of Tissue Train culture plates with two anchors (Flexcell International; Hillsborough, NC, United States). After 11 days of culture, constructs exposed to uniaxial displacement were stronger than unloaded counterparts, yet far weaker than native adult tendons (Garvin et al., 2003). The same bioreactor was used by Scott et al. (2011) to examine different strain protocols on bioartificial tendons, made of a cell-collagen mixture. Tenogenic gene expression (scleraxis and collagen I) in MSCs was increased by administering cyclic load (versus static load) with increasing magnitude (0, 2.5, 5, 7.5, or 10%), and with implementing 10s rest periods in between loading cycles and with increasing repetitions (10,100, or 1,000 cycles/day) (Scott et al., 2011). Bosworth et al. (2014) utilized the BOSE BioDynamic chamber 5110 (TA Instruments; New Castle, United Kingdom) for evaluating static (0%) versus cyclic loading (5% strain, 1 Hz for 1 h per day) on electrospun PCL yarns seeded with MSCs. Results showed increased cell proliferation, matrix deposition and gene expression in response to intermittent cyclic loading (Bosworth et al., 2014). Wu et al. (2017) cultured three different cell types

(tenocytes, AT-MSCs and HUVECs) on synthetic electrospun fibers (PCL/PLA scaffolds) and stated that dynamic culture in a custom-made mechanical stimulation device promoted collagen production and tenogenic differentiation (Wu et al., 2017). Atkinson et al. (2020) demonstrated with the use of a custom-designed bioreactor that equine tendon constructs within collagen I gels, analogous to other species, show improved mechanics when exposed to cyclic strain (Atkinson et al., 2020).

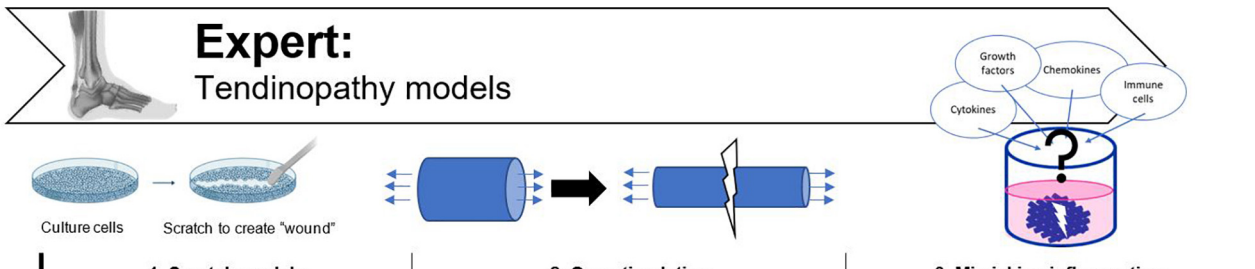
Stretch and Perfusion Models

As culture medium perfusion was incorporated in these bioreactors to mimic blood flow, these models are discussed separately. With the implementation of culture medium perfusion, cells have superior access to oxygen and nutrients which leads to increased proliferation rates (Wendt et al., 2006). Unidirectional perfusion is shown to create heterogeneous spreading of the cultured cells within 3D scaffolds, whereas bidirectional flow leads to more uniformly colonized scaffolds (Wendt et al., 2006; Du et al., 2009). Barber et al. (2013) braided nanofibrous scaffolds out of PLA and seeded them with BM-MSCs. Instead of replacing the differentiation medium every 2–3 days, active unidirectional perfusion (20 ml/min) was implemented (Barber et al., 2013). Hohlrieder and his group tackled the need to control exact environmental conditions to evaluate cell behavior. A new bioreactor was designed with 10 independent vessels to evaluate braided silk scaffolds for ligament graft design. Mechanical load and unidirectional perfusion were controlled per vessel (Hohlrieder et al., 2013). Talò et al. (2020) designed an oscillating stretch-perfusion bioreactor with programmable uniaxial strain for evaluating decellularized SDFT. Bidirectional perfusion was administered after MSC seeding, and the effect of stretching cycles was evaluated. The bioreactor has been declared unique and cost-effective for the incorporation of multiple chambers for controlling different biological and mechanical protocols (Talò et al., 2020).

Conclusive, different custom-made and commercially available bioreactors are being used with different mechanical loading protocols. The variety within these studies complicates translational research and hampers scientific progress (Beldjilali-Labro et al., 2018). For a universal tendon model, an optimal bioreactor with a multiple-chamber, multiple-sample set-up should be designed and used across laboratories, since different experimental conditions and appropriate controls are required (Jaiswal et al., 2020). The stretch-perfusion bioreactors are the most advanced and the most suitable for this application, but a universal loading protocol is lacking.

EXPERT: AVAILABLE TENDINOPATHY MODELS

Two major techniques are available to provide a pathophysiological tendon model, namely scratch models and overstimulation-based approaches (**Figure 7**). To mimic the tendinopathy environment with its physical damage and mechanical disruption, researchers have supplemented various cytokines and enzymes to different *in vitro* cultures and animal models. It is evident, however, that when the physiological tendon model does not mimic *in vivo* tendon tissue regarding complexity and functionality, the "-pathy" models so far available are not very representative either. A main drawback is the lack of nutrition *in vitro*, i.e., vascular supply, to provide inflammatory cytokines and (immune) cells, which normally



	1. Scratch models	2. Overstimulation	3. Mimicking inflammation
Advantages	Suitable for preliminary experiments Acute disruption	Acute & chronic tendinopathy mimicking 2D & 3D models ECM/ fiber disruption	2D & 3D models Standardization by dosage
Limitations	Only 2D models Lack of structural complexity Small defect No ECM/fiber disruption Difficult standardization No chronic tendinopathy mimicking	No protocol available Not (yet) suitable for 3D scaffolds "Breakage" is acute, hard to capture	Lack of structural complexity No ECM/fiber disruption Only environment mimicking

2D: Two-dimensional; 3D: Three-dimensional; ECM: Extracellular matrix.

FIGURE 7 | Advantages and disadvantages of tendinopathy models currently used. Adjusted from Reaction Biology Corp. (n.d.).

regulate the *in vivo* repair process (Dirks and Warden, 2011; Adekanmbi et al., 2017).

Scratch Models

A popular way to assess cell motility in 2D cultures is by a scratch assay. A scratch defect can be created in confluent cell monolayers with a sterile pipette microtip and can then be monitored for several hours-days until closure of the scratch (Fessel et al., 2014). This technique is most frequently used in wound healing studies (Ranzato et al., 2009; Bussche et al., 2015; Liu et al., 2020), but can also represent an acute tendon injury (Fessel et al., 2014; Randelli et al., 2016; Adekanmbi et al., 2017). While *in vivo* all fibers are disrupted, in the scratch model only a small defect is made in the cell layers without the influence of ECM or fiber disturbance. Another issue with this method is standardization, as the defect is mostly applied manually and in contrast to the width of the scratch, which is more fixed as one size of pipette tips is chosen (e.g., a 2–200 μL pipet tip), trying to maintain a certain length is much more variable (Liang et al., 2007). Other culture-inserts [e.g., the Ibidi Culture-Inserts (Huang et al., 2019)] are available to perform more reproducible experiments. However, when these inserts are incorporated in a culture dish, cells grow separated by a cell-free gap and are not disrupted after applying a scratch, and as such, are less representative for tendon injury. Adekanmbi et al. (2017) created a tendon scratch model by simulating a tendon tear with a needle scratch on rat tail tendon fascicles to study the effect of high frequency, low magnitude loading. They standardized the scratch length with marker dots 1cm apart on the petri dish (Adekanmbi et al., 2017). Finally, the scratch model only represents an acute disruption, so no insights in chronic tendinopathy mechanisms can be evaluated, and these assays are usually performed in 2D models, lacking the complexity required for a fundamental *in vitro* model.

Overstimulation

To mimic a tendinopathy and the accompanying inflammation response *in vitro*, overstimulation can be realized by applying a high mechanical load, a high stretch rate, or continuous cyclic duration. As there is currently no consensus on the optimal mechanical stimulation protocol for tendon homeostasis, it is difficult to define what is "too much." Different studies report loading with excessive strain (>9%), which *in vitro* results in integrity damage, cell apoptosis and increased collagen III production (Legerlotz et al., 2013; Wang et al., 2013, 2017; Zhang and Wang, 2013). Dudhia et al. (2007) on the other hand, dissected the SDFT of horses of different ages to study the effect of aging on matrix metalloproteinases activity in a bioreactor with cyclic loading at 5% strain (1 Hz for 24 h). They concluded that their cyclic loading protocol decreased tendon tensile strength and function, and this effect was most pronounced in the group containing the older horses (19 years versus 3 years) (Dudhia et al., 2007). Wang et al. (2003) showed an increase in prostaglandin E_2 secretion, an inflammatory mediator of tendinopathy, with increasing strain magnitude (4, 8, and 12%) in human tenocytes cultures on micro-grooved silicone dishes (Wang et al., 2003). Recently, Kubo et al. (2020) studied the short-term influence of 5% cyclic uniaxial stretching with a frequency of 1 or 2 Hz on primary tenocytes, cultured on PDMS chambers. The 1 Hz group expressed significantly more collagen I and had a higher cell proliferation compared to the 2 Hz or the non-stretched control group. Moreover, the group stimulated with 2 Hz displayed several metabolic changes, such as significant more cell apoptosis, reduced cell viability, and increased matrix metalloproteinase secretion. Their findings contribute to the identification of an appropriate overstimulation stretching profile (Kubo et al., 2020).

Mimicking Inflammation

The actual impact of inflammatory processes on tendon disease is controversial. Whilst the early inflammation phase is clearly present in subacute injured tendons, persistent inflammation results in fibrosis and impaired healing. Various studies have evaluated supplementation with growth factors, cytokines, and chemokines to mimic the acute inflammatory phase of tendon injuries and to evaluate their effect on tenocytes, tendon stem/progenitor cells, and tenogenic differentiation of MSCs. An overview of all cells/inflammatory molecules involved in tendon pathophysiology is beyond the scope of this review and current insights have been recently reviewed elsewhere (Tang et al., 2018; Chisari et al., 2020). Briefly, interleukin -1β treatment reduced tenogenic gene expression of injured tendon-derived stem/progenitor cells (scleraxis, tenomodulin, collagen I, collagen III, biglycan and fibromodulin) and inhibited adipogenic, chondrogenic, and osteogenic differentiation of tendon stem/progenitor cells (Zhang et al., 2015). Stolk et al. (2017) studied the response of human tenocytes on pro-inflammatory factors and macrophages in an *in vitro* inflammation model, and reported altered surface marker and cytokine profiles. Furthermore, macrophage polarization was influenced by the inflammatory environment (Stolk et al., 2017). Alternatively, different immune cell populations can also be introduced in an *in vitro* at different time points, as these cells emerge depending on the stage of the disease. For example, and while there are no macrophages present in normal tendon tissue, pro-inflammatory M1 macrophages are observed in subacute injured tendons, whereas immunosuppressive M2 macrophages are mainly observed with chronic tendinopathy (Tang et al., 2018). Brandt et al. (2018) mimicked tendon inflammation by adding interleukin-1β or tumor necrosis factor-α, and evaluated the effect of peripheral blood leukocytes on AT-MSCs differentiation. High cytokine concentrations decreased ECM production and intracellular tenogenic gene expression in both monoculture and static culture conditions (co-cultures with leukocytes). More importantly, when dynamic loading was incorporated in the co-cultures, a reduced effect of the inflammation-mimicking cytokines was observed (Brandt et al., 2018). As tendinopathy pathophysiology is not yet completely understood and it is still unclear whether or not the onset of inflammation is triggered by immune cells or tenocytes, further research is mandatory to identify the inflammatory components which should be introduced into the disease model. In conclusion, a mixture of all the above-mentioned injury-mimicking mechanisms should be evaluated in 3D scaffolds.

A STATE-OF-THE-ART *IN VITRO* TENDINOPATHY MODEL

Based on the many requirements for a representative physiological tendon and/or pathological tendinopathy model *in vitro*, described throughout this review, it becomes clear that in order to establish a state-of-the-art tendinopathy model to improve knowledge on MSC-based tendon healing, an appropriate biomaterial should be colonized with a relevant cell source, combined with a pertinent production technology and subsequently cultured in the most suitable bioreactor (Caddeo et al., 2017). Mimicking the exact tendon pathophysiology *in vitro* is mandatory to (i) make significant progress in our understanding of tendinopathy mechanisms, (ii) unravel MSC-associated tendon healing, (iii) evaluate novel, regenerative treatments, and (iv) perform pharmacological experiments, while reducing the number of animals used for biomedical research purposes and related costs. The following requirements should be fulfilled: (i) representative cellular growth as demonstrated by the spindle-shape morphology and tenocyte marker expression, (ii) production of ECM and cell–matrix interactions, (iii) supporting nanometric and axially aligned structure (anisotropy), (iv) responsive to physiological levels of uniaxial strain, (v) neuro-vascular supply, and (vi) mimicking micro-damage like acute injuries and chronic overuse (**Figure 8**).

Cells

The cells, relevant for the *in vitro* model, have to be capable of colonizing the scaffold similar to native tissue. In tendon tissue engineering, the most obvious cell type to utilize are tenocytes, as they are the most abundant cell type *in vivo* (Tan et al., 2015; Snedeker and Foolen, 2017; Wang et al., 2017; Schneider et al., 2018). Tenocytes can be easily isolated from adult tendons, but their unequivocal characterization is not evident as there is no unique tendon marker yet. Morphological characterization is usually performed, as tenocytes are spindle-shape, nicely aligned cells (Tan et al., 2015). It is generally accepted to confirm the tenogenic identity by demonstrating the presence of collagen I, collagen III, tenascin-C, scleraxis and tenomodulin, either on gene or protein level (Schweitzer et al., 2001; Zhu et al., 2010; Govoni et al., 2016; Wang et al., 2017). Furthermore, dedifferentiation of the tenocytes should be avoided by applying mechanical stimulation or other contact guidance cues (Nikolovski et al., 2003; Park et al., 2006; Kuo et al., 2010). Because adult cells have only a limited life span and have usually a low proliferation rate (Caddeo et al., 2017), another option is to use MSCs, which can be stimulated to differentiate into a tenocyte-phenotype (Lake et al., 2020). Similar to the *in vivo* situation, identifying the most appropriate cell source is challenging. BM-MSCs are believed to be the most suitable source to treat tendon injuries, but AT-MSCs or MSCs derived from neonatal sources are present in higher numbers and more easily accessible (Shojaee and Parham, 2019). Because tenogenic differentiation is not as straightforward to achieve and various stimulation parameters are involved, it is also possible to use the intrinsic stem cell population present in the tendon, called tendon stem/progenitor cells. These resident cells are responsible for tendon maintenance and repair. Furthermore, these cells are able to respond to inflammation following variable cytokine signals and growth factor profiles. When abnormal pathway activation occurs, tendon stem/progenitor cells differentiate into inappropriate cell types such as chondrocytes or adipocytes, resulting in calcification and metaplasia formation (Steinmann et al., 2020). Unfortunately, tendon stem cells are difficult to isolate because they are only present in small numbers *in vivo* (Wu et al., 2018).

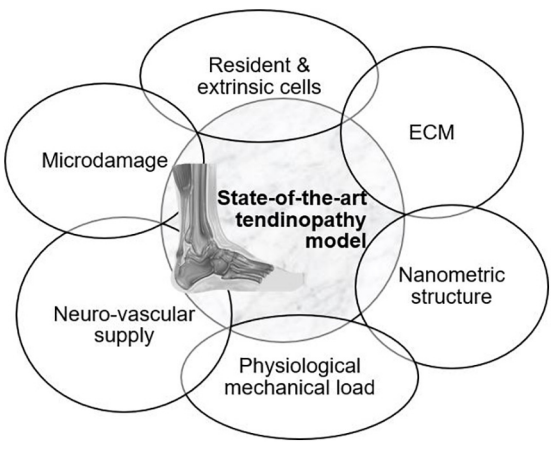


FIGURE 8 | Requirements for the state-of-the-art tendinopathy model.

In the healing process, both the intrinsic and extrinsic tendon cell populations, consisting of circulating cells and cells from nearby tissues, play a role. Therefore, to study the pathophysiological response in accordance to tendon injury, immunocompetent cells should also be incorporated in the model. Although the exact role of inflammatory and immune cells remains unclear (Jomaa et al., 2020), these cells are important for healing of the injured tendon (Caddeo et al., 2017; Tang et al., 2018; Nichols et al., 2019; Jomaa et al., 2020). Initial inflammation, accompanied by attraction of macrophages and neutrophils is crucial for healing. Pro-inflammatory M1 macrophages are mainly present during this early stage, but prolonged activity is reported to result in detrimental healing and increased scar tissue formation (Tang et al., 2018; Nichols et al., 2019). M2 macrophages, on the other hand, broadly described as anti-inflammatory, contribute to ECM deposition and remodeling. However, when inflammation and macrophage activity are completely inhibited, decreased mechanical properties of the healed tissue are observed. Nichols et al. (2019) reviewed the available research on cell populations during tendon healing phases, and frequently reported conflicting results. Therefore, inflammatory cell activity should first be clarified and subsequently balanced cell activity should be established *in vitro* to mimic the *in vivo* situation.

The interaction between resident cells (tenocytes, tendon stem cells, or MSCs), immune cells, growth factors and cytokines is crucial to gain new insights (Caddeo et al., 2017). These immunomodulatory cells/molecules are *in vivo* partially provided

through the blood stream, therefore incorporation of vascular supply, or at least endothelial cells is mandatory (see section "Neuro-Vascular Supply") (Tempfer and Traweger, 2015). Once the pathogenesis of tendinopathy is better understood, supplementing appropriate inflammatory signals and cells to the *in vitro* model to mimic the inflammation following injury, will further improve our insights in MSC healing mechanisms. In order to approximate the (patho-)physiological situation, one cell type will not be sufficient, but a balanced mixture of all of the above should be incorporated.

Extracellular Matrix (ECM)

As stated above, tendon ECM consists of collagen I and III, which provides structure and mechanical strength. The proteoglycans (e.g., decorin and lumican) are important in fibrillogenesis and provide the tendon its high resistance to compressive and tensile forces (viscoelasticity) (Tan et al., 2015; Schneider et al., 2018). For an *in vitro* model, ECM should be included as it represents 80% of the *in vivo* tendon composition. Furthermore, cell–matrix interactions should be enabled as ECM contains many different growth factors, cytokines, and chemokines (Kleinman et al., 2003; Buxboim et al., 2010). Simulating ECM using natural materials is the most straightforward way to establish a representative *in vitro* tendon model, but highly modified synthetic materials (e.g., after adding integrin-binding peptide sequences) represent a valuable alternative.

Supporting Nanometric Structure

There are three important matrix scales in tissue engineering: the macroscopic shape (cm–mm level), the pore structure regulating cell invasion and cell growth (μm level) and surface chemistry which controls cell adhesion and gene expression (nm level) (Kim and Mooney, 1998). For tendon tissue, this implies that fiber diameters should be varying between 2 μm, i.e., diameter of collagen fiber is 1–20 μm, and 200 μm, i.e., diameter of fascicles (**Figure 1**) (Liu Y. et al., 2008). When murine fibroblasts (C3H10T1/2) were cultured on fibers with larger diameter (>2 μm), an increased tenogenic gene expression was observed when compared to small (<1 μm) and medium fiber diameters (1–2 μm) (Cardwell et al., 2014). The maximum fiber diameter is mainly limited by oxygen diffusion capacity (max. 200 μm). *In vivo*, fascicles are surrounded by endotenon (**Figure 1**), which includes blood vessels providing nutrients and oxygen, and thus, the designed *in vitro* scaffolds also need to support nutrients and oxygen supply (Kim and Mooney, 1998; Lu et al., 2013). Furthermore, pore size is also critical, as it allows cell infiltration, nutrition, proliferation and migration (Sensini and Cristofolini, 2018; Luo, 2020). Besides superior cell characteristics, increased and interconnected porosity (usually >90%) also facilitates efficient nutrient and oxygen diffusion and waste removal (Loh and Choong, 2013). The lower limit of pore size is determined by cell size (±20 μm) whereas the upper limit is depending on biomaterial and cell combination (100–200 μm) (Freyman et al., 2001). However, the disadvantage of high porosity is the fact that mechanical properties are compromised due to the large amount of dead volume (Loh and Choong, 2013).

Because of the axially aligned nature of collagen fibrils *in vivo*, a tendon model should provide parallelly aligned fibers (Cardwell et al., 2014) to provide tensile strength during loading and cell proliferation stimuli (Kleinman et al., 2003; Dudhia et al., 2007). The microstructural anisotropy might even influence gene expression levels without modifying gene sequence (epigenetic role) (Wang et al., 2018). Therefore, an *in vitro* tendinopathy model should be designed by implementing electrospinning and extrusion-based 3D printing.

Physiologically Relevant Mechanical Load

The hierarchical anisotropic tendon structure typically enables its non-linear mechanical properties (Sensini and Cristofolini, 2018). When tendons are subjected to mechanical load, the collagen fibers stretch out, nullifying the unique crimp-pattern (called "toe region"). When the mechanical load is increased further, collagen molecules become more aligned, resulting initially in linear stretching, followed by microscopic damage and finally macroscopic tearing (Wang, 2006; Sensini and Cristofolini, 2018). The slope of the stress-strain curve represents the tendon stiffness, also known as Young's modulus. The linear phase of this curve in human tendons is quite short (2–6%) and similar for each tendon of the human body (**Figure 9**). The loading response of the equine SDFT results in a similar stress-strain curve with linear deformation between 3.6 and 10% (examined on an adult fore limb SDFT *in vitro*) which supports the claim for the equine SDFT as a model for human Achilles tendinopathy (**Figure 9**) (Patterson-Kane et al., 2012; Sensini and Cristofolini, 2018). Mechanical properties, however, are dependent on tendon cross-section and function (Sensini and Cristofolini, 2018). As the aim of the *in vitro* model is to generate a physiological response, the cultured cells should experience scaffold stiffness and strain comparable to the *in vivo* situation, resulting in similar cell behavior (Patterson-Kane et al., 2012).

As musculoskeletal loading is essential for maintaining tendon homeostasis, cyclic strain should be included in culture systems (Patterson-Kane et al., 2012). While subtle changes in mechanical loading result in an anabolic and anti-inflammatory response, both over- and under-stimulation can result in degeneration and remodeling of the ECM (Patterson-Kane et al., 2012; Wang et al., 2013; Youngstrom and Barrett, 2016). As already mentioned, it is challenging to identify a suitable protocol to mimic the *in vivo* load of tendon. Indeed, the tendon response to mechanical load is varying depending on the location in the body, the age of the human or animal, and the differentiation level of the cells (Patterson-Kane et al., 2012). In conclusion, mechanical loading should be within physiological range, i.e., (Achilles tendon: 2–6%, SDFT: 3–10%, **Figure 9**) in the context of translational research.

Neuro-Vascular Supply

In vivo, tendon is considered as a hypovascular tissue as blood supply is only present in the endo- and epitenon (Evans, 2012; Docheva et al., 2015; Tempfer and Traweger, 2015; Schneider et al., 2018; Sensini and Cristofolini, 2018; Costa-Almeida et al., 2019). Nevertheless, also in tendon, cells, growth factors

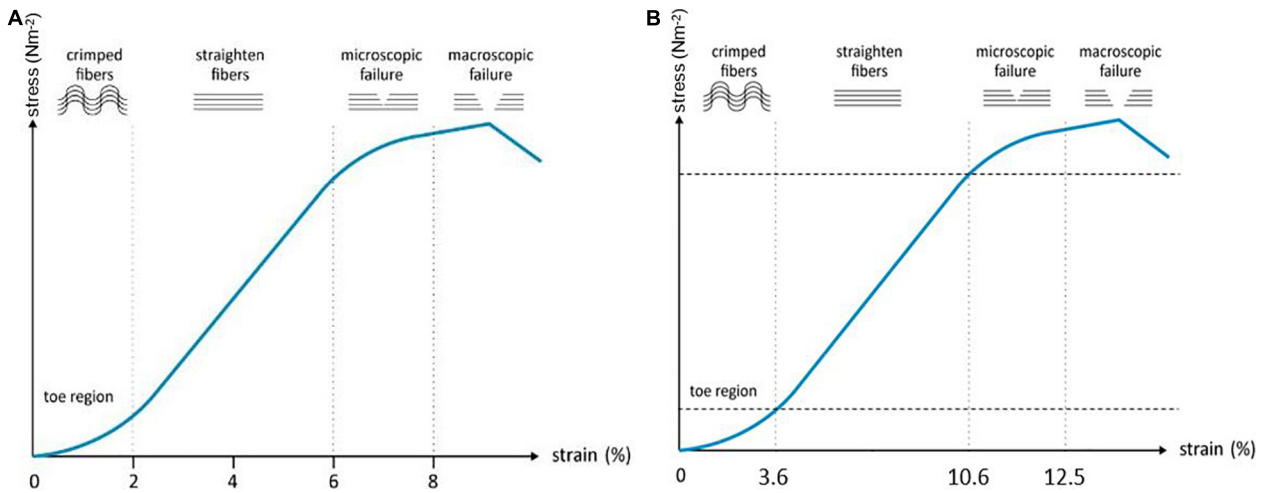


FIGURE 9 | (A) Stress-strain curve of the human Achilles tendon. **(B)** Stress-strain curve of the equine superficial digital flexor tendon. Stress: applied force over area, Strain: deformation in response to stretch, Elasticity: stress/strain ratio or how force results in a deformation of the tissue. Adjusted from Barfod (2014).

and cytokines should be delivered through the blood stream, besides nutrients and oxygen. Increased vascularization and scar tissue formation is observed with tendon injuries (Tempfer and Traweger, 2015). Therefore, vascularization should also be incorporated in an *in vitro* tendinopathy model which remains a major challenge in tissue engineering. 3D bioprinting, however, could offer a solution. As proposed by Richards et al. (2016), a mixture of tissue-specific and pro-vascular bioink should be printed with tenocytes and endothelial cells in exact spatial distribution. Wu et al. (2017) showed that the expression of tenogenic markers was upregulated when AT-MSCs, tenocytes, and HUVECS, were cultured together, illustrating that tenocytes also profit from providing vascularization.

Besides blood vessels, the endo- and epitenon contain nerves and lymphatic vessels to support the tendon cells in their function (Wang, 2006; Docheva et al., 2015; Tan et al., 2015; Schneider et al., 2018). During tendon inflammation, different neuronal mediators play an active role in regulating pain and the inflammation process (Ackermann, 2013; Tempfer and Traweger, 2015). However, the exact mechanisms are not yet elucidated. The incorporation of (induced) pluripotent stem cells capable of neural differentiation and MSCs or adult neural stem cells, in an *in vitro* model is of great importance (Maltman et al., 2011; Azari and Reynolds, 2016; Snedeker and Foolen, 2017), but is still in its infancy for both tendon and other types of tissue engineering.

Microdamage Leading to Failure

It is generally accepted that tendinopathy occurs due to chronic overuse. As a reaction to the matrix disruption, *in vivo* neovascularisation is observed. VEGF is highly secreted which results in increased matrix metalloproteinase secretion and further matrix degradation (Tempfer and Traweger, 2015). Petersen et al. (2004) demonstrated increased VEGF secretion in response to 1 Hz cyclic stretching and decreased expression if low frequency is used (0.5 Hz). As discussed above, by subjecting strain above physiological level, one should be able to create a tendinopathy model (Wang et al., 2003, 2013). The exact protocol should be defined as different responses will be observed depending on the (bio-)material of choice. Incorporation of inflammatory molecules and cells will represent the detrimental environment after the ideal mechanical protocol is defined.

CONCLUSION

After decades of research, it is clear that MSC-derived bioactive factors have great regenerative potential in healing tendon injuries. Yet, clinical application remains limited due to the unclarified pathogenesis of tendinopathy and MSCs' underlying mechanisms of action. The complexity of *in vitro* tendon engineering has been evolving the last decade, and substantial progress has been made in mimicking tendon physiology by switching from 2D tenocyte cultures to 3D jet writing. The appropriate biomaterials, bioreactor and production technology should be combined to create *in vitro* tendon (Govoni et al., 2016). However, currently used models are far from ideal, especially by the functional (neuro-)vascular supply which is still lacking (Richards et al., 2016). The major remark in all listed models is the lack of consistency. All research groups developed and used their own protocols, mono-materials and hybrids, making conclusions impossible and preventing scientific progress. Moreover, both natural and synthetic materials are used interchangeably, in different combinations, and in different proportions. A crucial step to success is identifying the optimal mix of materials, manufacturing techniques, and biological stimuli, to mimic representative tendon properties. To achieve this, a multidisciplinary approach is needed where experts in biotechnology, cell biology, molecular biology, material sciences, physics, physiology, bioengineering and polymer chemistry join forces (Siemionow, 2015; Aibibu et al., 2016).

We propose future models need to incorporate collagen or gelatin simulating the tendon ECM, either chemically modified or reinforced with a strong synthetic material, such as PCL, to mimic the cellular microenvironment (ECM, mechanical characteristics, . . .). As cell encapsulation is the most representative technique, resident cells (tenocytes, tendon stem cells, or MSCs), immune cells, growth factors and cytokines should be incorporated in a biomaterial-cell mixture for 3D-bioprinting. Applying mechanical stimulation in a stretch-perfusion bioreactor, once the appropriate strains have been identified, will enable to representatively mimic the tendon environment in both physiological and pathological conditions. In addition, vascularization should be present either by medium perfusion or by incorporating endothelial cells. A representative pathophysiological tendon model can be established by combining mild overstimulation for a longer period of time (e.g., 3 weeks), mimicking the chronic situation, and an acute extreme overloading and/or scratch, representing the acute injury. It is only a matter of time until tendon pathophysiology is unraveled as tendon tissue engineering strategies are rapidly evolving. Therefore, *in vitro* models can provide strategies to solve the MSC puzzle and evidence-based treatment protocols can be established which will quickly become available to tendinopathy patients, irrespective of the species.

AUTHOR CONTRIBUTIONS

MM performed the bibliographic research, drafted the manuscript, and created the figures. GV, SV, and CD revised the manuscript and supervised the process. CD finalized the manuscript. All authors contributed to the article and approved the submitted version.

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Mesangiogenic Progenitor Cells Are Tissue Specific and Cannot Be Isolated From Adipose Tissue or Umbilical Cord Blood

Serena Barachini[1], Marina Montali[1], Francesca M. Panvini[2], Vittoria Carnicelli[3], Gian Luca Gatti[4], Nicola Piolanti[5], Enrico Bonicoli[5], Michelangelo Scaglione[5], Gabriele Buda[1] and Paolo D. Parchi[5]*

[1] Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy, [2] Sant'Anna School of Advanced Studies, Institute of Life Sciences, Pisa, Italy, [3] Department of Surgical, Medical and Molecular Pathology and Critical Care Medicine, University of Pisa, Pisa, Italy, [4] Plastic and Reconstructive Surgery Unit, Azienda Ospedaliero-Universitaria Pisana, Pisa, Italy, [5] Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy

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***Correspondence:**
Serena Barachini
serena.barachini@med.unipi.it

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Mesangiogenic progenitor cells (MPCs) have been isolated from human bone marrow (BM) mononuclear cells. They attracted particular attention for the ability to differentiate into exponentially growing mesenchymal stromal cells while retaining endothelial differentiative potential. MPC power to couple mesengenesis and angiogenesis highlights their tissue regenerative potential and clinical value, with particular reference to musculoskeletal tissues regeneration. BM and adipose tissue represent the most promising adult multipotent cell sources for bone and cartilage repair, although discussion is still open on their respective profitability. Culture determinants, as well as tissues of origin, appeared to strongly affect the regenerative potential of cell preparations, making reliable methods for cell isolation and growth a prerequisite to obtain cell-based medicinal products. Our group had established a definite consistent protocol for MPC culture, and here, we present data showing MPCs to be tissue specific.

Keywords: MPCs, MSCs, bone marrow, adipose tissue, umbilical cord blood, tissue engineering, neovascularization

INTRODUCTION

Mesenchymal stromal cells (MSCs), first identified in bone marrow (BM) over 50 years ago (Friedenstein et al., 1968), are characterized by their differentiative potential, both *in vitro* and *in vivo* (Caplan, 1991; Pittenger et al., 1999). Subsequent investigation focused on MSC role in repairing and healing of skeletal tissues (Jethva et al., 2009; Xiao et al., 2010), whereas, further research sparkled interest in their therapeutic potential in the regeneration of a broad spectrum of injured organs (D'souza et al., 2015). However, harvesting of BM is considered an invasive and potentially painful procedure, which also exposes donors to site morbidity (Bain, 2003). Alternative sources for MSC-like cells were considered, leading to the evidence that they could be obtained from a wide range of adult tissues and their clinical potential was investigated (Brown et al., 2019). Adipose tissue (AT) being abundant, relatively easy to access, and usually collected from discarded material after cosmetic interventions showed a valuable supply of MSCs

(Zuk et al., 2001). Unlike BM, where MSCs represent a very rare population, AT can provide a high yield of cells with strong proliferative potential and therefore may be considered as a feasible source for cell therapy (Mushahary et al., 2018; Brown et al., 2019). The isolation of MSCs from AT is affected by donor's age, health, and site of collection. In search of more primitive MSCs, fetal and perinatal tissues, including human umbilical cord blood (UCB), were also investigated (Barachini et al., 2009; Ding et al., 2015; Bieback and Netsch, 2016) and the proliferative as well as differentiating potential of derived MSCs compared (Mushahary et al., 2018). Despite a considerable amount of studies on MSC biology and clinical application, decades of efforts moving from the benchtop to the bedside have brought no consolidated MSC-based therapy (Mastrolia et al., 2019). Small variations in the isolating and culturing procedures and reagents, such as centrifugation g-force, and basal media formulation, as well as serum quality and concentration, can significantly affect the yield and composition of the isolated MSC population (Brown et al., 2019). In addition, the heterogeneity of cell culture protocols hampers a definite assessment of *in vitro*, *in vivo*, and clinical results, thus impeding confirmation of the therapeutic potential of MSC-based treatments.

Pacini suggested that the heterogeneity of MSC preparations could be considered a consequence of the combined effects of stochastic fluctuations and deterministic variations, with apparently minimal modifications of culture determinants strongly affecting cell composition and regenerative potential of cell-based medicinal products (Pacini, 2014). As a consequence, the number of contradictory results, regarding efficacy of the MSC-based therapies, could be explained by the comparisons of data produced applying significantly different cell populations, erroneously grouped under the same acronym MSCs. For instance, in 2014, Pacini hypothesized that the co-isolation of the mesangiogenic progenitor cells (MPCs), described by our group in 2009, could be responsible for the controversial data regarding the genuine angiogenic potential of MSC cultures. Although these cells can be co-isolated with MSC culture, different protocols may determine a different yield of MPCs that has been demonstrated retaining higher angiogenic potential (Pacini and Petrini, 2014). MPCs have been identified in human BM mononuclear cell (BM-MNC) cultures using autologous sera as a supplement instead of standard fetal bovine serum (Petrini et al., 2009). High-purity-grade (> 95%) MPC cultures were obtained under selective culture conditions, including medium supplementation with 10% pooled human AB-type serum (PhABS) and no gas-treated hydrophobic plastics (Trombi et al., 2009; Montali et al., 2016a). MPCs attracted particular attention for their ability to efficiently differentiate into exponentially growing MSCs, activating the Wnt5/calmodulin signaling pathway (Fazzi et al., 2011). They also retained the ability to differentiate toward the endothelial lineage. More recently, we confirmed MPC genuine angiogenic potential both *in vitro* and *in vivo*, demonstrating the mesengenic and angiogenic potentials to be mutually exclusive (Montali et al., 2017). MPCs possess longer telomeres and express pluripotency-associated markers including Oct-4 and Nanog. In particular, nestin has been considered a marker for BM-derived MPCs (Pacini et al., 2010). Cell sorting experiments showed that a highly specific BM subpopulation, described as *Pop#8* and identified by the $CD64^{bright}CD31^{bright}CD14^{neg}CD45^{dim}$ phenotype, represents the only BM subpopulation able to generate MPCs in culture under selective conditions (Pacini et al., 2016). MPCs' ability to undergo dual lineage differentiation (mesengenesis vs. angiogenesis) underlines their great tissue regenerative potential and clinical value, especially in musculoskeletal tissues regeneration (Giannotti et al., 2013; Savelli et al., 2018).

With the aim of extending the range of tissue sources for MPCs herein we evaluated the efficacy of our MPC isolation and culture protocol using three candidate tissues, including BM, human stromal vascular fraction (SVF), and UCB.

MATERIALS AND METHODS

Cell Isolation and Culture From Human BM

BM aspirates were obtained from 32 patients (16M/16F, median age = 68 years, age range = 52–85 years) undergoing orthopedic surgery for hip replacement. A 20-mL syringe containing 500 IU of heparin was used to aspirate 10 mL of BM immediately after femoral neck osteotomy during femoral reaming; the samples were collected instead of being discarded as usual, without any alteration of the standard surgical procedures. BM-MNCs were isolated and expanded as previously published. In particular, we applied the exact protocol described in 2009 (Trombi et al., 2009), validated in 2016 (Montali et al., 2016a), and described below.

Fresh BM samples were diluted 1:4 in Dulbecco's modified phosphate-buffered saline (D-PBS; Thermo Fisher Scientific, Waltham, MA, United States) and gently layered on Ficoll-Paque™ PREMIUM (GE Healthcare, Uppsala, Sweden). Samples were centrifuged at 400 g for 25 min and MNCs harvested at the interface, filtered on 70-μm filters, and washed twice in D-PBS. Cells were plated at $8 \times 10^5/cm^2$ in hydrophobic T-75 flasks (GreinerBio-One, Kremsmünster, Austria) and cultured in low-glucose Dulbecco modified Eagle medium (DMEM; Thermo Fisher Scientific) supplemented with 10% pooled human AB type serum (PhABS), 2 mM Glutamax® (Thermo Fisher Scientific), and 100 μg/mL gentamicin (Thermo Fisher Scientific). PhABS batch was purchased from Lonza (Basel, Switzerland) and manufactured by the "off-the-clot" method from male sera only. The batch has been previously evaluated for its performance in MPC isolation from BM-MNCs. Validation criteria have been previously reported in Montali et al. (2016b). Culture medium was changed every 48 h. After 5–6 days, plates were morphologically screened for MPCs using an inverted microscope, cells detached by TrypLE Select® (Thermo Fisher Scientific) digestion and washed in D-PBS.

Cell Isolation and Culture From Human UCB

Donors undergoing delivery were recruited in the study. Samples were harvested from normal term pregnancies ($n = 26$) between

37 and 42 weeks of gestation, both after vaginal or cesarean section delivery. The umbilical blood was allowed to flow into heparinized tubes (5,000 IU/mL) and processed within 12 h. Samples were then diluted with D-PBS (Thermo Fisher Scientific) and MNCs collected by density gradient centrifugation using Ficoll-Paque™ PREMIUM (GE Healthcare) and cultured as described above applying the protocol validated for BM-MNCs and the same PhABS batch described above.

Cell Isolation and Culture From Human SVF

Adipose tissue was collected from patients undergoing cosmetic liposuction ($n = 7$), three from the abdominal area and four from the buttocks. In brief, 250-mL samples of liposuctioned material were extensively washed with equal volumes of D-PBS to remove erythrocytes and centrifuged for 5 min at 600 g to separate fat from oil and liquid phases. After washing, fat was combined vol/vol with 125 CDU/mL type IV collagenase (Thermo Fisher Scientific) and incubated for 1 h at 37°C in a shaking water bath. Samples were then filtered through a 100-μm filter and SVF harvested by centrifugation at 600*g* for 10 min. The resulting pellet was resuspended, and MNCs isolated and cultured under the MPC selective conditions, validated for BM-MNCs and described above taking care of applying the same PhABS batch.

After cell harvesting, cell yields have been calculated dividing absolute number of freshly detached cells by number of seeded cells, recorded as percentage (yield %) and reported as mean values ± SEM. Non-parametric Wilcoxon test for unmatched pairs was performed applying the GraphPad Prism® software (GraphPad Software, San Diego, CA, United States).

Flow Cytometry

MNCs from the three above sources (150,000 cells per sample) were incubated with REAfinity® anti-human CD64 (clone REA978) fluorescein isothiocyanate–conjugated, CD31 (clone REA730) PE/Cy7-conjugated, CD14 (clone REA599) VioGreen® -conjugated, and CD45 (clone REA747) VioBlue® -conjugated antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30' at 4°C in the dark, and washed twice in MACS Quant® Running Buffer (Miltenyi Biotec). Data were acquired using MACS Quant® flow cytometer and analyzed by MACS Quantify® Analysis Software (Miltenyi Biotec).

Flow cytometry of freshly detached cells from primary cultures was performed as described above using antihuman CD90 (clone DG3) FITC-conjugated, CD73 (clone AD2) PE-conjugated, CD31 PE/Cy7-conjugated, CD14 VioGreen® -conjugated, and CD45 VioBlue® -conjugated antibodies (Miltenyi Biotec).

Frequencies of cell populations were calculated on total events, after exclusion of cell debris on FSC vs. SSC density plots and doublets on FSC-A vs. FSC-H. Non-parametric Wilcoxon test for unmatched pairs was performed applying GraphPad Prism® software (GraphPad Software, San Diego, CA, United States).

Characterization of Cells From Primary Cultures

Cell characterization was performed according to the MPC identification protocol (Montali et al., 2016a).

Mesengenic Differentiation

Freshly detached cells from primary cultures were replated at 20,000 cells/cm^2 and let adhere in DMEM/10% PhABS for 24 h. Culture medium was then replaced with StemMACS® MSC Expansion Media XF (Miltenyi Biotec), and cells cultured up to 80% of confluence (usually 7–8 days) to obtain P1-MSCs. Cultures were then incubated for further 7–8 days to complete mesengenic differentiation (P2-MSCs). Cell osteogenic and adipogenic potential was tested. P2-MSCs were replated at 20,000 cells/cm^2 in TC-treated 6-wells plates and grown to confluence. Medium was then replaced with either StemMACS® OsteoDiff Media, StemMACS® AdipoDiff Media, or expansion medium (negative controls). Two to 3 weeks later, calcium deposits were revealed by staining with alizarin S (Sigma Aldrich) and lipid droplets revealed by staining with Nile red 200 nM (Thermo Fisher Scientific), according to manufacturer's. Imaging was performed on inverted fluorescence DM IRB Leica microscope (Leica, Wetzlar, Germany), equipped with LAS image acquisition software (Leica).

Sprouting Angiogenesis Assay

We generated a minimum of two spheroids per sample by the hanging drop method (1.5×10^4 cells/spheroid). Spheroids were let to sprout out on Geltrex® LDEV-free reduced growth factor basement membrane matrix (Thermo Fisher Scientific) in EGM-2 endothelial growth medium (Lonza). Spheroids were checked and imaged at 24 h and 7 days of culture, on inverted fluorescence DM IRB Leica microscope (Leica, Wetzlar, Germany), equipped with LAS image acquisition software (Leica). Quantification of sprouting distance was assessed independently by three examiners (S.B., M.M., and F.M.P.) using QWin® Image Analysis software (Leica); values were reported as mean values ± SEM and two-tailed unpaired *t* test was performed.

Nestin Detection and F-Actin Organization Analysis

Primary cultures were grown in 2-well Lab-Tek® Chamber slides. Cells were then fixed in 4% paraformaldehyde and permeabilized in 0.05% Triton X-100 for 30 min. Slides were incubated with mouse monoclonal antibody against nestin (1:150, clone 10C2, Abcam, Cambridge, United Kingdom) and after extensive washing nestin was revealed by AlexaFluor® 488 Goat Anti-Mouse SFX Kit (Thermo Fisher Scientific), according to manufacturer's. Slides were then stained with phalloidin AlexaFluor® 555-conjugated antibody (Thermo Fisher Scientific) for 30 min to reveal F-actin organization. Nuclei were detected by ProLong® Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific).

Gene Expression Profile of Cells From Primary Cultures

Gene expression analysis was performed on cells from five primary cultures for each of the three different tissue sources. Custom 96-well PrimePCR® Plates (BioRad, Hercules, CA, United States) including primer sets for 87 target genes, 5 reference genes (**Supplementary Table 1**), and 5 internal controls were used for gene expression profiling of P1-MSCs. Total RNAs were purified from freshly detached cells using Direct-zol RNA MicroPrep Kit (Zymo Research, Irvine, CA,

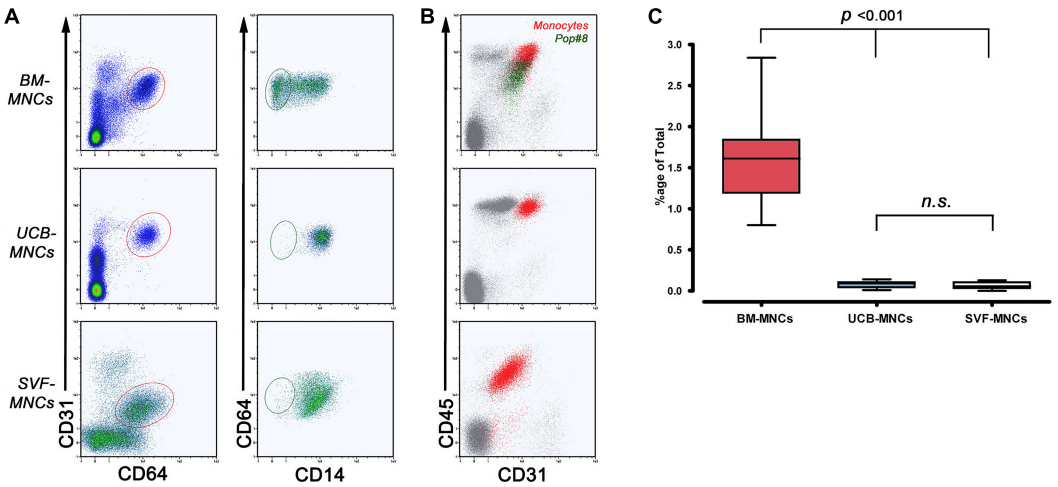

FIGURE 1 | Flow cytometry quantification of MPC in vivo progenitors (Pop#8). **(A)** To quantify Pop#8 population in the three different tissue sources, the specific gating strategy has been applied. In details, $CD64^{bright}CD31^{bright}$ events (elliptical region in red) were displayed on CD64 vs. CD14 density plots in order to quantify the $CD64^{bright}CD31^{bright}CD14^{neg}$ population (elliptical region in dark green), representing the genuine in vivo progenitor of the MPCs (Pop#8). This population was consistently detected in BM-MNCs only. **(B)** CD45 vs. CD31 dot plots confirmed the characteristic CD45 dim expression on Pop#8 (dark green dots) in contrast to the bright expression on $CD64^{bright}CD31^{bright}CD14^{+}$ monocytes (red dots). **(C)** Mean percentage of Pop#8 in BM-MNCs resulted in approximately 1.5% of the total. n.s., not significant.

United States) and quantified with Qubit 4 Fluorometer (Thermo Fisher Scientific) by Qubit RNA HS Assay Kit (Thermo Fisher Scientific). cDNAs were synthesized from 1 μg of total RNA using iScript gDNA Clear cDNA Synthesis Kit, according to manufacturers. Quantitative polymerase chain reaction (qPCR) was carried out with SsoAdvanced Unversal SybrGreen Supermix (BioRad), on iQ5 Real-Time PCR Detection System (BioRad), according to PrimePCR Array® instruction manual. Fold changes calculation by $\Delta\Delta C_t$ method and statistical analysis were assessed by PrimePCR® Analysis software (BioRad). According to the manufacturer, the p values reported on the results table are the result of unpaired t tests comparing the distributions of per well normalized expression (NE) values for the control sample (BM-MNCs) versus the test sample (UCB-MNCs). C_t values higher than 35, were considered as "no expression." After the analysis of the relative stability, two reference genes (*B2M*, *GAPDH*) were validated for normalization.

RESULTS

Flow Cytometry Quantification of MPC *in vivo* Progenitors (Pop#8)

We used multicolor flow cytometry to identify and quantify *Pop#8* MPC *in vivo* progenitors in freshly isolated MNCs from BM-MNCs, UCB-MNCs, and SVF-MNCs. The *Pop#8* immunophenotype was previously described as $CD64^{bright}CD31^{bright}CD14^{neg}CD45^{dim}$ (**Figures 1A,B**; Pacini et al., 2016). A $CD64^{bright}CD31^{bright}$ subpopulation was clearly detectable in both BM- and UCB-MNCs while SVF-MNCs expressed lower levels of CD31. However, we identified the genuine *Pop#8* immunophenotype defined as $CD14^{neg}CD45^{dim}$ in BM-MNCs only and quantification revealed consistent to previous results (1.60% ± 0.12%, **Figure 1C** and **Supplementary Table 2**). In UCB-MNCs almost the entire $CD64^{bright}CD31^{bright}$ population was represented by CD14-positive mature monocytes (red dots in **Figure 1B**). In SVF-MNCs the CD64/CD31-positive population expressed CD14 and CD45 although at lower intensities. Differences in expression could be ascribed to SVF-MNC different isolating procedure.

Morphology, Immunophenotype, and Yield of Cells From Primary Cultures Under MPC Selective Conditions

After 5–6 days of culture under MPC selective conditions, BM-MNCs generated rounded, highly refringent, firmly attached cells. Their high side scatter (SSC) signal and $CD14^{neg}CD45^{dim}CD31^{+}$ phenotype, lacking MSC-related antigens CD90 and CD73, allowed us to identify them as MPCs. UCB-MNC cultures generated fewer larger cells that, despite the MPC-like morphology, were identified as macrophages because of their $CD14^{+}CD45^{bright}CD31^{+}$ phenotype. The spindle-shaped morphology and $CD90^{+}CD73^{+}$ phenotype of SVF-MNC–derived cells were reminiscent of standard AT-MSCs (**Figure 2A**). Very rare $CD14^{+}CD31^{+}CD45^{+}$ rounded cells were also detected (**Figure 2A**, red arrowheads). MPC yield from BM-MNCs was consistent with previous data (0.97% ± 0.12%), while yield from UCB-MNCs was slightly lower (0.63% ± 0.13%). Significantly higher yield was evidenced from SVF-MNC cultures (3.02% ± 0.38%, $p < 0.001$) probably due to the proliferating nature of the MSC-like cells (**Figure 2B**), which represent more than 95% of cell population. Consistent with previous reports,

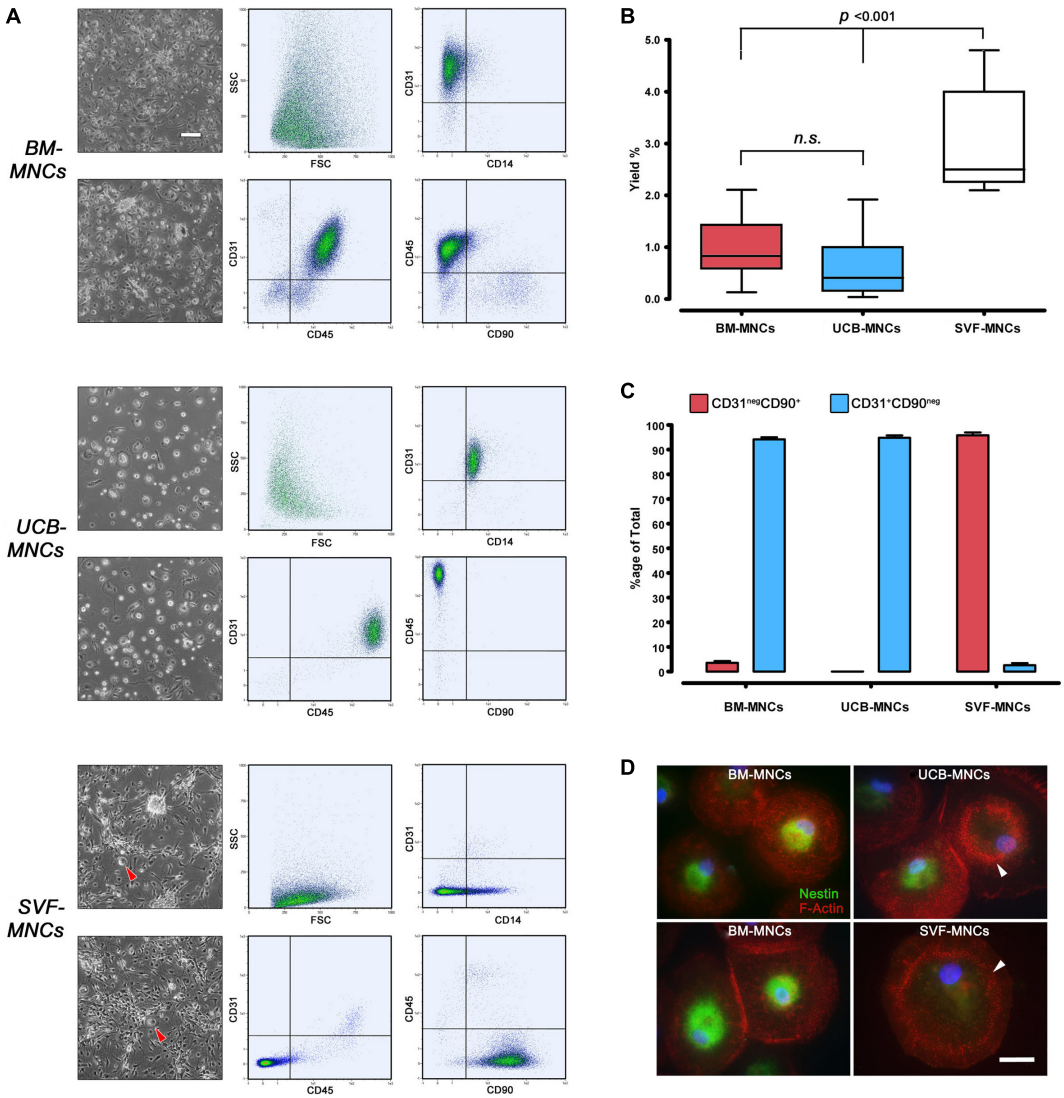

FIGURE 2 | Primary culture under MPC selective culture conditions. **(A)** After a week of culture in DMEM/10% PhABS on hydrophobic plastics, adherent cells from BM- and UCB-MNCs showed similar rounded refringent morphology. However, cells from UCB-MNCs appeared larger and flattened, with very rare interspersed polar elongated cells frequently detected in BM-MNC cultures. SVF-MNC cultures resulted in an almost confluent layer of fibroblastoid MSC-like cells with sporadic rounded refringent cells (red arrowheads). $CD31^{+}CD45^{dim}CD14^{neg}CD90^{neg}$ phenotype of BM-MNC culture generated cells was distinctive of MPCs at a difference with the macrophagic $CD14^{+}CD45^{bright}CD31^{+}$phenotype displayed by most UCB-derived cells. **(B)** Cell recovery was significantly higher from SVF-MNCs possibly due to the expansion of proliferating cells similar to MSCs as demonstrated by their $CD31^{neg}CD90^{+}$ phenotype (**C**, red bars). A very small population of MSC-like cells was detected also in BM-derived cultures. **(D)** Nestin (green) was found in the vast majority of cells from BM-MNCs, showing dispersed podosomes (red). Most cells from UCB-MNCs were nestin-negative and characterized by a "belt" distribution of podosomes, similarly to the rare rounded cells detected in SVF cultures. n.s., not significant.

a small population of MSC-like cells (3.51% ± 0.78%) was also detected in BM-MNC cultures, at a difference with UCB-MNCs (**Figure 2C**).

Most cells from BM-MNC primary cultures expressed nestin and showed dispersed podosome-like structures as revealed by F-actin dotted pattern of expression, characteristic of MPC phenotype (Pacini et al., 2013). A significant number of nestin-negative cells, showing "belt" distribution of podosomes, were detected in UCB-MNC cultures. The rare rounded cells co-isolated in SVF-MNC cultures were all nestin-negative and showed the "belt" podosome pattern (**Figure 2D**, white arrowheads).

Differentiation Potential of Cells From Primary Cultures

We analyzed the mesengenic potential of cells from primary cultures by the two step protocol previously described (Fazzi et al., 2011). We were able to obtain P2-MSCs from BM- and SVF-MNC primary cultures, whereas, cells isolated from UCB-MNCs failed to differentiate. They kept their round

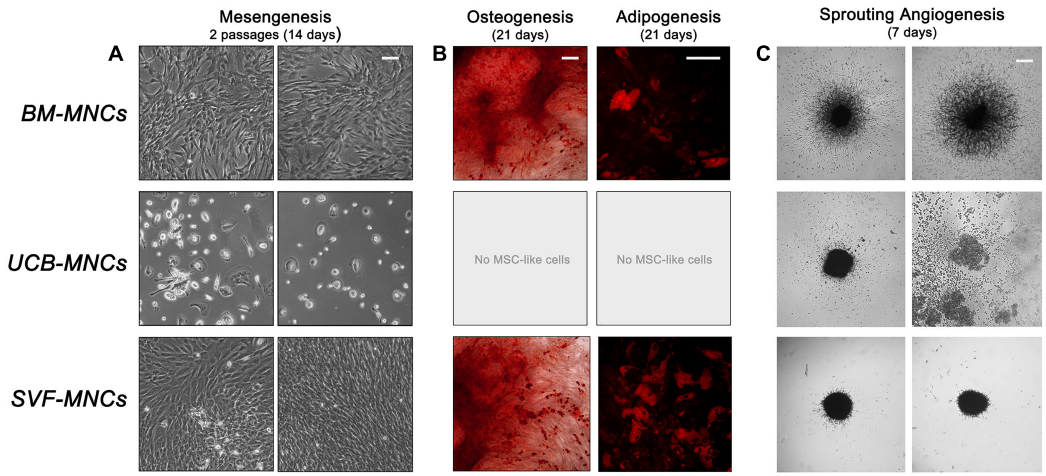

FIGURE 3 | Mesangiogenic potential of cells from primary cultures. **(A)** After 2 weeks of mesengenic induction the rounded refringent MPCs from BM-MNCs differentiated into proliferating fibroblastoid MSCs. Conversely, cells from UCB-MNCs maintained their morphology with no sign of differentiation. Spindle-shaped cells from SVF-MNC primary cultures could be expanded with unaltered morphology. **(B)** Terminal osteogenic or adipogenic differentiation confirmed the MSC-like nature of the cells from BM and SVF after mesengenic induction. **(C)** Only BM-derived MPCs showed a consistent sprouting activity under angiogenic stimulus, confirming their mesangiogenic potential.

morphology and did not proliferate at all, notwithstanding the 14-day culture in differentiating conditions (**Figure 3A**). The MSC nature of BM- and SVF-derived P2-MSCs was definitely demonstrated by their terminal differentiation into either osteoblasts or adipocytes. After further 3 weeks of culture under osteogenic or adipogenic induction, extracellular calcium deposits, and intracellular lipid droplet accumulation were revealed by alizarin S and Nile red stains, respectively (**Figure 3B**).

Sprouting angiogenesis assay revealed that only MPCs from BM-MNCs retained angiogenic potential with more than 300 μm sprouting from 3D spheroids (325.1 ± 29.9 μm). Cells from UCB-MNCs gave origin to few loose cell aggregates, which lacked the mechanical properties required for handling. As a consequence, the spheroids disaggregated during seeding, and no sign of ECM degradation was reported. Compact spheroids were obtained from AT-derived cells without evidence of significant sprouting activity (27.8 ± 9.1 μm, $p < 0.0001$), under vascular endothelial growth factor (VEGF) stimulus (**Figure 3C**).

Gene Expression Profile of Cells From Primary Cultures

Unsupervised cluster expression analysis of 87 target genes in cells from primary cultures revealed three main clusters (gene clusters A–C in **Figure 4A**). Cluster A included a number of angiogenesis- and lymphoangiogenesis-associated genes (*FLT4*, *LYVE1*, *DLL4*, *KDR*, *VWF*, and *EMCN)* as well as pericyte markers *(RGS5* and *MCAM)*. Cluster B included MSC-related genes (*DES*, *DKK1*, *NT5E*, *SOX9*, *EGFR*, and *PDGFR*), while most genes in cluster C were associated to MPCs (*SPP1*, *ITGB2*, *SOX15*, and *FBX15*, in particular). Cells derived from BM- and UCB-MNC cultures showed increased expression of gene cluster C and reduced expression of gene cluster B. Conversely, gene expression profile of SVF-MNCs was characterized by up-regulation of cluster B and down-regulation of cluster C. Comparison of single-gene expression between BM- and UCB-MNCs revealed substantially lower levels of some genes of interest, in the latter. In particular, *MCAM* reduction was approximately 50-fold (0.0394 ± 0.0117 vs. 2.0903 ± 1.8011, $n = 5$), *WNT5B* approximately 30-fold (0.0306 ± 0.0348 vs. 0.9842 ± 1.0254, $n = 5$), *TEK* almost 50-fold (0.0069 ± 0.00743 vs. 0.3461 ± 0.2615, $n = 5$), and *SIGLEC1* close to 10-fold (0.0943 ± 0.0301 vs. 0.7012 ± 0.2243, $n = 5$, **Figure 4B**). Drastic reduction in the expression of EGF (-1,486.4), PDGF (-253.6), FGF-2 (-91.2), VEGF (-30.9) receptor genes, and *CXCL12* (-200.6) was also detected in UCB-MNCs, although data were too variable for statistical significance.

DISCUSSION

MSCs and their *in vivo* ancestors hold great promise for the treatment of bone and cartilage defects (Lin et al., 2017) as shown by their ability to enhance bone repair in a wide range of animal model systems (Pacini et al., 2007; Jafarian et al., 2008; De Schauwer et al., 2013). To date, BM-MNCs and AT-derived SVF are still the main sources of adult multipotent cells for autologous cell–based therapies (Hoogduijn and Dor, 2013; Shariatzadeh et al., 2019). Both BM- and AT-MSCs have been used to repair various bone defects (Marcacci et al., 2007; Mesimaki et al., 2009; Gimble et al., 2010; Lindroos et al., 2011). Regeneration of articular cartilage has been achieved by applying both BM- and AT-MSC in models of osteochondral defect (Ishihara et al., 2014; Murata et al., 2015; Itokazu et al., 2016; Li et al., 2018).

Bone marrow- and AT-MSCs share a number of features, including morphology and cell surface markers. However, significant biological differences have been found in their

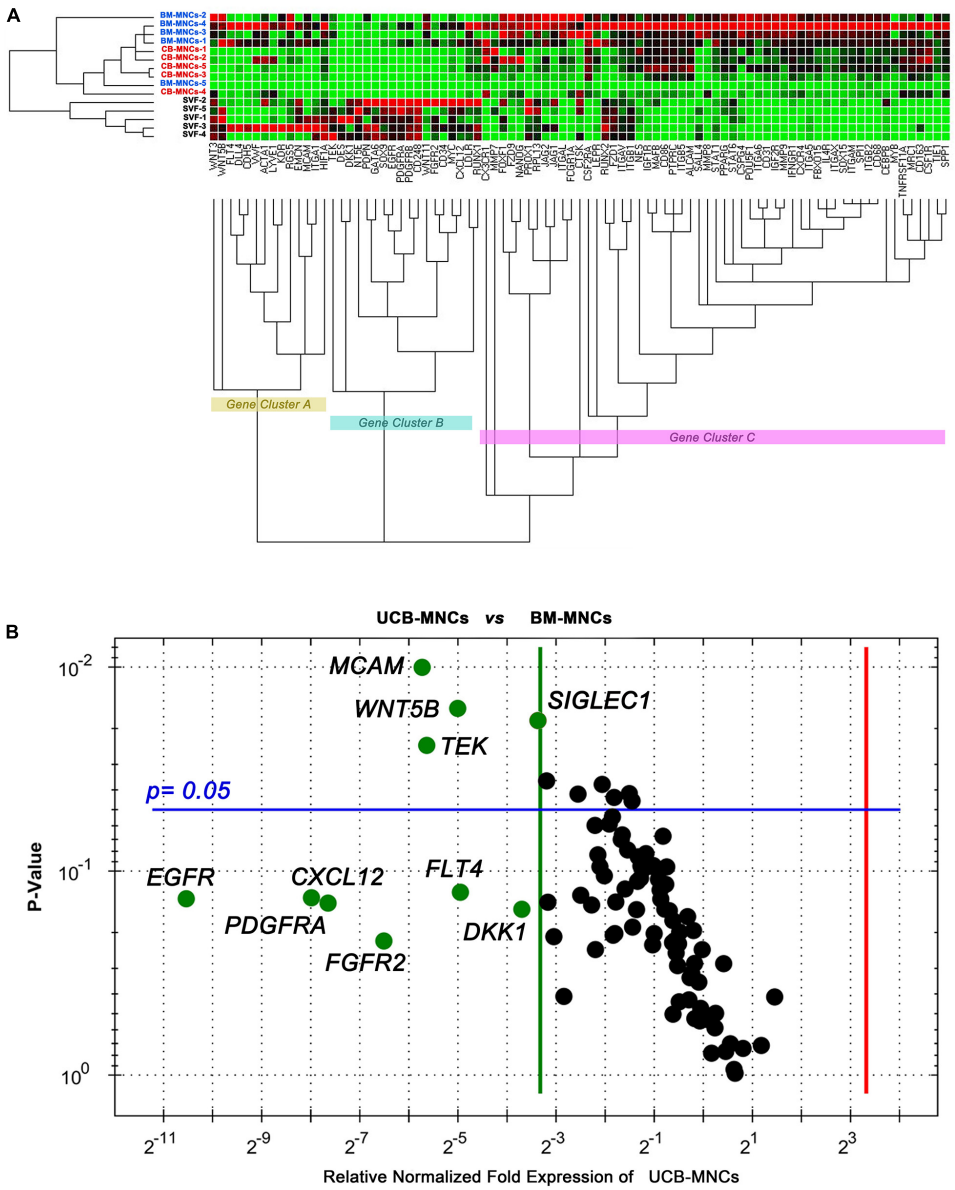

FIGURE 4 | Gene expression profiling of cells from primary cultures. **(A)** BM- and UCB-derived cells showed parallel expression profiles for the 87 genes analyzed. Angiogenesis/lymphangiogenesis (gene cluster A) and MPC-related genes (gene cluster C) were significantly up-regulated in these cells, whereas, MSC-related genes (cluster B) resulted up-regulated in SVF-derived cells. **(B)** Single-gene expression analysis revealed significant lower expression of MCAM, WNT5B, TEK, and SIGLEC1 in UCB-derived cells as compared to BM-derived cells. Relevant reduction of stromal growth factor receptor gene expression was also detected.

proliferation/differentiation properties (Danisovic et al., 2009), and the discussion on their respective regenerative potential is still open (Huang et al., 2005; Elman et al., 2014; Rasmussen et al., 2014). Despite remarkable improvements in isolation, expansion, and characterization of adult multipotent cells, clinical and preclinical trials often showed disappointing outcomes with lack of efficacy in long-lasting consolidated repair (Mastrolia et al., 2019; Shariatzadeh et al., 2019). A primary reason of such unsatisfactory results could be lack of or inefficient vascularization in newly formed tissues (Chung and Shum-Tim, 2012).

Nonetheless, BM-MSCs still represent the most applied cells for the engineering of cell-based medicinal products (CBMPs) (Mastrolia et al., 2019), with a number of preclinical studies showing BM-derived cells to be more effective in the regeneration and repair of skeletal tissues than alternative sources (Brennan et al., 2017). AT-MSCs demonstrated inferior *in vivo* osteogensis and superior angiogenesis as compared to BM stromal cells (Brennan et al., 2017), casting doubts on AT-MSC use in bone repair because of their limited osteogenic differentiation potential. In the present study we showed AT-MSCs not to possess intrinsic vasculogenic potential, corroborating the idea

that their contribution to new vessel formation would be exerted exclusively by the secretion of specific angiogenic factors. Thus, vascularization of AT-MSC engineered implants strictly depends on perfusion of the surrounding microenvirorment. This represents a further limiting factor in regenerating naturally low vascularized tissues, as bone and cartilage, or compromised injured sites as non-union fractures.

Our results demonstrated that MPCs are tissue specific and, in accordance with what previously reported (Montali et al., 2016a, 2017), $CD64^{bright}CD31^{bright}CD14^{neg}CD45^{dim}$ *Pop#8* MPC progenitors were consistently detected exclusively in BM-MNCs leading to the isolation of MPCs under selective culture conditions. Extended *Pop#8* characterization revealed CD45 to be mildly expressed while most of the antigens feasible for prospective isolation of MSCs from BM remained unexpressed (Pacini et al., 2016). In particular, the lack of both CD146 and CD271 expression suggests that *Pop#8* should be considered distinct from the $CD146^{bright}$ pericytes found in the subendothelial layer of sinusoids (Sacchetti et al., 2007), from the trabecular bone-lining $CD271^{+}CD146^{neg}$ cell population (Tormin et al., 2011) and from the stromal reticular cells as well (Omatsu et al., 2010), all of them described as *in vivo* MSC progenitors in the BM, sustaining the idea of a multiple origin of MSCs. Interestingly, similar $CD146^{bright}$ perivascular cell population has been found in SVF from the AT (Crisan et al., 2008; Corselli et al., 2012) suggesting that BM and AT could share a common perivascular progenitor for the MSCs, whereas, *Pop#8* is exclusively detected in BM and at significantly higher frequency respect to pericytes.

Here, we hypothesize that BM concentrates and BM-MSC superior performances in skeletal tissue regeneration, could be explained by the presence of MPCs and/or *Pop#8* progenitors. Their essential chondrogenic and osteogenic potential would couple with their capability to trigger new blood vessel formation in implant early phases. Interestingly, specific endothelial cells were found in tight relation with chondrocytes and osteogeoprogenitors in the growth plate of long developing bones (Kusumbe et al., 2014). According to the "developmental engineering" paradigm (Lenas et al., 2009), vascularization is vital to bone tissue regeneration, and conception of new CBMPs should take it into consideration. Researchers and clinical community rely on the increasing knowledge of angiogenic and vasculogenic processes stimulating a clinically relevant vascular network formation within the implanted engineered constructs. In this view, clinical application of MPC-based CBMPs could take advantage from the unique features of these adult multipotent cells. MPCs are found at frequencies from one to two logs higher than other BM-MSC progenitors and vast numbers could be readily isolated in 4–6 days from 10 to 15 mL of fresh BM using a cheap GMP-compliant culture method (Montali et al., 2016a). The lack of requirement for *in vitro* cell expansion minimizes culture times and carries significant advantages in terms of reduced risk of cell transformation, cellular senescence, and exposition to bacterial and viral contamination. Moreover, the application of undifferentiated MPCs could also provide beneficial effects on producing functional long-lasting healing of target tissues.

DATA AVAILABILITY STATEMENT

All relevant data is contained within the article. The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author. Moreover, datasets are available on request and the raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of *Azienda Ospedaliero-Universitaria Pisana – Comitato Etico di Area Vasta Nord Ovest* (CEAVNO) (committee approval number: 48812/07). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SB and MM were responsible for the conception and design, acquisition, analysis, interpretation of data, and drafting the article. FMP was responsible for acquisition and analysis of data. VC was responsible for qPCR data acquisition. GLG was responsible for adipose tissue sample collection. NP and EB were responsible for bone marrow sample collection. MS and GB have critically revised the manuscript. PP was responsible for bone marrow sample collection and approved the final version to be published. All authors read and approved the final manuscript.

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REVIEW
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Mesenchymal Stem Cells in Treatment of Spinal Cord Injury and Amyotrophic Lateral Sclerosis

Eva Sykova[1], Dasa Cizkova[1,2]* and Sarka Kubinova[3]*

[1] Institute of Neuroimmunology, Slovak Academy of Sciences, Bratislava, Slovakia, [2] Centre for Experimental and Clinical Regenerative Medicine, University of Veterinary Medicine and Pharmacy in Kosice, Kosice, Slovakia, [3] Department of Optical and Biophysical Systems, Institute of Physics of the Czech Academy of Sciences, Prague, Czechia

Preclinical and clinical studies with various stem cells, their secretomes, and extracellular vesicles (EVs) indicate their use as a promising strategy for the treatment of various diseases and tissue defects, including neurodegenerative diseases such as spinal cord injury (SCI) and amyotrophic lateral sclerosis (ALS). Autologous and allogenic mesenchymal stem cells (MSCs) are so far the best candidates for use in regenerative medicine. Here we review the effects of the implantation of MSCs (progenitors of mesodermal origin) in animal models of SCI and ALS and in clinical studies. MSCs possess multilineage differentiation potential and are easily expandable *in vitro*. These cells, obtained from bone marrow (BM), adipose tissue, Wharton jelly, or even other tissues, have immunomodulatory and paracrine potential, releasing a number of cytokines and factors which inhibit the proliferation of T cells, B cells, and natural killer cells and modify dendritic cell activity. They are hypoimmunogenic, migrate toward lesion sites, induce better regeneration, preserve perineuronal nets, and stimulate neural plasticity. There is a wide use of MSC systemic application or MSCs seeded on scaffolds and tissue bridges made from various synthetic and natural biomaterials, including human decellularized extracellular matrix (ECM) or nanofibers. The positive effects of MSC implantation have been recorded in animals with SCI lesions and ALS. Moreover, promising effects of autologous as well as allogenic MSCs for the treatment of SCI and ALS were demonstrated in recent clinical studies.

Keywords: mesenchymal stem cells, cell therapy, spinal cord injury, amyotrophic lateral sclerosis, neurodegenerative diseases, conditioned medium, exosomes, biomaterials

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Laura N. Borodinsky,
University of California, Davis, United States
Stavros Malas,
University of Nicosia, Cyprus

****Correspondence:***
Eva Sykova
sykovae@gmail.com
Dasa Cizkova
cizkova.dasa@gmail.com

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INTRODUCTION

Adult stem cells and their secretomes play an important role in physiological conditions and in pathological states throughout our lives. Among adult stem cells, mesenchymal stem cells (MSCs) (Caplan, 1991) (progenitors of mesodermal origin) are of particular interest since they can be easily isolated from the bone marrow (BM), umbilical cord blood, umbilical Wharton jelly, and placenta. They can be separated, expanded *in vitro*, and implanted from autologous or allogenic sources. For these reasons, implantation of expanded MSCs or isolated secretomes or concentrated conditioned media from MSC cultivation can be used for treatment in animal models of degenerative diseases as well as in human clinical studies. Their beneficial effects on disease time course, accompanying

symptoms, and life expectancy have been shown in neurodegenerative diseases such as Alzheimer's disease (Nakano et al., 2020), spinal cord injury (SCI) (Pego et al., 2012; Liau et al., 2020), and amyotrophic lateral sclerosis (ALS) (Forostyak and Sykova, 2017; Barczewska et al., 2020).

In the last 10 years, the mechanism of MSC action has been gradually clarified. According to the International Society for Cellular Therapy position statement, MSCs are defined as cells which (1) adhere to plastic in culture conditions, (2) express CD105, CD73, and CD90 but not CD45, CD34, CD14, CD11b, CD79alpha, CD19, and HLA-DR surface molecules, (3) are able to differentiate *in vitro* into osteoblasts, adipocytes, and chondroblasts [see Dominici et al. (2006)]. Using specific procedures, MSCs have also been reported as being able to differentiate into neural cells and to express neuronal markers (Mezey et al., 2000b; Tropel et al., 2006; Park et al., 2012; Taran et al., 2014; Ullah et al., 2015). Although MSCs have the ability to differentiate into a variety of tissues, most of their effects are attributed to their paracrine action. MSCs produce and release a variety of biomolecules and soluble factors called secretomes. They are released from cells in the form of extracellular vesicles (EVs), i.e., lipid bilayer particles, which include microvesicles, apoptotic bodies, and exosomes. The proteomic analysis of MSC secretomes derived from the BM, adipose tissue, and fetal tissue has revealed trophic factors and cytokines as growth factors, immunomodulators, and antioxidants (Shin et al., 2021). Important growth factors are vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF-1), platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF). Anti-inflammatory factors such as interleukin 10 (IL-10) or transforming growth factor β1 (TGF-β1) have been shown to be involved in tissue repair and regeneration (Discher et al., 2009). MSC paracrine factors, therefore, have different functions, e.g., protecting against fibrosis, apoptosis, and oxidative damage, promoting angiogenesis, and conducting immunomodulatory and neuroprotective action.

Depending on the cellular microenvironment, MSCs can secrete neuroprotective growth factors in neural tissue, which can protect neurons and glial cells. Thus, secreted nerve growth factors like glia-derived nerve growth factor (GDNF), brain-derived growth factor (BDNF), VEGF, IGF-1, nerve growth factor (NGF), ciliary neurotrophic growth factor (CNTF), and neurotropin-3 (NT-3) promote different aspects of neural regeneration. Neurodegenerative diseases and traumatic brain or SCI caused by various pathologies and accidents often lead to permanent disabilities or death. MSCs and their secretomes are, therefore, accessible therapeutic tools for regenerative medicine, also including central nervous system (CNS) pathologies.

Damaged neural tissue is typically accompanied by cavitation induced by neural cell death, axonal degeneration, and tissue necrosis. This inevitably leads to scar formation, composed of inflammatory immune cells, fibroblasts, extracellular matrix (ECM) deposits, and astrocytes. MSCs have been shown to be anti-inflammatory, anti-apoptotic, and ECM modulatory (Marconi et al., 2012). Nevertheless, in chronic disease states, cavities and scars are impermeable barriers for tissue regeneration. For these reasons, tissue engineering and scaffold development often accompany cell therapies. Natural and synthetic scaffolds are developed to bridge the tissue defects. Moreover, various carriers for stem cell delivery and protection can enhance their effects.

In this review, we shall give examples of preclinical and clinical studies in SCI and motoneuronal disease or ALS (MND/ALS), focusing on the regenerative potential of MSCs, modulation of scar formation, ECM composition, and plasticity in CNS. We shall review recent development in scaffolds and tissue bridges. We are, of course, aware that MSC treatment can also be a useful strategy in the treatment of other neurodegenerative pathologies such as Alzheimer's disease, stroke, or brain trauma.

SPINAL CORD INJURY

Injury of the spine is a life-threatening neurodegenerative disorder leading to partial or complete loss of motor, sensory, and autonomic function below the injury. Spinal cord mechanical insult results in initial primary injury, when the spinal cord undergoes disruption caused by contusion, compression, transection, or stretching of the spinal column (Rosenzweig and McDonald, 2004; Ramer et al., 2005; Wang and Pearse, 2015). Secondary processes develop within minutes of the mechanical insult, manifested in subsequent progressive hemorrhaging, edema, thrombosis, ionic changes, ischemia, release of free radicals, lipid peroxidation, excitotoxicity, and apoptotic and necrotic cell death. All these together contribute to uncontrolled inflammation and immune response. It is necessary to understand that ongoing secondary mechanisms negatively affect the cells which survive within the primary injury site as well as those in the surrounding tissue, leading to enlargement of the lesion into adjacent spinal cord segments in rostro-caudal directions (Ramer et al., 2005). Moreover, progressing axonal degeneration together with tissue necrosis and cavity and scar formation ultimately preclude functional recovery (Silver and Miller, 2004; Forostyak et al., 2014; Bradbury and Burnside, 2019).

In this context, the diversity of secondary processes and the complexity of the spinal cord cyto-architecture together with its limited regenerative capacity in mammals are a major obstacle for finding an effective therapy (Sharif-Alhoseini et al., 2017). Better understanding of SCI pathophysiology, including genomic and proteomic profiles, may therefore provide opportunities for minimizing secondary pathological processes in adjacent healthy tissue (Ramer et al., 2005; Devaux et al., 2016).

Conventional and Experimental Treatment

Patients with an injured spinal cord usually undergo standard neurosurgical procedures allowing for the safe decompression and stabilization of the spinal cord. This operational approach protects the nearest structures but has almost no impact on the progression of a secondary injury (Ahuja et al., 2017; Rath and Balain, 2017). Subsequently used pharmaceutical treatments are mainly aimed at suppressing a limited range of pathological

processes of the same origin, for example, reducing inflammation and swelling, an approach which is neuroprotective but often ineffective overall and can leave patients paralyzed for the rest of their lives (Fawcett, 2009).

On the other hand, some positive effects have been achieved in SCI by combined neuro-protective–regenerative scaffold-based strategies such as therapeutic hypothermia, stem cells, biomaterials, and long-term targeted neuro-rehabilitation, all of which are being considered for experimental and clinical trials (Ahuja et al., 2017). Their multifactorial mechanisms of action may effectively protect injured tissue, enhance regeneration, and improve neurological function. Despite this possibility, however, traumatic SCI still results in severe or irreversible loss of function.

Recently, studies using different types of stem cells [neural stem cells, induced pluripotent stem cells (iPS), and MSCs] or their conditioned media have been undergoing extensive research. One of the most commonly used therapies involves adult MSCs because MSCs are well recognized for releasing bioactive molecules, such as growth factors and cytokines, which have immunomodulatory, anti-inflammatory, anti-stress, angiogenic, and anti-apoptotic effects (**Figure 1**). However, the main shortcomings of MSC therapies lie in their unsatisfactory translation from small animal experimental models (mice and rats) into human clinical practice. It is necessary, therefore, to evaluate these therapies on animals which more closely resemble the anatomy of the spinal cord and immune response in humans and, at the same time, allow the performance of long-term follow-up studies (dogs, pigs, and primates) (Vikartovska et al., 2020). Moreover, it is important to bear in mind that no animal experimental model can completely match a human study.

Stem Cell-Based Therapies

Strategies in regenerative medicine including cell-based nanotechnologies (Kubinová and Syková, 2010), spinal electric stimulation devices (Angeli et al., 2018; Gill et al., 2018), and targeted rehabilitation (Musselman et al., 2018) have shown progress in experimental and clinical therapies for CNS injuries (Kubinova and Sykova, 2012; Sykova and Forostyak, 2013). However, many years of research and practical experience have led to the conclusion that complex aspects need to be considered for successful treatment, for example, optimal timing/therapeutical window, delivery routes, the age and health condition of patients with SCI, and the use of a suitable source of stem cells or, alternatively, their EVs and their combination with advanced biomaterials (Grulova et al., 2015).

Bone Marrow Mesenchymal Stem Cells

Bone marrow mesenchymal stem cells (BMSCs) were the first cells used for traumatic injury treatment in experimental and clinical trials alike (Syková et al., 2006a). Primary studies based on the thoracic spinal cord contusion and BMSC transplantation in rats showed partial improvements in motor, sensory, and autonomic functions as well as in tissue sparing (Syková et al., 2006b). Interestingly, similar beneficial effects were detected when BMSCs were administered locally into the cavity of the spinal cord (Nandoe et al., 2006), intrathecally (Cizkova et al., 2011) or systemically (Cízková et al., 2006; Osaka et al., 2010). In a rat SCI model, as in balloon-induced compression lesion (Vanický et al., 2001), BMSCs were grafted intravenously at 1 week after injury. Behavioral testing revealed a significant improvement in motor and sensory tests (Jendelová et al., 2004).

In these studies, magnetic resonance imaging (MRI) was used as a non-invasive method of studying the progress of transplanted cells in SCI *in vivo* (Jendelová et al., 2003, 2004; Syková and Jendelová, 2005). Superparamagnetic iron-oxide nanoparticles were inserted into adult BMSCs during their cultivation prior to their transplantation into the animals with SCI. The BMSCs were then visible in the MRI images of SCI as hypointensive signals persisting for more than 4 weeks (**Figures 2A,B**). *Ex vivo* Prussian blue histological staining for iron confirmed iron-positive cells at the lesion site (see **Figures 2C–F)** (Syková and Jendelová, 2005; Sykova and Jendelova, 2007). Chronic SCI is characterized by tissue loss (cavity formation and spinal atrophy), resulting in a stable functional deficit. It is therefore necessary to bridge any spinal cavity by implanting a functionalized scaffold (see section "Biomaterials In Combination With MSCs In SCI Treatment"). Partial recovery of motor and sensory function was found in chronic SCI after the implantation of hydrogel seeded *in vitro* with BMSCs (**Figure 3**) (Hejcl et al., 2010).

These promising preclinical studies initiated a series of I/II clinical trials delivering BMSCs (autologous and allogenic) or mononuclear fractions in patients with acute, sub-acute, or chronic SCI. In summary, the results from these clinical trials demonstrate that BMSCs are safe and without adverse effects. One of the first studies used all mononuclear cells from BM (Syková et al., 2006a). Partial improvement in the American Spinal Injury Association (ASIA) score and recovery of motor-evoked potentials and somato-sensory evoked potentials were observed in several patients when treated during the acute or sub-acute phase. Subsequently, a number of phase I/II clinical studies were launched in Korea, Japan, India, Egypt, China, Brazil, Chile, and Switzerland; an overview of these studies can be found on the clinicaltrial.gov website. The results of these studies are modest but promising. A comprehensive review of these studies has been published (Forostyak et al., 2013; Muthu et al., 2020; Silvestro et al., 2020), and it is beyond the scope of this review to list the results here. However, regardless of the promising results achieved, larger groups of patients are required before any practical statements can be drawn (Forostyak and Sykova, 2017).

Adipose Tissue Mesenchymal Stem Cells

Because BM isolation requires specialist intervention and there are certain limitations for donors, fat cells are presently being taken into account as well. They can be obtained more easily by means of liposuction or other surgical interventions (Vishnubalaji et al., 2012). Adipose tissue mesenchymal stem cells (ATMSCs) seem to have some similar characteristics with BMSCs, such as cell surface antigens expression, but they have different proliferation and multilineage capacities (Danisovic et al., 2009; Petrenko et al., 2020). Interestingly, contradictory data have been published in the relevant studies. Some studies indicate that ATMSCs are more effective than BMSCs, while others report that BMSCs are superior to ATMSCs (Elman et al., 2014).

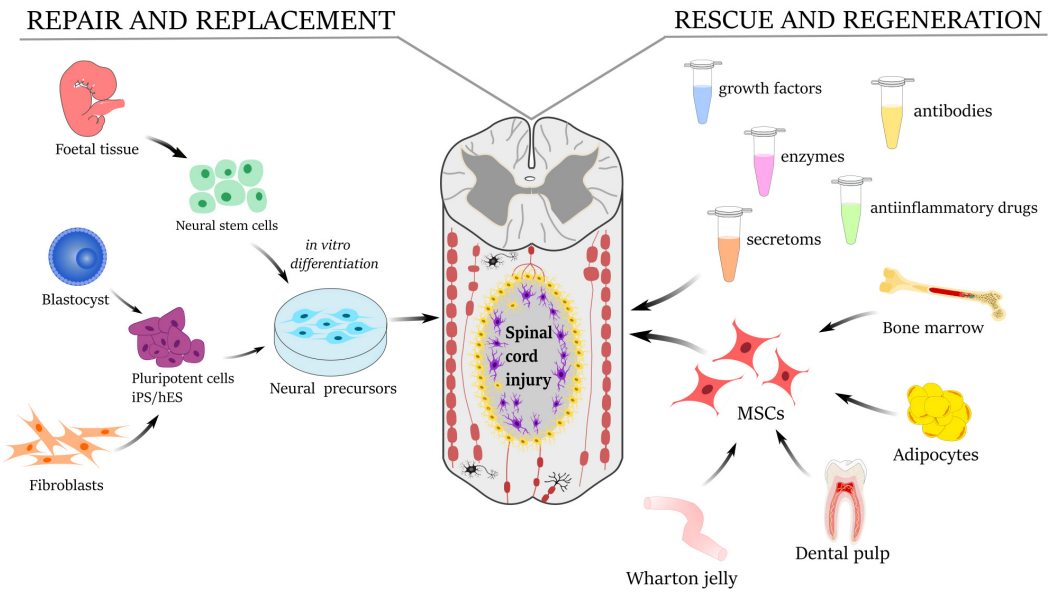

FIGURE 1 | Schematic illustration describing the strategies for the treatment of spinal cord injury. Repair and replacement of damaged tissue by neural precursors derived either from fetal neural tissue or from induced pluripotent stem cells (iPS/hES). The mesenchymal stem cells derived from bone marrow, adipose tissue, dental pulp, or umbilical cord Wharton's jelly have a rescue-and-regeneration effect mediated by paracrine action through releasing secretomes, growth factors, anti-inflammatory molecules as well as enzymes and antibodies.

Differences in the results can be obtained due to the fact that these cells differ in cytokine release, chemokine receptor expression, and apoptosis (Ahmadian Kia et al., 2011; Hsiao et al., 2012; Ruzicka et al., 2017). Furthermore, ATMSCs show a higher proliferative activity and are capable of secreting higher levels of IGF-1, VEGF-D, and interleukin-8 (Hsiao et al., 2012). In contrast, BMSCs are characterized by a slower proliferation but higher osteogenesis and chondrogenesis, and they secrete VEGF-A, angiogenin, bFGF, NGF, stem cell-derived factor-1, and HGF at comparable levels with ATMSCs (Ahmadian Kia et al., 2011). Due to these findings, ATMSCs tend to be preferred for stimulating angiogenesis. Thus, both types of stem cell have a range of biological activities and immunomodulatory properties which need to be considered when selecting these cells for a specific clinical trial (Huang et al., 2005; Zhou et al., 2020).

The ATMSC mechanisms underlying inflammatory suppression may be mediated by blocking the infiltration of ED1 macrophages as well as attenuating Notch1 signaling (Leu et al., 2010; Zhou et al., 2020). In a mouse model of SCI, the delivery of ATMSCs immediately after contusion led to decreased neuronal death and improvement in locomotion. Because transplanted ATMSCs do not differentiate into glial or neural cells, other processes may be responsible for this beneficial effect, such as the downstream factors of attenuated Notch1 signaling, including Jagged1, NICD, and RBP-JK (Zhou et al., 2020).

Follow-up clinical trials using intrathecal implantation of autologous ATMSCs in patients ($n = 14$) with SCI (at the cervical–thoracic and lumbar level) proved to be safe and revealed mild improvements in ASIA motor and sensory scores at 8 months of follow-up. Adverse events were observed in three patients, who suffered with urinary tract infection, headache, nausea, and vomiting (Hur et al., 2016). Similarly, a recently published case report from a phase 1 trial (CELLTOP study) declared that intrathecal autologous ATMSC delivery was feasible and safe with signs of an improved neurological condition (Bydon et al., 2020).

Umbilical Cord Wharton's Jelly-Derived MSCs

Variability in experimental models of SCI and limited efficacy of adult stem cells may contribute to the final failure in clinical practice. A lot of effort has therefore been put into finding a more embryonic-like source of stem cells. Accordingly, the therapeutic potential of umbilical cord Wharton's jelly-derived MSCs (WJMSCs) has emerged (Balasubramanian et al., 2013). These cells possess more embryonic-like properties with increased proliferation, reduced immunogenicity, and no tumorigenicity (Zhou et al., 2011; Kim et al., 2013). They secrete high levels of NGF, neurotrophic factors NT-3, NT-4, bFGF, and GDNF, and other molecules associated with neuroprotection, and they stimulate neurogenesis and angiogenesis (Balasubramanian et al., 2013). In a recent study, the repeated intrathecal delivery of WJMSCs into a rat ischemic–compression model of SCI showed the potentiated regeneration (Krupa et al., 2018) of the spinal cord in a dose-dependent manner. Histochemistry, in particular, indicated that higher doses of the delivered WJMSCs enhanced the number of GAP43-positive fibers, sparing the nerve tissue and reducing glial scar (Krupa et al., 2018).

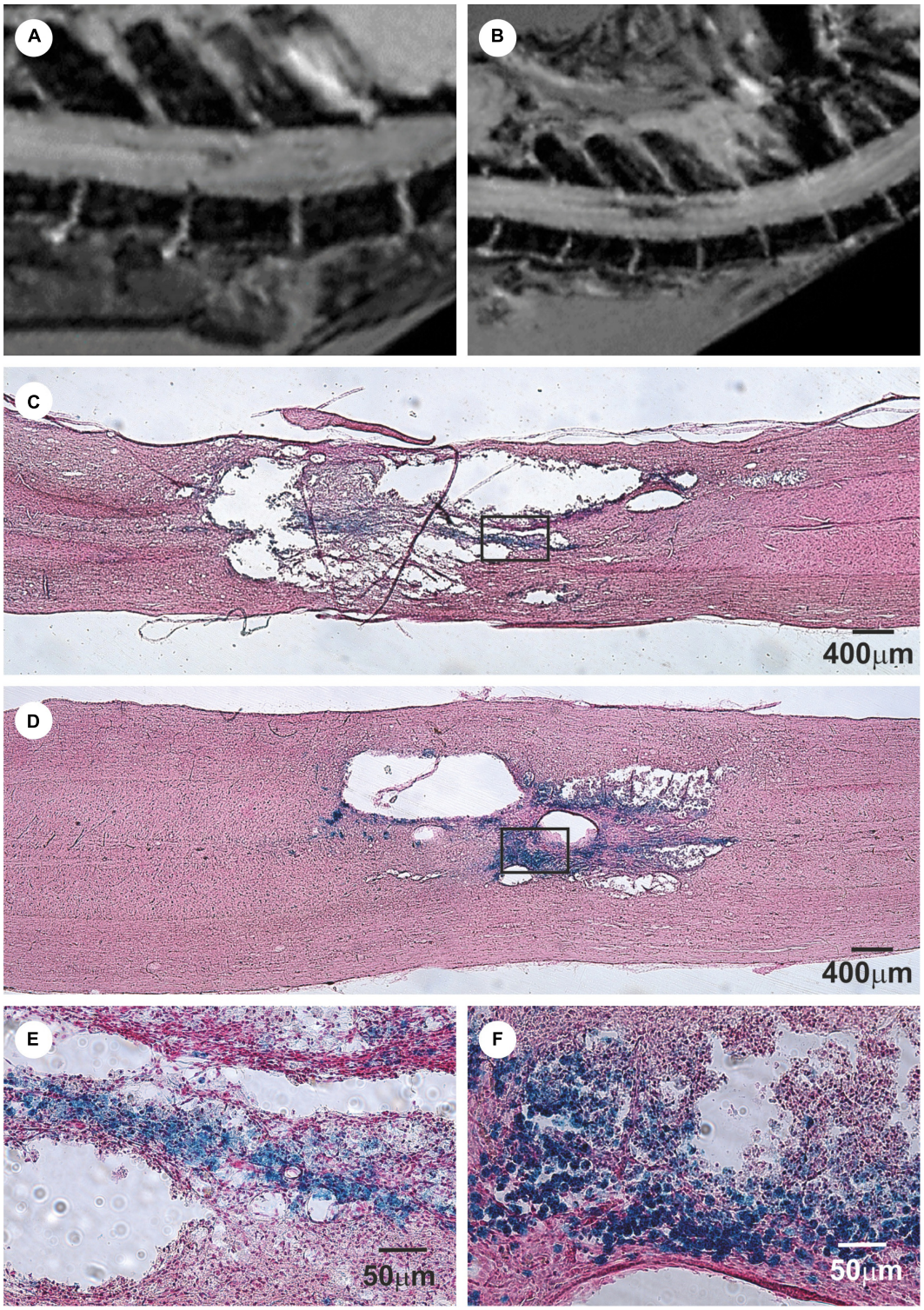

FIGURE 2 | Bone marrow mesenchymal stem cells (BMSCs) labeled with iron-oxide nanoparticles implanted into rat with acute balloon-induced spinal cord compression lesion. **(A,B)** Longitudinal MRI images of spinal cord lesion. **(A)** At 5 weeks after compression the lesion was detected as a hyperintensive area with a weak hypointense signal. **(B)** Entire lesion populated by intravenously injected magnetically labeled BMSCs at 4 weeks after implantation is visible as a dark hypointensive area. **(C)** Prussian blue staining for iron of a spinal cord lesion in control animal. **(D)** Prussian blue staining for iron of a spinal cord lesion at 4 weeks after labeled BMSCs implantation. Note the smaller lesion size in the animal with implanted BMSC. **(E)** Prussian blue staining in detail shows a staining for hemoglobin. **(F)** The lesion is populated with Prussian blue-positive cells. Modified from Jendelová et al. (2004).

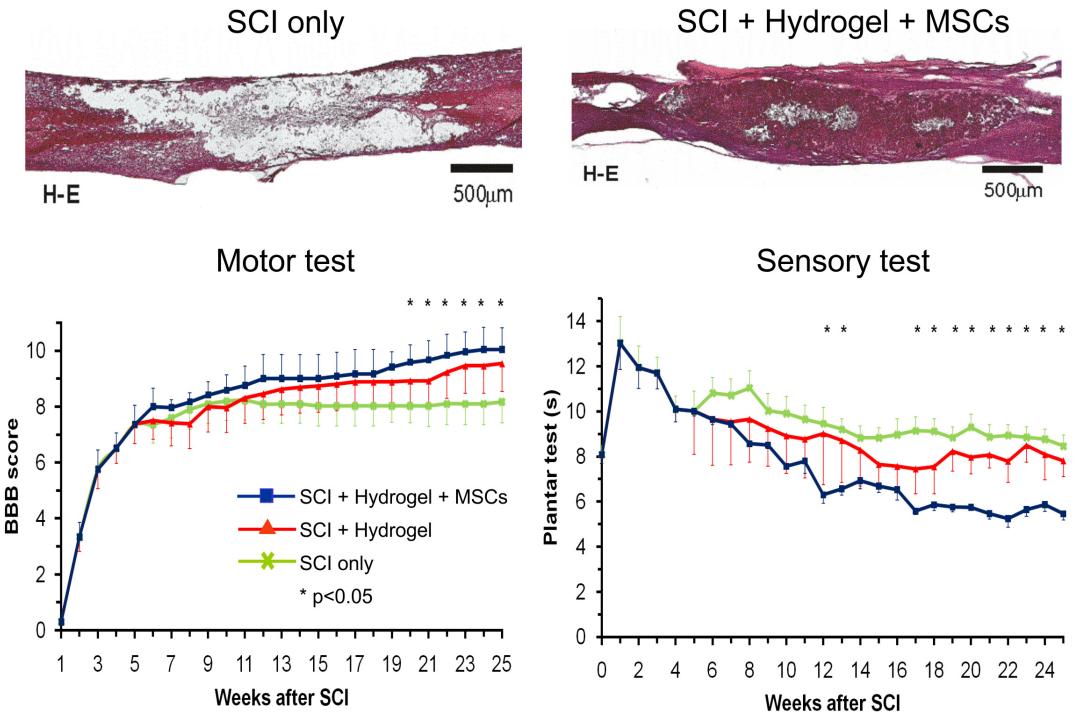

FIGURE 3 | Hematoxylin–eosin staining of a rat spinal cord with acute balloon-induced spinal cord compression lesion. Spinal cord injury (SCI) only: longitudinal section of the spinal cord with a cavity in the control animal at 6 months after SCI. SCI + hydrogel + mesenchymal stem cells (MSCs): spinal cord at 6 months after a lesion with MSC-seeded HPMA-arginine–glycine–aspartic acid hydrogel implanted at 5 weeks after SCI. Motor test and sensory test: A comparison of motor test (Basso, Beattie, and Bresnahan score) and sensory test (plantar test) in 1–25 weeks after SCI in rats with SCI only, SCI and hydrogel implantation, and SCI with implantation of hydrogel seeded with bone marrow mesenchymal stem cells. Modified from Hejcl et al. (2010).

MSC-Conditioned Media

As an alternative to cell-based therapies, conditioned media (CM) represent a cell-free product which can reduce undesirable immune issues, with effective mass production and storage and off-the-shelf availability. Similarly as with cell transplantations, the intrathecal administration of CM from BMSCs in a rat SCI model stimulated the intrinsic factors of spinal regeneration, resulting in tissue repair and motor function improvement (Kanekiyo et al., 2018). CM may be produced from various cells, such as BMSCs (Cizkova et al., 2018), dental pulp-derived MSCs (Asadi-Golshan et al., 2018), endothelial progenitor cells, or WJMSCs (Chudickova et al., 2019; Vawda et al., 2020). BMSC-CM contain anti-apoptotic, proinflammatory, neuromodulator, and angiogenic factors (Kanekiyo et al., 2018). When delivered for SCI treatment, they supported axonal regrowth and the recovery of locomotor function, reduced the lesion cavity, and promoted vascular stabilization (Cantinieaux et al., 2013). The content of CM has a limited concentration, so multiple injections over a longer time should be considered. CM may be delivered as intrathecal injections (Kanekiyo et al., 2018) or locally into the injured spinal cord tissue by means of an osmotic pump (Cantinieaux et al., 2013). Moreover, a systemic delivery of four intravenous injections of allogenic MSC-conditioned medium to dogs with chronic SCI proved to be safe and well tolerated (Vikartovska et al., 2020). MSC-CM in dogs, in combination with comprehensive and targeted physiotherapy, resulted in the improvement of the hind limb function and bladder control. This pilot study suggests that non-invasive, repeated injections of allogenic stem cell CM may substitute cell-based therapy and support spinal cord regeneration. However, to confirm the safety and efficacy of this treatment, it is necessary to involve a larger number of dogs and placebo controls during a long-term study (Vikartovska et al., 2020).

The most recently published report on high-throughput conditioned medium-secretome derived from umbilical cord matrix cells (HUCMCs) and BMSCs, as well as fibroblasts derived from newborn and adult tissue, compared their efficacy in a rat model of spinal clip compression injury. Data from this study indicate HUCMC-derived CM as being superior than the others tested due to the limitation of vascular pathology and participation in immune cell migratory pathways (MAPK/ERK, JAK/STAT) (Vawda et al., 2020).

Exosomes

Besides the growing interest in the beneficial effects of conditioned media on SCI, recent data highlight the therapeutic potential of cell-derived exosomes (Mendt et al., 2019). Exosomes are defined as small EVs composed of the lipid bilayers of a cell donor membrane, with a diameter of 50–150 nm (Théry et al., 2002). They are released through exocytosis by various cell types and can be detected in all body fluids (Murgoci et al., 2018). Interestingly, the intravenous delivery of MSC-derived

exosomes in a rat model of SCI mitigated the severity of injury and enhanced functional recovery (Lankford et al., 2018). Most probably, MSC exosomes can mediate the transfer of miRNAs or release of trophic factors at the injury site and play a key role in intercellular communication (Li et al., 2018). Recently, it has been reported that MSC-derived exosomes migrated solely to the contused regions of the spinal cord and were associated with M2 macrophage-expressing CD206 (Lankford et al., 2018). Detailed analyses of exosomal content confirmed a complex cargo consisting of proteins, lipids, and short and long forms of RNA and DNA. The miR-133b found in MSC exosomes showed a therapeutic benefit in CNS trauma as well as in SCI (Li et al., 2018). The treatment of rats with miR-133b exosomes reduced spinal cavity volume, protected neuronal cells, and stimulated neurite outgrowth following SCI (Li et al., 2018). This may be attributed partially to the stimulation of ERK1/2, STAT3, and CREB and the attenuation of RhoA expression (Li et al., 2018). In a clinical study, miR-21 and miR-19b delivered by means of human MSC-derived EVs regulated the apoptosis and differentiation of neurons in patients with SCI (Xu et al., 2019). According to these findings, MSC-derived exosomes may treat SCI through angiogenic properties, stimulating axonal regeneration and suppressing the development of glial scar. These exosomes, similarly as the conditioned medium, present no risk of immune rejection, are more stable, and may be stored for a longer period than cells (Li et al., 2018).

Many years of studies using stem cells in regenerative medicine lead to the conclusion that the most efficient therapy for SCI can be based on a combination of biomaterial, stem cell, CM, or exosome therapies and molecule delivery (Xu et al., 2019). Despite the enormous scientific efforts being made so far in the research into SCI, the ultimate clarification of regeneration processes is still missing. However, care for SCI patients has significantly improved, and innovated surgical approaches together with supporting treatment and targeted rehabilitation can restore functionality to varying degrees and improve their quality of life.

BIOMATERIALS IN COMBINATION WITH MSCs IN SCI TREATMENT

It is generally accepted that transplanted MSCs do not differentiate into neuronal or glial cells, but their therapeutic effects are associated with their ability to release a variety of antiapoptotic, neurotrophic, and anti-inflammatory molecules (Urdzikova et al., 2014; Ruzicka et al., 2017; Krupa et al., 2018; Chudickova et al., 2019; Petrenko et al., 2020). In line with this concept, we and others have previously demonstrated the regenerative effects and functional recovery in SCI after intrathecal MSC transplantation, without the transplanted cells being integrated into the damaged tissue (Urdzikova et al., 2014; Krupa et al., 2018). Moreover, a comparable therapeutic effect has also been shown after the intrathecal delivery of conditioned media containing a complex of MSC-secreted products, developed as a cell-free alternative to cell therapy (Amemori et al., 2015; Cizkova et al., 2018; Chudickova et al., 2019). In contrast to intrathecal application, intralesional cell transplantation may provide higher cell retention and more localized and focused cell effects at the site of delivery. However, the efficacy of intralesional transplantation is often limited by poor cell survival in the unfavorable microenvironment of the injured neural tissue.

Various biomaterials of synthetic [e.g., 2-hydroxyethylmethacrylate, hydroxypropylmethacrylamid, poly L-lactic acid, poly(lactic-co-glycolic acid), poly-L-lysine, and polyethylene glycol] as well as natural origin [e.g., Hyaluronic acid (HA), alginate, chitosan, collagen, fibrin, and ECM scaffolds] have been developed to bridge the lesion and provide a stimulatory microenvironment to support the survival and efficacy of transplanted cells (**Figures 3**, **4**) (Hejcl et al., 2010; Kubinova and Sykova, 2012; Liu et al., 2019). In fact, scaffolds promote MSC adhesion and their survival. The effects of cell-seeded biomaterial scaffolds on improved axonal regrowth or enhanced functional outcomes after SCI than scaffolds alone have been shown in many studies (Hejcl et al., 2010; Kim et al., 2016; Blasko et al., 2017; Peng et al., 2018; Zaviskova et al., 2018) and reviewed in Libro et al. (2017) and Yousefifard et al. (2019).

The composition of scaffolds together with their physical properties, such as stiffness, pore size, and porosity, or three-dimensional structures should mimic the target tissue and allow appropriate scaffold integration into the site of transplantation. Additionally, the development of different types of scaffolds for 3D culture enables the generation of *in vitro* neural-like tissue as a new approach for modeling and tackling diseases of the brain and CNS (Murphy et al., 2017).

Hydrogels

Hydrogels have proved to be the most convenient material, especially due to their ability to retain high levels of water and potential to adjust their mechanical properties to imitate soft nervous tissue. The benefit of the hydrogels is their injectability, which enables *in situ* gelation in lesion cavities of irregular shape together with cell or drug encapsulation. Hydrogels for CNS repair are commonly based on ECM, such as HA, collagen, or gelatine. HA, as a component of the ECM, is a biomaterial which is widely used in various clinical settings; it is biocompatible, biodegradable, and non-immunogenic. Native HA does not form a gel or promote cell adhesion, so various physical and chemical crosslinking methods have been developed to prepare injectable HA hydrogels. We previously demonstrated the neuroregenerative potential of an enzymatically crosslinked hydroxyphenyl derivative of HA modified with the integrin-binding peptide arginine–glycine–aspartic acid in the case of subacute spinal cord hemisection (Zaviskova et al., 2018). The hydrogels filled the lesion, promoting vascularization and axonal ingrowth into the lesion, and this effect was further potentiated in combination with human Wharton's jelly-derived MSCs (Zaviskova et al., 2018).

Extracellular Matrix Scaffolds

Other interesting materials are biological ECM scaffolds prepared by means of tissue decellularization, which removes cellular components from the tissue. These materials recapitulate the

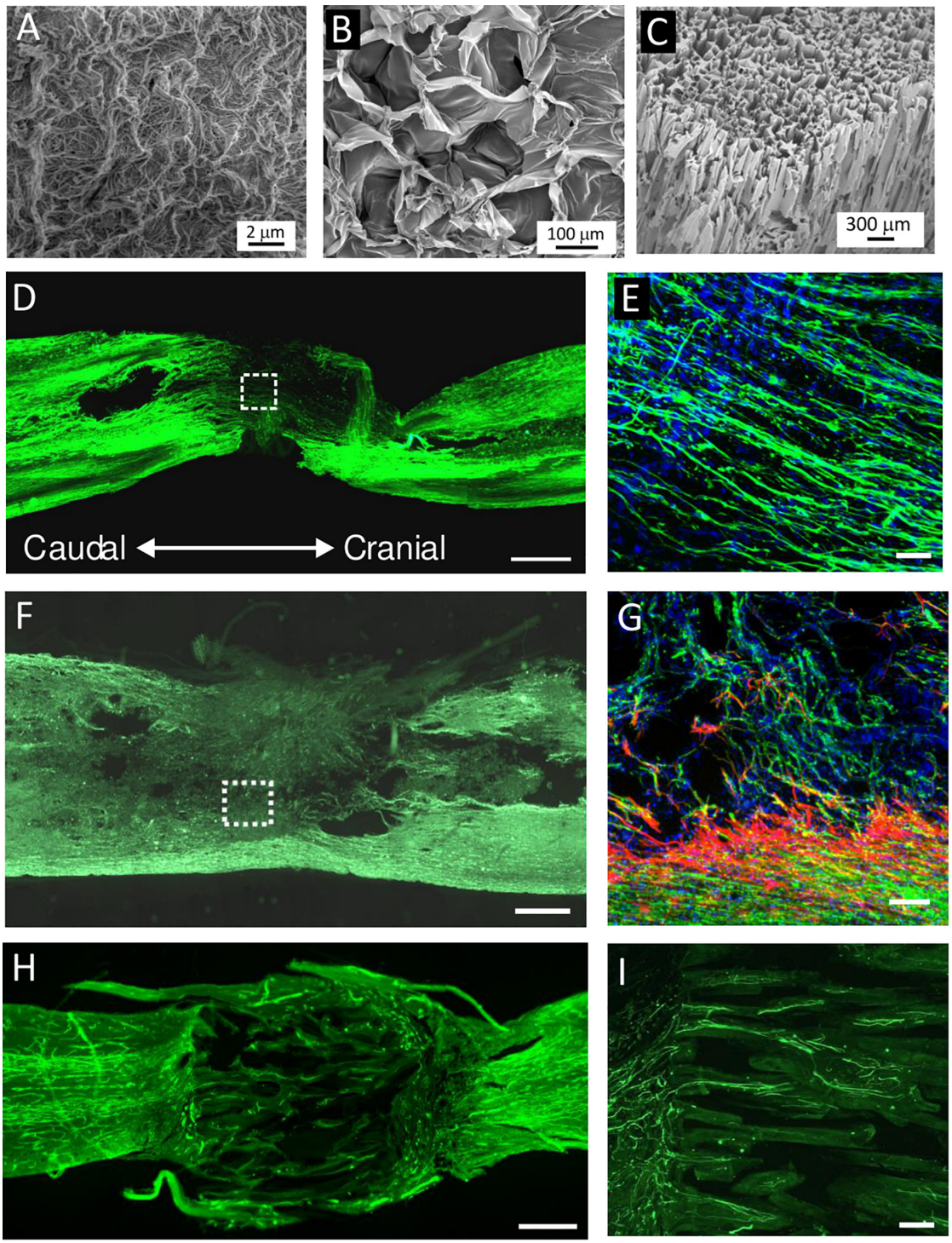

FIGURE 4 | (A–C) SEM micrographs of **(A)** extracellular matrix (ECM) hydrogel, **(B)** hyaluronic acid (HA) hydrogel modified with arginine–glycine–aspartic acid (RGD), and **(C)** highly superporous SIKVAV-modified superporous poly (2-hydroxyethyl methacrylate) hydrogel scaffolds with oriented pores. **(D–I)** Representative images of the longitudinal sections of the spinal cord lesion after hydrogel injection or implantation into the hemisection cavity. **(D,E)** Immunofluorescence staining for neurofilaments (NF-160, green) and **(E)** cell nuclei (DAPI, blue) at 2 weeks after the injection of ECM hydrogel derived from porcine spinal cord. **(F,G)** Immunofluorescence staining for neurofilaments (NF-160, green), **(G)** astrocytes (GFAP, red), and cell nuclei (DAPI, blue) at 8 weeks after HA–RGD hydrogel implantation; the square in panel **(F)** is shown under the higher magnification inset in panel **(G)**. **(H,I)** Immunofluorescence staining for **(H)** blood vessels (RECA) and **(I)** neurofilaments (NF-160, green) at 2 months after the implantation of SIKVAV-modified superporous poly (2-hydroxyethyl methacrylate) hydrogel with parallel-oriented pores. Scale bar: **(D,F,H)** 500 μmm, **(E,G)** 50 μmm, and **(I)** 100 μmm. Modified from **(A)** Koci et al. (2017), **(D,E)** Tukmachev et al. (2016), **(B,F,G)** Zaviskova et al. (2018), and **(C,H,I)** Kubinova et al. (2015).

complex biochemical composition of ECM and mimic the native cell environment. The composition of ECM scaffolds can vary between tissues, but the most abundant compounds are collagen, glycosaminoglycans, fibronectin, or laminin (Costa et al., 2017). Generally speaking, decellularized scaffolds can be prepared as solid fibrous structures, sponges, or sheets and can also be further solubilized into the form of injectable ECM hydrogel (Costa et al., 2017). The advantage of ECM hydrogels is their ability to physically crosslink *in situ* at physiological pH and temperature, which allows their non-invasive injection into the lesion or cavity (Kubinova, 2017).

For CNS repair, ECM hydrogels derived from porcine brain, spinal cord, or urinary bladder has been evaluated in *in vitro* as well as *in vivo* studies (Crapo et al., 2014; Hong et al., 2020). We demonstrated that ECM hydrogels derived from decellularized porcine spinal cord and urinary bladder tissues filled the lesion, had an effect on the immune response, and created a stimulatory substrate for *in vivo* neural tissue repair after SCI. On the other hand, no significant changes were found in chemotactic or neurotrophic properties *in vitro* or *in vivo* between CNS-derived and non-CNS-derived ECM hydrogels, which do not indicate any detectable tissue-specific effect of the neural ECM (Tukmachev et al., 2016). Remarkably, using adult CNS tissues as a source of ECM matrix might be limited due to the presence of factors which suppress axonal growth, such as chondroitin sulfate proteoglycans and myelin-associated molecules. In this context, it has been demonstrated that decellularization in the adult optic nerve selectively removes the inhibitory compounds of the CNS tissue and preserves some axon-promoting ECM proteins, including collagen IV and laminin (Sun et al., 2020). It should therefore be emphasized that, for the development of CNS-derived ECM scaffolds, the extent of tissue decellularization must maintain an optimal balance between the effective clearance of myelin and myelin-related inhibitory factors while retaining compounds with neurotrophic properties.

Besides xenogeneic or allogeneic cadaveric tissues, ECM derived from fetal human tissue, such as the umbilical cord, represents a promising source for tissue engineering due to its human origin, easy accessibility, and absence of ethical constraints. We developed efficient and reproducible decellularization protocols for the production of ECM-based hydrogel derived from human umbilical cord tissue and proved its *in vitro* biocompatibility and similarity with ECMs derived from porcine tissues such as urinary bladder, spinal cord, and brain (Tukmachev et al., 2016; Koci et al., 2017). Moreover, the mechanical strength and bio-stability of ECM hydrogels can be further improved by crosslinking with genipin (Vyborny et al., 2019).

Conduits for Axonal Guiding in SCI Repair

Injectable hydrogels are suitable carriers for cell or drug delivery, but during *in situ* gelation, they usually do not allow controllable microporosity, which could guide regenerating axon growth through the lesion. For this purpose, various types of solid fibrous or multichannel polymer conduits have been proposed as providing directional support for regrowing axons together with cell or drug delivery (Liu et al., 2017). Such conduits are mostly implanted into the complete spinal cord transection after removal of the spinal segment. Importantly, micro/nano-structures in nerve conduits have proved to be essential in tuning a large variety of post-implantation effects (Sun et al., 2019).

For example, Chen et al. (2020b) showed the regenerative effect of BMSCs seeded into a chitosan tubular scaffold combining the two architectures of a single H-shaped central tube and several microchannels. The scaffold was implanted to bridge the 5-mm defect of a complete transverse lesion in the thoracic spinal cord of rats, and when compared with the empty scaffold, the BMSC enhanced functional improvement and the number of regenerating axons and elicited antiapoptotic effects (Chen et al., 2020b). Peng et al. (2018) showed that rat MSCs combined with a nerve-guide collagen scaffold inhibited chronic scar formation, provided linear guidance for the nerves, and promoted M2 polarization to form an anti-inflammatory environment in a hemisected SCI rat model (Peng et al., 2018). Deng et al. (2020) transplanted human WJMSCs on collagen scaffolds into complete spinal cord transection in rats and dogs. The transplantation improved motor scores, enhanced amplitude, shortened the latency of motor evoked potential, and decreased the lesion area, which was further potentiated when the scaffold was used in combination with stem cells (Deng et al., 2020).

We previously tested SIKVAV-modified superporous poly (2-hydroxyethyl methacrylate) hydrogel with oriented pores (Kubinova et al., 2015). The hydrogels, either empty or seeded with rat MSCs, were implanted in the spinal cord transection. However, MSCs seeded in the scaffold did not enhance tissue infiltration into the pores, and only rare axons crossing the hydrogel bridge were observed after 6 months, which suggests that this type of scaffold did not provide an optimal environment for neural tissue repair (Hejcl et al., 2018).

To support the effects of MSCs, combined strategies have been proposed to further stimulate axonal growth and tissue regeneration. For example, the effect of rat BM-MSCs in a multichannel polymer poly (lactic-co-glycolic acid) scaffold was enhanced by their co-transplantation with Schwann cells, which promoted MSC survival and differentiation into neuron-like cells and resulted in the regeneration of axons and functional recovery after complete spinal cord transection (Yang et al., 2017).

Clinical Trials Using Scaffolds in SCI Repair

Despite the number of preclinical studies using various scaffolds for SCI repair, only a few of them are currently approved for clinical trials. The safety and benefit of implantation of poly(lactic-co-glycolic acid)-b-poly(L-lysine) scaffold (neuro-spinal scaffold) has been evaluated in patients with thoracic AISA A spinal cord injury at a level of injury of T2–T12 (NCT02138110). No adverse effects related to acute scaffold implantation were reported in the 6-month study (Layer et al., 2017).

In another clinical study, a collagen scaffold with linearly aligned pores and functionalized with neuroactive factors

(NeuroRegen scaffold) was loaded with autologous BM mononuclear cells and transplanted into the surgically cleaned lesion in seven patients with acute complete SCI. No adverse symptoms were present in the 3-year follow-up period. In some patients, partial sensory and autonomic nervous functional improvements, but no motor function recovery, were observed (Chen et al., 2020a). In the following study, a NeuroRegen scaffold was loaded with human WJMSCs and implanted into the surgically cleaned lesion in eight patients with chronic complete SCI. No adverse events were reported during 1 year of follow-up. In some patients, increase of sensation level and motor evoked potential-responsive area, enhanced finger activity and trunk stability, defecation sensation, and autonomic neural function recovery were found (Zhao et al., 2017).

Deng et al. (2020) published the results of a phase I clinical trial on 40 patients with acute complete cervical injuries. The group of patients (n = 20) obtained collagen scaffolds seeded with MSCs derived from umbilical cord tissue. No serious complications were reported during the 12-month follow-up. In the treatment group, an improvement in neurological functions was observed over the follow-up period, while no neurological functions were improved in the control group of patients (Deng et al., 2020).

Encouraging first clinical results indicate the safety and feasibility of MSC-seeded scaffold-based therapy in SCI repair; however, the observed weak functional recovery suggests the need to develop more advanced combinatorial approaches which would further target the inhibitory environment of the adult CNS tissue as well as the limited regenerative ability of the long-track axons.

MESENCHYMAL STROMAL CELLS FOR THE TREATMENT OF ALS

Amyotrophic lateral sclerosis or MND is a devastating, rapidly progressing, and fatal neurodegenerative disease which attacks motoneurons (MNs) in the anterior horn of the spinal cord. Patients exhibit atrophy of the spinal cord and often also atrophy of cerebral gray and white matter. The disease is characterized by muscle weakness and atrophy, fasciculations, spasticity, and paralysis, which are leading to death usually within 3–5 years after the onset of clinical symptoms. Some patients exhibit a slower time course of the disease. About 90% of all cases are sporadic, while 10% of patients suffer from a familial disease. Researchers and clinicians worldwide have been searching for an effective treatment of this devastating disease for many years. Poor prognosis and symptomatic treatment are so far the only prospect for patients. The pharmaceutical treatments used in all patients include glutamate inhibition with riluzole (Bensimon et al., 1994), which only extends survival by about 3 months, or free radical scavenger edaravone (Cho and Shukla, 2020). Both of these drugs only delay the symptomatic and pathological progression of ALS. Apart from this, patients can get some relief from secondary complications through neurorehabilitation.

Stem cell-based therapies are potentially effective treatments for ALS patients (Forostyak and Sykova, 2017; Goutman et al., 2019). There are generally two strategies in using stem cells: firstly, achieving the replacement of lost motoneurons or pathological astrocytes, in particular, using a ready source of induced pluripotent stem cells (iPSCs) (Sareen et al., 2014; Goutman et al., 2019) and, secondly, using adult stem cells which can play a supportive role and provide a neuroprotective environment. This purpose may be served by autologous or allogenic undifferentiated MSCs of various origin, derived from the BM, umbilical cord blood, adipose tissue, or Wharton's jelly. MSCs from different sources show some differences in growth rate, molecular phenotype, cell marker expression, and ability to differentiate into neuronal- or glial-like phenotypes (Blondheim et al., 2006; Zhu et al., 2008; Forostyak et al., 2016b), but they generally share some common features: they grow extensively in culture and differentiate *in vitro* into chondrocytes, osteocytes, adipocytes, and muscle cells, and they can be obtained for autologous application (Prockop, 1997; Mezey et al., 2000a; Krause, 2002; Forostyak et al., 2016a).

Mesenchymal stem cells of a different origin have been tested in rodent models to treat diseases such as ALS. Numerous preclinical studies with mutant superoxide dismutase 1 (SOD1) in mouse or rat have been performed. These preclinical studies have demonstrated that the intrathecal, intraspinal, intravenous, or combined intraspinal and intravenous administration of MSCs is a safe procedure which is able to slow down motor impairment, decrease inflammation, and stimulate the secretion of specific cytokines and growth factors which promote cell survival and enable symptomatic transgenic animals to survive longer. The single or repeated transplantation of MSCs induces the secretion of BDNF, VEGF, NGF, GDNF, and IGF-1, which play a crucial role in neuroregeneration (Uccelli et al., 2011; Li et al., 2002; Zhang et al., 2004; Vercelli et al., 2008; Gu et al., 2009). Their paracrine action rather than cell replacement supports the resistance of neurons and glia to apoptosis due to the release of anti-apoptotic and trophic factors, thus maintaining a neuroprotective microenvironment.

We found that engraftment of human MSCs into symptomatic ALS rats was able to preserve MNs. It decreased the extent of apoptosis in motor neurons, supported the survival of larger-sized neurons, and modified the affected ECM and cytokine homeostasis (Forostyak et al., 2011, 2014). The MSCs in these animals had anti-inflammatory and neuroprotective effects, and due to their ability to remodel the gene expression profile of the recipient, they activated CNS plasticity. *Wisteria floribunda* agglutinin (WFA) fluorescence intensity, measured in the ventral horns of the cervical and lumbar spinal cord, revealed greater numbers of perineuronal nets (PNNs) in the MSC-treated animals when compared with the control group. In our preclinical study, the MSCs were delivered intrathecally into symptomatic SOD1 G93A transgenic rats, and survival in the MSC-treated group was prolonged by 13.6 days compared with the control group. The cell-treated rats showed better motility and grip strength test results; there were significantly greater numbers of motoneurons compared to non-treated animals and less apoptotic activity (TUNEL assay). Applying quantitative analyses of WFA fluorescence intensity, we found preserved PNNs (Forostyak et al., 2011, 2014). PNNs have been shown to affect CNS plasticity and to protect neurons

during injury and neurodegeneration (Sorg et al., 2016; Fawcett et al., 2019; Chelyshev et al., 2020; Yang, 2020). Moreover, the concomitant intraspinal and intravenous transplantation of rat MSCs resulted in neuroprotective effects also due to decreased inflammation, suppressed proliferation of microglial cells, and reduced expression of COX-2 and NOX-2, which increased motor activity and extended the lifespan of ALS rats (Boucherie et al., 2009; Forostyak et al., 2011).

A similar positive effect on motor activity and longer animal survival was found after the intravenous application of human umbilical cord blood and MSC in asymptomatic rat models of ALS (Mazzini et al., 2004; Garbuzova-Davis et al., 2008; Vercelli et al., 2008; Kim et al., 2010).

MSCs in Clinical Trials for ALS

There is a spectrum of stem cells which can be considered for human clinical trials in neurodegenerative diseases. In ALS BM mononuclear cells, olfactory ensheathing cells, iPSCs, and fetal neural precursor cells have been used. An overview of the clinical studies in ALS can be found on the clinicaltrial.gov website. Here we review human ALS clinical trials using MSCs. It is evident that the number of phase I/II clinical trials is increasing annually. The majority of approved clinical trials employ autologous MSCs derived from BM. They can be easily obtained by BM aspiration and then expanded *ex vivo*. This is a minimally invasive procedure, and manipulation with autologous cells from patients, legal issues, and the long history of clinical application of BM-derived cells make them ideal candidates for stem cell therapy. These cells are quite unique, especially for their paracrine properties. Autologous MSC application does not require any immunosuppression, and there is no evidence of malignant transformation (Forostyak and Sykova, 2017). MSC implantation in animal models has revealed the paracrine production of growth factors and cytokines (see above). Furthermore, since CNS neuroinflammation plays an important role in neurodegenerative diseases, the anti-inflammatory influence of MSC can also explain their beneficial effect in clinical trials. Similarly as in SCI, different methods of MSCs and stem cell delivery have been used (Forostyak et al., 2014).

Successful experiments with rodent models of ALS have established a platform for clinical trials involving patients (Vercelli et al., 2008). Current and future clinical trials using stem cells for ALS treatment have been summarized in several reviews (Forostyak and Sykova, 2017; Goutman et al., 2019; Sharma et al., 2019). These clinical trials are mostly safety studies involving small numbers of patients. Majority of these trials do not present enough details about the types of cell used, dosage of stem cells, and criteria for patient monitoring or do not sufficiently report the study outcomes. Proper interpretation of the data is impossible, thus complicating its further clinical application. Most of the trials performed so far did not include patient follow-up for longer than 24 months. The first long-term outcome was studied after 5 years of monitoring 19 ALS patients treated with MSCs. These patients were enrolled in two phase I clinical trials, but no clear clinical benefits of MSC implantation was found. However, the collected data show support for the implantation of autologous BMSCs into the spinal cord, as no structural changes, tumor formation, or deterioration in psychosocial status were found, and all patients coped well with the procedure (Mazzini et al., 2003, 2010, 2011). Another study injected a mononuclear CD133(+) fraction from autologous stem cells isolated from the peripheral blood and into the frontal motor cortex of ALS patients (Martinez et al., 2009). This application of mononuclear cells prolonged the survival of the treated patients and increased the quality of their life compared with the control (untreated patients). Deda et al. (2009) reported the results of a 1-year follow-up of patients with the implantation of BM-derived hematopoietic progenitor stem cells into the anterior part of the spinal cord. From 13 patients with a bulbar form of ALS, nine patients became much better compared with their pre-operative status; one patient was stable, without any decline or improvement in his status, and three patients died at 1.5, 2, and 9 months after the stem cell therapy due to lung infection and myocardial infarction (Deda et al., 2009). Petrou et al. (2016) performed a safety study with MSCs secreting a neurotrophic factor (Petrou et al., 2016).

Our prospective, non-randomized, open-label clinical trial has been performed in Prague, Czechia (Sykova et al., 2017). This study concentrated on the safety and efficacy assessment of autologous multipotent MSC application in the treatment of patients with a confirmed diagnosis of ALS. The trial involved 26 patients with sporadic ALS, who received a single intrathecal dose of autologous MSCs applied into the cerebrospinal fluid. The intrathecal application seems to be preferable than intravenous administration, where the cells can be trapped in different organs. Intrathecally implanted cells immediately spread in the CSF around the brain and spinal cord, without the need to cross the blood–brain barrier. Compared to previous MSC trials, this study included the largest group of ALS patients and had a longer pre- and post-treatment assessment period, and only a single dose of stem cells was used. In the 18-month follow-up period, potential adverse reactions were assessed by means of clinical, laboratory, and MRI examination. The clinical outcome was evaluated using ALS functional rating scale (ALSFRS), Norris spinal and bulbar scale, forced vital capacity (FVC), and weakness scale. After MSC application, 30% of patients experienced mild/moderate headache, typically observed as resembling the headache after a standard lumbar puncture. No suspected serious adverse reactions or a cerebrospinal pathology was found during the MRI examinations. In almost 80% of patients, the FVC values remained above 60% for a time period of 12 months. In a group of 12 patients with a remarkable pretreatment decline in functional scales, we found a significant mitigation/stabilization in their total functional score decline at 3 months after application, which was less pronounced at 6 and 9 months (**Figure 5**).

Another small study using autologous BMSCs applied either intrathecally or intravenously similarly showed a slower deterioration in ALSFRS-R score, with the FVC remaining stable for about 6 months, and longer survival (Karussis et al., 2010; Prabhakar et al., 2012; Oh et al., 2015; Rushkevich et al., 2015; Sharma et al., 2015). Repeated intrathecal MSC application with similar positive and longer-lasting effects was reported by Oh et al. (2015, 2018). It is an important finding that some

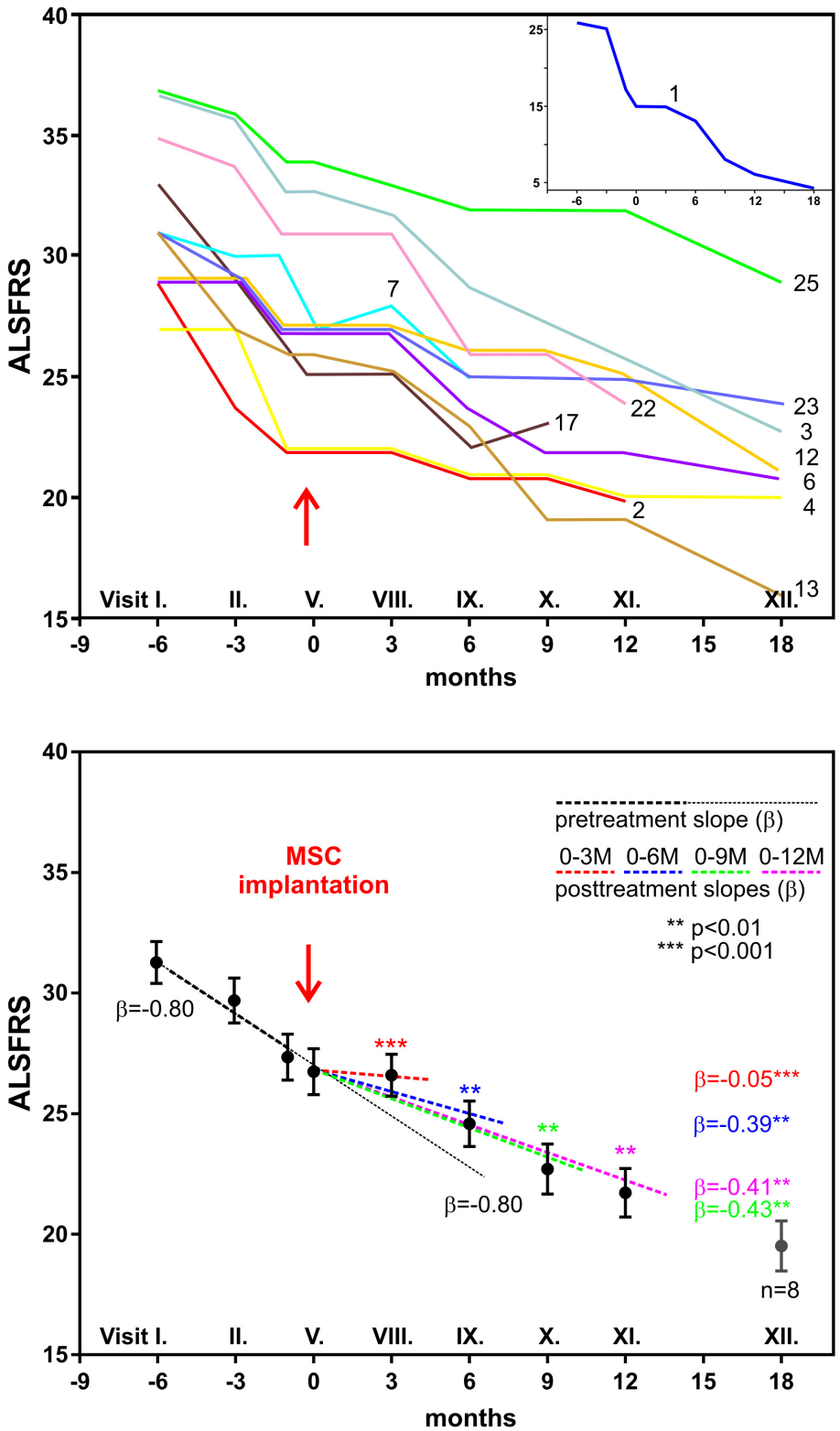

FIGURE 5 | Clinical analysis of 12 amyotrophic lateral sclerosis patients with fast decline of functional rating scale (ALSFRS) scores 6 months before and 12 months after autologous bone marrow mesenchymal stem cell (BMSC) application. The upper panel shows the time courses of ALSFRS scores in individual patients; patient no. 1 is shown in the inset. The lower panel shows the regression analysis of ALSFRS scores before and after BMSC application. The solid line with β = –0.80 is the predicted time course without BMSC treatment. Modified from Sykova et al. (2017).

studies report a better and longer-lasting outcome after repeated applications of MSCs. A recent and larger study by Barczewska et al. (2020) used umbilical cord MSCs in a case–control study involving 67 patients (Barczewska et al., 2020). The patients were treated with WJMSCs, with three intrathecal injections every 2 months at a dose of 30×10^6 cells. The authors report that median survival time increased twofold in all patients, and in some patients, there was a decrease in progression rate. Sharma et al. (2020) found in a case study that cell therapy, along with intensive physical rehabilitation, significantly improved the outcomes in a 40-year-old male ALS patient suffering for the preceding 4 years and who underwent multiple doses of cell therapy (Sharma et al., 2020).

CONCLUSION

To conclude, even though all the above-mentioned studies report similar outcomes, thus corroborating the safety of the procedure, there is a need for more extensive multicenter trials. Even though a small series of experiments involving patients suggests an improvement in motor and sensory functions after the administration of MSCs, significant obstacles remain before these findings can be translated into novel therapies. In particular, we need to better understand the mechanisms of MSC action and the behavior of the transplanted stem cells in the pathological environment of CNS. More clinical trials with larger and more homogeneous groups of patients and with a longer follow-up are needed to enable better evaluation of stem cells treatments (Lindvall and Kokaia, 2006). It is also necessary to recall the fact that neuroprotective effects after cell-based therapy have been achieved in trials employing different routes of application, so the combination of different methods of cell delivery might produce even better results related to survival and motor functions. Finally, the development of specific markers enabling an early disease diagnosis will be of great importance for the evaluation of a possible effect of cell-based therapy. It is particularly important because, at the beginning of neurodegeneration, stem cells might produce more benefits in rescuing neurons from death, influence changes in ECM, and stimulate plasticity.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Corrigendum: Mesenchymal Stem Cells in Treatment of Spinal Cord Injury and Amyotrophic Lateral Sclerosis

Eva Sykova[1]*, *Dasa Cizkova*[1,2]* and *Sarka Kubinova*[3]

[1] Institute of Neuroimmunology, Slovak Academy of Sciences, Bratislava, Slovakia, [2] Centre for Experimental and Clinical Regenerative Medicine, University of Veterinary Medicine and Pharmacy in Kosice, Kosice, Slovakia, [3] Department of Optical and Biophysical Systems, Institute of Physics of the Czech Academy of Sciences, Prague, Czechia

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A Corrigendum on

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In the original article, there was a mistake in the caption of **Figure 2** as published. The description of part (A) was incorrect. The corrected caption appears below.

Figure 2 | Bone marrow mesenchymal stem cells (BMSCs) labeled with iron-oxide nanoparticles implanted into rat with acute balloon-induced spinal cord compression lesion. **(A,B)** Longitudinal MRI images of spinal cord lesion. **(A)** At 5 weeks after compression the lesion was detected as a hyperintensive area with a weak hypointense signal. **(B)** Entire lesion populated by intravenously injected magnetically labeled BMSCs at 4 weeks after implantation is visible as a dark hypointensive area. **(C)** Prussian blue staining for iron of a spinal cord lesion in control animal. **(D)** Prussian blue staining for iron of a spinal cord lesion at 4 weeks after labeled BMSCs implantation. Note the smaller lesion size in the animal with implanted BMSC. **(E)** Prussian blue staining in detail shows a staining for hemoglobin. **(F)** The lesion is populated with Prussian blue-positive cells. Modified from Jendelová et al. (2004).

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Joan Oliva,
Emmaus Medical Inc., United States

Correspondence:
Eva Sykova
sykovae@gmail.com
Dasa Cizkova
cizkova.dasa@gmail.com

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REVIEW
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From Mesenchymal Stromal Cells to Engineered Extracellular Vesicles: A New Therapeutic Paradigm

Jancy Johnson[1,2†], Mozhgan Shojaee[1†], James Mitchell Crow[1] and Ramin Khanabdali[1]*

[1] Exopharm Ltd., Melbourne, VIC, Australia, [2] Department of Biochemistry and Pharmacology, University of Melbourne, Parkville, VIC, Australia

Mesenchymal stromal cells (MSCs) are multipotent cells obtained from many tissues including bone marrow, adipose tissue, umbilical cord, amniotic fluid, and placenta. MSCs are the leading cell source for stem cell therapy due to their regenerative and immunomodulatory properties, their low risk of tumorigenesis and lack of ethical constraints. However, clinical applications of MSCs remain limited. MSC therapeutic development continues to pose challenges in terms of preparation, purity, consistency, efficiency, reproducibility, processing time and scalability. Additionally, there are issues with their poor engraftment and survival in sites of disease or damage that limit their capacity to directly replace damaged cells. A key recent development in MSC research, however, is the now widely accepted view that MSCs primarily exert therapeutic effects via paracrine factor secretion. One of the major paracrine effectors are extracellular vesicles (EVs). EVs represent a potential cell-free alternative to stem cell therapy but are also rapidly emerging as a novel therapeutic platform in their own right, particularly in the form of engineered EVs (EEVs) tailored to target a broad range of clinical indications. However, the development of EVs and EEVs for therapeutic application still faces a number of hurdles, including the establishment of a consistent, scalable cell source, and the development of robust GMP-compliant upstream and downstream manufacturing processes. In this review we will highlight the clinical challenges of MSC therapeutic development and discuss how EVs and EEVs can overcome the challenges faced in the clinical application of MSCs.

Keywords: mesenchymal stromal cells, MSCs, extracellular vesicles, MSC-EVs, EV therapeutics, engineered EVs

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Vaijayanti Prakash Kale,
Symbiosis International University, India
Pavel Makarevich,
Lomonosov Moscow State University, Russia

***Correspondence:**
Ramin Khanabdali
ramin.khanabdali@exopharm.com

† These authors have contributed equally to this work

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INTRODUCTION

Mesenchymal stromal cells (MSCs) were identified more than 5 decades ago and have captured substantial attention among the scientific community since then due to their potential clinical applications (Friedenstein et al., 1970; Haynesworth et al., 1992). The ability of MSCs to differentiate into various cell types, and to support tissue function and repair, was quickly recognized. Since their initial discovery in bone marrow, MSCs from many other organs and tissues including adipose tissue, placenta, umbilical cord, Wharton's Jelly, peripheral blood, skeletal muscle, skin, heart, liver, and the brain have also been isolated and studied (Baksh et al., 2004).

Mesenchymal stromal cells' particular biological properties, including their self-renewal, proliferation and differentiation potential, make them attractive stem cell resources for pre-clinical and clinical studies that focus on the repair and/or regeneration of damaged tissue

(Uccelli et al., 2008). The beneficial attributes of MSCs include their high resistance to oxidative stress, potent differentiation capacity, unique immunophenotypic characteristics, limited ethical constraints, low risk of tumor formation and powerful immunomodulatory activity (Trounson and McDonald, 2015). The medical interest in MSCs in indications ranging from cardiac conditions to neurodegenerative diseases has so far led to more than 1200 clinical trials being listed on clinicaltrials.gov.

The path from pre-clinical promise into clinical use has not been smooth, however. Multiple MSC therapeutics have failed to demonstrate efficacy in early or late clinical trials, in indications ranging from amyotrophic lateral sclerosis to stroke (Levy et al., 2020; FDA, 2021). Despite more than 30 years of work very few MSC therapeutic products have been approved for clinical use in any jurisdiction, indicating the significant pre-clinical and clinical challenges that remain to be resolved.

Despite the setbacks the field has experienced, the scale of effort still being applied to MSC-based therapeutics development is testament to the clinical promise that MSCs and their derivatives still hold. MSCs continue to show an excellent safety profile and remain the most studied stem cell population, particularly in the context of therapeutic use.

One of the most significant shifts in the MSC research field over the past decade has been the growing recognition that, rather than acting by engrafting and differentiating in sites of disease or injury to directly replace damaged cells, MSCs primarily exert their therapeutic effects via the release of paracrine factors (Yang et al., 2013). One of the major paracrine effectors are extracellular vesicles (EVs); nanoscale lipid-wrapped packages of lipids, proteins and nucleic acids that possess the same therapeutic properties as their parent cells (Bruno et al., 2009; Lai et al., 2010). As a cell-free MSC product, EV therapeutics pose few of the complications hampering the development of MSC cell therapies.

This review aims to highlight the ongoing issues associated with the development of MSC therapeutics and also to discuss how EVs isolated from MSCs overcome these issues to replace or complement cell-based therapies.

CHALLENGES IN THE CLINICAL APPLICATION OF MSCs

Most MSC clinical studies have produced disappointing findings despite encouraging results in pre-clinical animal studies. Although 10 MSC therapeutics have received market approval in some jurisdictions (**Table 1**), in general low efficacy continues to blight MSC clinical trials. Some of the reasons proposed for this low efficacy are discussed below.

MSC Diversity

One factor that can lead to unexpected clinical outcomes is the significant heterogeneity that can exist between MSCs from different sources. MSCs isolated from different tissues show differences in their proliferative behavior and differentiation capacity in both *in vitro* and *in vivo* studies (Hass et al., 2011). Even when isolated from the same tissue type, significant differences in MSC populations have been observed between individual donors, with the characteristics of MSCs varying according to factors such as the donor's age, health, sex, and body weight. For example, the age-associated deficits observed for MSCs include loss of key attributes such as proliferation and differentiation potential (Zhou et al., 2008). A study of aged bone marrow derived MSCs (BM-MSCs) recorded increased senescence, and a loss of bone formation capability (Stolzing et al., 2008). The decline of MSC function with age has significant implications for autologous use–particularly when considering that ill health itself can impair MSC function (van Rhijn-Brouwer et al., 2018).

Donor sex can also have an impact on the characteristics and function of harvested MSCs. In a rat model of lung inflammation, Female BM-MSCs reduced inflammation more effectively than BM-male MSCs (Sammour et al., 2016). A meta-analysis of human adipose tissue derived MSCs showed significant differences in the gene expression of cells from males and females, with the changes predicted to affect processes including inflammation, differentiation and cell communication (Bianconi et al., 2020).

MSC Manufacturing Challenges

Once harvested, MSCs often need to be expanded to generate sufficient cells to be formulated into therapeutic doses. Treating a condition such as graft-versus-host disease may require tens of millions of cells per dose (Introna et al., 2014). Low cell harvest yield is particularly acute for BM-MSCs (Pittenger et al., 1999). Scale-up to a cell number sufficient for clinical use usually involves their proliferation in a large batch culture system. This process is lengthy and costly and therefore commercially unattractive. Additionally, MSC expansion and long-term culture to generate sufficient MSCs for clinical studies is often associated with increasing cell senescence and decreasing potency (Wagner et al., 2009).

Cost of MSC product manufacture and delivery is a significant barrier to its commercial viability. Depending on production scale and dose size, the cost of goods (COG) per dose varies dramatically, from US$485 to US$111,488 (Chilima et al., 2018). Technological advances such as bioreactors have been proposed to alleviate COG issues. This development may have the potential to improve MSC manufacturing output, and lower production costs (Chilima et al., 2018) but may not sufficiently address the COG issues. For example, hollow-fiber bioreactors were recently shown to be the least cost-effective manufacturing method due to high consumables and equipment costs with a COG almost double that required for a product to be commercially viable (Mizukami et al., 2018).

Culture medium development is another challenge for MSC production at the clinical level. Culture and expansion of MSCs has traditionally required media enriched with serum, but the complex and variable nature of this mixture of nutrients, growth factors and other constituents poses further challenges for maintaining product consistency. The development of serum-free media or chemically defined media is encouraging but they generally do not perform as well, especially for longer

TABLE 1 | List of regulator-approved MSC therapeutic products.

Drug name	MSC type	Indication	Country of approval (date)
Queencell	Autologous adipose MSC	Subcutaneous tissue defect	South Korea (March 2010)
Cellgram-AMI	Autologous bone marrow MSC	Acute myocardial infarction	South Korea (July 2011)
Cartistem	Allogeneic umbilical cord MSC	Knee articular cartilage defects	South Korea (January 2012)
Cupistem	Autologous adipose MSC	Crohn's fistula	South Korea (January 2012)
Prochymal	Allogeneic bone marrow MSC	Graft-versus-host disease	Canada (May 2012); New Zealand (June 2012)
Neuronata-R	Autologous bone marrow MSC	Amytrophic lateral sclerosis	South Korea (July 2014)
Temcell HS Inj	Allogeneic bone marrow MSC	Graft-versus-host disease	Japan (September 2015)
Stempeucel	Allogeneic bone marrow MSC	Critical limb ischemia in Buerger's Disease.	India (May 2016)
Alofisel	Allogeneic adipose MSC	Complex perianal fistulas in Crohn's disease	Europe (March 2018)
Stemirac	Autologous bone marrow MSC	Spinal cord injury	Japan (December 2018)

passages and scaling-up. They also put upward pressure on COG (Jung et al., 2012).

BIOENGINEERING TO BOOST THE CLINICAL POTENTIAL OF MSCs

The potential for clinical-scale MSC manufacture has been restricted by limited yield of donated cells, quality variation, and biosafety concerns regarding potential transmission of pathogens. An ideal cell source for industrial-scale MSC product manufacture should offer easy and unrestricted availability, have regulatorily acceptable provenance, present no biological safety risks, and be amenable to unlimited expansion while retaining its original "as harvested" phenotype. In a step toward this ideal, bioengineering approaches to increase both the yield and the homogeneity of MSCs are now being explored. For example, differentiation of induced pluripotent stem cells (iPSCs) to MSCs can be expanded to produce large quantities of cells thereby generating large quantities of highly homogenous MSCs (Ozay et al., 2019). Although concerns have been raised regarding the teratogenic potential of iPSC-derived cells, MSCs produced from iPSCs have been shown not to form teratomas or to show pro-tumor potential (Qingguo et al., 2015). After a series of pre-clinical studies, in indications including critical limb ischemia, asthma and organ transplant rejection, this approach was recently assessed in a Phase I clinical trial for acute steroid-resistant graft-versus-host disease (Bloor et al., 2020). No adverse events were reported during this study. In a related process, researchers have used a CRISPR/Cas9-based strategy to temporarily immortalize BM-MSCs, to readily expand these cells in long-term culture without the phenotypic changes that typically accumulate in high passage MSCs, before reversing the immortalization for potential therapeutic use (Hu et al., 2017). The safety of these reversibly immortalized cells now needs to be investigated before being used in clinical trials.

Despite the potential of engineered MSC sources to overcome specific limitations in MSC production, additional issues associated with MSC therapeutics persist. Immortalized live cell therapies may retain the issues associated with potential immunogenicity and tumorigenicity, and their engraftment qualities post-administration remain to be elucidated.

EVs AS A NOVEL CLASS OF THERAPEUTIC

Although it was previously believed that the therapeutic potential of MSCs was due to cell-to-cell contact or engraftment and differentiation of transplanted MSCs, it has become increasingly clear that MSC therapeutics' prime mode of action is to release paracrine factors (Timmers et al., 2008; Caplan and Correa, 2011). In one non-human primate study of MSC infusion following total body irradiation, MSC engraftment levels were found to be below 3% (Devine et al., 2003). Similarly, in a mouse model of emphysema, Katsha et al. (2011) found that intratracheal injection with fluorescent labeled MSCs restored lung function, yet few engrafted MSCs and no differentiated cells could be detected in the lung beyond day 7 post-injection. The observed therapeutic effect was attributed to MSC paracrine factor release. Seminal papers reported that administration of conditioned media from cultured MSCs exerted the same beneficial effects as with whole cells (Gnecchi et al., 2006; Lai et al., 2010). Paracrine factors within the media were shown to be taken up by cells in the damaged tissue or by immune cells to promote cellular rejuvenation and restoration of tissue function. Prominent among the paracrine factors exerting these effects are EVs. Isolated MSC-EVs have shown to possess the same therapeutic potential as their parent cells (Bruno et al., 2009). This discovery has contributed to the interest in, and development of, EVs as medicines. As next generation, cell-free, MSC-based therapeutics, EVs have significant advantages in overcoming the limitations and risks associated with MSC-based cell therapy, as discussed below.

EVs as a Cell-Free Application of MSCs

Extracellular vesicles are lipid bilayer-wrapped vesicles approximately 30–200 nanometers in size, released by virtually every cell type in the body. Once thought to be a mechanism for cellular refuse disposal, EVs are now known to be key mediators of cell-to-cell communication, delivering a cargo of lipids, proteins and nucleic acids that reflects their cell of origin. Although significantly simpler than live MSCs, EVs are still highly complex in composition and exert their effects by either transferring bioactive cargo such as functional protein and miRNAs that alter cell fate, or by modulating cell surface

receptors and triggering intracellular signaling pathways (Xin et al., 2012; Guo et al., 2017).

In the case of MSC-derived EVs, they have been found to share many attributes such as content, immunophenotype and unique homing ability (tropism) as their parental MSCs (Wei et al., 2021). As a result, their intrinsic therapeutic ability has been heavily investigated in recent years. MSC-EVs have been demonstrated as a potential treatment for several clinical indications including graft-versus-host disease (Kordelas et al., 2014), kidney injury (Nassar et al., 2016), myocardial infarction (Lai et al., 2010), systemic lupus erythematosus (Liu et al., 2015) and wound healing (Guo et al., 2017), and represent a novel therapeutic modality that could overcome roadblocks faced by MSC-based cell therapies (**Figure 1**).

The Advantages of EVs Compared to Cell-Based Therapies

The nature of EVs helps overcome several technological challenges faced by MSC-based therapy. Firstly, due to their phospholipid bilayer EVs are more resistant to damage caused by freeze-thaw cycles (Jeyaram and Jay, 2017) and possess high *in vivo* stability (Dang et al., 2020). In mice, intravenously injected MSC-EVs labeled with a bioluminescent dye were found to have an EV plasma half-life of 1.2–1.3 min due to their rapid uptake into tissues, where they were still detectable 24 h post-injection (Shelke et al., 2018). Secondly, as EVs do not self-replicate, they bypass the risk of endogenous tumor-formation that accompanies MSC-based therapy (Volarevic et al., 2018). Moreover, their small size as well as low or lack of expression of membrane histocompatibility complexes reduces the risk of inducing immune responses (Reis et al., 2016).

Extracellular vesicles also represent a novel strategy for hard-to-treat diseases for which there is a high unmet medical need. For example, in the treatment of neurological disorders some studies have shown that MSCs have difficulty transmigrating across the blood–brain barrier (BBB). In one study, MSCs were found to be retained in the lungs (Wang et al., 2010), while another reported that MSCs required a permeabilizing agent to cross the BBB (Cerri et al., 2015). Conversely, MSC-EVs have been shown to cross the BBB offering solutions in terms of route of administration and dosage (Moon et al., 2019). An example of their potential utility demonstrates that they can induce immunomodulatory and neuroprotective effects in a 3xTg model of Alzheimer's disease (Losurdo et al., 2020) after systemic administration. MSC-EVs can also regulate neuronal cell apoptosis *in vitro* (Wei et al., 2020).

As EVs can be continually harvested from a MSC population, they can potentially overcome some of the issues of limited supply associated with MSCs themselves. As MSCs are highly amenable to modification, EV production can potentially be boosted to improve scalability. For example, the application of various stimuli to the parent cells, including physical stressors such as hypoxia or mechanical forces, or the addition of various small molecule modulators, can increase EV release (Zhu et al., 2017; Wang et al., 2020). MSCs have also been shown to produce significantly higher yields of EVs when grown in three-dimensional bioreactors rather than two-dimensional cell culture, and the EVs produced in these reactors can show enhanced therapeutic properties (Yan and Wu, 2020).

From the perspective of drug manufacturing, EVs offer several logistical advantages over cell-based therapies. These includes the possible addition of sterile filtration of the drug substance before aseptic filling, unfeasible for whole cells (Gimona et al., 2017). EVs are also suited to a wider range of storage conditions including flexibility in storage buffers as well as preservation techniques (Kusuma et al., 2018). Methods such as lyophilisation has been successfully utilized to store EVs (Frank et al., 2018) leading to an extended shelf-life and reduced costs due to a simplified cold chain (Kusuma et al., 2018). These characteristics suggest EV therapeutics could be developed as "off-the-shelf" products.

Bioengineering to Broaden EV Therapeutic Utility

The therapeutic application of MSC-derived EVs is not limited to naïve, i.e., naturally secreted, EVs. The parent cell or its secreted EVs can be bioengineered to generate an EV product with enhanced or altered therapeutic properties. EV engineering strategies can be divided into two broad categories. Firstly, EVs can be engineered to alter their tropism and bias their uptake toward a target cell type. Alternatively, or in addition, EVs can be loaded with a particular therapeutic cargo.

Engineering EVs for Altered Tropism

A key factor determining the potential therapeutic utility of MSC-EVs is their fate once infused into the body. Several biodistribution studies have used dye- or bioluminescent-labeled naïve EVs to observe EV uptake by different tissues following infusion into animals (Wiklander et al., 2015; Shelke et al., 2018).

In a study on MSC-EVs labeled with a near-infrared lipophilic dye, more than 70% of EVs were found to accumulate in the liver when systemically injected into mice (Wiklander et al., 2015). Other animal studies have analyzed labeled naive EVs from a range of cell sources and shown that the EVs distribute widely through the body but accumulate primarily in the liver and spleen (Charoenviriyakul et al., 2017; Shelke et al., 2018). This natural tropism may be exploited to treat conditions associated with these organs. However, to enhance EV accumulation in other organs, EV engineering may be considered. This approach has been received favorably by some biopharma companies who are incorporating it into their developmental pipeline for "hard-to-treat disease," e.g., cancer (Lewis et al., 2021).

Engineered EV (EEV) *in vivo* tropism may be altered by incorporating selected surface proteins onto their surface. One method for conferring targeting capabilities onto EVs is to manipulate the parent cell. One route is to create fusion proteins, in which the protein tail attaches or inserts into the EV membrane, and the head binds a receptor on the target cell type. In one early example, a fusion protein created between EV membrane protein Lamp2b and the rabies viral glycoprotein (RVG) peptide which binds the acetylcholine receptor on brain cells. These EEVs were used to target neurons, microglia and oligodendrocytes in the brain after systemic injection (Lydia et al., 2011). More recently, Lu et al. showed that a glycoprotein-g

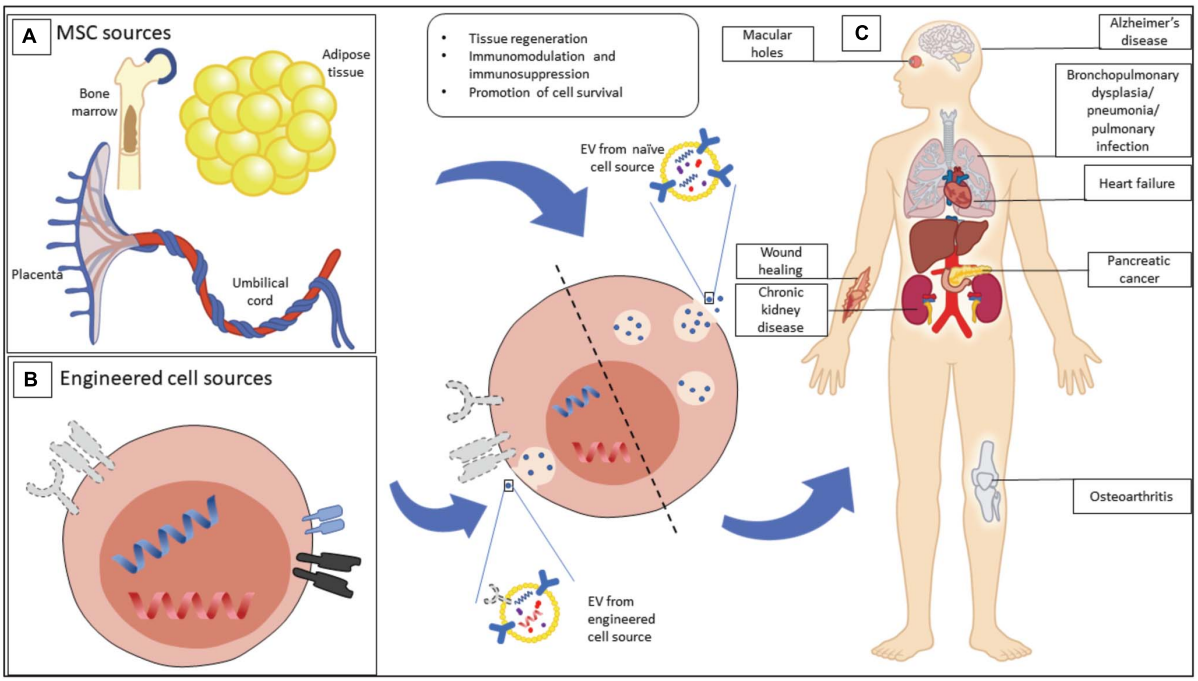

FIGURE 1 | Mesenchymal stromal cell (MSC) EVs have therapeutic potential for a range of possible indications. **(A)** MSCs, as a source of MSC-EVs, can be isolated from a wide range of tissues, including bone marrow, adipose tissue, and birth-associated tissue. **(B)** MSCs can be engineered in order to enhance their output of EVs, or to produce EVs with particular properties. **(C)** Beneficial properties of EVs from naive and engineered sources are being explored for a range of clinical indications.

originally identified on the vesicular stomatitis virus, VSV-G, can be readily incorporated into nascent EV's membrane by the parent cell. In addition, a large section of the VSV-G protein can be substituted for a "tropic" protein (Meyer et al., 2017). This approach has been shown to target EEVs to specific cell types including B cells, neurons and tumor cells (Yang et al., 2018).

Targeting proteins can also be incorporated onto EVs after their isolation from cell culture. Tian et al. (2021) recently showed that fusion proteins that bind the phosphatidylserine component of the EV lipid membrane can be used to target EVs to ischemic brain tissue after systemic injection, relieving inflammation. The targeting protein was attached to EVs by simple incubation. A similar approach has also been used to target tumor cells in a mouse model of glioblastoma (Zhilan et al., 2018).

As well as altering EV tropism, proteins can be added to the vesicle surface for a range of other applications. For example, EEVs with protein receptors on their surface have been developed as decoys to capture target molecules such as the pro-inflammatory cytokine IL-6, as a potential therapeutic for chronic inflammatory diseases (Conceição et al., 2021). Another study demonstrated that EEVs engineered to express CD47 on their surface have reduced systemic clearance, which extends circulation time to improve targeting of pancreatic cancer cells (Kamerkar et al., 2017).

Engineering EVs as Therapeutic Delivery Vehicles

Mesenchymal stromal cell-EVs are known to possess a cargo that can promote regeneration in damaged tissues, for example by lowering inflammation and inhibiting apoptosis to promote healing. EV engineering allows the possibility of loading EVs with a defined therapeutic cargo that could enhance this capability. MSCs have been engineered to overexpress microRNA-let7c, to generate EEVs which contain elevated levels of this microRNA. Infusion of these MSC-derived EEVs attenuated renal fibrosis in a mouse model of unilateral ureteral obstruction (Wang et al., 2016). More recently, MSC engineered to overexpress bone morphogenetic protein 2 produced EEVs with an enhanced capacity to promote bone regeneration (Chun-Chieh et al., 2020).

However, EV drug loading methodologies also enable the potential development of EEV therapeutics for a wide range of indications beyond tissue regeneration and healing. The key characteristics of EVs–non-immunogenic capsules able to deliver cargo to a target cell type–appear to make them ideal as drug delivery vehicles, such as for targeting tumor cells.

Many therapeutics, from small molecule drugs to RNAs, need to be shepherded to their site of action, either to overcome unfavorable pharmacokinetics, as protection from metabolic breakdown, or to reduce off-target effects. Synthetic delivery systems such as liposomes or nanoparticles have been limited by poor stability in storage, toxicity, poor efficacy and a limited capability to deliver cargo to target tissues other than the liver (Setten et al., 2019).

As knowledge surrounding EV biogenesis and mechanisms of cargo sorting has increased, new strategies have emerged to generate EEVs that contain a defined therapeutic cargo.

The techniques associated with the generation of EEVs include incubation with drugs for delivery (Pascucci et al., 2014) or transfection of the parent cells to allow specific small RNA and small molecules to be incorporated into their EVs. For example, MSCs have been engineered to produce EVs enriched in the microRNA miR-379, a potent tumor suppressor. In a mouse model of breast cancer, systemic administration of the miR-379 EEVs significantly reduced tumor growth–whereas administration of the engineered parental MSCs themselves had no impact on tumor growth (O'Brien et al., 2018).

In head-to-head testing, EVs were recently shown to be superior to liposomes for RNA transfer, delivering a cargo of RNA several orders of magnitude more efficiently into cells (Murphy et al., 2021). The biggest hurdle, the researchers noted, was to efficiently load the RNA cargo into EVs–but emerging technologies can offer highly effective ways to accomplish EV RNA loading. Nguyen and Ferguson at the University at Buffalo identified RNA sequences that are specifically loaded by cells into EVs. By attaching therapeutic RNA to these sequences, up to 100-fold gains in loading of the therapeutic RNA into EVs was achieved (Nguyen and Ferguson, 2018).

Extracellular vesicle loading can also take place after EV isolation from cell culture. Techniques including freeze-thawing, sonication, electroporation, osmotic shock and saponin permeabilization have been developed to temporarily disrupt the EV membrane sufficient for uptake of a therapeutic cargo. However, as the cargo loading method itself may alter the biophysical characteristics or biological function of treated EVs, and the loading efficiency can vary between different types of cargo, care must be taken to determine the appropriate cargo-loading methodology in each case (Franziska et al., 2021).

Clinical Application of EVs and EEVs

The progress made in EV pre-clinical studies and the additional advantages of EVs relative to whole cell therapeutics has led to a growing number of MSC-EV human clinical trials. Over 80 studies have been registered at the www.ClinicalTrials.gov database to assess the therapeutic effects of EVs in several therapeutic arenas. MSC-EVs have demonstrated acceptable safety and tolerability profiles, and promising signs of therapeutic efficacy (**Table 2**).

In one early study, for example, repeated injections of MSC-EVs demonstrated significant improvement in a patient with severe therapy-refractory acute graft-versus-host disease. The patient's pro-inflammatory cytokine levels and disease symptoms improved markedly after MSC-EVs therapy, and the patient's improvement remained stable for at least 4 months after treatment (Kordelas et al., 2014). In a Phase II/III placebo-controlled clinical trial, umbilical cord blood derived MSC-EVs administration was shown to be safe, and to modulate inflammation and improve kidney function, in patients with chronic kidney disease (Nassar et al., 2016).

Studies currently underway include a safety study of the use of bone marrow MSC-EVs to treat bronchopulmonary dysplasia, a chronic lung disease that mainly affects premature babies (NCT03857841); a Phase I study of MSC-EVs loaded with a siRNA therapeutic for patients with pancreatic cancer (NCT03608631); and a study to assess MSC-EV administration for improvement of disability in patients with acute ischemic stroke (NCT03384433).

There are also studies underway to test inhaled MSC-EVs in patients with acute respiratory distress syndrome (ARDS) associated with severe cases of COVID-19 (NCT04602442; NCT04602104). **Table 2** summarizes clinical trials that have been performed using EVs isolated from MSCs.

Extracellular vesicles from other sources than MSCs have also been used in clinical trials. Significantly, in addition to the early phase trials conducted by academic groups, several trials of experimental EV therapeutic products developed by biopharmaceutical companies are now underway, reflecting the increasing maturity of the therapeutic EV field. These trials include platelet derived EVs tested by Exopharm in two Phase I clinical trials for wound healing applications (ANZCTR, 2019, 2020). Codiak Biosciences is currently investigating engineering the HEK293 cell line for their clinical applications, and in late 2020 commenced two Phase I clinical trials testing the safety and efficacy of EEVs for certain cancers (Clinicaltrials.gov, 2020).

Despite rapid development in the EV field and the commencement of clinical trials, several challenges remain and should be resolved before EVs can achieve widespread clinical utility. These challenges are discussed in the following section.

CHALLENGES IN THE CLINICAL APPLICATION OF EVs

Although regulatory approval and safety of clinical application of EVs appears feasible, and may be simpler than cell-based therapy, important barriers that need to be addressed for clinical GMP-grade production of EVs include selection of the starting cellular material and optimizing cell culture, purification, quantification, and quality control (Gowen et al., 2020). Understanding and determining the quantity, purity and potency of EVs, by the development of appropriate assays, will be an important part of the quality control criteria (Gimona et al., 2021). Further challenges include issues of EV preservation and long-term stability, as we also go on to describe below.

Identifying Ideal EV Cell Sources

As EV are a secreted product of cells, their manufacture is heavily dependent on the ability to produce large quantities of cells in ways that do not alter their phenotype. However, the opportunities for producing large quantities of stem-cell based conditioned medium with which to undertake meaningful scale-up of EV production are limited.

Generally, for stem cell derived EVs, the potential cell sources for EV production are either MSCs, or pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) or iPSCs derived lineages. In each case there are advantages and disadvantages associated with their clinical use, which are summarized in **Table 3**.

Primary or somatic MSCs may not be an ideal cell source for EV manufacture at clinical scale due to their limited lifespan,

TABLE 2 | List of registered clinical trials using MSC derived EVs.

EV source	Application	Clinical phase	Status	References
BM-MSC	Graft-versus-host disease	Individual patient	Completed	10.1038/leu.2014.41
Wharton's jelly MSC	Chronic ulcer wounds	Phase I	Completed	NCT04134676
AD-MSC	COVID-19 associated pneumonia	Phase I	Completed	NCT04276987
MSC	COVID-19 associated pneumonia	Phase I/II	Completed	NCT04491240
UC-MSC	Chronic kidney disease	Phase II/III	Completed	10.1186/s40824-016-0068-0
UC-MSC	Macular holes	Early Phase I	Ongoing	NCT03437759
AD-MSC	Periodontitis	Early Phase I	Ongoing	NCT04270006
MSC	Pancreatic cancer	Phase I	Ongoing	NCT03608631
BM-MSC	Tolerance study on aerosol inhalation of MSC-EVs in healthy volunteers	Phase I	Ongoing	NCT04313647
MSC	Cerebrovascular disorders	Phase I/II	Ongoing	NCT03384433
AD-MSC	Pulmonary infection	Phase I/II	Ongoing	NCT04544215
AD-MSC	Alzheimer's disease	Phase I/II	Ongoing	NCT04388982
UC-MSC	Dry eye in patients with chronic graft-versus-host disease	Phase II	Ongoing	NCT04213248
BM-MSC	Bronchopulmonary dysplasia	Phase I	Active, not recruiting	NCT03857841
BM-MSC	COVID-19 associated acute respiratory distress syndrome	Expanded access protocol	Available	NCT04657458
UC-MSC	Type I diabetes	Phase II/III	Unknown	NCT02138331
AD-MSC	Osteoarthritis	n/a	Not yet recruiting	NCT04223622
MSC	Multiple organ dysfunction syndrome following aortic dissection repair	n/a	Not yet recruiting	NCT04356300
AD-MSC	Osteoarthritis	Phase I	Not yet recruiting	NCT04223622
BM-MSC	Dystrophic epidermolysis bullosa	Phase I/II	Not yet recruiting	NCT04173650
BM-MSC	COVID-19 associated acute respiratory distress syndrome	Phase II	Not yet recruiting	NCT04493242

TABLE 3 | Comparison of cell sources for large scale production of EVs.

	Somatic tissue derived MSCs	ESCs	iPSCs	ESC/iPSC derived MSCs	HEK293
Quantity	Variable, depending on donors	Reliable, can be derived from a single hPSCs line	Reliable, can be derived from a single hPSCs line	Reliable, can be derived from a single hPSCs line	Reliable, can be derived from a single hPSCs line
Pathogens	Possible and hard to control from sources	Rare and easy to control from sources	Rare and easy to control from sources	Rare and easy to control from sources	Rare and easy to control from the sources
Cell number	Limited	Unlimited	Unlimited	Unlimited	Unlimited
Cell Homogeneity	Medium	Medium	Medium	High	High
Cost	High	Medium	Medium	Medium	Low
Differentiation efficiency	High	High	High	Low	Low
Proliferation	Slow	Fast	Fast	Fast	Fast
Immunomodulatory effects	High	Low	Low	High	?
Potency	Medium	High	High	High	?
Senescence	Faster	Slow	Slow	Slow	Slow
Genome editing	Hard	Easy	Easy	Easy	Easy
Level of risk overall	Low	High	High	Medium	Medium
References	Mastrolia et al., 2019	Thomson et al., 1998	Keisuke et al., 2007; Akira et al., 2013	Xing-Liang et al., 2018; Ozay et al., 2019; Bloor et al., 2020	Jing et al., 2016; Magdalena et al., 2020

heterogeneity and batch-to-batch or donor-to-donor variations. One approach to increase the yield of EVs from MSCs is to immortalize MSCs using the hTERT method or a CRISPR/Cas9-based strategy, to enable long term cell culture and scaled up EV production. Recently, EVs from immortalized MSCs were shown to be non-tumorigenic both *in vitro* and *in vivo*, neither promoting nor inhibiting tumor growth (Tan et al., 2021).

Alternatively, pluripotent cells could be exploited as source cells. As stated previously, the ideal cell source for industrial-scale EV production should have unrestricted availability, regulatorily

acceptable provenance, present no biological safety risks, and be amenable to unlimited expansion while retaining its original "as harvested" phenotype. The only cell types which can have most or least some of these characteristics are ESCs and iPSCs. The main advantage of these cells is their unlimited capacity and growth. The main disadvantages of these cells are the ethical concern in the case of ESCs, and the likelihood of immune responses and risk of teratoma for iPSCs.

An approach to exploit the benefits of pluripotent cells, but navigate their disadvantages, could be to incorporate iPSCs as a source of MSCs for EV manufacture. This approach might increase the yield and the homogeneity of the cell source for industrial scale production of EVs. The same scenario can also be applied to ESCs, by directly differentiating ESCs to MSCs. The MSCs derived from these sources have shown similar properties to adult MSCs, including their differentiation potential and immunomodulatory effect (Bloor et al., 2020). Several studies have demonstrated that these cell lines have superior efficacy over MSCs derived from somatic tissues (Jiang et al., 2019). The unique advantages of PSC derived MSCs over adult MSCs include unlimited supply, high purity, lower cost and most importantly, scalable production. The process of manufacturing clinical GMP-grade MSCs involves donor identification, screening, tissue harvest, cell isolation, purification and expansion which require a theater and clinician involvement as well as GMP facilities each time. Since this whole process must be repeated several times due to the limited expansion of adult MSCs compared with iPSC derived MSCs, this can increase the cost of GMP-grade production of adult MSCs compared with iPSC derived MSC line. These factors are all important for the development of a cell system for MSC-EV manufacture.

Alternate EV Cell Sources for EEV Applications

For therapeutic applications in which EVs are employed for their drug delivery capabilities rather than for their inherent regenerative properties, a broader range of EV cell sources can be considered. One potentially important cell source being explored for the mass production of EEVs are human stable cell lines such as HEK293. Currently, HEK293 cells are predominantly used for recombinant protein production and vector transfection. HEK293 cell derived EVs are a promising platform from which to produce EEVs modified specifically for the clinical indication of interest.

The main advantages of HEK293 are that this cell line proliferates rapidly, can be easily grown in serum-free suspension culture, and importantly is FDA-approved as a human cell line for recombinant protein production. As such, this cell type has become a particular focus for biotechnology companies developing EEV therapeutics. For instance, Codiak Bioscience is using HEK293 to produce engineered EVs as a potential cancer therapy and is conducting clinical trials using HEK293 engineered cells as an EEV source for the treatment of cutaneous T cell lymphoma and solid tumors (Dooley et al., 2021). In addition, ILIAS Biologics researchers and their collaborators have used HEK239-derived EEVs as a platform for several experimental therapeutics. These EEVs were recently used to deliver an inhibitor of transcription factor NF-κB, to prolong pregnancy in a mouse model of pre-term birth (Sheller-Miller et al., 2021).

Although the HEK293-derived EEV production system may offer potential manufacturing advantages, the potency, efficacy and associated biological risk factor of EV generated from this cell line need to be evaluated in both *in vitro* and *in vivo* systems.

Meeting GMP-Grade Processing Requirements for Clinical EV Production

Once a reliable cell source for industrial-scale EV production is identified, issues remain in the upstream and downstream processing and quality control of the resulting EVs.

Purity is one significant issue that needs to be addressed for GMP-grade production of EVs. Research labs engaged in EV research, development and pre-clinical studies predominantly use media formulations containing fetal bovine serum (FBS) for cell culture and EV collection, which cannot be applied for clinical application (Gottipamula et al., 2013; Pachler et al., 2017). Although ultracentrifuged, EV-depleted FBS is often used by researchers and academician, not all the EV contaminants can be removed (Zhiyun et al., 2016; Shelke et al., 2018). The use of human platelet lysate (hPL) instead of FBS may solve the problem (Torreggiani et al., 2014). Ideally when manufacturing GMP-grade EVs, serum-free and chemically defined media are safest for EV collection, preventing the inadvertent introduction of contaminating EVs in the source serum (Pachler et al., 2017). However, switching from serum to serum-free cell culture media causes cell stress which in turn may lead to production of EVs with different cargo and profile. Therefore, cell culture conditions must be carefully considered. Large-scale culture systems that align with GMP requirements for the production of master and working cell banks also need to be adopted. Hollow fiber and stirred-tank bioreactors are the more promising approaches because they are closed, scalable, GMP-compatible systems that provide a high surface-to-volume ratio for cell growth (Mendt et al., 2018).

Large-scale isolation and purification of EVs is one of the most significant challenges in downstream processing for clinical GMP-grade EV production. Ultracentrifugation (UC) remains the most popular method of EV isolation, despite ongoing concerns over product purity, and that UC protocols are not directly scalable for clinical-scale manufacture (Casulli et al., 2018; Colao et al., 2018). Alternative purification methods such as those based on scalable and high throughput ion exchange protocols and size exclusion chromatography with membrane filtration such as tangential flow filtration (TFF), are being investigated as a promising way around this significant roadblock (Law et al., 2021). EV purification should ideally utilize methods such as filtration and chromatography that are currently available for the manufacture of biologics, as these methods are already demonstrated to be GMP-compliant.

As different isolation and purification methods have an impact on EV characteristics and produce different population of EVs, the need for standardized GMP is emphasized (Agnes et al., 2018). MISEV guidelines lay out basic standards for EV markers, but specific standardized universal markers are still lacking for

EV characterization, and further investigation is required to fully characterize EV populations to establish their identity.

Quantification of EVs is another fundamental issue which remains challenging. How to accurately measure and assess EV purity is a critical issue when evaluating EV dosage for both pre-clinical and clinical applications. At present, there is no single method that can accurately measure EVs. One of the most common approaches to measuring EVs is determining the total amount of proteins and particle numbers. There are several methods and instruments measuring EV numbers including nanoparticle tracking analysis (NTA), resistive pulse sensing (RPS), and dynamic light scattering (DLS). However, copurification of other proteins by measuring total protein concentration and current particle tracking approaches might be biased toward a designated EV size range which does not discriminate EVs from other nanoparticulate materials. Recent developments in this field include nanoflow cytometry, imaging flow cytometry, and ExoCounter with optic disk technology to quantify EVs (Sotiris et al., 2018; Hartjes et al., 2019). A combination of flow cytometry-based methods for analyzing the membrane surface markers and quantification of EVs may represent improvements in the assessment of EV purity and yield.

For GMP compliance, fill and finish of the final product should be performed in a closed system. This might present as a logistical challenge for small biotech companies and academic labs operating at research scale (Rohde et al., 2019). Overall, the currently available methods and technologies for purification, quantification and characterization of EVs are inconsistent and GMP standards need to be developed that are reproducible, practical and scalable for clinical GMP-grade EV application. However, with better know-how and strategic decisions early on in development, GMP-grade EV preparations can be successfully produced for clinical administration (Mendt et al., 2018).

Finally, storage and long-term stability of EVs is another important issue if EVs are to be used as a third party "off the shelf" product. EV quantity decreased in a dose-dependent manner at room temperature, at 4°C, −20°C, but not when frozen in −80°C, therefore most protocols store EVs at −80°C long term without any reported changes in EV profile and structure (Lõrincz et al., 2014). Preservation condition and storage solution buffer is also very important as EVs are sensitive to shifts in pH. The majority of published studies have used phosphate-buffered saline (PBS) and some have used sucrose and trehalose buffers (Steffi et al., 2016; Busatto et al., 2018; Li et al., 2019). Preservation conditions need to be further optimized and validated in both *in vitro* and *in vivo* assays to ensure post-thaw potency of the EVs product is retained (Chung et al., 2021). Alternatively, EV lyophilization has been successfully utilized to store EVs and may offer a simpler option for transport and storage (Frank et al., 2018; Kusuma et al., 2018). Standardized GMP-grade methods to define specific storage condition including temperature, buffer solution, pH and duration must be addressed for clinical applications of EVs to be advanced.

CONCLUSION

Experimental MSC therapies continue to show promise in pre-clinical research. This promise, however, is still to translate into widespread clinical use. The inherent complexity of a live cell product presents considerable challenges to successful therapeutic translation. Although cell engineering approaches may address some of these challenges, the potential risks associated with cell-based therapeutics, such as tumorigenicity and undesired differentiation, and limitations such as rejection of cells and poor engraftment, will remain.

Mesenchymal stromal cell-EVs, as a cell-free product, offer a potential pathway through these challenges. An increasing number of studies have shown that the therapeutic effects of MSC-EVs are equal to those of the MSCs. The use of EVs rather than MSCs protects against issues of tumorigenicity, immunogenicity, and poor engraftment. In addition, EV-based therapeutic manufacture, as well as transport and storage, promises to be more readily scalable at lower cost than live cell MSC therapeutics. However, to achieve their full clinical potential and to avoid some of the pitfalls that have contributed to clinical trial failures of MSC therapeutics, EVs need to be thoroughly investigated in terms of purification, quantification, characterization and potency before their clinical use. Genetic modification of the source cells for large scale manufacture of MSC-EVs may serve as an effective strategy to further improve the therapeutic effect of MSC derived cell-free products. The increasing sophistication with which EVs from a variety of cell sources can be engineered, to enhance their homing capabilities and bolster their therapeutic cargo, further demonstrates the considerable potential of this versatile therapeutic platform.

AUTHOR CONTRIBUTIONS

RK, JJ, JMC, and MS designed and wrote the content, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Decreased Insulin Sensitivity in Telomerase-Immortalized Mesenchymal Stem Cells Affects Efficacy and Outcome of Adipogenic Differentiation *in vitro*

Konstantin Kulebyakin[1,2], Pyotr Tyurin-Kuzmin[1], Anastasia Efimenko[1,2], Nikita Voloshin[1], Anton Kartoshkin[1], Maxim Karagyaur[1,2], Olga Grigorieva[2], Ekaterina Novoseletskaya[2], Veronika Sysoeva[1], Pavel Makarevich[1,2] and Vsevolod Tkachuk[1,2]*

[1] Department of Biochemistry and Molecular Medicine, Faculty of Medicine, Lomonosov Moscow State University, Moscow, Russia, [2] Institute for Regenerative Medicine, Medical Research and Education Center, Lomonosov Moscow State University, Moscow, Russia

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Katrina Adlerz, University of Texas MD Anderson Cancer Center, United States

***Correspondence:**
Konstantin Kulebyakin
konstantin-kuleb@mail.ru

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Modern biomedical science still experiences a significant need for easy and reliable sources of human cells. They are used to investigate pathological processes underlying disease, conduct pharmacological studies, and eventually applied as a therapeutic product in regenerative medicine. For decades, the pool of adult mesenchymal stem/stromal cells (MSCs) remains a promising source of stem and progenitor cells. Their isolation is more feasible than most other stem cells from human donors, yet they have a fair share of drawbacks. They include significant variability between donors, loss of potency, and transformation during long-term culture, which may impact the efficacy and reproducibility of research. One possible solution is a derivation of immortalized MSCs lines which receive a broader use in many medical and biological studies. In the present work, we demonstrated that in the most widely spread commercially available hTERT-immortalized MSCs cell line ASC52telo, sensitivity to hormonal stimuli was reduced, affecting their differentiation efficacy. Furthermore, we found that immortalized MSCs have impaired insulin-dependent and cAMP-dependent signaling, which impairs their adipogenic, but not osteogenic or chondrogenic, potential under experimental conditions. Our findings indicate that hTERT-immortalized MSCs may present a suboptimal choice for studies involving modeling or investigation of hormonal sensitivity.

Keywords: mesenchymal stem/stromal cells, osteogenic differentiation, chondrogenic differentiation, adipogenic differentiation, insulin signaling, protein kinase A signaling

INTRODUCTION

Adult human mesenchymal stem/stromal cells (MSCs) were initially isolated from the bone marrow by Friedenstein et al. (1966); subsequently, MSCs have been isolated from most other tissues (Caplan and Correa, 2011). These cells play a pivotal role in maintaining normal tissue function via the support and formation of a stromal environment for tissue-specific cells. This function relies on

Abbreviations: MSC, Mesenchymal stem/stromal cells; hTERT, Human telomerase reverse transcriptase; IBMX, 3-isobutyl-1-methylxanthine; PKA, Protein kinase A.

the 1) differentiation potential of MSCs, which includes several lineages and multiple mature cell types: osteoblasts, adipocytes, chondroblasts, or fibroblasts 2) the production of tissue matrix components and paracrine regulatory factors.

This activity has also become the foundation for MSC application in regenerative medicine in its cell therapy field. Multiple experimental studies in damage models have demonstrated that MSCs participate in repair of various tissues and their recovery after injury (Qian et al., 2008; Natsumeda et al., 2017). Indications for MSC-based therapies include a wide range of conditions from bone fractures (Harris et al., 2004; Wang X. et al., 2018), cardiovascular diseases (Duran et al., 2013) to the restoration of male fertility (Sagaradze et al., 2019), and control adipose tissue function and systemic insulin sensitivity (Wang M. et al., 2018). The latter relies on the fact that MSCs are the precursors for the majority of newly formed adipocytes (Sanchez-Gurmaches and Guertin, 2014). That makes the MSCs a relevant object for both therapy and preclinical studies in light of the global spread of obesity and associated metabolic syndrome (Kopelman, 2000).

Understanding the participation of MSCs in tissue renewal, repair, and potential modulation of their physiological properties for regenerative medicine demands appropriate and relevant *in vitro* models to investigate MSC biology (Jimenez-Puerta et al., 2020). However, as an object in biomedical research, cultured primary MSCs show certain disadvantages: donor-to-donor variability and limitations during large-scale expansion which are accompanied by the fact that MSCs isolated from different tissues also exhibit significant variability (Elahi et al., 2016). A possible solution of listed problems is application of immortalized lines derived from primary human MSCs. These cells occupy a good intermediate position between highly variable primary cell cultures and lines derived from tumor cells characterized by significantly altered physiology.

One of the methods to obtain an immortalized cell line is introducing the gene encoding telomerase (TERT), an enzyme that provides restoration for telomere regions of chromosomes and thereby increases the number of possible cell divisions (Bodnar et al., 1998). Lines of hTERT-immortalized MSCs have recently become spread in biological and medical research as a substitute for primary MSCs. They were used in studies of MSC function in maintaining tissue homeostasis (Pitrone et al., 2017; Maj et al., 2018) and to create scaffolds for tissue engineering (Zitnay et al., 2018). In most studies using hTERT-immortalized MSCs, authors assumed them to be generally similar to primary human MSCs. At the same time, despite the active introduction of hTERT-immortalized MSCs in research few studies focus on functional similarities and differences between hTERT-MSC and primary MSCs and how these differences may affect the outcome of experimental studies.

We have shown that hTERT-MSC exhibited altered hormonal sensitivity compared to primary MSC culture obtained from healthy donors: in particular, they have significantly reduced sensitivity to noradrenaline (Tyurin-Kuzmin et al., 2018). As far as the sensitivity of MSCs to hormones plays a decisive role in control of their differentiation, the question of how differentiation properties of MSCs change when they are immortalized is yet to be answered. In the present work, we compared the phenotype and functional properties of hTERT-MSC and primary MSCs, focusing on differentiation to "classical" (adipogenic, chondrogenic, and osteogenic) directions.

MATERIALS AND METHODS

Cell Cultures

hTERT-immortalized, adipose-derived mesenchymal stem cells (ASC52telo, ATCC® SCRC-4,000^{TM}) were maintained in the medium supporting the growth of undifferentiated mesenchymal progenitor cells (Advance Stem Cell Basal Medium; HyClone, Logan, UT, United States) containing 10% of supplement (Advance Stem Cell Growth Supplement, HyClone) and 100 U/ml of penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Waltham, MA, United States). The medium was changed every 2–3 days. Cells were passaged at ≈80% confluency. All experiments were performed with cells from 15 to 25 passages.

Primary cells used in the presented study were obtained from four donors who gave their informed consent. The local ethics committee of the Medical Research and Education Center of Lomonosov Moscow State University (Moscow, Russia) approved the study protocol (#4, 04.06.2018). All donors were younger than 55, with BMI $\leq$ 25. Subcutaneous adipose tissue samples (0.5–5 ml) harvested during surgery were homogenized and digested in collagenase I (200 U/ml; Worthington Biochemical; Lakewood, NJ, United States) and dispase (40 U/ml; Sigma-Aldrich, St. Louis, MO, United States) solution under agitation for 30–40 min at 37°C. The tissue was then centrifuged at 200 × *g* for 10 min, and the supernatant was discarded. The pellet containing ADSC was lysed to destroy erythrocytes, filtered through a sieve (BD Falcon Cell Strainer, 100 μm; BD, Franklin Lakes, NJ, United States), and centrifuged at 200 × *g* for 10 min. The final pellet was resuspended in a culture medium. Cells were cultured in the medium supporting the growth of undifferentiated mesenchymal progenitor cells (Advance Stem Cell Basal Medium, HyClone) containing 10% of supplement (Advance Stem Cell Growth Supplement, HyClone), 100 U/ml of penicillin/streptomycin (Gibco), and 0.292 mg/ml L-glutamine (Pen Strep Glutamine, Gibco) at 37°C in a 5% CO_2 incubator. The medium was changed every 3–4 days. Cells were passaged at 80% confluence using a HyQTase solution (HyClone). All experiments were performed with cells within five passages. MSC quantity and viability were assessed using the Countess cell counter (Invitrogen; Thermo Fisher Scientific). Proliferation activity for both cell lines was compared in the automated IncuCyte® ZOOM Live Cell Analysis System (Essen Bioscience, Ann Arbor, MI, United States).

Cell Proliferation Assay

Adipose tissue-derived MSCs of the first passage were placed in a six-well plate (100,000 cells/well). The plate was placed in an automated IncuCyte® ZOOM Live Cell Analysis System (Essen Bioscience) for *in vitro* imaging of the cell culture. The survey was carried out at a frequency of once per hour for 96 h (16 fields of view/well). The device's built-in software makes it possible to

estimate the area occupied by cells by applying a "mask" to the images obtained and thus calculate the percentage of cell culture confluence. An increase in confluence directly correlates with an increase in the number of cells, making it possible to evaluate the growth rate of a cell culture by the calculated parameters.

Flow Cytometry

MSC immunophenotype was analyzed using flow cytometry. After medium harvesting, cells were detached from culture dishes using the Versene solution and stained with antibodies against CD73, CD90, and CD105 (MSC Phenotyping Kit; Miltenyi Biotec, Auburn, CA, United States), as described in the manufacturer's protocol. IgGs of appropriate isotype were used as a negative control. Stained cells were analyzed using FACS ARIA III cell sorter (BD).

For analysis of expression of the insulin receptor, cells were stained with antibodies to INSR alpha subunit (Thermo Fisher Scientific).

MSC Differentiation Assays

The ability of MSC to differentiate to osteogenic, adipogenic, and chondrogenic lineages was tested *in vitro* using standard differentiation and analysis protocols described elsewhere. Briefly, cells were cultured in 12-well culture plates up to 100% confluence in all differentiation experiments. Adipogenic differentiation was induced by incubation in the growth medium based on DMEM with low glucose (HyClone) containing 10% of FBS (HyClone), 1 μMdexamethasone, 200 μM insulin, and 0.5 mM 3-isobutyl-1-methylxanthine (Millipore, Billerica, MA, United States) for 21 days. The medium was changed every 2 days. Alternatively, the adipogenic differentiation medium contained 1 mkM dexamethasone, 200 μM insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 100 μM indomethacin—agonist of PPARγ (Millipore) (Puhl et al., 2015) for 21 days. The medium was changed every 2 days.

Osteogenic differentiation was induced by incubating MSCs on collagen- and vitronectin-covered plates in a growth medium containing StemPro Osteogenesis Differentiation Kit (Thermo Fisher Scientific) for up to 21 days. The medium was changed every 2 days. Alternatively, osteogenic differentiation was induced by incubation in the growth medium based on DMEM with low glucose (HyClone) containing 10% of FBS (HyClone) and 10 μM dexamethasone + 0.2 mM ascorbic acid + 10 mM of glycerol-2-phosphate (Millipore) for up to 21 days. The medium was changed every 2 days. Differentiation efficiency was analyzed using Alizarin Red S staining. Chondrogenic differentiation was induced by incubating pelleted MSCs in StemPro Chondrogenic Differentiation Medium (Thermo Fisher Scientific) for 28 days. The medium was changed every 2 days.

Staining of Differentiated Cultures

Cell cultures were stained with Oil Red O (Millipore) to determine the efficiency of adipogenic differentiation. Cells were fixed with 4% paraformaldehyde for 30 min. They were then stained with 0.4% Oil Red O in 60% isopropanol for 1 h.

Alizarin Red was used to estimate the efficiency of osteogenic differentiation. Cells were fixed with 10% paraformaldehyde for 30 min. They were then stained using the Osteogenesis Quantitation Kit (Millipore) following the manufacturer's instructions.

Toluidine blue staining was used to visualize chondrogenic differentiation. Cells were fixed with 4% paraformaldehyde for 30 min. They were then stained with 0.4% Toluidine Blue in 0.2 M acetate buffer at pH 4.0.

Obtained samples were analyzed via Leica AF6000 microscope with a DFC 420C camera. To evaluate the effectiveness of adipogenic differentiation, we analyzed the percentage of cells bearing lipid droplets to the total number of cells in 12 independent fields of view for each experiment using the ImageJ software (NIH, Bethesda, MD, United States).

RT-PCR

The RNeasy Mini Kit (Qiagen) was used to extract RNA. cDNA was synthesized from 500 ng RNA with the MMLV Reverse Transcription Kit (Evrogen, Moscow, Russia) according to the manufacturer's instructions. The relative expression of gene-markers of osteogenic (RUNX2, osteocalcin) and adipogenic (PPARγ, adiponectin) differentiation was analyzed by quantitative real-time PCR. The following equipment were used: qPCR mix-HS SYBR + LowROX (Evrogen) reagents and CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States). The gene of 60S Ribosomal protein P0 (RPL0) was used as a housekeeping gene. Quantification and normalization of expression levels of the target genes and the reference gene (RPLP0) were calculated using the comparative threshold cycle (CT) method. Primers for PCR were picked using the NCBI Primer Designing Tool. Primer sequences are presented in the **Table 1**.

Gel Electrophoresis and Western Blotting

Cell protein samples were obtained via cell lysis in a sample buffer (62.5 mM Tris-HCl pH 6.8, 2.5% SDS, 0.002% Bromophenol Blue, 5% β-mercaptoethanol, 10% glycerol). Proteins were divided by the SDS-PAGE method. Afterward, proteins were transferred from polyacrylamide gel to the PVDF membrane by Western blotting. TBS containing 0.1% Tween-20 and 5% BSA (PanEco, Moscow, Russia) was used to prevent non-specific binding. The next step was overnight staining of the membrane with protein-specific antibodies to phosphorylated T308 Akt [Anti p-Akt (Thr308) (244F9) Rabbit mAb #4056; Cell Signaling Technology Inc., Danvers, MA, United States] and Vinculin (Anti Vinculin Rabbit antibody V4139; Sigma-Aldrich). Unbound antibodies were then washed away, and the rest were incubated with antibodies for total rabbit immunoglobulins conjugated with peroxidase (P-GAR Iss; IMTEK, Moscow, Russia) for 1 h. Amplified chemiluminescence was used as a visualization method with a Clarity ECL detection kit (Bio-Rad). Image registration was carried out using the ChemiDoc Touch gel documenting system (Bio-Rad). Image analysis and volume measurements were performed using the Image Lab Software (Bio-Rad). Total Akt staining volume readings were normalized to the respective vinculin level, and then volume readings for p-Akt were compared with respective normalized Akt.

TABLE 1 | List of primers used in the present work.

Target	Gene	Primers	Amplicon size (bp)
60S Ribosomal protein P0	RPL0	F: GCTGCTGCCCGTGCTGGTG R: TGGTGCCCCTGGAGATTTTAGTGG	130
Adiponectin	ADIPOQ	F: GACCAGGAAACCACGACTCA R: TTTCACCGATGTCTCCCTTAGG	199
Peroxisome proliferator-activated receptor gamma	PPARγ	F: TCAGGTTTGGGCGGATGC R: TCAGCGGGAAGGACTTTATGTATG	147
Runt-related transcription factor 2	RUNX2	F: TCTTAGAACAAATTCTGCCCTTT R: TGCTTTGGTCTTGAAATCACA	136
Osteocalcin	OCN	F: AGCAAAGGTGCAGCCTTTGT R: GCGCCTGGGTCTCTTCACT	63

Registration of PKA Activity Using PKA-Spark Sensor

To deliver the cAMP sensor to hTERT-MSCs, the lenti-PKA-SPARK construct was used as described earlier (Tyurin-Kuzmin et al., 2020). Lentiviral particles (LVPs) were assembled in HEK293T cells using the standard PEI transfection protocol (Longo et al., 2013). The conditioned medium containing LVPs was collected 48–72 h post-transfection and separated from cell debris by centrifugation at 4,000 × *g*, 4°C for 15 min. Protamine sulfate (50 μg/ml) was added to the medium containing LVPs to increase transduction efficiency. Cells were cultured on 24-well plates, incubated in the medium containing LVPs, and subjected to centrifugation at 800 × *g* RT for 1.5 h to assist virus entry. After centrifugation, the medium was changed to the standard culture medium, and cells were incubated for ∼8 h. The transduction procedure was repeated two times. Between transductions, the virus stock was stored at + 4°C.

To register PKA activation with PKA-Spark, we grew transduced cells in 24- or 48-well plates at low densities to prevent cell-to-cell communications during imaging of signaling. Before the experiment, the growth medium was changed to Hanks Balanced Salt Solution (PanEco) with 20 mM Hepes (HyClone). Cells were treated with 10^{-6} M of forskolin (Abcam, Cambridge, United Kingdom) or 10^{-4} M of direct activator of protein kinase A 6-Bnz-cAMP (Biolog, Bremen, Germany) after registration of the basal activity of the cells. Activation of PKA was measured in individual cells using an inverted fluorescent microscope Nikon Eclipse Ti equipped with an objective CFI Plan Fluor DLL 10 × /0.3 (Nikon, Tokyo, Japan) and digital cooled monochrome CCD camera Nikon DS-Qi1 (Nikon). We used the simultaneous measuring of 6 × 6 fields of view in Large Image mode to increase the number of analyzed cells. Movies were analyzed using NIS-Elements (Nikon) and ImageJ software (NIH).

Statistical Data Analysis

Experimental data were expressed as means ± standard deviation (SD). Because of the size of experimental groups, we used the Mann–Whitney *U* test, which was performed with the Statistica 6.0 software (StatSoft, Tulsa, OK, United States), and statistical significance was accepted at $p < 0.05$.

RESULTS

hTERT-MSCs Demonstrate Immunophenotype Similar to Primary MSC and a Higher Proliferation Rate

Both adipose-derived primary MSCs and hTERT-MSCs showed spindle-like shape morphology (**Figure 1A**) in culture and shared a similar immunophenotype characteristic for multipotent MSC (Dominici et al., 2006; **Figure 1B**). As expected, hTERT-MSCs had significantly higher proliferation activity than primary MSCs: from 1.25- up to a 3-fold decrease of population doubling time (PDT). Still, the growth curve shapes were comparable for both studied cell lines (**Figure 1C**).

hTERT-MSCs Demonstrate Reduced Adipogenic, but Not Osteogenic or Chondrogenic Potential Compared to Primary MSC

Both cell lines were effectively differentiated into osteogenic and chondrogenic lineages under specific inductive conditions (**Figure 2A**). There were no detectable differences between primary MSCs and hTERT-MSCs supported by similar dynamics of respective marker gene expression in both cell lines (**Figure 2B**).

However, when subjected to adipogenic inductive conditions, hTERT-MSCs demonstrated a significantly impaired ability to differentiate into adipocytes compared to primary MSC. After 21 days of induction, hTERT-MSCs contained <10% of morphologically differentiated adipocytes (**Figures 3A,B**), whereas in primary MSC culture >65% of cells already accumulated lipid droplets. Marker gene expression concordantly showed a dramatic difference between hTERT-MSCs and primary MSC (**Figure 3C**). The latter demonstrated a significant stable increase in expression of both PPARγ (a master-gene of adipogenic differentiation) and adiponectin (an adipokine upregulated in several orders of magnitude during differentiation to adipocytes). In hTERT-MSCs, PPARγ expression remained relatively stable throughout the differentiation timeline. However, it was significantly lower than in primary MSC at Day 21 of the experiment despite increased baseline (Day 0) expression vs. primary MSC without induction of adipogenesis.

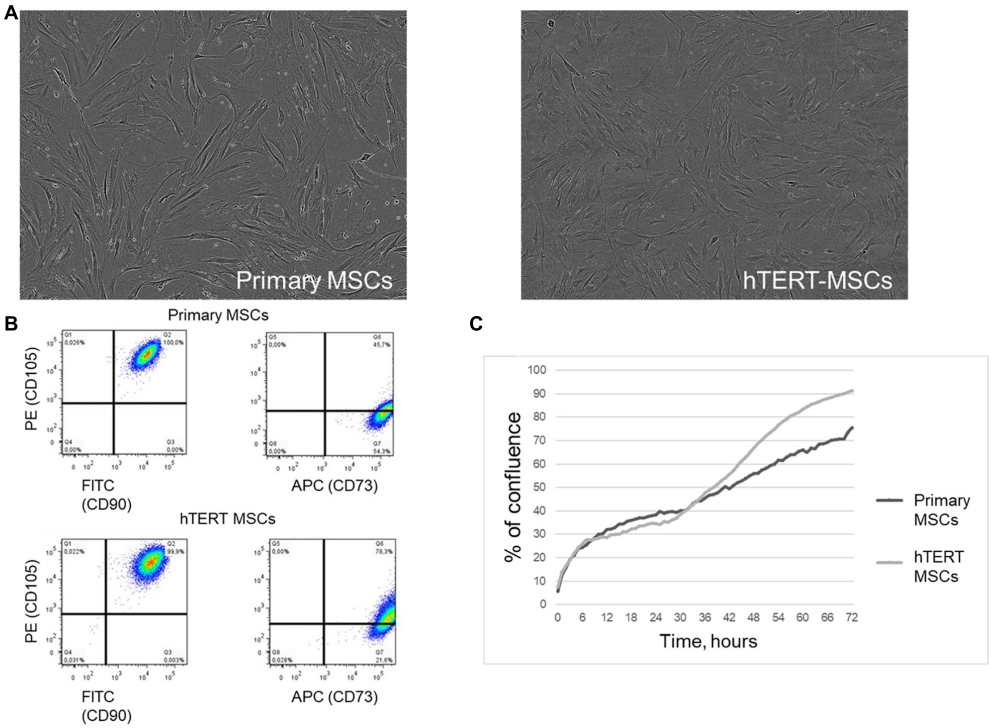

FIGURE 1 | Human adipose-derived primary MSCs and hTERT-MSCs share similar morphology and immunophenotype characteristics of multipotent MSCs. **(A)** Morphology of cultivated MSCs and hTERT-MSCs, phase contrast microscopy, magn. ×200. **(B)** Evaluation of MSC markers by flow cytometry. More than 99% of MSCs express CD73, CD90, and CD105. **(C)** Proliferation curves of primary MSCs and hTERT-MSCs over a 72-h time-lapse in IncucyteZOOM. Presented data refer to the $n = 8$ independent experiments on primary MSCs from four different donors.

At day 21, adiponectin expression in hTERT-MSCs was also significantly lower than in primary MSCs.

Overall, comparison of differentiation potential in primary and immortalized MSCs suggested that hTERT-based immortalization of these cells impaired their potential for adipogenic, but not osteogenic or chondrogenic, differentiation under conventional inductive conditions.

In hTERT-MSC PPARγ Agonist, Indomethacin Restores Their Ability for Adipogenic Differentiation

Observed decline of adipogenic potential in hTERT-MSC may result from two putative factors: (1) decreased number of cells capable for adipogenic differentiation or (2) disrupted hormonal sensitivity, which reduced cells' sensitivity to factors inducing adipogenesis.

To address the first hypothesis, cells were subjected to adipogenic differentiation in the presence of indomethacin—a direct PPARγ agonist (Puhl et al., 2015) able to stimulate adipogenesis bypassing hormonal signaling pathways that regulate it under physiological conditions (Cristancho and Lazar, 2011).

As seen in **Figures 4A,B**, indomethacin effectively abolished differences between hTERT-MSC and primary MSC in adipogenic differentiation. After 21 days of differentiation, both cell lines demonstrated similar lipid droplet accumulation and the percentage of differentiated cells (**Figure 4B**). While the dynamics of adiponectin expression became similar between primary and hTERT-immortalized MSCs, PPARγ expression still differed dramatically between cell lines (**Figure 4C**). These results correspond with indomethacin mechanism of action. It has been shown that direct PPARγ activators (such as indomethacin and rosiglitazone), while promoting the expression of PPARγ targets such as adiponectin, did not increase expression of this transcription factor itself (Muhlhausler et al., 2009). An increase in PPARγ expression requires the activation of the classical insulin/PI3K/Akt signaling pathway (Rieusset et al., 1999). This suggests that disrupted hormonal sensitivity was a more probable cause of differences in adipogenesis in these cell lines.

Insulin-Dependent and cAMP-Dependent Signaling Are Impaired in hTERT-MSC

To address the hormonal sensitivity of hTERT-MSCs, we evaluated the activity of two major adipogenic pathways in these cells, namely, insulin-dependent and cAMP-dependent signaling. Insulin is a primary adipogenic hormone *in vivo* and an indispensable component in most differentiation protocols (Accili and Taylor, 1991; Cristancho and Lazar, 2011; Scott et al., 2011).

We found no differences in expression of insulin receptors between primary MSC and hTERT MSC

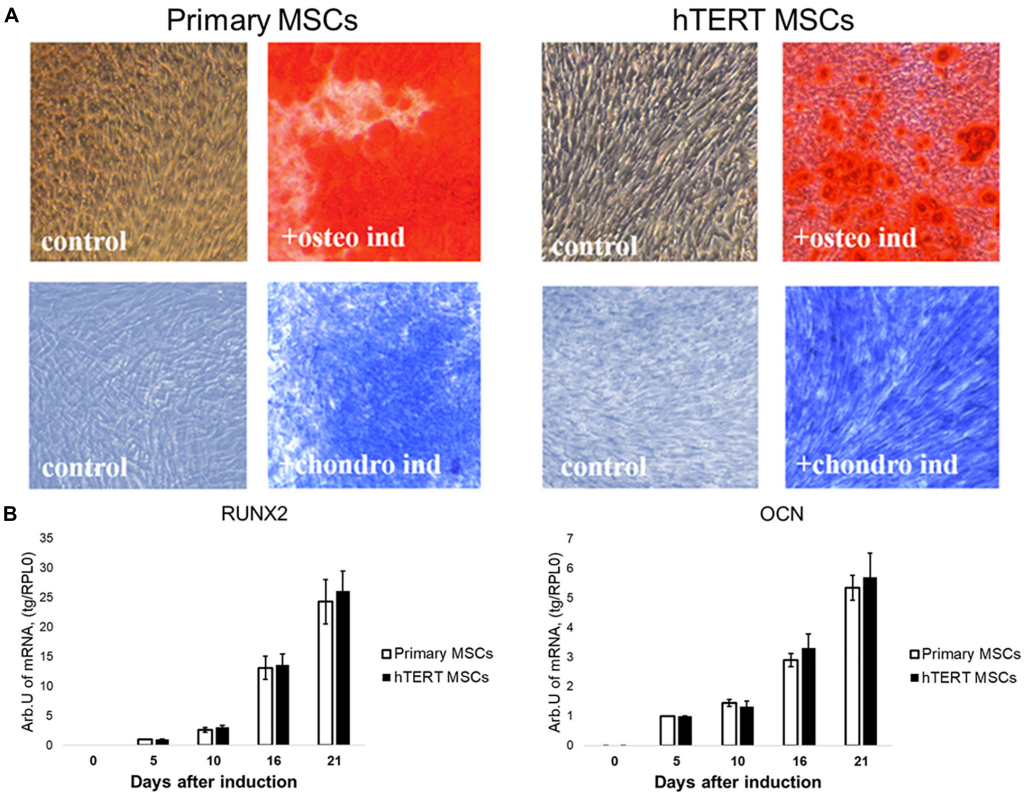

FIGURE 2 | Human adipose-derived primary MSCs and hTERT-MSCs show comparable osteogenic and chondrogenic differentiation potential. Both studied MSCs differentiated into osteocytes (Alizarin Red S staining, "osteo ind") and chondrocytes (Toluidine Blue staining, "chondro ind") under inductive conditions. Groups "control" related to the cells cultured in standard conditions without differentiation induction. **(A)** Representative images, phase contrast microscopy, 10× objective. **(B)** Temporal dynamics of marker gene expression during differentiation in MSCs and hTERT-MSC; RT-PCR. Results are presented as mean ± SD, $n = 6$ independent experiments on primary MSCs from three different donors analyzed with Mann–Whitney *U* test.

(**Supplementary Figure 1**). As seen from **Figure 5**, primary MSCs responded to insulin by increased Akt phosphorylation. At the same time, hTERT-MSCs failed to react similarly after treatment by insulin in the same concentration. Furthermore, hTERT-MSCs demonstrated a significantly higher basal level of phosphorylated Akt. Therefore, the lack of p-AKT increase in response to a respective hormonal stimulus in hTERT-MSCs can be described as insulin resistance. Interestingly, in hTERT-MSC pattern of Akt activation corresponded with PPARγ expression—a high basal level was accompanied by the lack of upregulation after insulin treatment. In light of Akt being a crucial activator of PPARγ expression (Peng et al., 2003), this may explain the inability of hTERT-MSC to upregulate PPARγ expression during differentiation (**Figures 3C**, **4C**).

Another significant component in the medium used for adipogenic differentiation is IBMX (3-isobutyl-1-methylxanthine), which inhibits cellular phosphodiesterase and increases cAMP concentration (Parsons et al., 1988), resulting in activation of PKA. We used the PKA-Spark sensor to evaluate the status of cAMP-dependent signaling in hTERT-MSCs (**Figure 6A**).

As seen from **Figure 6B**, only a small percentage of cells in hTERT-MSCs (solid black bars) can respond via the cAMP-dependent pathway resulting in PKA activation. For example, a direct activator of adenylyl cyclase forskolin invoked response in 21% ± 4.3% of cells, and an activator of PKA 6-Bnz-cAMP leads to response of 29% ± 5.2% of cells in the population. In contrast primary, MSCs (white bars on **Figure 6B**) showed 48.2% ± 4.7% sensitivity to forskolin and 91.2% ± 2.8% to 6-Bnz-cAMP as we reported earlier (Tyurin-Kuzmin et al., 2020).

Presented data suggest that in hTERT-MSCs both insulin-dependent signaling and cAMP-dependent signaling are significantly impaired, which explains their reduced ability for adipogenesis after stimulation by hormones in particularly, insulin—a major regulator of metabolism and adipose tissue turnover.

DISCUSSION

Immortalized MSCs are becoming widespread as a model object for fundamental research in stem cell biology, tissue engineering, and regenerative biology (Nardone et al., 2017; Maj et al., 2018; Sim et al., 2018; Zitnay et al., 2018). From a practical perspective, they possess many features that allow their reproducible and feasible use in research. Immortalized MSCs are devoid of

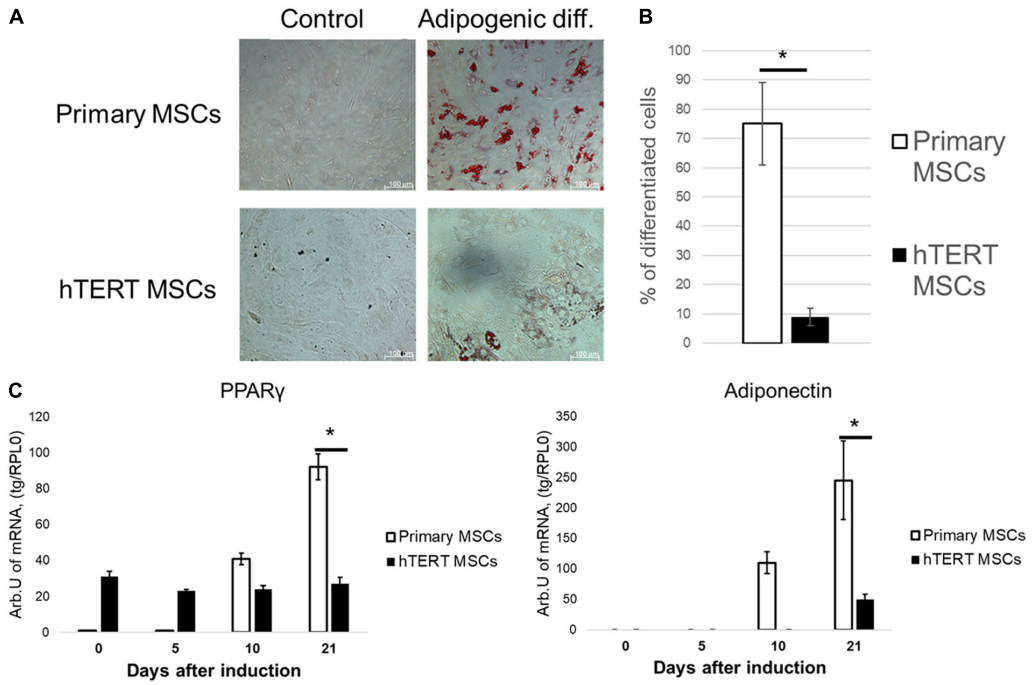

FIGURE 3 | Human adipose-derived primary MSCs and hTERT-MSCs demonstrate significant differences in adipogenic potential. MSC differentiated into adipocytes (Oil Red O staining, "Adipogenic diff.") under inductive conditions. Groups "control" related to the cells cultured in standard conditions without differentiation induction. **(A)** Representative images, phase-contrast microscopy. **(B)** Relative efficiency of adipogenic differentiation of primary MSCs and hTERT-MSCs. **(C)** Temporal dynamic of marker gene expression during differentiation of MSCs and hTERT-MSC. Results are presented as mean ± SD, $n = 8$ independent experiments on primary MSCs from four different donors. *Mann–Whitney U test, $p < 0.05$.

disadvantages existing in primary MSCs: variability between cell samples obtained from different donors, limited ability to retain their physiological properties during passage in culture, and well-known resistance to most non-viral gene delivery approaches which limits their modification. This makes immortalized MSCs a promising object for practical applications in tissue engineering and a potentially valuable object for experimental models that can be used to establish molecular mechanisms of tissue renewal, particularly the differentiation of adult multipotent cells.

Notably, accumulating evidence supports the idea that MSCs could be a promising tool for cell-based therapy, primarily due to their multipotency, anti-inflammatory, and immunomodulatory properties as well as interactions with their immediate surroundings to provide regenerative cell-based responses. Furthermore, the medical utility of MSCs continues to be investigated in more than 1,000 clinical trials with a broad spectrum of diseases (reviewed in detail in Pittenger et al., 2019). Immortalized MSC advantages suggest they would benefit translational studies and overcome limitations associated with primary MSCs (Piñeiro-Ramil et al., 2019). Specifically, these cells might be preferred for studies investigating the therapeutic potential of MSC secretome. Thus, we recently reported that the extracellular matrix, produced by hTERT-immortalized MSCs, could potentiate the sensitivity of the primary stem cells to differentiation stimuli and hold a promising option to functionalize biomaterials for tissue engineering (Novoseletskaya et al., 2020).

In this work we found that the most widely commercially available immortalized MSC cell line ASC52telo shows significant alteration of differentiation potential compared to primary adipose-derived MSCs. While retaining osteogenic and chondrogenic differentiation in hTERT-MSCs comparable to primary cells, their ability to differentiate to adipocytes was significantly reduced (**Figures 3A,B,C**). This is probably an outcome of the TERT-based immortalization procedure. Addition of indomethacin, a direct activator of an adipogenic master regulator (PPARγ), restored hTERT-MSCs adipogenic potential (**Figures 4A,B,C**) indicating that hTERT-MSCs possess an ability for adipogenic differentiation, which corresponds with previous studies of hTERT-MSC characteristics in which indomethacin was used as an additional stimulator of adipogenic differentiation (Wolbank et al., 2009).

We also found that in hTERT-MSCs, two pivotal signaling cascades determining adipogenic differentiation, namely, insulin signaling and cAMP-dependent cascade, are markedly impaired. Western blotting analysis showed that hTERT-MSCs have significantly upregulated the basal level of Akt phosphorylation and were unable to increase Akt phosphorylation in response to insulin. The latter presents a phenomenon generally observed in insulin-resistant cells (Liu et al., 2009). This abnormal Akt phosphorylation pattern may explain the differences in adipogenic differentiation between primary MSCs and hTERT-MSC. Usually, during adipogenesis, insulin upregulates PPARγ through Akt, which mediates the expression of a

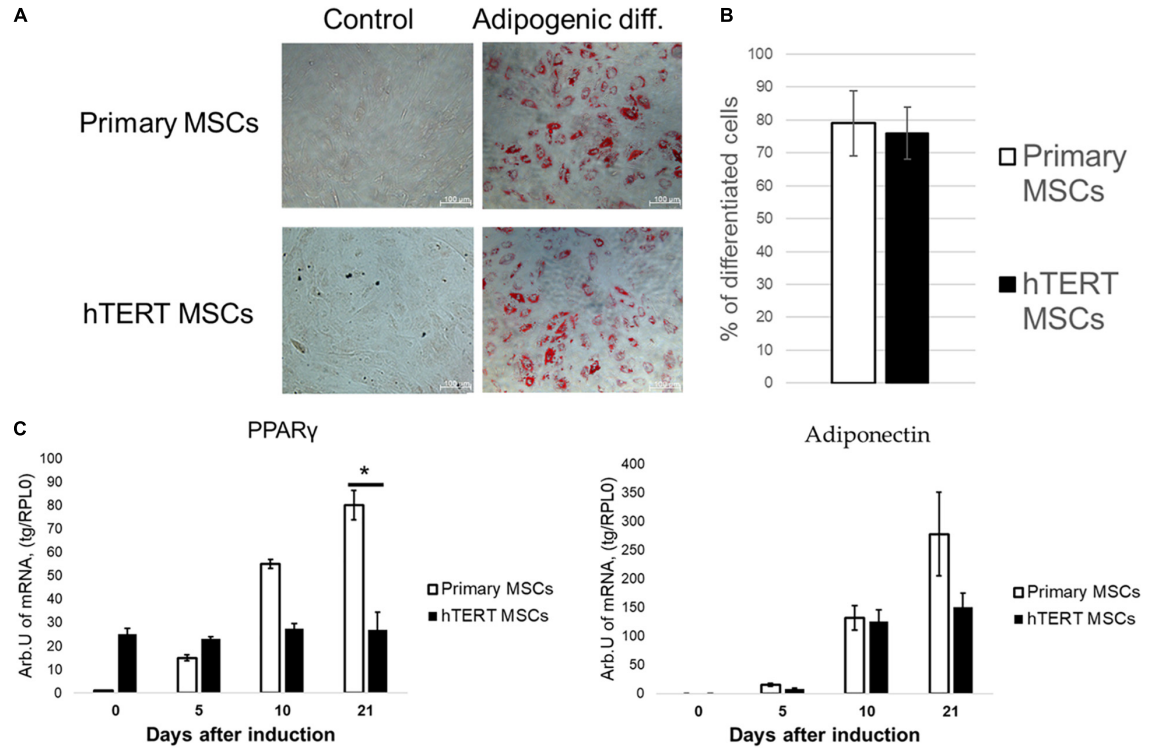

FIGURE 4 | Indomethacin reduces differences in adipogenic potential between human adipose-derived primary MSCs and hTERT-MSCs. MSC differentiated into adipocytes (Oil Red O staining, "Adipogenic diff.") under inductive conditions in the presence of indomethacin. Group "control" related to the cells cultured in standard conditions without differentiation induction. **(A)** Representative images, phase-contrast microscopy. **(B)** Relative efficiency of adipogenic differentiation of primary MSCs and hTERT-MSCs. **(C)** Temporal dynamic of marker gene expression during differentiation in MSCs and hTERT-MSC. Results are presented as mean ± SD, $n = 8$ independent experiments on primary MSCs from four different donors. *Mann–Whitney U test, $p < 0.05$.

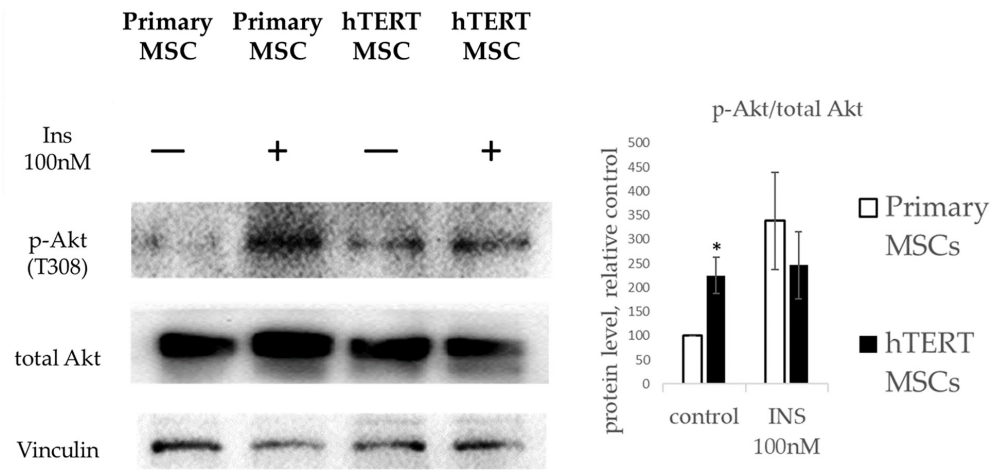

FIGURE 5 | Insulin-dependent Akt phosphorylation in primary MSC and hTERT-MSC. P-Akt levels in cells are normalized to p-Akt levels in untreated primary MSCs (control). Results are presented as mean ± SD, $n = 4$ independent experiments on primary MSCs from four different donors. *Mann–Whitney U test, $p < 0.05$.

variety of adipogenic genes (Rieusset et al., 1999; Peng et al., 2003; Zhang et al., 2009; Martinez et al., 2020). We propose that, in hTERT-MSCs, this process is severely dysregulated, resulting in a lack of adipogenesis in insulin-based differentiation protocol. In presence of direct PPARγ activator (indomethacin) however, hTERT-MSC can undergo adipogenesis, bypassing the required activation of PPARγ by insulin. However, this process is not accompanied by canonical upregulation of PPARγ expression but only by increased expression of PPARγ-regulated genes. This corresponds with

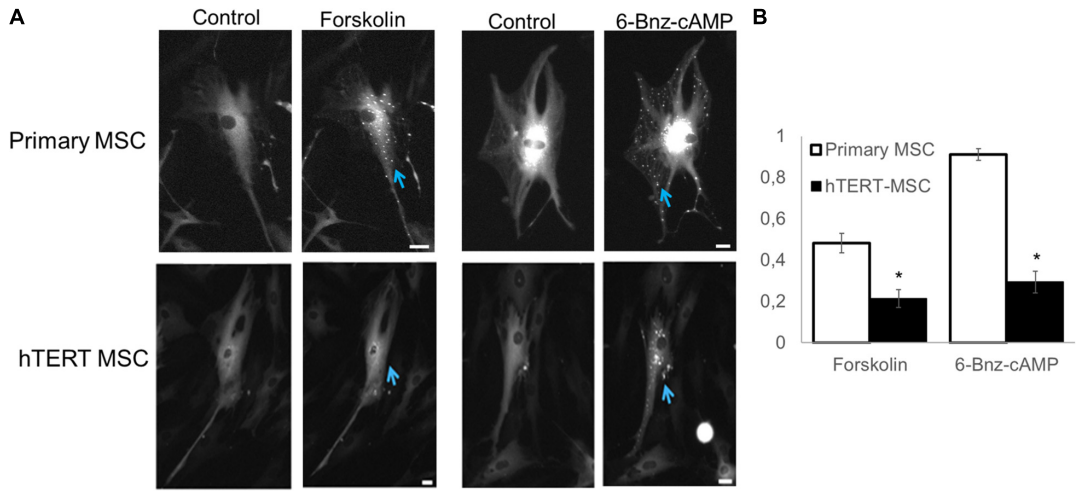

FIGURE 6 | Detection of PKA activation using PKA-Spark sensor**. (A)** Representative images of sensor response to different stimuli. **(B)** Comparing the percentage of responsive cells in primary and hTERT-immortalized MSC culture. Results are presented as mean ± SD, $n = 4$ independent experiments on primary MSCs from two different donors. *Mann–Whitney U test, $p < 0.05$.

described effect of direct PPARγ activators on its own expression (Muhlhausler et al., 2009).

Using a genetically encoded PKA activity sensor, we found that the cAMP-dependent signaling pathway is also significantly disrupted in hTERT-MSCs. Typically, all cells in the total population of primary MSCs bear functional PKA, activated by 6-Bnz-cAMP treatment (Tyurin-Kuzmin et al., 2020). However, in hTERT-MSCs, 6-Bnz-cAMP induces a specific response only in 30% of cells. Using forskolin, a direct activator of adenylyl-cyclase, we observed that in primary MSCs, 50% of cells carry a functional adenylyl-cyclase, while in hTERT-MSC, less than 25% of the population. Suggesting the decreased ability of hTERT-MSC to respond to cAMP-dependent hormonal stimuli.

Dysregulation of hormonal signaling may explain why adipogenic differentiation of MSC was exclusively affected by immortalization. The majority of commonly used protocols for osteogenic or chondrogenic differentiation relies not on activation of membrane receptors and downstream signaling pathways but, on the application of steroid hormones which activate transcription factors and promote extracellular matrix production via the addition of direct activators of key enzymes (e.g., the addition of ascorbic acid for prolyl-hydroxylase). To some extent, this may be expanded to the induction of adipogenesis. Indeed, using a protocol with direct activation of transcription factor PPARγ by indomethacin, we effectively differentiated both primary and hTERT-immortalized MSCs. However, a "more physiological" approach with hormonal stimulation by insulin has proven ineffective for hTERT-MSC.

The decrease in the hormonal sensitivity of MSCs after immortalization may be related to the procedure used to establish the ASC52Telo line. To do such, primary adipose tissue MSCs have been subject to retroviral transduction to deliver the hTERT gene with subsequent clonal cell selection by an antibiotic-like compound G418 (Wolbank et al., 2009). In recent years, the heterogeneity of MSCs and their role in their functional activity have been actively discussed. Moreover, published data describe specific populations of MSCs that take the regulatory role and control differentiation processes in surrounding cells (Schwalie et al., 2018).

Our group has previously described distinct subpopulations of MSCs with dramatic differences in hormonal sensitivity and associated changes of functional activity, including adipogenic differentiation (Sysoeva et al., 2017; Ageeva et al., 2018). Furthermore, during selection whithin heterogeneous cell populations, only clones with high proliferative potential gain advantage resulting in marginal elimination of subpopulations with slower proliferation (Shakiba et al., 2019). In case of hTERT-immortalized MSC, this may have resulted in loss of MSC subpopulations that determine hormonal sensitivity and differentiation of surrounding cells. Thus, despite their convenience hTERT-MSC as a tool to investigate certain aspects of MSCs physiology, should be used with caution to evaluate hormonal signaling and control of differentiation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the local ethics committee of the Medical Research and Education Center of Lomonosov Moscow State University (Moscow, Russia). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KK, PT-K, and AE: Conceptualization. KK, PT-K, AE, OG, EN, and VS: Data curation. KK, PM, and VT: Funding acquisition. KK, PT-K, NV, AK, and EN: Investigation. MK, OG, and VS: Methodology. KK, PT-K, AE, PM, and VT: Writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.662078/full#supplementary-material

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Regenerative Medicine for the Treatment of Ischemic Heart Disease; Status and Future Perspectives

Babak Arjmand[1,2†‡], Mina Abedi[1], Maryam Arabi[1], Sepideh Alavi-Moghadam[1‡], Mostafa Rezaei-Tavirani[3†‡], Mahdieh Hadavandkhani[1‡], Akram Tayanloo-Beik[1†‡], Ramin Kordi[4], Peyvand Parhizkar Roudsari[2] and Bagher Larijani[5*†‡]*

[1] Cell Therapy and Regenerative Medicine Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran, [2] Metabolomics and Genomics Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran, [3] Proteomics Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [4] Sports Medicine Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran, [5] Endocrinology and Metabolism Research Center, Endocrinology and Metabolism Clinical Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran

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Chunying Li,
Georgia State University, United States

***Correspondence:**
Babak Arjmand
barjmand@sina.tums.ac.ir
Bagher Larijani
emrc@tums.ac.ir

†ORCID:
Babak Arjmand
orcid.org/0000-0001-5001-5006
Mostafa Rezaei-Tavirani
orcid.org/0000-0003-1767-7475
Akram Tayanloo-Beik
orcid.org/0000-0001-8370-9557
Bagher Larijani
orcid.org/0000-0001-5386-7597

‡Scopus ID:
Babak Arjmand
Scopus ID: 24485067100
Sepideh Alavi-Moghadam
Scopus ID: 57204949703
Mostafa Rezaei-Tavirani
Scopus ID: 57199354719
Mahdieh Hadavandkhani
Scopus ID: 57204931916
Akram Tayanloo-Beik
Scopus ID: 57204860185
Bagher Larijani
Scopus ID: 57193118609

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Cardiovascular disease is now the leading cause of adult death in the world. According to new estimates from the World Health Organization, myocardial infarction (MI) is responsible for four out of every five deaths due to cardiovascular disease. Conventional treatments of MI are taking aspirin and nitroglycerin as intermediate treatments and injecting antithrombotic agents within the first 3 h after MI. Coronary artery bypass grafting and percutaneous coronary intervention are the most common long term treatments. Since none of these interventions will fully regenerate the infarcted myocardium, there is value in pursuing more innovative therapeutic approaches. Regenerative medicine is an innovative interdisciplinary method for rebuilding, replacing, or repairing the missed part of different organs in the body, as similar as possible to the primary structure. In recent years, regenerative medicine has been widely utilized as a treatment for ischemic heart disease (one of the most fatal factors around the world) to repair the lost part of the heart by using stem cells. Here, the development of mesenchymal stem cells causes a breakthrough in the treatment of different cardiovascular diseases. They are easily obtainable from different sources, and expanded and enriched easily, with no need for immunosuppressing agents before transplantation, and fewer possibilities of genetic abnormality accompany them through multiple passages. The production of new cardiomyocytes can result from the transplantation of different types of stem cells. Accordingly, due to its remarkable benefits, stem cell therapy has received attention in recent years as it provides a drug-free and surgical treatment for patients and encourages a more safe and feasible cardiac repair. Although different clinical trials have reported on the promising benefits of stem cell therapy, there is still uncertainty about its mechanism of action. It is important to conduct different preclinical and clinical studies to explore the exact mechanism of

action of the cells. After reviewing the pathophysiology of MI, this study addresses the role of tissue regeneration using various materials, including different types of stem cells. It proves some appropriate data about the importance of ethical problems, which leads to future perspectives on this scientific method.

Keywords: heart diseases, ischemia, regenerative medicine, stem cells, tissue engineering

INTRODUCTION

Ischemic heart disease (IHD) mostly appears as myocardial infarction (MI) and is one of the most fatal factors for patients around the world (Sahoo and Losordo, 2014; Sharma et al., 2017; Johnson et al., 2019). MI is a pathologic state of the heart that leads to the death of myocytes because of poor blood supply in ischemic conditions (Frangogiannis, 2011). In chronic hypoxic conditions of IHD, some cardiomyocytes are replaced by scar tissue and the others attempt to reduce their energy demands. Moreover, their contractility power also decreases (Fisher et al., 2016). In recent years, regenerative medicine has been widely utilized as a treatment for IHD to repair the lost part of the heart by using stem cells (Fisher et al., 2016). Transplantation of stem cells to the infarcted site can lead to the production of new cardiomyocytes (Michler, 2018). Regenerative medicine is an interdisciplinary approach to restoring, replacing, or repairing the damaged parts of various organs in the body (Glotzbach et al., 2011). Accordingly, different types of stem cells can be used for cardiac cell therapy, including xenogeneic stem cells from nonhuman species, allogeneic ones from human donors, and autologous cells from and implanted into the same person (Alrefai et al., 2015). In other words, it seems that by cell therapy, paracrine factors such as cytokines, chemokines, growth factors, etc. provide an anti-apoptotic and anti-fibrotic state in addition to enhancing endogenous cardiac regeneration and power of contractility (Michler, 2018). Although the therapeutic efficiency of cell therapy makes it a suitable choice for cardiac repair, there is no evidence to show the mechanism of its benefit in the production of heart muscle cells and vessels in humans. It is, therefore, important to explore regenerative medicine and understand the ways it can help in treating cardiac disease. The next step in this field is to find an efficient way of assessing the pros and cons of cardiac cell therapy and recognize if it improves IHD or not. Different categories of strategies are available to evaluate the progression, by using equipment like magnetic resonance imaging (MRI), positron emission tomography (PET), and single-photon emission CT (SPECT) (Dorobantu et al., 2017).

Abbreviations: 3D, three-dimensional; ADSCs, adipose derived stem cells; AMI, acute myocardial infarction; bFGF, basic fibroblast growth factor; BM, bone marrow; BMD, bone marrow derived; BMSC, bone marrow derived stromal cell; BMP4, bone morphogenetic protein 4; CABG, coronary artery bypass graft; CSCs, cardiac stem cells; CST, clusterin; CLK, Lin–c-Kit+; CPCs, cardiac progenitor cells; CRP, C reactive protein; DDR2, discoidin domain receptor 2; DKK1, Dickkopf1; EMA, European Medicines Agency; EU, European Commission; EMSCs, embryonic mesenchymal stem cells; ECs, endothelial cells; ECM, extracellular matrix; EPCs, endothelial progenitor cells; EVs, extracellular vesicles; FAK, focal adhesion kinase; FGF1, fibroblast growth factor 1; Flt-3, Fms Like Tyrosine Kinase 3; GelMA, gelatin methacryloyl; GMP, good manufacturing practice; GSK3, Glycogen Synthase Kinase HCT/Ps: Human Cells, Tissues, or Cellular and Tissue-Based Products; HGF, hepatocyte growth factor; HIF-1, hypoxia inducible factor 1; HP, hypoxic preconditioning; HSCs, hematopoietic stem cells; MRI, magnetic resonance imaging; IC, intracoronary; ICAM-1, intercellular adhesion molecule 1; MSCs, mesenchymal stem cells; IFN-alpha, interferon-alpha; IHD, ischemic heart disease; iPSCs, induced pluripotent stem cells; MI, myocardial infarction; LAD, left anterior descending artery; LDL, low density lipoprotein; LV, left ventricular; MACE, major adverse cardiac events; MAGIC, myoblast autologous grafting in ischemic cardiomyopathy; MAPK, mitogen activated protein kinase; MMPs, matrix metalloproteinases; MPNPs, magnetic polymeric nanoparticles; MVs, micro vesicles; NTproBNP, N-Terminal Pro-Brain Type Natriuretic Peptide3; PEG, polyethylene glycol; PET, positron emission tomography; IGF-1, insulin-like growth factor 1; HGF, hepatocyte growth factor; TEP, tissue engineered products; TNF, tumor necrosis factor; RCSCs, resident cardiac stem cells; SCF, stem cell factor; SMCs, smooth muscle cells; SPECT, single-photon emission CT; SPIO, superparamagnetic iron oxide; VCAM, vascular cell adhesion protein; VEGF, vascular endothelial growth factor; VSMCs, vascular smooth muscle cells; hFCs, human fetal cardiomyocytes; hFVs, human ventricular cells; rFCs, rat fetal cardiomyocytes; EBs, embryoid bodies; hMSCs, human mesenchymal stem cells; mESCs, mouse embryonic stem cells; LVEF, left ventricular ejection fraction; PI3K, phosphatidyl-inositol 3- kinase; BCL-2, B-cell lymphoma 2; hERL, human estrogen receptor ligand; F-FES, 16α-18F fluoro-17β-estradiol.

BASIC PATHOLOGY OF ISCHEMIC HEART DISEASE

Ischemic heart disease is one of the first non-communicable diseases in the world and it will become more common in the future. Initial prevention of IHD is not well established. Currently, it accounts for around 45% of all deaths. IHD is a set of syndromes that are closely related and myocardial ischemia is the hallmark of this condition. Atherosclerosis, coronary microvascular dysfunction, inflammation, and vasospasm all play a part in the pathophysiology of IHD (**Figure 1**; Severino et al., 2020). Because of atherosclerosis, the majority of IHD patients have narrowed epicardial coronary arteries (Buja, 2013; Buja and Vander Heide, 2016). Indeed, IHD is the product of a demand-supply imbalance. In other words, the myocardial tissue does not supply enough blood (Buja and Vander Heide, 2016). Moreover, IHD covers stable and unstable angina, MI, heart failure, and arrhythmia. Herein, typical symptoms are chest pain, cold sweating, nausea, and vomiting (Buja, 2013; Buja and Vander Heide, 2016).

Inflammatory Response After Ischemia

Endothelial dysfunction, vascular wall inflammation, and lipid accumulation are significant factors in the progression of atherosclerosis (as the most usual causes of myocardial ischemia). The first level of atherosclerosis is the aggregation of LDL and inflammation of the arterial wall. Both the carotid and coronary arteries reach this level. Herein, the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule (ICAM)-1, *P*-selection, and *E*-selection along with

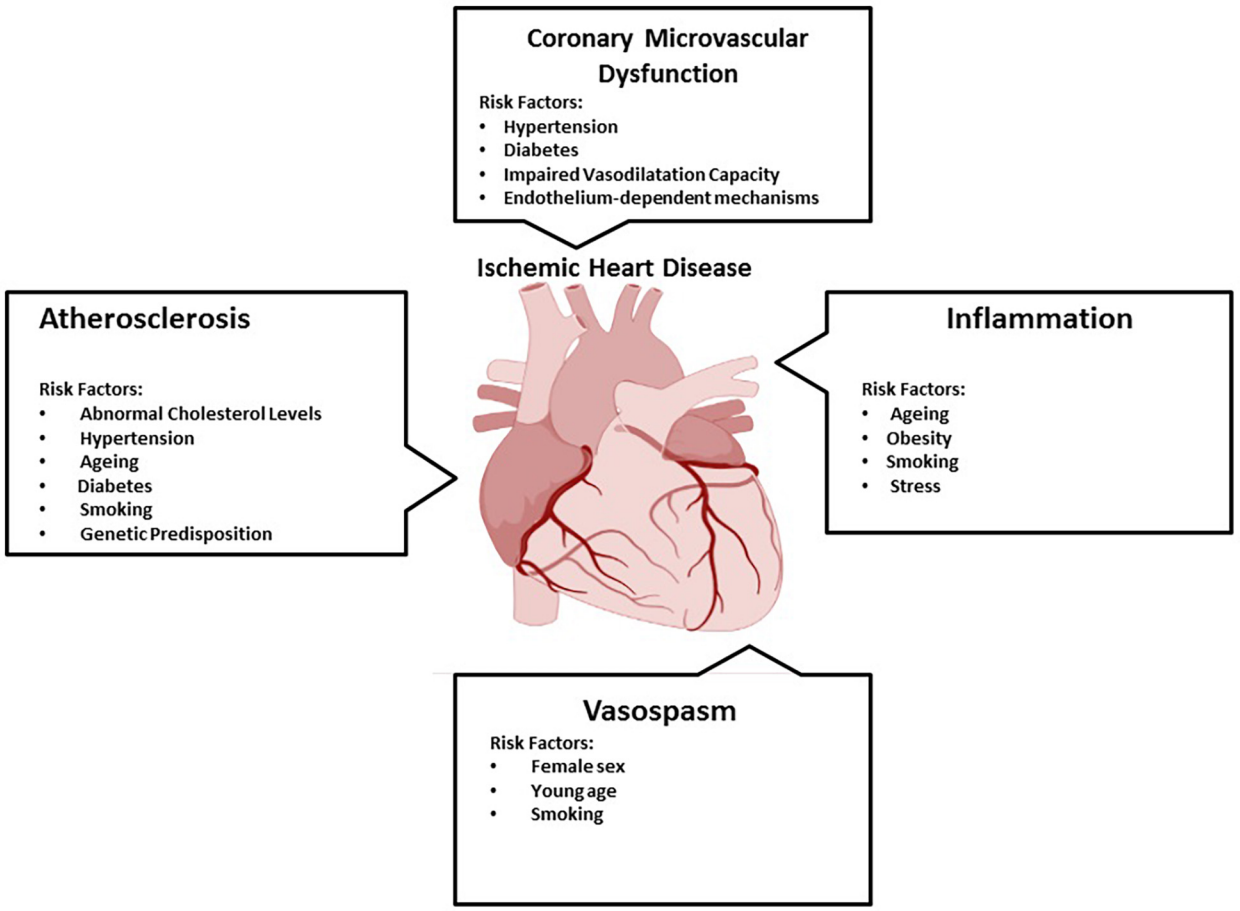

FIGURE 1 | Multifaceted pathophysiology of ischemic heart disease. Ischemic heart disease has multifaceted pathophysiology. Herein, atherosclerosis (the accumulation of fats, cholesterol, etc. in and on the walls of the arteries), coronary microvascular dysfunction (when the small blood vessels in the heart dilate and constrict abnormally), inflammation (an important component of the immune system's response to infection and injury), and vasospasm (vasoconstriction, or narrowing of the arteries) play a role (Severino et al., 2020).

oxidative stress conditions are induced on endothelial cells during inflammatory responses. The persistence of oxidizing atherosclerotic inducements and pro-inflammatory responses contributes to the activation of T and B cells, more macrophages, and mast cells. Their activation can increase vascular lesions and release cytokines [such as interleukin (IL) 6, IL-1β, interferon (IFN)-α, and tumor necrosis factor (TNF)-α] to enhance monocyte migration into the sub- intimal region (Varbo et al., 2013).

ENHANCING ENDOGENOUS CARDIAC REGENERATION

Recent studies of myocyte turnover have been performed. It is worth noting that the possibility of reconstructing the damaged myocardium after its ischemia is very low. The elements involved in endogenous repairs such as inflammatory cells include macrophages and T cells, cytokines, growth factors, and cardiac progenitor cells (CPCs). Some of the methods that can enhance endogenous cardiac regeneration include modulation of macrophage, regulatory T cell function, induction of myocardial proliferation (it never responds to injuries in adult mammals) (Lauden et al., 2013; Aurora and Olson, 2014; Zacchigna and Giacca, 2014). Additionally, it can be promoted by the administration of fibroblast growth factor-1 (FGF-1), p38 mitogen-activated protein (MAP) kinase, and blocking the Hippo pathway (which includes transcriptional coactivators, serine/threonine kinases, and transcription factors) (Engel et al., 2006; Morikawa et al., 2015).

THE HISTORY OF REGENERATIVE MEDICINE STUDIES IN THE FIELD OF ISCHEMIC HEART DISEASE

In recent decades, stem cell therapy has been a very impressive and superior scientific investigational topic. The most promising point in regenerative medicine is that it can present drug-free or

surgical-free options for the treatment of patients with chronic pain and severe injuries (Fisher et al., 2016). In the field of IHD, some studies have suggested using stem cells for treatment. Among the different kinds of stem cells, the application of mesenchymal stem cells (MSCs) in the treatment of IHD is more significant and many preclinical and clinical studies have dealt with it (Golpanian et al., 2016). Hereupon, some in vitro findings show that the injection of MSCs to infarcted myocardia can inhibit fibrosis (Zhang et al., 2019). In this respect, bone marrow-derived stromal cell (BMSCs) transplantation along with a combination of bone morphogenetic protein (BMP)-2 and salvianolic acid B (Sal-B) can result in better differentiation of BMSCs to myocardial cells (Lv et al., 2017). In another *ex vivo* study, genetically engineered rat MSCs (modified with Akt) were transferred to ischemic rat myocardium and findings indicated that tissue remodeling was inhibited and most parts of the myocardia were regenerated (Mangi et al., 2003). He et al. (2019) utilized adipose-derived mesenchymal stem cells (ADSCs) for C57BL/6J mice with ischemic myocardia. Additionally, they used resistin-treated ADSCs [ADSC-resistin (adipose tissue-specific secretory factor)] or vehicle-treated ADSCs (ADSC-vehicle) and found that ADSC-resistin had a positive effect on ejection fraction (EF) and reduction of myocyte apoptosis (He et al., 2019). Clinical trials also show that the application of MSCs could be considered a new method in the field of IHD treatment, for instance, in a phase I study by Joshua et al. in which autologous BMSCs were injected into patients with *trans* myocardial revascularization (TMR) ($n = 10$) and coronary artery bypass graft surgery (CABG) ($n = 4$). After 1 year following the patients, regional contractility had been improved in the areas that had cell injection, in comparison to baseline (Chan et al., 2020). In another study, MSCs were injected to patients with chronic myocardial ischemia, left ventricular ejection fractions (LVEFs) of $\leq$35%, and those who had reversible perfusion defects and were not candidates for revascularization. After following up with the patients for 2 years, significant improvements were detected from baseline to month 12 in several parameters including LVEF, LV end-systolic volume, 6-min walk test and, NYHA functional class (Guijarro et al., 2016). Moreover, an investigation on cell-based therapies for IHD BMSCs led to a reduction in the death of participants who were followed for at least 12 months. In conclusion, as opposed to those who did not receive stem cells, those who received stem cell-based treatments had fewer heart attacks and arrhythmias (Varbo et al., 2013).

PATIENT SELECTION (COMORBIDITIES AND CO-SELECTION)

When researchers design clinical trials about the effects of regenerative medicine in IHD, they have to pay attention to ethical and safety issues such as patient selection (Morikawa et al., 2015). Cautious attention must be given to patient-specific cardiovascular risk factors including age, gender, diabetes, hypertension, smoking, dyslipidemia, depression, psychological problems, and medical help they need (Beckerman et al., 2003; De Groot et al., 2003; Madonna et al., 2016). Studies indicate there is less information about comorbidities and cell therapy in this field (Madonna et al., 2016).

CONTROLS, DATA REPRODUCIBILITY, STANDARDIZATION ISSUE, AND DATA QUALITY

The definition of tissue-engineered constructs and combination of cells and biomaterials is considered as an advanced therapy medicinal product definition by European Medicines Agency (EMA) classification (EC No. 1394/2007). There is another definition by the Food and Drug Administration in the United States that covers Human Cells, Tissues, or Cellular and Tissue-Based Products (HCT/Ps). In both of these definitions and to guarantee standardization, safety, traceability, and potency of the final product, it is important to provide criteria according to Good Manufacturing Practice (GMP) production under a manufacturing authorization. Due to the diverse nature of advanced therapy medicinal products, including the tissue-engineered products (TEP), the European Commission (EU) has provided guidelines (e.g., Guidelines on Safety and Efficacy Follow-up: Risk Management of Advanced Therapy Medicinal Products - EMEA/149995/2008) on how to (1) ensure the quality of the production process, (2) evaluate potential risks and, and (3) demonstrate potency and efficacy of the final product via *in vitro/in vivo* tests (Madonna et al., 2019). In the case of cell/material combinations, specific testing of biodegradation and mechanical factors should be done based on the guidelines available, meaning an evaluation of long-term patient/graft interactions needs to be performed (Madonna et al., 2019). For example, there are some recommendations about setting myocardial therapy by using cellular patches. These products should be able to release therapeutic cells without there being inflammatory responses secondary to material degradation. This issue requires patches to be made of fully bio-absorbable materials. These patches have to be resistant to biodegradation (Madonna et al., 2019). Finally, a responsible regulatory authority should observe these products to ensure that the required manufacturing and preclinical strategies have been performed (Madonna et al., 2019).

DIFFERENT TYPES OF MESENCHYMAL STEM CELLS USED IN THIS FIELD

The selection of an appropriate population of cells due to the aim of producing different components of a specific natural tissue – tissue matrix, connective tissue, native tissues- depends on the target structure (Alrefai et al., 2015). Different types of cells have been used in tissue engineering, from stem cells (especially adult stem cells) to terminally differentiated cells but there have been more favorable responses toward using stem cells (Ott et al., 2008; Alrefai et al., 2015) because they can be harnessed more easily than other common cell types (Alrefai et al., 2015; Paschos et al., 2015). In preclinical studies including in vitro and animal experiments, there has been a willingness

TABLE 1 | Selected mesenchymal stem cell based clinical studies for ischemic heart disease.

Study	Disease	LVEF	MSC type	Cell origin	Results	References
Chan, 2020	Post revascularization	≤50%	BM	Autologous	↑regional contractility ↑quality of life ↓angina scores at 1 year post-treatment	Chan et al., 2020
Hare, 2020	Chronic IHD	Reduced	BM	Allogenic	↓Infarct Scar Size ↓Peak Oxygen Consumption ↑Six-minute Walk Test ↑MACE ↑Treatment Emergent Adverse Event ↑LVEF ↓Abnormal ECHO Reading	Florea et al., 2017; Hare, 2020
Qayyum, 2019	Chronic IHD	Preserved	AD	Autologous	NS LVEF NS myocardial mass NS stroke volume NS left ventricle end-diastolic volume NS left ventricle end-systolic volume NS amount of scar tissue	Qayyum et al., 2019b
Qayyum, 2019	Chronic IHD	Preserved	AD	Autologous	NC exercise performance ↓performance in METs ↓angina CCS class	Qayyum et al., 2019a
Kastrup, 2017	IHD	≤ 45%	AD	Allogenic	↔ inflammatory parameters ↑cardiac function ↓LVESV ↑left ventricular ejection fraction ↑exercise capacity	Kastrup et al., 2017
Bartolucci, 2017	Chronic IHD	≤40%	UC	Allogenic	↑expression of hepatocyte growth factor (involved in myogenesis, cell migration, and immunoregulation) ↑LVEF ↑New York Heart Association Functional Class ↑Minnesota Living with Heart Failure Questionnaire score	Bartolucci et al., 2017
Teerlink, 2017	Chronic IHD	<35%	BM	Autologous	↓LVEDV ↓LVESV	Teerlink et al., 2017

BM: Bone Marrow, AD: Adipose-derived, NS: Not Significant, NR: Not reported, IHD: Ischemic Heart Disease, MET: Metabolic Equivalents of Task, CCS: Canadian Cardiovascular Society, UC: Umbilical Cord, MACE: Major Adverse Cardiac Events, LVESV:left ventricular end systolic volume, LVEDV:left ventricular end diastolic volume.

toward using stem cells. Among all stem cell types, mesenchymal stem cells are one of the most commonly used cell types in this field. Considering preclinical studies, MSC injection in necrotic tissue in acute/chronic MI conditions leads to increased cell proliferation, cell protection against apoptosis and induces angiogenesis in the affected area (Abd Emami et al., 2018; Lu et al., 2019; Sadraddin et al., 2019; Tu et al., 2019; Zhang et al., 2019; Lin et al., 2020). Some studies have applied MSCs in combination with other components such as Nestin and Asprosin, etc. (Lu et al., 2019; Zhang et al., 2019). In some cases, MSCs are used not only to help regeneration in the necrotic area but also to prevent ventricular arrhythmia post-MI (Sadraddin et al., 2019). Studies have shown that applying Insulin-like growth factor 1 (IGF-1) overexpressing MSCs can cause higher rates of cell proliferation and increase cell survival by protecting cells from apoptosis through lowering β-catenin expression (Lin et al., 2020). Thereby, when Asprosin is used in combination with MSCs, it prevents cell apoptosis by reducing oxidizing agents in the hypoxic area (Zhang et al., 2019). Some studies have emphasized the efficacy of Human CD271+ MSCs in preventing post-MI arrhythmia as well as reducing local inflammation (Sadraddin et al., 2019; Sasse et al., 2019). Some other studies have reported the facilitative role of TGF-β, BMP-2, and sal-B in cardiomyocyte differentiation of MSCs (Lv et al., 2017, 2018). Considering the different types of stem cells that are used in clinical experiments, three main types are more commonly applied: Xenogeneic stem cells from nonhuman species; the allogeneic ones from human donors; and autologous cells from the same individual (Alrefai et al., 2015).

TABLE 2 | Some of the mesenchymal stem cells-based preclinical studies for ischemic heart disease.

Study	Type of study	Disease	MSC type	Cell origin	Animal model	Results	References
Lin, 2020	*In vitro*	AMI	BMSCs overexpressing IGF-1	–	–	↑cell proliferation rate ↑migration capacity ↑stemness ↓apoptosis ↑cell survival ↓β-catenin expression	Lin et al., 2020
Gottipati, 2019	*In vitro*	AMI	GFP+ BMMSC	–	–	≠predisposing BMMSCs to aggregation ≠increasing BMMSC susceptibility to phagocytosis ≠heightened immune response ↑cell retention	Gottipati et al., 2019
Zhang, 2019	Animal + *in vitro*	CMI	ASP pretreated BMMSC	Allogenic	C57BL/6 mice	↑LVEF ↓myocardial fibrosis ↑homing of transplanted MSCs NS MSC proliferation and migration ↓H2O2-induced apoptosis ↑SOD2 enzyme ↓H2O2-induced ROS generation ↓apoptosis	Zhang et al., 2019
Shi, 2019	*In vitro*	AMI	serum deprived BMMSCs+Ulinastatin	–	–	↑cell viability ↓apoptosis ↓caspase-3 activation ↓expression levels of Bcl-2, Bcl-extra large and Bcl-associated X protein	Shi et al., 2019
Lu, 2019	Animal	AMI	Nestin positive MSC	Allogenic	Nestin-GFP transgenic mice	↑survival ↑LVEF ↑endogenous CECs ↑chemokine levels	Lu et al., 2019
Tu, 2019	Animal	AMI	BMMSC transfected with miR-15a/15b inhibitors	Allogenic	Luciferase transgenic FVB/N mice	↑proliferation ↓apoptosis ↑VEGFR-2 expression and survival ↑VEGFR-2/PI3K/AKT signaling pathway	Tu et al., 2019
Sasse, 2019	Animal	AMI	H-BM CD271 + MSC	Xenogeneic	SCID beige mice	↓inflammatory cytokines	Sasse et al., 2019
He, 2019	Animal	AMI	ADMSC + resistin	Allogenic	C57BL/6J mice	↑LVEF ↓fibrosis ↓atrial natriuretic peptide/brain natriuretic peptide ↓Apoptosis ↑angiogenesis ↑Cell proliferation and migration	Shi et al., 2019
Sadraddin, 2019	Animal	VA post-MI	H-BM CD271+MSC	Xenogeneic	Immunocompromised Rag2–/– γc–/– mouse strain	↓VA post MI NS scar reduction	Sadraddin et al., 2019
Ciuffreda, 2018	Animal	CMI	BMMSC+ H-HG	Allogenic	Sprague Dawley rats	↓ventricular remodeling ↑neo-vasculogenesis ↑MSC engraftment ↑cardiac function	Ciuffreda et al., 2018
Chen, 2018	Animal	AMI	S1P-treated ADMSC	allogenic	C57BL/6J mice	↑AT-MSCs migration ↓AT-MSCs apoptosis ↑post-MI cardiac function ↑post-MI cardiac remolding	Chen et al., 2018
Abd Emami, 2018	Animal	AMI	BM, AD	Autologous	New Zealand rabbit	↓scar size ↑LVEF	Abd Emami et al., 2018
Lv, 2018	*In vitro*	AMI	BMMSC+TGF-β+Sal-B	-	-	↑expression of cardiac-specific markers ↑The amount of cardiomyocytes differentiated from MSCs	Lv et al., 2018
Lv, 2017	*In vitro*	AMI	BMMSC+BMP-2+ Sal-B	–	–	↑expression of cardiac-specific markers ↑The amount of cardiomyocytes differentiated from MSCs	Lv et al., 2017

(Continued)

TABLE 2 | (Continued)

Study	Type of study	Disease	MSC type	Cell origin	Animal model	Results	References
Qin, 2017	Animal	AMI	Transfected BMMSCs with hERL and VEGF165	Allogenic	SD rat	hERL/ F-FES could be used as a reporter gene/probe system	Qin et al., 2017

AD: Adipose Derived, S1P: sphingosine 1-phosphate, AMI: Acute Myocardial Infarction, BM: Bone Marrow, ADMSC: Adipose Derived Mesenchymal Stem Cell, BMMSC: Bone Marrow Mesenchymal Stem Cell, TGF-SS: Transforming Growth Factor beta, Sal-B: salvianolic acid B, IGF-1:insulin-like growth factor-1, BMP-2: Bone morphogenetic protein 2, HG: Hydrogel, CMI: Chronic MI, ASP: Asprosin, SOD-2: Superoxide dismutase 2, GFP: green fluorescent protein, CEC: Circulating Endothelial Cells, VA: Ventricular Arrythemia, SCID: Severe Combined Immunodeficiency, BCL-2: B-cell lymphoma 2, hERL: human estrogen receptor ligand, VEGF: Vascular endothelial growth factor, F-FES: 16a-18F fluoro-17SS-estradiol.

Allogenic graft transplantation tends to be more successful than the Xeno type, which is due to the immunologic rejections that cause more controversies over using Xenografts. Regarding this issue, more recent autologous grafts have been the subject of increasing attention especially skeletal myoblasts, ADSCs, resident cardiac stem cells (RCSCs), and bone marrow-derived (BMD) stem cells- such as CD34+ cells-, induced pluripotent stem cells (iPSCs), multipotent adult progenitor cells, endothelial progenitor cells (EPCs) and the MSCs that are the main focus of this issue (Alrefai et al., 2015). When coming to clinical studies, the most common function of MSCs is increasing LVEF. Other outcomes are reducing Left Ventricular End Systolic/Diastolic Volume (LVES/DV), better performance in exercise tests as well as promoting cardiac function post-MI (Bartolucci et al., 2017; Florea et al., 2017; Kastrup et al., 2017; Teerlink et al., 2017; Qayyum et al., 2019a). Hepatocyte Growth Factor (HGF) is one of the involved factors in MSC cardiomyocyte differentiation, and cell migration, etc., and is upregulated in MSC transplantation (Bartolucci et al., 2017). Experimental data on the applications of MSC and preclinical studies from 2017 until now is provided in more detail in **Tables 1**, **2** below. The main allogeneic and autologous cell types and their characteristics will be discussed in more detail later in this paper.

Allogeneic Sources

Fetal Cardiomyocytes

Some characteristics make this kind of stem cell an unfavorable candidate for regenerative therapy including immunogenicity, malignant potential, ethical questions, and limited availability (Li et al., 1996; Alrefai et al., 2015).

Embryonic Mesenchymal Stem Cells

Cells of three embryonic germ layers and intact cardiomyocytes can be produced by human (ESCs) derived MSCs (EMSC) differentiation (Xu et al., 2002; Alrefai et al., 2015). However, there are some negative aspects of using EMSCs in cardiology, including teratoma formation in rodent models (Lee et al., 2009; Alrefai et al., 2015), malignant transformation, and the legal issues surrounding their use.

Human Umbilical Cord Blood-Derived Cells

These kinds of cells contain a large number of non-hematopoietic stem cells with two suitable properties for the treatment of bone marrow illnesses, which are (1) that they contain less class II human leukocyte antigens and (2) that they do not trigger an immune response (Li et al., 1996; Alrefai et al., 2015). Some experiments have shown that intramyocardial injection of human cord blood-derived cells could act positively in reducing the infarction size (Alrefai et al., 2015).

Autologous Sources

Adipose Derived Stem Cells

Adipose derived stem cells contain a heterogeneous mixture of MSCs, hematopoietic stem cells, and EPCs. Their favorable characteristics are availability, easy harvesting, and low cost, which make them effective in improving ventricular function in animal models of MI, and neoangiogenesis activity is also hypothesized in them (Valina et al., 2007; Wang et al., 2009; Alrefai et al., 2015).

Skeletal Myoblasts

These kinds of cells can make myotubules after engraftment and improve cardiac function in the infarcted myocardium (Taylor et al., 1998; Alrefai et al., 2015). Some trials, such as the Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial, discuss the feasibility of myoblast injection into the epicardium as well as CABG surgery. These trials have shown that this process could be possible, with likely functional benefits (Dib et al., 2005; Menasché et al., 2008; Alrefai et al., 2015). The only problem in using this cell type is its interference with the electrical activity of the heart's native tissue due to its belonging to skeletal muscle lineage, i.e., the new graft can make some islands of skeletal muscle cells between cardiomyocytes that cause arrhythmia (Alrefai et al., 2015).

BMD Stem Cells

Bone marrow derived are the kind of adult stem cells that have been tested and given profitable results after their transplantation in cases of ischemic heart failure due to their versatility and ease of collection (Orlic et al., 2001; Alrefai et al., 2015).

Induced Pluripotent Stem Cells

Some adult cells can be programmed in a way that expresses embryonic genes and changes into pluripotent cells. They can produce specific types of cytokines that make it possible for the iPSCs to differentiate into smooth muscle cells (SMCs), cardiomyocytes, and vascular endothelial cells (ECs) (Alrefai et al., 2015).

Resident Cardiac Stem Cells

Resident cardiac stem cells have recently been isolated with the ability to differentiate into several cell types like cardiomyocytes or vascular SMCs (VSMCs). This is opposed to the notion that there is a lack of self-renewal in cardiomyocytes (Beltrami et al., 2003; Messina et al., 2004; Alrefai et al., 2015). If the harvesting technique is perfect, an improvement in left ventricular (LV) function can be observed in rodent models of MI (Alrefai et al., 2015). The most well-studied RCSCs are c-kit+/Lin– cells which show all the properties of induced stem cells. Besides, they are capable of restoring the cardiac pattern and function in animal models of MI as well as promising improvements in patients with ischemic cardiomyopathy (Beltrami et al., 2003; Linke et al., 2005; Urbanek et al., 2006; Bearzi et al., 2007; Tang et al., 2010; Bolli et al., 2011; Alrefai et al., 2015).

THE IMPORTANCE OF EXTRACELLULAR MATRIX (ECM) IN REGENERATING NORMAL CARDIAC TISSUE

Extracellular matrix plays an important role in the function and homeostasis of the tissue. Accordingly, any kind of microenvironment provided in different regenerating approaches has to mimic normal cardiac ECM. For example, three-dimensional (3D) hydrogel scaffolds are generated from decellularized cardiac ECM (Yanamandala et al., 2017; Madonna et al., 2019). Bio-mimicking and the bioactive properties of ECM proteins make them capable of being used as a hydrogel-based scaffold in cardiac tissue engineering, such as in collagen (Tiburcy et al., 2017; Madonna et al., 2019), fibrin (Hirt et al., 2014; Madonna et al., 2019), gelatin (Nawroth et al., 2018; Madonna et al., 2019), hyaluronic acid (Camci-Unal et al., 2013; Madonna et al., 2019), and alginate (Shin et al., 2013; Madonna et al., 2019). There are some shortcomings in the use of these materials; one of them is the possibility of activating post modification fibrosis due to the emerging stiffness-sensitivity of myocardial resident stromal cells (Mosqueira et al., 2014; Madonna et al., 2019). In this respect, 'bio-ink' materials, such as gelatin methacryloyl (GelMA) hydrogels have been introduced, that have the quality of sufficient levels of degradation to control the viscoelastic properties of the used materials and as a result, avoid changes in myocardium compliance (Yue et al., 2015; Bejleri et al., 2018; Madonna et al., 2019).

MYOCARDIAL TISSUE ENGINEERING METHODS

There are several obstacles in the way of tissue engineering in ischemic heart diseases such as low cellular survival, poor localization to the target area. To prolong the graft activity, several approaches have been arranged, including (1) seeding of cells on preformed scaffolds, (2) self-assembly of cells in hydrogels, and (3) cell sheet engineering (Zimmermann, 2009; Weinberger et al., 2017; Madonna et al., 2019). Some of these approaches are discussed in the following section (**Figure 2**).

Hydrogels

In this technique, hydrophilic structures, made of synthetic or natural polymers are used (Camci-Unal et al., 2013; Madonna et al., 2019). There are some requirements in this 3D network: the exchange of oxygen, nutrients, and metabolites through their pores, in addition to including growth factors and other molecules to mediate the cross-talk between cells (Wang et al., 2010; Madonna et al., 2019).

Engineered Myocardial Tissue

There are two main approaches of tissue engineering in cardiology; one of them aims to promote cardiac function and the other is to develop 3D *in vitro* models capable of mimicking the native heart muscles. Some characteristics need to be considered in these approaches: (1) native-like biochemical, electrophysiological, and mechanical cell-ECM and cell-cell interactions, (2) dynamic in vivo like conditions such as fluid flow and shear stress, and (3) correct cell characteristics and morphologies and structural micro-architectures (Madonna et al., 2019).

Cell Sheets

Temperature-responsive polymer surfaces are used in this approach as they make the release of cell monolayers possible. These free-floating sheets of cohesive cells can be placed on the epicardium with or without stitches. In the same way, 3–4 monolayers can be fused without palpable core necrosis (Madonna et al., 2019). Different types of cells can be used in this approach, such as cardiomyocytes for contractile support and non-myocytes for the delivery of secreted factors (Miyahara et al., 2006; Masumoto et al., 2012; Narita et al., 2013; Madonna et al., 2019). Some shortcomings of this approach include the frailty of sheets that cause folding or tearing, as well as the limited number of sheets that can be stacked on each other without cell death (Madonna et al., 2019).

Biofabrication

In this approach, to provide a cellular microenvironment to serve as a structural platform, an appropriate cellular scaffold is needed. We follow some purposes by producing such a formation: delivering biochemical factors, providing a suitable environment for cell attachment, migration, and differentiation (Alrefai et al., 2015; Madonna et al., 2019). The main limitation of this approach is that only inhomogeneous cell densities can be achieved because of the cell's propensity to remain at the scaffold's surface, so only weakly contracting cardiac tissues can be used in the fabrication method. Accordingly, there has been a lot of effort to make hierarchical tissue-like structures. This specific printing method is a kind of 3D printing (Groll et al., 2016; Cui et al., 2018; Madonna et al., 2019) that produces multiple cell layers out of different cell types (Madonna et al., 2019). There are some standardization issues with this approach, such as correct cellular composition, the positioning of various cell types and materials,

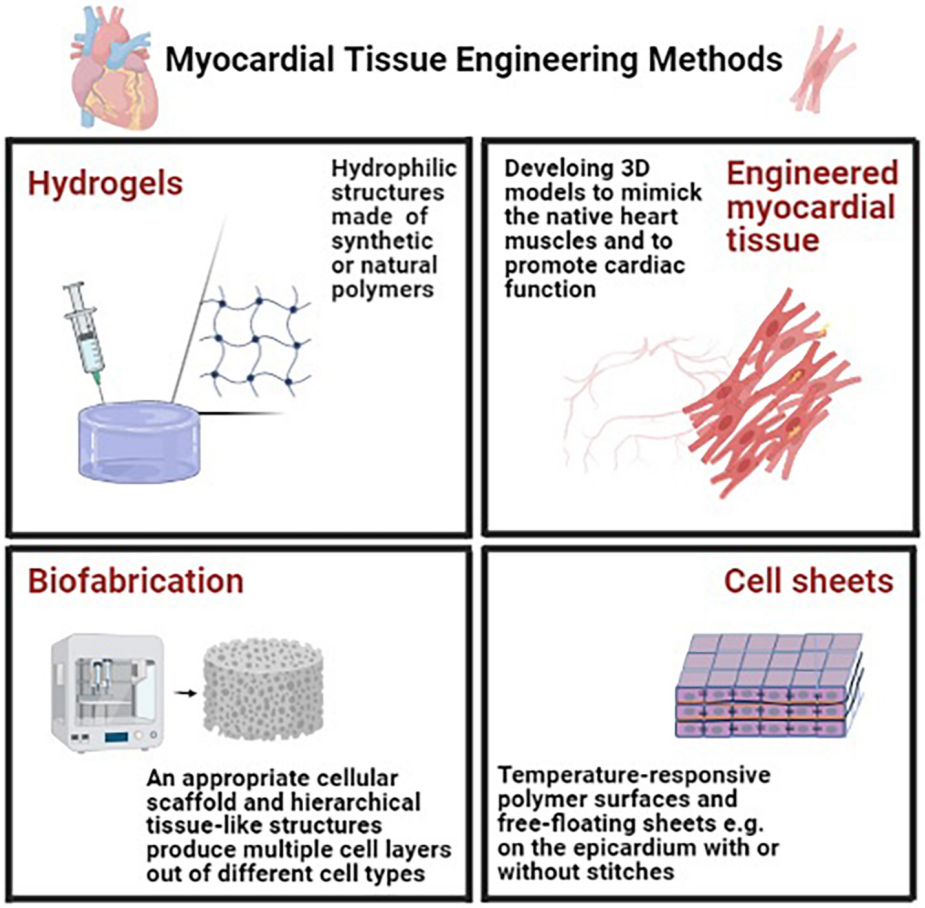

FIGURE 2 | Myocardial tissue engineering methods in order to prolong the graft activity for tissue engineering in ischemic heart diseases several approaches have been arranged including utilizing hydrogels (using hydrophilic structures synthetic or natural polymers), engineered myocardial tissue [developing three-dimensional (3D) *in vitro* models to mimic the native heart muscles], cell sheets (using temperature-responsive polymer surfaces that make the release of cell monolayers possible and can be placed for example on the epicardium with or without stitches) and biofabrication (requiring an appropriate cellular scaffold to provide a cellular microenvironment to serve as a structural platform) (Madonna et al., 2019).

vascularization, and the incorporation of bioactive substances (Madonna et al., 2019).

DELIVERY SYSTEMS AND ADMINISTRATION ROUTES

There are several techniques to deliver stem cells into the myocardium; from direct syringe injection to the left ventricle under visual control to guided percutaneous transendocardial injection to the ischemic area of the left ventricle guided by the NOGA system, which is minimally invasive (Litwinowicz et al., 2018). The direct surgical myocardial injection can be used in hypokinetic myocardial areas, which are not suitable for CABG (Pavo et al., 2014; Litwinowicz et al., 2018). Studies have shown that the largest stem cell retention in the injured myocardium can be provided when this surgery is operated immediately after left anterior descending artery (LAD) occlusion has happened (Elhami et al., 2013; Litwinowicz et al., 2018). Another approach is the intracoronary (IC) delivery of therapeutic agents. Significant drug retention in heart muscle is not provided in this method, but the point is that the extent of stem cell retention in the human myocardium is about 2 h for 1% and 18 h for 5% of the cells, which is very noticeable compared to this extent in the spleen, liver, lung, lymph nodes, and bone marrow (BM) (Litwinowicz et al., 2018). Intramyocardial delivery is a method that has two components: transcutaneous left heart catheterization for transendocardial injection, which is minimally invasive (Tse et al., 2003; Litwinowicz et al., 2018), and direct transepicardial injections under direct visualization during sternotomy (Hamano et al., 2001; Litwinowicz et al., 2018) or left small thoracotomy (Konstanty-Kalandyk et al., 2018; Litwinowicz et al., 2018). Minimally invasive access to the heart muscle is a safe procedure but its main limitation is that it makes safe access to only the anterior and anterolateral wall of the heart possible (Litwinowicz et al., 2018). Full sternotomy has some shortcomings, too. For example, it is associated with some perioperative complications

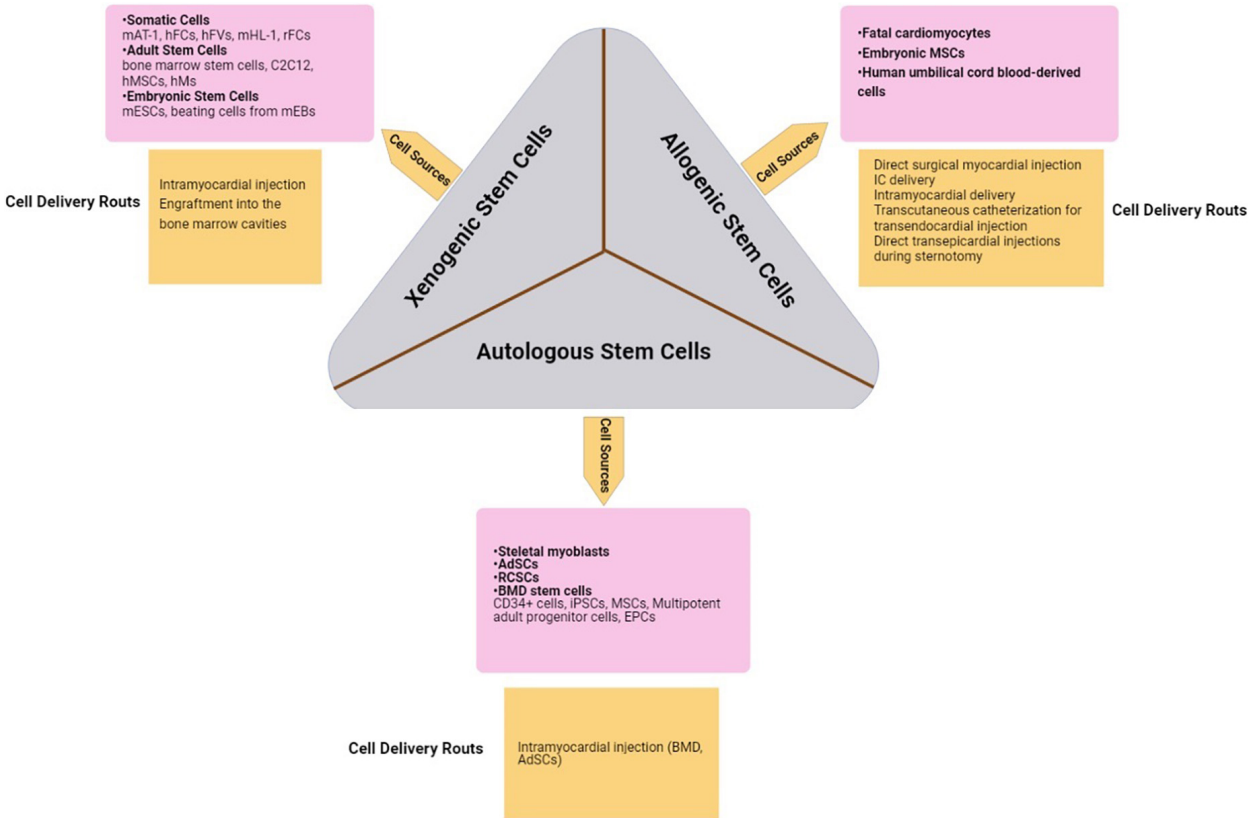

FIGURE 3 | Stem cells are collected from appropriate cell sources. The collected cells are replicated in suitable cultures in order to reduce the risk of tissue rejection. Finally, the generated tissues will be transported to the target organ via different tissue delivery routes due to their availability, invasiveness, and the target tissue's characteristics (Xiao et al., 2004; Beeres et al., 2007a; Rodrigo et al., 2013; Alrefai et al., 2015; Henry et al., 2017; Konstanty-Kalandyk et al., 2018; Litwinowicz et al., 2018). hFCs, human fetal cardiomyocytes; hFVs, human ventricular cells; hMSCs, human mesenchymal stem cells; hMs, human myoblasts; mEBs, mouse embryoid bodies; mESC, mouse embryonic stem cells; rFCs, rat fetal cardiomyocytes; IC, intracoronary; MSCs, mesenchymal stem cells; AdSCs, adipose-derived stem cells; RCSCs, resident cardiac stem cells; BMD, bone marrow-derived stem cells; iPSCs, induced pluripotent stem cells; EPCs, endothelial progenitor cells.

such as bleeding, infections, abnormal sternal healing, or respiratory complications (Litwinowicz et al., 2015, 2016, 2018). Recent animal studies have shown that intracoronary stem cell delivery decreased absolute myocardial blood flow and consequently an increase in myocardial expression of the oxidative stress marker matrix metalloproteinase-2. It reduced the number of CXCR4 receptors and myocardial homing and angiogenic factor release in comparison to intramyocardial cell delivery (Litwinowicz et al., 2018; Zlabinger et al., 2018; **Figure 3**).

STEM CELL HOMING

Homing is the ability of stem cells to find their destination in the target organ when moving in the bloodstream (Zhao and Zhang, 2016; Tao et al., 2018). Signaling factors direct cells toward their destination, making homing possible (Tao et al., 2018). The new tissue's phenotype is directed by different signaling factors that can be discovered by observing those involved in native tissue formation. Cell metabolism, migration, and organization will be influenced by these factors (Alrefai et al., 2015). These factors are molecules like chemokines or growth factor receptors on the surface of stem cells to which chemokines, adhesion molecules, growth factors, and the enzymes released from a specific tissue or organ bind (Tao et al., 2018). There are two major types of stem cell homing: endogenous homing and exogenous one after cell transplantation (Tao et al., 2018).

Endogenous Homing Mechanism

In this mechanism, stem cells mobilize toward their destination while inflammatory factors released after MI direct them. For example, activating proteolytic enzymes [like matrix metalloproteinases (MMPs)] can direct EPCs isolated from bone marrow, toward the infarcted part of the heart (Aicher et al., 2005; Tao et al., 2018). Relevant studies have shown that Hematopoietic stem cells (HSCs) will be mobilized very soon post-acute myocardial infarction (AMI). Consequently, the number of erythroid burst-forming units and granulocyte-macrophage colony-forming units will increase after MI (Wojakowski et al., 2012; Tao et al., 2018). Du et al. (2012) have shown that ESCs homing would be impossible

without CD18 and its ICAM-1 (Wu et al., 2006; Tao et al., 2018).

Exogenous Homing Mechanism

In a specific experiment, MSCs were transduced with human IGF-1 and injected into a rat heart model of AMI. It was concluded that more expression of IGF-1, causes BMSCs to be mobilized through the paracrine activation of stromal cell-derived factor 1 (SDF1)/CXCR4 signaling (Haider et al., 2008; Tao et al., 2018). Another study showed that when vascular endothelial growth factor (VEGF) -expressing MSCs are injected into the infarcted myocardium, endogenous cardiac stem cells (CSCs) will be mobilized through SDF1α/CXCR4 signaling (Tang et al., 2011; Tao et al., 2018).

Homing-Related Molecules and Signaling Pathways

There are several cytokines involved in the process of stem cell homing. The most important one is the SDF-1/CXCR4 signaling pathway, which plays a crucial role in EPCs, BMSCs, MSCs, and CSCs stem cell populations (Wang et al., 2006; Chavakis et al., 2008; Zhao et al., 2009; Sharma et al., 2010; Herrmann et al., 2015; Sun et al., 2016; Tao et al., 2018). This signaling cascade, phosphatidyl-inositol 3-kinase (PI3K)/Akt, and mitogen-activated protein kinase (MAPK) are involved (Ratajczak et al., 2006; Tao et al., 2018). FMS like tyrosine kinase 3 (Flt-3) ligand, stem cell factor (SCF), IL-6, HGF, IL-3, and as a result CXCR4 protein, are kinds of cytokines for which surface expression increases in the process of MI (Shi et al., 2007; Tao et al., 2018). SDF-1 is another signaling factor that is expressed more than normal conditions, while MI is happening in rats (Pillarisetti and Gupta, 2001; Askari et al., 2003; Tao et al., 2018). The heart's response to MI is an increase in hypoxia-inducible factor 1 (HIF-1) and VEGF levels, resulting in stem cell mobilization (Mirahmadi et al., 2016; Tao et al., 2018). In an experiment designed by Huang et al. (2014) the increased expression level of VEGF and SDF-1α, angiogenesis, as well as decreased cardiomyocyte apoptosis at the peri-infarct site were reported in post-MI rats' hearts, transplanted by MSCs (Huang et al., 2014; Tao et al., 2018). Another factor that plays an important role in the migrating and homing process of 10 cardiosphere-derived Lin–c-Kit+ progenitor cells (CLK) is hypoxic preconditioning (HP), which promotes CXCR4 expression through the SDF-1/CXCR4 axis (Tang Y. L. et al., 2009; Tao et al., 2018). HP expression in BMSCs activates focal adhesion kinase (FAK) by Kv2.1 mediation. The previous process is responsible for the migration and homing of BMSCs (Hu et al., 2011; Tao et al., 2018). In an experiment, a CXCR4 antagonist [AMD3100 (AMD)] was used and it was used. Accordingly, an increase in the EPCs population was reported due to the MMP-9 expression downregulation by VEGF when using AMD (Jujo et al., 2010; Tao et al., 2018). Some other cytokines and signaling factors will be discussed in the following. Another factor that increases its serum levels after MI, is clusterin (CST, a stress-responding protein) (Trougakos et al., 2002; Tao et al., 2018). This factor stimulates the migration of cardiac progenitor cells (CPCs). In addition, a study showed that CXCR4 expression will rise in fetal canine heart CPCs, which are transfected by CST. This study was designed on CST-expressing CPCs, which showed that their stimulation by SDF-1 promotes migration through the PI3K/Akt pathway *in vitro* (Li et al., 2010; Tao et al., 2018). Tang J. et al. (2009) showed that wortmannin, which is the inhibitor of PI3K/Akt, can block VEGF-induced CSC migration (Tao et al., 2018). Another study showed that the process of Sca-1+ CSCs migration when injecting peri-infarct myocardium can be promoted by basic fibroblast growth factor (bFGF), through activating the PI3K/Akt pathway (Ling et al., 2018; Tao et al., 2018). There are also some down regulators for the migration process, which are discussed in the following section of this paper. Hyperglycemia suppresses ERK1/2 and p38 MAPK activities and can prevent CSCs from migrating (She et al., 2012; Tao et al., 2018). Hyperhomocysteinemia suppresses NFkB, ERK1/2, and p38MAPK- related to SCF/c-Kit signaling pathway- activities that cause decreased SCF protein expression leading to migration suppression (Wan et al., 2011; Tao et al., 2018). In summary, there are several pathways involved in the stem cell migrating process but the quality of responding to these processes is under question (Tao et al., 2018).

QUANTIFICATION OF HOMING EFFICIENCY

There have been several methods introduced to quantify the homing efficacy such as species mismatch (Jiang et al., 2006; Tao et al., 2018), radioactive labeling (Kraitchman et al., 2005; Li and Hacker, 2017; Tao et al., 2018), fluorescent labeling (Kawada et al., 2004; Tao et al., 2018), and transduction of cells with reporter genes, such as green fluorescent protein gene (Devine et al., 2001; Tao et al., 2018), superparamagnetic iron oxide (SPIO) labeling (Jasmin de Souza et al., 2017; Tao et al., 2018). There are two main methods: (1) quantifying the level of radioactivity in the target organ (2) quantifying the number of cells labeled by fluorescent, which are discussed in detail in the following section (Tao et al., 2018).

Quantifying the Level of Radioactivity

In this method, imaging techniques are required such as magnetic resonance imaging (MRI), bioluminescence and fluorescence imaging, positron emission tomography (PET), and single-photon emission CT (SPECT) (Meleshina et al., 2015; Tao et al., 2018). MRI has some merits and demerits similar to other modalities. It has a high spatial resolution (Kraitchman et al., 2003; Seeger et al., 2007) and provides beneficial 3D anatomical information, but has lower sensitivity than PET/SPECT (Kraitchman et al., 2005; Tao et al., 2018). PET/SPECT can trace stem cells through radioactive labeling (Bansal et al., 2015; Tao et al., 2018). One of the limitations of this method is the possibility of causing injury to target cells (Brenner et al., 2004; Tao et al., 2018). Labeled gene tracing is possible using both methods (Sheikh et al., 2007; Tao et al., 2018).

Quantifying the Number of Labeled Cells

One of the methods used in this field is optical imaging in which fluorescent labeling is involved. In this method, the average number of labeled cells in a fixed microscopic field is counted in a short time and highly sensitive way (Tao et al., 2018). A new study has shown that magnetic polymeric nanoparticles (MPNPs) can be used as a labeling agent for MSCs (Supokawej et al., 2015; Tao et al., 2018) since they are not toxic toward cells (Beeres et al., 2007b; Tao et al., 2018).

STRATEGIES TO IMPROVE CELL HOMING, SURVIVAL, AND THE PREVENTION OF TISSUE REJECTION

The efficacy of cell therapy depends on the quality of the graft's homing, cell recruitment, and coupling in the target tissue. Accordingly, some methods are necessary in order to promote the graft's homing or cells' coupling (Seeger et al., 2007).

Cell Homing and Survival Improvement

Some cytokines are increased in amount during ischemic heart diseases such as SDF-1 or VEGF. These factors mediate cell recruitment. However, the best cell incorporation when infusing stem cells into the coronary arteries is 10% (Seeger et al., 2007). It is documented that low energy shock waves can exacerbate cytokine expression in the target tissue (Seeger et al., 2007). One of the critical issues in investigating the quality of stem cell homing is the time at which the infraction happened. A study (S. Dimmeler, unpublished data) designed on tracing labeled EPCs when infusing them into an infarcted tissue showed that the EPC uptake in an old infarcted tissue is lower than that of an acute infarcted one (Seeger et al., 2007). SDF-1 is a promoting factor that can play a critical role in chemotaxis and cell migration since it stimulates the CXCR4 receptor, which is expressed on EPC and BMC, as well as the ability to retain proangiogenic cells in the target area (Grunewald et al., 2006; Seeger et al., 2007). It was documented that insulin-like growth factor 1 with biotinylated peptide nanofibers into the infarcted heart muscle, can promote homing efficacy since this method changes the target environment (Davis et al., 2006; Seeger et al., 2007).

Prevention of Tissue Rejection

One of the most challenging issues in cardiac tissue engineering is to prevent foreign-body host immune response. To address this problem, novel biocompatible, immunomodulatory biomaterials have been introduced, as well as smart biomaterials that prevent this issue by doing immunomodulation. The physicochemical properties of these materials have been modified in a way that can promote biomaterial integration and interaction with reparative immune cells such as macrophages and MSC, as well as the controlled delivery of anti-inflammatory small molecules and cytokines (Vishwakarma et al., 2016; Madonna et al., 2019).

CELL DIFFERENTIATION PROTOCOLS

In order for human EMSCs to differentiate into cardiomyocytes, BMP4 and recombinant human activin A have to be added. This induces cardiac mesoderm to reproduce the main foundations of embryonic development (Laflamme et al., 2007; Burridge Paul et al., 2012; Alrefai et al., 2015; Yue et al., 2015). There is a protocol by Laflamme et al. (2007) and Alrefai et al. (2015) which produces cardiomyocyte populations with over 50% cardiac purity (consisting of nodal cells, ventricular cells, and atrial cells) by adding BMP4 and activin A. In this protocol, first of all, the expression of Wnt ligands is induced and subsequently in order to have a successful cardiac differentiation, this process should be inhibited (Paige et al., 2010; Alrefai et al., 2015). By doing this procedure a biphasic signaling profile will be formed that can be used to evaluate the efficacy of cardiac differentiation. The purity of produced cardiomyocytes based on this protocol is 50% (Alrefai et al., 2015). Yang et al. (2008) and Alrefai et al. (2015) proposed another protocol. In this protocol, small cell groups called "embryoid bodies" are used, mimicking the 3D setting of an evolving embryo. These cells are exposed to bFGF, activin A, BMP4, VEGF and Dickkopf1 (DKK1). Following this protocol, after 4 days, a progenitor population expressing kinase insert domain receptor and platelet-derived growth factor receptor-alpha can be isolated, as well as low levels of ECs (CD31+), fibroblasts [discoidin domain receptor 2 (DDR2)+], and VSMCs (Alrefai et al., 2015). In another approach, to activate Wnt/β-catenin signaling and mesoderm differentiation, the glycogen synthase kinase 3 (GSK3) inhibitor is used which leads to more than 80% of cardiomyocyte purity (Alrefai et al., 2015).

CELL AND TISSUE REJUVENATION

As aging has several effects on cells and tissues, decreasing the number and function of both resident and circulating cells can be noted as its comorbidities (Madonna et al., 2016). Therefore, scientists and even the public community have become interested in using regenerative therapies for tissue rejuvenation, especially in the field of cardiac diseases such as acute MI as an irreversible loss of cells (Povsic and Zeiher, 2016). Nowadays, different pharmacological and physiological approaches recommend that epigenetic factors, signaling pathways, and proteins can be utilized to reverse the process of aging (Rando and Chang, 2012; Rando and Wyss-Coray, 2014; Madonna et al., 2016). One of the examples is the pro-viral integration site for Moloney murine leukemia virus-1 (Pim-1) kinase, the over expression of which influences the elongation of telomerase in CPCs (Siddiqi and Sussman, 2013), as well as its anti-aging and anti-apoptotic properties in CSCs and MSCs (Siddiqi and Sussman, 2013; Madonna et al., 2016). In addition, when telomerase and myocardin genes are overexpressed, it is more possible for cells to survive and their proliferation increases (Madonna et al., 2016).

Cell-Free Approaches

Different studies have shown that the benefits of transplanted stem cells were mostly because of their paracrine effects and

cytokines, in comparison with cell differentiation. Thus, releasing factors from transplanted stem cells attracted researchers to focus on them (Jung et al., 2017; Johnson et al., 2019) and try to imitate the beneficial effects of cell therapy through cell-free *in situ* approaches to cardiac regeneration (Madonna et al., 2016; Micheu, 2019). Mediators causing paracrine effects might embrace episomes, growth factors, and non-coding RNAs. Recently, cell-free approaches have been developed, including extracellular vehicles (EVs) secreted from almost all types of cells including cardiomyocytes that contain exosomes and microvesicles (MVs) (Huang et al., 2016; Madonna et al., 2016). Exosomes are small double-bound vesicles that transfer different secreted substances like proteins, lipids, and mRNAs as communicating vehicles between cells in both physiological and pathological conditions (Sahoo and Losordo, 2014; Huang et al., 2016; Jung et al., 2017; Johnson et al., 2019), such as cardiac repair (Sahoo and Losordo, 2014; Madonna et al., 2016).

HOW TO ASSESS THE CLINICAL BENEFIT OF CELL THERAPY?

Recently, by using stem cell therapy for cardiac disease, it seems necessary to utilize assessments and endpoints that evaluate the impact of this therapy. They are categorized into some groups, including structural, biological, functional, physiological, major adverse cardiac events (MACE), or quality of life.

Structural endpoints contain different items that are (Dorobantu et al., 2017):

- Left ventricular ejection fraction (LVEF) and left ventricular volumes which mostly are LVEF and are measured as the endpoint of stem cell transplantation (Madonna et al., 2016; Dorobantu et al., 2017). Different methods are applied to measure LVEF and LV volume but MRI and recently, 3D-echography are the most accurate (Hung et al., 2007; Dorobantu et al., 2017).
- Myocardial deformation techniques like strain/strain rates, tissue Doppler echocardiography are used and seem to find more sensitive markers than LVEF after acute MI.
- Infarct size is another structural end-point. Several direct and indirect methods are used to quantify the infarct area but contrast enhanced MRI is considered as the gold standard.
- Myocardial viability is measured by PET and SPECT, low-dose dobutamine echocardiography, and MRI. The PET-CT is the most accurate.
- Myocardial perfusion is the last item of structural end-points. MRI, nuclear imaging including SPECT and PET, and contrast echocardiography can quantify myocardial perfusion.

Biological endpoints include biomarkers such as N-terminal pro-brain type natriuretic peptide (NTproBNP) and inflammatory markers like IL-6, CRP (C reactive protein), and TNF-α. Functional capacity is based on patient performance status and exercise tests like the 6-min walk test and treadmill test, etc. (Dorobantu et al., 2017).

COMBINATION OF TISSUE ENGINEERING AND CELL THERAPY

Only cells or cells and biomaterials are widely used as a way of improving cardiac regeneration and recovery (Kim et al., 2018). Several studies show that a mixture of cells and biomaterials improves cell survival in stem cell transplantation in ischemic heart disease (Huang et al., 2016; Micheu, 2019). They are divided into two groups: in vitro tissue engineering and in situ tissue engineering (Christman and Lee, 2006; Hsiao et al., 2013; Ye et al., 2013; Madonna et al., 2019). *In vitro* tissue engineering manufacture scaffolds that contain cells and biomaterials together. Materials are mostly protein-based like collagen, fibrin, and alginate or synthetic polymers such as polyglycolic acid, polyglycerolsebacate, and polyethylene glycol (PEG) (Ye et al., 2013; Huang et al., 2016). In situ tissue engineering includes scaffold-free strategies that make it possible to directly inject a combination of cells and biomaterials into the injured myocardium. Biomaterials of this group commonly are fibrin glue, chitosan, Matrigel, alginate, self-assembling peptides, collagen, and decellularized extracellular matrices (ECMs) (Hsiao et al., 2013; Huang et al., 2016).

SAFETY AND ETHICAL ISSUES

Based on the classification of EMA, the combination of cells and biomaterials are in the group of advanced therapy medicinal products. Therefore there are some guidelines about the certainty of production process quality, evaluation of potential risks, and utilizing *in vitro* and *in vivo* tests to show the final product's potency and efficacy (Madonna et al., 2019). About ethical issues of clinical trials, researchers should consider that commercial interests do not influence them to start the trial before being ready to start it. They should also note that patients' expectations may make them interested to be in the group that gets interventions. It is also significant to predict the probable risks and challenges and consider suitable endpoints for the study (Niemansburg et al., 2013; Madonna et al., 2016).

CONCLUSION

In recent years the interdisciplinary and novel approaches of regenerative medicine have been utilized in the treatment of IHD, using stem cells to repair the lost part of the heart. There are some recommendations about using cellular patches setting for myocardial therapy. These include using therapeutic cell releasing products whilst avoiding inflammatory responses secondary to material degradation or biodegradation resistant patches. A responsible regulatory authority should be established to ensure that the required manufacturing and preclinical strategies have been carried out and upheld.

Stem cells can be harnessed more easily than other common cell types and are therefore mostly used in tissue engineering.

MSCs are one of the most commonly used cell types in this field. In preclinical studies, MSC injection in necrotic tissue in acute/chronic MI conditions leads to enhanced cell proliferation, cell protection against apoptosis and induces angiogenesis in the stimulated area. Moreover, studies have illustrated that applying MSCs with several factors and substances, for instance, Nestin, Asprosin, IGF-1, and the presentation of cell apoptosis, post-MI arrhythmia. Various types of stem cells containing xenogeneic, allogeneic, and autologous stem cells can be used for cardiac cell therapy. Accordingly, allogenic sources include fetal cardiomyocytes, EMSCs, human umbilical cord blood-derived cells, and autologous sources containing ADSCs, skeletal myoblasts, BMD stem cells, iPSCs, and RCSCs. ECM plays an important role in the function and homeostasis of the tissue. The bio-mimicking and bioactive properties of ECM proteins make them capable of being used as a hydrogel-based scaffold in cardiac tissue engineering, such as collagen and fibrin. On the other hand, one of the shortcomings is the possibility of activating post modification fibrosis due to the emerging stiffness-sensitivity of myocardial resident stromal cells. Furthermore, there are several obstacles in the way of tissue engineering in ischemic heart diseases such as low cellular survival and poor localization to the target area.

To prolong the activity of the graft, several approaches have been arranged, including (1) seeding of cells on preformed scaffolds, (2) self-assembly of cells in hydrogels, and (3) cell sheet engineering and (4) bio-fabrication. Moreover, there are several techniques to deliver stem cells into the myocardium; from direct syringe injection to the left ventricle, direct surgical myocardial injection, IC delivery of therapeutic agents. Minimally invasive access to the heart muscle and full sternotomy are suitable approaches but have some limitations, as after stem cell deliverance, the issue of cell homing arises. Signaling factors direct cells toward their destination, making homing possible. These factors are molecules like chemokines on the surface of stem cells to which chemokines, adhesion molecules, growth factors, and enzymes released from a specific tissue or organ bind. There are two major types of stem cell homing: endogenous homing and exogenous one after cell transplantation. After that, we should quantify the homing efficacy such as the level of radioactivity and the number of labeled cells. For human EmSCs to differentiate into cardiomyocytes, BMP4 and recombinant human activin A have to be added. This induces cardiac mesoderm to reproduce the main foundations of embryonic development. There are several protocols relating to this issue such as the Laflamme protocol and Yang protocol.

Another use of regenerative medicine is for tissue rejuvenation, especially in the field of cardiac diseases such as acute MI. Several studies show that a mixture of cells and biomaterials improves cell survival in stem cell transplantation in ischemic heart disease, which is divided into two groups: in vitro tissue engineering and in situ tissue engineering. Finally, it is significant to predict the probable risks and challenges and consider suitable endpoints for the study.

FUTURE PERSPECTIVES

Ischemic heart disease is still one of the major causes of mortality in human society. Cell-based therapy and regenerative medicine represent a new way to be hopeful about the treatment of IHD. It seems necessary to find the best source of stem cells and the most efficient way of importing them to the injured area of the myocardium. Recently, using biomaterials alone as a cardiac therapy has been discussed (Pascual-Gil et al., 2015; Huang et al., 2016) and more studies are needed to clarify the efficacy of this method. Studies show that the need for discoveries of molecular mechanisms that can be utilized in clinical practice dramatically increases because of low cell attachment to the graft. Moreover, researchers are working to increase the effectiveness of regenerative medicine by a combination of cell and gene therapy. Furthermore, cell sheet therapy is another novel approach that can increase the number of transplanted stem cells in the heart tissue. Pharmacological treatments may also affect the molecular pathways related to the increase in the qualification of cell therapy.

AUTHOR CONTRIBUTIONS

BA supervised the project from the scientific view of point and advised on study design. MiA, MaA, and SA-M drafted the manuscript. PPR and MH designed the figures and tables. AT-B participated in the study design and provided final approval of the version to publish. MR-T and RK participated in the study design and interpretation. BL supervised the project and participated in critical review. All authors read, provided feedback, and approved the final manuscript.

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REVIEW
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The Relationship Between Mesenchymal Stem Cells and Tumor Dormancy

Linxian Zhao[1†], Kai Zhang[1†], Hongyu He[2], Yongping Yang[1], Wei Li[1], Tongjun Liu[1] and Jiannan Li[1]*

[1] Department of General Surgery, The Second Hospital of Jilin University, Changchun, China, [2] Operating Theater and Department of Anesthesiology, The Second Hospital of Jilin University, Changchun, China

Tumor dormancy, a state of tumor, is clinically undetectable and the outgrowth of dormant tumor cells into overt metastases is responsible for cancer-associated deaths. However, the dormancy-related molecular mechanism has not been clearly described. Some researchers have proposed that cancer stem cells (CSCs) and disseminated tumor cells (DTCs) can be seen as progenitor cells of tumor dormancy, both of which can remain dormant in a non-permissive soil/niche. Nowadays, research interest in the cancer biology field is skyrocketing as mesenchymal stem cells (MSCs) are capable of regulating tumor dormancy, which will provide a unique therapeutic window to cure cancer. Although the influence of MSCs on tumor dormancy has been investigated in previous studies, there is no thorough review on the relationship between MSCs and tumor dormancy. In this paper, the root of tumor dormancy is analyzed and dormancy-related molecular mechanisms are summarized. With an emphasis on the role of the MSCs during tumor dormancy, new therapeutic strategies to prevent metastatic disease are proposed, whose clinical application potentials are discussed, and some challenges and prospects of the studies of tumor dormancy are also described.

Keywords: mesenchymal stem cells (MSCs), cancer stem cells (CSCs), disseminated tumor cells (DTCs), tumor dormancy, anti-tumor treatment

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Simone Pacini,
University of Pisa, Italy

Reviewed by:
Miranda Clements,
National Institutes of Health (NIH), United States
Margherita Cortini,
Rizzoli Orthopedic Institute (IRCCS), Italy

****Correspondence:***
Jiannan Li
jnli@ciac.ac.cn

[†] These authors have contributed equally to this work and share first authorship

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INTRODUCTION

Although clinical treatments are developing, cancer is still a significant challenge due to recurrence and metastasis. The unbridled proliferation and uncontrollable dormancy have been regarded as two main hallmarks of cancer (Yeh and Ramaswamy, 2015). Recent studies have discovered a direct association between cancer progression and tumor dormancy (Boire et al., 2019). In other words, tumor dormancy is one of the leading causes of tumor outgrowth, relapse, and metastasis. In 1934, tumor dormancy was firstly defined by Willis as an intricate phenomenon that tumor

Abbreviations: CSCs, cancer stem cells; DTCs, disseminated tumor cells; MSCs, mesenchymal stem cells; HSCs, hematopoietic stem cells; DCCs, dormancy-competent cancer stem cells; DICs, dormancy-incompetent cancer stem cells; DTCs, Disseminated tumor cells; CTCs, circulating tumor cells; BMSCs, bone marrow-derived mesenchymal stem cells; AMSCs, adipose tissue-derived mesenchymal stem cells; TA-MSCs, tumor-associated mesenchymal stem cells; MAPK, Mitogen-activated protein kinase; TGF-β, transforming growth factor-β; BMPs, bone morphogenetic proteins; hUCMSCs, human umbilical cord-derived MSCs; CXCL12, chemokine ligand 12; Gas6, growth arrest-specific protein 6; CXCR4, C-X-C motif chemokine receptor 4; HSCs; hematopoietic stem cells; EVs, Extracellular vesicles; BCCs, breast cancer cells.

cells stop proliferating but remain capable of malignant progression (Willis, 1953). In recent years, tumor dormancy has been extensively investigated. Tumor dormancy can occur in various phases of tumor progression, which is a primary cause of tumor recurrence even the primary tumor has been resected for decades (Kleffel and Schatton, 2013). Therefore, a deep understanding of the mechanisms of tumor dormancy is needed to develop more promising therapeutic strategies to eliminate dormant cells.

Based on the physiological traits, tumor dormancy can be divided into two categories: cellular dormancy and tumor mass dormancy. Cellular dormancy, a quiescence of solitary cells, is characterized by each cancer cell residing in the G0-G1 phase of the cell cycle. Besides, the underlying mechanisms of cellular dormancy, governed by the extracellular matrix, nutriture, and metastatic microenvironment, are complicated (Dittmer, 2017; Endo and Inoue, 2019). Tumor mass dormancy is characterized by entire neoplasm cells entering a balance between cell division and apoptosis, mediated by angiogenic dormancy and immunologic dormancy (Gao et al., 2017; Hen and Barkan, 2020). With the changes in the surrounding environment, these cessative cells can re-enter the cell cycle, leading to cancer recurrence. Likewise, the progression of cancer occurs in tumor mass dormancy when angiogenic dormancy and immune dormancy are reawakened by surrounding stimuli, after which the balance between proliferation and apoptosis will be broken (Yeh and Ramaswamy, 2015), leading to cancer recurrence. Besides, tumor dormancy is not a single state but an equilibrium determined by cell-intrinsic characteristics and surrounding niche (Senft and Jeremias, 2019).

Stem cells are units of biological organization with clonogenic potential and self-renewal capacity and can be differentiated into multiple functional cell lineages (Dulak et al., 2015). Up to now, stem cells have not been clearly defined due to elusive identities. Therefore, the classifications of stem cells are varied. There are two main classification criteria. In terms of development potential, stem cells can be divided into totipotent stem cells, pluripotent stem cells, and pluripotent stem cells. Totipotent stem cells are capable of differentiating into various kinds of cells and developing into complete human beings (Zakrzewski et al., 2019). Pluripotent stem cells are accompanied with the potential of differentiating into multi-tissue but don't possess the ability to develop into a complete individual (Abu-Dawud et al., 2018). Unipotent stem cells, derived from further differentiation of pluripotent stem cells, can only differentiate into one or two closely related types of cells (Dulak et al., 2015).

On the other hand, it has been proposed that the main sources of stem cells include embryonic tissues, adult tissues, fetal tissues, and differentiated cells with genetically reprogrammed (Qin et al., 2016). Therefore, based on their origins, stem cells can be divided into four categories: embryonic stem cells, fetal stem cells, adult stem cells, and cord blood stem cells (Li et al., 2020). Embryonic stem cells possess the capability of totipotent stem cells, which have a good potential for differentiation (Dou et al., 2020); Fetal stem cells have a higher potency of multi-differentiation than adult stem cells and can differentiate into numerous cell phenotypes (Bacakova et al., 2018). Adult stem cells are multipotent and are capable of differentiation into only a limited number of cell types, and therefore can only be used to repair and regenerate elected tissue (Han et al., 2019). Cord blood stem cells include hematopoietic stem cells (HSCs) and MSCs (Mohr and Zwacka, 2018). HSCs are generally isolated from bone marrow and have been successfully used in clinical treatment for 50 years, especially in hematological malignancies (Bujko et al., 2019). In recent years, MSCs also have shown great application potential and are mainly applied to treat tumors and various systemic diseases (Pajarinen et al., 2019). Details about MSCs are summarized in Chapter 4.

The research interest in stem cells is due to the potential application in a wide range of clinical treatments, including tissue engineering, cell replacement therapy, gene therapy, system reconstruction, and anti-aging treatment. However, the underlying mechanisms have not been thoroughly studied, which also promotes more researchers to focus on these unknown fields. In recent years, an increasing number of studies have been performed to investigate the interaction between stem cells and tumor development. Especially, with the discovery of cancer stem cells, the roots of uncurable cancer are gradually being ascertained. Among all stem cells, MSCs have been one of the most promising ones in cancer treatment. This review summarizes the mechanisms of MSC-mediated modulation of tumor dormancy and discusses the relationship between cancer stem cells and dormant tumor cells.

CANCER STEM CELLS AND DISSEMINATED TUMOR CELLS

In 1997, Bonnet et al. first isolated CSCs from patients of acute myeloid leukemia (Bonnet and Dick, 1997), and discovered that these CSCs show similar characteristics with normal stem cells, which are heterogeneous and also possess the capacity of self-renewal and multi-differentiation (Desai et al., 2019). During the next decades, it has been further confirmed that CSCs are a small subset of cancer cells that are resistant to conventional therapies and can initiate tumor formation (Eltoukhy et al., 2018b). Nowadays, it has been reported that the mechanisms of CSCs formation can be explained in two ways, namely the gene mutations of normal stem cells and the dedifferentiation of progenitor cells (Scheel and Weinberg, 2012; Crea et al., 2015; Eltoukhy et al., 2018b), also showing that CSCs are not a single subpopulation but a group of multi-source cells.

Based on the differentiation capacity, CSCs can be divided into dormancy-competent CSCs (DCCs) and dormancy-incompetent CSCs (DICs) (Talukdar et al., 2019). In this review, DCCs are mainly discussed as being capable of escaping immune clearance and entering dormancy and being responsible for tumor progression, metastasis, chemoresistance, as well as recurrence (Jones et al., 2012; De Angelis et al., 2019). Further studies have discovered that there are many similar characteristics and signaling pathways between DCCs and dormant tumor cells (Talukdar et al., 2019). For instance, it has been reported that DCCs and dormant cancer populations both can maintain quiescence, delay cancer recurrence, resist tumor therapy, and

evade immunologic clearance (Kleffel and Schatton, 2013), indicating that there may be a direct relationship between DCCs and tumor dormancy. In other words, DCCs may be the main subtype of dormant tumor cells. Interestingly, besides DCCs-derived dormant tumor cells, non-DCCs-derived ones have also been discovered in many cancers, also suggesting that non-CSC subpopulations also have the capacity of becoming quiescent (Talukdar et al., 2019).

Disseminated tumor cells (DTCs) were first discovered in the bone marrow. These cells not only play a pivotal role in the growth, dormancy, early metastasis, and late recurrences of minimal residual disease (Goddard et al., 2018), but also mark the early stage of cancers and have been used to monitor tumor progression (Synnestvedt et al., 2012). To date, the specific source of DTCs is still unclear. However, recent studies proposed that DTCs could be derived from circulating tumor cells (CTCs) and CSCs at all stages of tumor progression (Crea et al., 2015; De Angelis et al., 2019). In addition, by analyzing cell phenotype and function, researchers have discovered that DTCs and CSCs both possess similar dormant stem-cell-like abilities, such as immune evasion, chemoresistance, and the ability to increase the likelihood of recurrence (Hen and Barkan, 2020). By definition, DCCs and DTCs, the progenitor cells of tumor dormancy, both have the potential ability to become dormant tumor cells (Jahanban-Esfahlan et al., 2019; Talukdar et al., 2019). For example, the latest research has found that the CSCs of breast cancer could lurk in the bloodstream and premetastatic sites as CTCs and DTCs during the early stage of the primary tumor for two decades (De Angelis et al., 2019).

Moreover, DTCs are involved in the formation of tumor dormancy mediated by the organ microenvironment and immune system (Sosa et al., 2014; Risson et al., 2020). For instance, the dormant DTCs can evade immune system recognition and seed in bone marrow or distant tissues as the root of future metastasis (Hosseini et al., 2016; Risson et al., 2020), suggesting that tumor metastases may occur during the early phases of tumor progression. In conclusion, these findings indicate that DTCs and DCCs, like many sleeping seeds, are undetectable but can switch between the state of sleeping and proliferation (Hen and Barkan, 2020). Besides, the cell intrinsic or microenvironmental factors can also determine whether a niche is permissive or not. The bone marrow, a kind of particularly permissive microenvironment for tumor cells, can maintain DCCs in a dormant state for many decades before reactivation and ultimate metastatic outgrowth (Hen and Barkan, 2020). To prevent cancer metastasis, in future studies, what initiates CTCs/DTC quiescence and what promotes them to re-enter the cell cycle need to be clarified.

THE MECHANISMS OF TUMOR DORMANCY

Up to now, the mechanisms of tumor dormancy have only been partly illuminated, which can be divided into two categories: intracellular factors such as genetic alterations and the changes of signaling pathway; and extracellular factors such as tumor microenvironments, acidosis, hypoxia, angiogenic switch, as well as immunologic dormancy (Triana-Martinez et al., 2020). Therefore, only by deciphering mechanisms underlying MSC-mediated regulation of tumor dormancy, can more efficient targeted therapies be developed and the safe clinical application of MSCs be ensured.

Intracellular Mechanisms

Genetic Alterations

Genetic alteration not only contributes to tumorigenesis but also induces dormancy of cancer cells. It has been proposed that the mechanisms of DCCs entering dormancy are reversible genetic alterations due to microenvironmental stress such as hypoxic, lack of nutrition, and exogenous conditions. However, these alterations must be controlled within a reasonable range. If overaccumulation of mutations happens, DCCs will lose the dormancy potential and gradually become inadaptable to the bone marrow niche, leading to unrestrained tumor growth and multi-therapy-resistant cancers (Husemann et al., 2008; Crea et al., 2015). Furthermore, recent studies reported that some genetic alterations correlated with cell proliferation and/or differentiation which are involved in initiating and maintaining the dormant phenotype. For example, the upregulated KiSS1 gene can maintain the dormant state of melanoma, ovarian cancer, and breast cancer in preclinical cancer models (Gomatou et al., 2021).

Intracellular Signaling Mechanisms

As another key player of tumor dormancy, Mitogen-activated protein kinase (MAPK) pathways can instruct cells to respond to extracellular stimuli. MAPK family is a paramount mediator during cellular processes such as cell growth, differentiation, migration, proliferation, survival, and innate immunity (Johnson et al., 2005; Krens et al., 2006). Based on the composition of the signal transduction pathway, the MAPK family can be divided into three signaling cascades subfamilies: the extracellular signal-regulated kinases (ERK MAPK, Ras/Raf1/MEK/ERK), which is one of the most essential cascades for cell proliferation (Fang and Richardson, 2005), and is mainly activated by growth factors (Garrington and Johnson, 1999); the c-Jun amino-terminal kinases, which mainly regulates cell growth and cell apoptosis (Ip and Davis, 1998), and is activated by stress factors, differentiation factors, and growth factors (Weston and Davis, 2007); the p38 MAP kinases, which consist of p38α, p38β, p38γ and p38, all of which can be activated by stress factors and are essential for cell proliferation and differentiation (Nebreda and Porras, 2000; Krens et al., 2006). In terms of tumor dormancy, the effect of the different signaling pathways of the MAPK family is different. For instance, the activated p38 MAPK signaling pathway can induce DTCs into a quiescent status (Sosa et al., 2011) while the activated Jun N-terminal kinase pathway can promote tumor proliferation and outgrowth (Sui et al., 2014). Besides, Jo et al. have proposed that tumor cells lacking nutrients could enter into quiescence via reducing PI3K-AKT signaling (Jo et al., 2008). In addition, the extracellular signals also regulate tumor dormancy, such as the transforming growth factor-β (TGF-β) family and bone morphogenetic proteins (BMPs), which will be discussed in the rest of this paper.

Extracellular Mechanisms

Tumor Microenvironments

In comparison, extracellular factors are more complicated. It has been suggested that tumor dormancy is mainly regulated by tumor microenvironments, a complex network including extracellular matrix, bone marrow stem cell niche, perivascular niche, CSC niche, and so forth (Gay and Malanchi, 2017; Talukdar et al., 2019). Figuratively speaking, the tumor microenvironments are similar to the 'soil' in the 'seed and soil' theory, which was first proposed by James Paget at the end of the 19th Century (Phan and Croucher, 2020), and only when the 'soil' is fertile and suitable for the tumor cell (the 'seed'), will the dormant tumor cells start to re-proliferate. How these niches induce and maintain tumor dormancy and promote cell cycle arrest is not fully understood. The hypothesis is proposed that the bone marrow may provide a pro-dormancy signal to tumor cells. For example, it has been reported that most of the DTCs residing in the tumor niche are successfully eradicated by anti-tumor treatments and a few of them resided in bone marrow could enter into the state of quiescence and be mediated by bone marrow niches (Widner et al., 2018). Noteworthy, the microenvironmental factors may be one of the most complicated mechanisms of tumor dormancy, including many biochemical and biophysical factors, which can influence tumor dormancy by affecting interactions among myriad molecular (Talukdar et al., 2019). In conclusion, tumor dormancy is regulated by a series of complex cues, which are still unclear due to contextual molecular mechanisms and various undiscovered factors despite a series of plausible theories and hypotheses have been proposed. It has shown that tumor cells possess a mass of mutated signaling pathways to interact with cytokines secreted by various cells or tumor microenvironment.

Acidosis

The acidity of the extracellular microenvironment is the main characteristic of solid tumors, which is also correlated with the poor prognosis of many malignancies (Peppicelli et al., 2017). In recent years, more studies have focused on the relationships between the extracellular acidosis microenvironment and tumor dormancy. The underlying mechanisms of how extracellular acidosis may contribute to tumor cell dormancy has also been discussed. Current studies have reported that acidic tumor microenvironment was capable of promoting tumor dormancy through various mechanisms, such as increasing the percentage of cells in the G0 phase, regulating growth factor signaling and Raf/ERK pathway, increasing phosphorylation metabolism, leading to high resistance to apoptosis and autophagy, as well as hiding from immune surveillance (Peppicelli et al., 2017). In addition, the sustained extracellular acidity can reduce the intracellular pH from neutral to below pH 6.5, thus directly affecting both tumor cell and normal cell cycle through downregulating cyclin-dependent kinase 1-cyclin B1 activity (Putney and Barber, 2003). Interestingly, acidity can also awake the dormant tumor cells with genetic or epigenetic changes, leading to tumor recurrence, local invasion, and metastasis (Peppicelli et al., 2017). In MSCs, another study found that MSCs can be induced to secrete high levels of TGFβ family members in an acidic microenvironment, thus contributing to tumor dormancy (Peppicelli et al., 2015). Moreover, a low pH can facilitate tumor-recruited MSCs to secrete pro-tumorigenic cytokines. For example, in osteosarcoma, acidosis can lead to the reprogramming of tumor-recruited MSCs to the secretion of osteosarcoma-supporting mediators, such as IL6, IL8, and NF-κB inflammatory pathway (Di Pompo et al., 2021).

Hypoxia

A hypoxic microenvironment is very common in various malignancies, especially in solid tumors caused by rapid tumor cell growth and disorganized angiogenesis.

Previous study has reported that hypoxia could lead tumor cells to resist traditional treatment and induce a malignant tumor phenotype (Pouyssegur et al., 2006). In recent years, to better mimic hypoxic microenvironment and investigate the relationship between hypoxia and tumor dormancy, many *in vitro* dormancy models have been performed, such as hypoxic chamber, adding iron-binding/substitute agents, as well as imposing diffusion-limited hypoxia culturing cells in 3D hydrogel systems (Butturini et al., 2019). Further study proposed that the hypoxia-resistant breast cancer cell line could enter into a reversible dormant status in reoxygenation conditions, which might be caused by cells autophagy (Carcereri de Prati et al., 2017). A similar phenomenon was also observed in colorectal cancer and urothelial carcinoma (Endo et al., 2014). Other studies also have proposed that hypoxia was involved in regulating tumor dormancy by influencing the expression of molecules, such as angiostatin, thrombospondin, epoxyeicosatrienoic acids, as well as vascular endothelial growth factor (Gomatou et al., 2021).

Angiogenic Switch

In addition to the above mechanisms, the angiogenic switch has been proved to play a key role in regulating tumor dormancy (Shaked et al., 2014). With the progression of the tumor, the angiogenic capacity must be matched to the rate of tumor cell growth (Folkman, 1971). In other words, if tumor mass cannot acquire sufficient angiogenic potential during the tumor progression, these tumor cells will enter into a dormant status. The angiogenic dormancy can be reckoned as a stage of tumor progression when the pro- and anti-angiogenic roles achieve a balance. The angiogenic switch refers to the transition from the inability of angiogenesis to the acquisition of angiogenic potential, resulting in growing vascularized tumors (Hanahan and Folkman, 1996). It has been further confirmed that the angiogenic switch is regulated by angiogenic factors and oncogene expression (Ribatti et al., 2007; Natale and Bocci, 2018). These angiogenic factors are derived from tumor cells or other cells residing in the tumor microenvironment. The expression of these factors can be influenced by environmental stress and genetic changes, such as activation of oncogenes and inhibition of tumor suppressor genes (Kerbel, 2008). For example, it has been reported that hypoxia signaling and oxygen species are involved in the regulation of tumor dormancy by generating growth factors such as urokinase receptor, focal adhesion kinase, and epidermal growth factor receptor (Talukdar et al., 2019).

Immunologic Dormancy

Plenty of studies have reported that the immune system plays critical roles in regulating and maintaining tumor dormancy (Kleffel and Schatton, 2013; Hu et al., 2020). Current studies have found that T lymphocytes and CD8+ T cells are involved in regulating immune-mediated cancer dormancy at the metastatic site (Jahanban-Esfahlan et al., 2019). Particularly, an association of immune cells and their inflammatory mediators are involved in immunological dormancy. For instance, it has been proposed that the immune cells residing in the bone-tumor microenvironment could produce pro-inflammatory cytokines and anti-inflammatory cytokines, called the "bone-tumor-inflammation network" to mediate inflammatory responses (Hu et al., 2020). Another study found that DTCs were capable of escaping from immune-surveillance and resulted in tumor escape (Endo and Inoue, 2019). Accordingly, we can improve the efficacy of tumor immunotherapy by utilizing immunotherapy treatments to target disseminated cancer dormancy, aiming to control cancer growth and even eliminate tumors.

MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) are a subset of stromal cells that possess the ability of multi-lineage differentiation and self-renewal (Ding et al., 2011). To be more specific, MSCs are reckoned as one subtype of adult stem cells, which not only have the capability of normal stem cells, but also show some unique multipotent properties (Kim and Kim, 2019) such as immune privilege, tumor homing feature, and anti-tumor ability (Eltoukhy et al., 2018b), making MSCs the most promising stem cells for curing cancer and ensuring safe clinical applications and trials. It has also been suggested that the effect of MSCs on tumor growth is contradictory, which can play anti-tumor or pro-tumor roles on tumor development (Bartosh et al., 2016). It can be concluded that MSCs are the most commonly used stromal cells in anticancer experimental or clinical researches because of their great therapeutic potential.

Current studies have shown that MSCs could interact with tumor cells and their niches through the paracrine pathway and physical mechanisms (Cammarota and Laukkanen, 2016), to mediate the growth of a primary tumor and the spread of distant metastasis (Ridge et al., 2017; Ahn, 2020). For example, the bone marrow-derived MSCs (BMSCs) can induce breast cancer cells into dormancy via the paracrine route to activate the JAK/STAT3 signaling pathway (Leyh et al., 2015), which partly illuminates the reasons why the metastatic latency period of breast cancer can prolong to 25 years after primary tumor resection. Studies have also found that whether MSCs promote or inhibit tumor progression and formation (Timaner et al., 2020) depends on the type of tumor, the source of MSCs, as well as tumor microenvironment. For example, relative studies have discovered that adipose tissue-derived MSCs (AMSCs) could promote the growth of brain tumors, ovarian tumors, gastric tumors, and breast tumor (Lim et al., 2016; Li et al., 2020), but inhibit the growth of melanoma both *in vitro* and *in vivo* (Ahn et al., 2015). Another study has proposed the allogeneic MSCs could induce melanoma cells into a malignant tumor (Djouad et al., 2003).

In 1986, Dvorak first proposed that the tumors are wounds that do not heal (Dvorak, 1986). In recent years, with the further research of cancer, it has been considered that MSCs are capable of regenerating and promoting wound healing (Lyu et al., 2020). However, tumor "wound" is different from conventional tissue wound, and MSCs can aggravate these non-healing wounds by directly or indirectly interacting with the tumor wound microenvironment (Li et al., 2019). For example, tumor-associated MSCs (TA-MSCs) usually promote conventional tissue wound healing via accelerating inflammation response and suppressing adaptive immunity. However, these two mechanisms are beneficial for tumor progression, resulting in cancer wound overhealing (Li et al., 2019). On the other hand, the bidirectional interaction between MSCs and tumor cells can result in constant stroma renewal, which is similar to a wound that never heals (Cammarota and Laukkanen, 2016).

In addition to the above studies, studies on the relationship between MSCs and tumor dormancy have also been extensively concluded in the antitumor field. During the migration into the tumor niche, MSCs can crosstalk with tumor cells and shape cancer phenotype (Spaeth et al., 2008). In this paper, the ways of different sources of MSCs influence tumor dormancy are summarized, providing more opportunities and challenges for researchers to focus on this field.

THE INFLUENCE OF MSCS ON TUMOR DORMANCY

Mesenchymal stem cells have been discovered in most organs, including the menses blood, endometrial polyps, bone marrow, fetal tissues, umbilical cord matrix, and adipose tissue (Ding et al., 2011; Murphy et al., 2013; Li et al., 2020), but current studies have shown that different sources of MSCs possess distinct characteristics. These differences also partly illustrate why MSCs with different sources can play even opposite effects on tumor progression. It has been discussed in this paper that MSCs could either inhibit or promote tumor growth, but the specific mechanism is still controversial (Galderisi et al., 2010). Notably, tumor microenvironment plays an important role in regulating tumor dormancy, including the inhibition and promotion effects (**Figure 1**). For instance, TA-MSCs and BMSCs have different phenotypes, which may be influenced by tumor microenvironment factors, such as cancer cytokines and secretory protein (Cammarota and Laukkanen, 2016). In this review, the effects of BMSCs, hUCMSCs, TA-MSCs, and AMSCs on tumor dormancy are summarized. **Table 1** shows the effect of MSCs on tumor dormancy.

Bone Marrow-Derived Mesenchymal Stem Cells

In 1970, Friedenstein et al. (1970) reported the BMSCs which were derived from bone marrow for the first time. In the next few years, subsequent studies further proposed that BMSCs could also derive from other organs and tissues, such as teeth, adipose

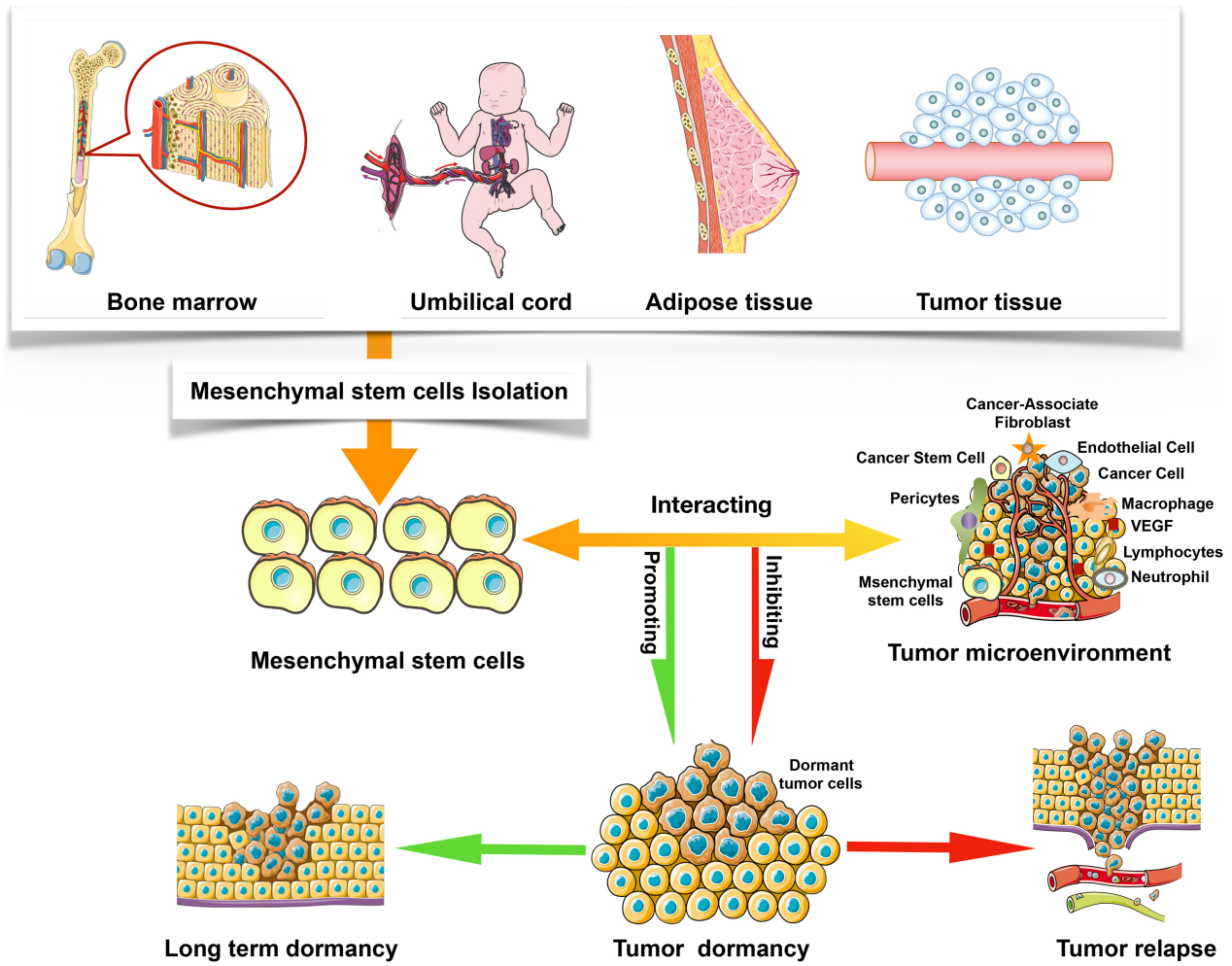

FIGURE 1 | The sources of MSCs and the interaction between MSCs and tumor microenvironment.

tissue, skin, and amniotic fluid (Mushahary et al., 2018; Mastrolia et al., 2019). In the present review, we mainly focus on the role of BMSCs from bone marrow on tumor dormancy. In addition to BMSCs, the bone marrow niche also includes osteoblasts, osteoclasts, as well as endothelial cells, all of which could regulate tumor dormancy (Fornetti et al., 2018; Widner et al., 2018). Thus, Hu et al. (2020) have regarded the bone marrow as a "bone-tumor microenvironment" including various immune cells and cytokines that directly mediate tumor progression. Noteworthy, it has been proposed that bone marrow is the most common tissue where dormant tumor cells re-enter the cell cycle, leading to metastatic disease recurrence (Widner et al., 2018). Pantel et al. (2009) further reported that bone marrow might serve as a common homing site for dormant tumor cells, and can increase the interaction between tumor cells and BMSCs. It has also been suggested that DTCs could crosstalk with BMSCs in the bone marrow (Bliss et al., 2016; Cammarota and Laukkanen, 2016), and there are abundant secreted factors such as all-trans retinoid acid and BMP-7 in the bone marrow microenvironment, which can inhibit the growth of DTCs and drive tumor cells into dormancy (Linde et al., 2016; Gay and Malanchi, 2017). The BMSCs mainly reside in the bone marrow stem cell niche, perivascular niche, and endosteal niche (Phan and Croucher, 2020). Therefore, BMSCs can interact with these niches and DTCs/CSCs directly or indirectly (Patel et al., 2014; Casson et al., 2018), including producing factors, secreting exosomes and extracellular vesicles and exosomes, cellular cannibalizing BMSCs, as well as inducing cellular morphology changes. All in all, once the DTCs enter into the bone marrow, the BMSCs will be recruited to interacting with these tumor cells (Walker et al., 2016).

Producing Factors

Current studies have reported that BMSCs can secrete a variety of microRNAs and various cell cytokines such as TGF-β family molecules, chemokine ligand 12 (CXCL12), growth arrest-specific protein 6 (Gas6), and so on (Decker et al., 2017; Talukdar et al., 2019; Hu et al., 2020). These substances can directly interact with target cells or be indirectly stored inside exosomes, leading to the dormancy of DTCs and CSCs. TGF-β

TABLE 1 | The effect of MSCs on tumor dormancy.

MSCs types	The effect on tumor dormancy	Factor/signal	Mechanism	Brief introduction	References
BMSCs	Dual role	TGF-β1	(1).Promoting breast cancer cell proliferation by inhibiting the angiogenic dormancy (2).Inducing squamous cell carcinoma latency by regulating cell-cycle gene transcription to control a reversible G1 cell-cycle arrest.	In terms of different tumors, TGF-β1 can play distinct roles on tumor dormancy.	Ghajar et al., 2013; Brown et al., 2017; Jahanban-Esfahlan et al., 2019
	Promoting	TGF-β2 GAS6	(1).Driving CSCs and DTCs into quiescence by inducing low ERK/P38 signal ratio (2). Induced tumor dormancy by crosstalking with AXL and GAS6 (3). GAS6 can regulate tumor dormancy by being combined with TAM receptors such as AXL and Tyro3	The dormant tumor cells can express abundant TGF-β2 to maintain the dormant state. GAS6 can be derived from BMSCs or osteoblasts	Taichman et al., 2013; Yumoto et al., 2016; Goddard et al., 2018; Axelrod et al., 2019; Hen and Barkan, 2020
	Regulating	TGF-β3	Promoting the proliferation and metastasis of head and neck cancer by inducing matrix-specific protein periostin	TGF-β family members are involved in tumor cells proliferation, differentiation, and tumor dormancy.	Qin et al., 2016
	Promoting	atRA	Increasing the expression of TGF-β2 via activating p38 and p27 MAPK-dependent pathways	TGF-β2 mainly provokes dormancy during tumor progression	Linde et al., 2016
	Promoting	BMP4	Inducing breast cancer dormancy via activating SMAD1/5 signaling	BMP is one subgroup of the TGF-β family, which can influence the induction of tumor dormancy	Gao et al., 2012
	Promoting	BMP7	Inhibit tumor cell growth and drive CSCs into dormancy by mediating the expression of N-myc downstream-regulated gene 1 (NDRG1) and activating p38 MAPK and p21	BMP7 could induce dormancy of prostate cancer	Kobayashi et al., 2011; Widner et al., 2018
	Promoting	CXCL12	(1).Inducing BMSCs to migrate to cancer site (2). Triggering DTC dormancy by promoting the exchange of cell-cell information and cellular adhesion between MSCs and DTCs (3). Regulating tumor dormancy by mediating the tumor inflammatory responses	CXCL12 is a classic chemokine that can promote tumor cells in the bone marrow to enter dormancy.	Balkwill, 2004; Widner et al., 2018; Susek et al., 2018
	Inhibiting	IL-10	Inhibit the growth of lymphoma and leukemia cells by reducing the secretion of interleukin IL-10	MSCs can regulate the expression of IL-10 to induce tumor dormancy	Lee et al., 2019
	Regulating	NE	(1).Binding with β2-adrenergic receptors (2).Modulating the expression levels of GAS6	The neurons can regulate tumor dormancy through releasing NE	Widner et al., 2018
	Promoting	MiR-127 MiR-197 MiR-222 MiR-223 MiR-23b	(1).Driving breast cancer cells into quiescence through reducing the expression of CXCL12 (2).The DTCs can promote BMSCs to express abundant distinct miRNAs such as miR222/223 and miR23b, all of which can result in the dormancy of certain DTCs by suppressing the TGF-b pathway	Dormant breast cancers could promote MSC to release exosomes including distinct miRNA such as miR-127, -197, -222, and -223 The quiescent phenotype of tumor cells can be reversed by antagomiR-222/223	Guasch and Blanpain, 2004; Ono et al., 2014; Bliss et al., 2016

(Continued)

TABLE 1 | (Continued)

MSCs types	The effect on tumor dormancy	Factor/signal	Mechanism	Brief introduction	References
	Prompting	Cell cannibalism	The cannibalism of MSCs could also drive MDA-MB-231 BCCs to enter dormancy under demanding conditions	The cannibalized MDA-MB-231 BCCs obtain a similar cell phenotype with dormant tumor cells	Bartosh et al., 2016
	Promoting	TWIST1	Inducing tumor micrometastatic dormancy by activating tumor growth-inhibitory signals pathway	The expression of TWIST1 is upregulating after being co-cultured with BMSCs in 3D non-adherent culture platforms	Tran et al., 2011
	Promoting	LOX JNK p38	Driving MDA-MB-231 BCCs into dormancy through cooperating with TWIST1	TWIST1 can regulate micrometastatic dormancy by interacting with LOX, JNK, and p38	El-Haibi et al., 2012
	Promoting	SASP	(1).Activating cytokine and chemokine signaling (2).Inhibiting cell proliferation and vascular development (3).Initiating inflammatory/immune response	There is an obviously upregulating of the expression of CXCL1, CXCL2, GCSF IL-1α, IL-1β, IL-8, and PAI-1, all of which are integral to the expression of the senescence-associated secretory phenotype (SASP)	Özcan et al., 2015; Bartosh, 2017
	Promoting	Cellular morphology	Inducing tumor cell dormancy by regulating the changes of extracellular matrix such as hypoxia and ECM detachment	Cellular morphology changes show many analogous features with cell cannibalism	Sosa et al., 2013
	Promoting	JNK SAPK FTIs	(1).The breast tumor cell morphology change makes these cells enter into dormancy through activating the JNK/SAPK signaling pathway (2).FTIs can induce breast cancer cells into reversible dormancy by undergoing morphology	There are direct and indirect links between morphology changes and tumor dormancy.	Chatterjee and van Golen, 2011
hUCMSCs	Promoting	β-catenin c-Myc Wnt	(1).Driving lung cancer cells to be arrested in the G0/G1 phase (2).Driving hepatocellular cancer cells are arrested in the S phase (3).Downregulating the expression of β-catenin and c-Myc	The detailed mechanisms of hUCMSCs on tumor dormancy may include inducing cell cycle arrest, promoting tumor cell apoptosis, as well as inhibiting the migration of cancer cell	Yuan et al., 2018
TA-MSCs	Promoting	TRAIL CXCL12 TGF-β MMPs microRNAs	(1).Drive epithelial tumor cells to enter dormancy during the tissue remodeling stage (2).Inhibit angiogenesis by expressing inhibitory factors (3).Initiating a cell cannibalism behavior	TA-MSCs often play an important role in the progression of tumor growth and metastasis.	Lee et al., 2012; Li et al., 2019
AMSCs	Promoting	miRNAs Wnt TGF-β	(1).Regulating the tumor dormancy of breast cancer by secreting multiple circulating miRNAs (2).Arrested dormant BCCs in G0/G1 phase and S phase	The AMSCs are capable of transporting the exosomes carrying miRNAs to BCCs, which can target the Wnt and TGF-β signaling pathways, thus regulating tumor dormancy.	Mohd Ali et al., 2020

MSCs, mesenchymal stem cells; BMSCs, bone marrow-derived mesenchymal stem cells; hUCMSCs, Human umbilical cord mesenchymal stem cells; TA-MSCs, tumor-associated mesenchymal stem cells; AMSCs, adipose tissue-derived mesenchymal stem cells; CSCs, cancer stem cells; DTC, disseminated tumor cells.

family members are very common in various cells and organs, which not only directly affects inflammation response and tissue repair, but also regulates cell proliferation, differentiation, and tumor dormancy (Seoane and Gomis, 2017). The roles of TGF-β family members on tumor progression are different at distinct stages (Prunier et al., 2019). It has been reported that TGF-β family members could suppress tumor cell proliferation at an early stage but promote tumor metastasis at an advanced stage (Papageorgis, 2015), which may be caused by different subtypes of TGF-β can activate distinct signal pathways and produce diverse chemokines. For example, TGF-β1 has a dual role in tumor dormancy, promoting breast cancer cell proliferation by inhibiting the angiogenic dormancy (Ghajar et al., 2013; Jahanban-Esfahlan et al., 2019) while inducing tumor cell latency of squamous cell carcinoma (Brown et al., 2017). However, TGF-β2 mainly provokes dormancy during tumor progression, which can drive CSCs and DTCs into quiescence by inducing a low ERK/P38 signal ratio (Goddard et al., 2018; Hen and Barkan, 2020), and conversely, these dormant tumor cells can express abundant TGF-β2 to maintain the dormant state (Johnson et al., 2016; Yumoto et al., 2016). Notably, during this induced dormancy progress, TGFβ2 is required to crosstalk with AXL and GAS6 (Yumoto et al., 2016).

Growth arrest-specific protein 6 can be derived from BMSCs or osteoblasts and plays an important role in the progression of tumor cells dormancy and reactivation by being combined with TAM receptors such as AXL and Tyro3 (Taichman et al., 2013; Axelrod et al., 2019). Decker et al. (2017) have proposed that neurons could also govern tumor dormancy through releasing norepinephrine, which can bind with β2-adrenergic receptors and modulate the expression levels of GAS6, thus promoting or inhibiting cancer cell proliferation (Widner et al., 2018). Moreover, it has been reported that all-trans retinoid acid could increase the expression of TGF-β2 by activating p38 and p27 MAPK-dependent pathways (Linde et al., 2016). In contrast, there are few reports about the influence of TGF-β3 on tumor dormancy, which proposed that TGF-β3 could promote the proliferation and metastasis of head and neck cancer by inducing matrix-specific protein periostin (Qin et al., 2016).

Bone morphogenetic proteins, one subgroup of the TGF-β family, also influence the induction of tumor dormancy. Especially, BMP4 and BMP7 are two common objects of dormancy study with mouse models, in which it has been discovered that BMP4 could induce dormancy of breast cancer (Gao et al., 2012) and BMP7 could induce dormancy of prostate cancer (Kobayashi et al., 2011). Furthermore, BMP4 was capable of inducing tumor dormancy via activating SMAD1/5 signaling (Gao et al., 2012) and BMP7 can inhibit tumor cell growth and drive CSCs into dormancy by mediating the expression of N-myc downstream-regulated gene 1 and activating p38 MAPK and p21 signaling pathways (Kobayashi et al., 2011; Widner et al., 2018). It has also been suggested that TGFβ and BMP both can stimulate MSCs to differentiate into osteoblasts, which were involved in the formation of the bone-tumor microenvironment (Chen et al., 2012).

In addition to BMSCs, other bone marrow stromal cells such as endothelial cells and osteoblasts can also secrete CXCL12, acting as an inducer for BMSCs to migrate to cancer sites (Widner et al., 2018). CXCL12 is capable of binding with the C-X-C motif chemokine receptor 4 (CXCR4) expressed on the surface of DTCs, directly triggering DTC dormancy through promoting the exchange of cell-cell information and cellular adhesion between BMSCs and DTCs (Balkwill, 2004; Widner et al., 2018). In addition, the CXCR4/CXCL12 axis has been reckoned as an important marker and mediator of tumor cells homing to bone (Reagan and Rosen, 2016). The bone marrow was capable of secreting a high level of CXCL12, which could be regulated by TGF-β family members (Yu et al., 2017) and microRNAs (Dittmer, 2017). For instance, connexin 43-based gap junction, a channel connected between breast cancer and BMSCs, can be used to exchange microRNAs, thus suppressing the synthesis of CXCL12 (Lim et al., 2011). Further studies have discovered that hematopoietic stem cells (HSCs) can also express CXCR4 (Sugiyama et al., 2006). Therefore, HSCs can be home to bone marrow and remain dormant with the combination of CXCL12 and CXCR4 (Hoggatt et al., 2016). On the other hand, CXCL12 also mediates the tumor inflammatory responses by interacting with various immune cells, which can either promote or suppress tumor dormancy (Susek et al., 2018).

Producing Exosomes and Extracellular Vesicles

Extracellular vesicles (EVs) are defined as cell structures composed of proteins, miRNAs, nucleic acid, and biological signaling molecules (vakhshiteh et al., 2019). Exosomes are one subtype of EVs with diameters between 20 and 100 nm (Susek et al., 2018). EVs can be derived from various subcellular compartments, and each type of exosome or EVs includes specific molecular constituents and plays distinct roles in tumor development (El Andaloussi et al., 2013; Pitt et al., 2016). For example, the fibroblast-secreted exosomes are involved in the migration of breast tumor cells via regulating autocrine Wnt–PCP (planar cell polarity) signaling pathway (Luga et al., 2012). In addition, the cancer cell-derived exosomes can promote tumor formation and metastasis by regulating the functions of surrounding noncancerous cells (Peinado et al., 2012), promoting angiogenesis, and neutrophil infiltration (Ono et al., 2014). Furthermore, the exosomes derived from stromal cells are capable of transferring their contents into tumor cells, thus influencing the progression of cancer (Luga et al., 2012; Roccaro et al., 2013). The emphasis in this paper is the effects of BMSC-derived exosomes and EVs on tumor dormancy. The BMSC-derived EV cargo is composed of tumor-supportive molecules and various miRNAs such as miR-205, miR-31, miR23b, and miR21, all of which are key mediators in tumor dormancy (Vallabhaneni et al., 2017; Casson et al., 2018).

Firstly, it has been demonstrated that BMSCs can directly crosstalk with tumor cells via these BMSC-derived EVs (Ono et al., 2014). In other words, the exosomes and EVs can be utilized to transfer proteins and RNAs from BMSCs to tumor cells. BMSC-derived exosomes and EVs are like a two-edged sword, which can either promote or inhibit tumor progression (vakhshiteh et al., 2019; Zhao et al., 2020). According to the latest discoveries, tumor-derived EVs are capable of promoting tumor cell proliferation (Cappariello and Rucci, 2019). However, the

BMSC-derived EVs can transfer dormancy initiating factors such as miRNAs into tumor cells (Widner et al., 2018), thus leading to the formation of tumor dormancy.

Although components of exosomes and EVs are multitudinous, the most commonly used in tumor dormancy researches is miRNAs, which can regulate tumor dormancy through modulating the expression of CXCL12 and the duration of TGF-β signaling. For example, current studies have discovered that dormant breast cancers could promote MSC to release exosomes including distinct miRNA such as miR-127, -197, -222, and -223, driving breast cancer cells into quiescence through reducing the expression of CXCL1 (Hanahan and Folkman, 1996; Bliss et al., 2016). Besides, the DTCs and MSCs can easily crosstalk within bone marrow as their anatomical locations are near, and the DTCs can promote BMSCs to express abundant distinct miRNAs such as miR222/223 and miR23b (Ono et al., 2014), and conversely, these miRNAs can result in the dormancy of certain DTCs by suppressing the TGF-b pathway (Guasch and Blanpain, 2004). On the other hand, it has been suggested that the quiescent phenotype can be reversed by antagomiR-222/223 (Bliss et al., 2016). More importantly, another study has proposed that the miR222/223 can also result in tumor cell drug resistance (Hanahan and Folkman, 1996), and there are abundant miR222/223 in the BMSC-derived exosomes and EVs, indicating that BMSCs can induce drug resistance of cancer (Ruksha, 2019). Based on these discoveries, antagomir-222/223 can be used to target dormant breast cancer cells, which may be a promising therapeutic strategy.

There are many interactions between cancer cells and BMSCs in the bone marrow via paracrine secretion or gap junctional intercellular communication, which can also be used to transport miRNAs. For instance, it has been suggested that miRNAs can be exchanged between BMSCs and BBCs through gap junctional intercellular communication established by bone marrow stroma[48], which directly controls the proliferation of breast cancer cells. In addition to BMSC-derived exosomes and EVs, many other circulating exosomes and EVs such as OS-derived, cancer-derived, and bone-derived, all of which can also be used as a promising clinical tool for clinical management and monitoring, as well as an index to screen therapeutic efficiency (Cappariello and Rucci, 2019).

Cannibalizing Bone Marrow-Derived Mesenchymal Stem Cells

Cell cannibalism can occur among the homotypic type of cells or heterotypic types of cells (He et al., 2013). Cell cannibalism is also thought to be a live-cell feeding behavior, which is not just about obtaining resources from other cells and is distinct from traditional macrophages phagocytosis, a live-cell entosis, as well as cytoplasm emperipolesis (Overholtzer et al., 2007). Bartosh et al. have discovered that MDA-MB-231 breast cancer cells (BCCs) could cannibalize BMSCs in 3D co-cultures, and the cannibalism of MSCs could also drive MDA-MB-231 BCCs to enter dormancy under demanding conditions (Bartosh et al., 2016). During the cannibalism of this progress, BMSCs quickly encapsulate clusters of MDA-MB-231 BCCs and change their phenotype of tumor cells, and then BMSCs are internalized (Bartosh et al., 2016). Further studies have indicated that the cannibalized MDA-MB-231 BCCs obtain a similar cell phenotype with dormant tumor cells (Bartosh et al., 2016). At the same time, BMSCs could also drive MDA-MB-231 BCCs into quiescence/dormancy through producing factors and exosomes. In addition to secreting various factors such as microRNA and signal proteins, a significant increase of expression of transcription factor TWIST1 after being co-cultured with BMSCs in 3D non-adherent culture platforms has also been observed, which can activate tumor growth-inhibitory signals pathway, and thus leading to the micrometastatic dormancy (Tran et al., 2011; Bartosh et al., 2016). Besides, the expression of lysyl oxidase and Jun N-terminal kinase also increased in these tumor cells, both of which can cooperate with TWIST1 to drive MDA-MB-231 BCCs into dormancy (El-Haibi et al., 2012). In other words, TWIST1 can regulate micrometastatic dormancy by interacting with lysyl oxidase, Jun N-terminal kinase, and p38. According to MDA-MB-231BCCs phenotype analysis, there is an obvious up-regulation of various cytokines/chemokines such as CXCL1, CXCL2, GCSF IL-1α, IL-1β, IL-8, and PAI-1 (SERPINE1), all of which are integral to the expression of the senescence-associated secretory phenotype, an important mediator in regulating tumor dormancy and relapse, which could induce tumor dormancy via activating cytokine and chemokine signaling, inhibiting cell proliferation and vascular development and initiating inflammatory/immune response (Özcan et al., 2015; Bartosh, 2017).

Cell cannibalism and dormancy represent cell survival status, in which cell growth might arrest or slow down. But there is no doubt that the cannibalism of BMSCs directly associates with tumor dormancy in tumor niches. However, the characteristics of cannibalistic tumor cells are distinct from traditional CSCs, indicating that these dormant cells may be a new cell population (Mitra et al., 2015). Further studies have proposed that the cannibalistic BCCs can recover the proliferation ability once they return to suitable surroundings (Bartosh et al., 2016). Moreover, one study has suggested that BMSCs with different sources might show distinguish cannibalism effects, and the detailed cues are still unclear (Castellone et al., 2013). Likewise, our observed results are linked to cell cannibalism, but the pathophysiological effect is not completely understood. Cell cannibalism, which has been proposed for many years was initially used to assess the deterioration of cancer (Gupta and Dey, 2003). Furthermore, current studies have also found that cell cannibalism could promote tumor formation and tumor gene transfer (Gupta and Dey, 2003; Bartosh et al., 2016). In conclusion, cell cannibalism may be just one of the ways of cellular interaction. With the development of the co-culture model, more information about the properties of BMSCs and cell cannibalism will be discovered, contributing to new methods of cancer treatment.

Inducing Cellular Morphology Changes

Compared with cell cannibalism, cellular morphology change is a self-degradative process occurring inside the cell to avoid apoptosis through degrading organelles such as mitochondria (Levine and Kroemer, 2008). Cellular morphology changes show

many analogous features with cell cannibalism (Walker et al., 2016). Some studies have proposed that morphology could result in cell dormancy, which is regulated by extracellular matrix (Kai et al., 2019). For example, when being in microenvironmental stresses such as hypoxia and extracellular matrix detachment, tumor cells are capable of quickly entering into a dormancy-like state (Sosa et al., 2013). These dormant tumor cells usually reside in secondary sites, resulting in tumor recurrence after a long time. For instance, Chatterjee et al. discovered that breast tumor cell morphology changes make these cells enter into dormancy by activating the JNK/SAPK signaling pathway. Besides, farnesyl transferase inhibitors can induce breast cancer cells into reversible dormancy by undergoing morphology, which has also been used in clinical anti-tumor research (Chaterjee and van Golen, 2011).

Further study proposes that autophagy is involved in the formation of the "stemness" of tumor cells (Talukdar et al., 2018), which also explains why normal tumor cells can show similar characteristics with CSCs. In conclusion, these studies suggest there are direct and indirect links between morphology changes and tumor dormancy. However, whether MSCs can induce tumor dormancy through cellular morphology changes is still unclear, and the study on the relationship between MSCs and morphology changes is rare. To better understand the mechanisms of tumor dormancy, how MSCs regulate morphology should be investigated in future studies.

Human Umbilical Cord Mesenchymal Stem Cells

Human umbilical cord mesenchymal stem cells (hUCMSCs), a type of adult stem cell stemming from the umbilical cord matrix, have been discovered to possess the ability to inhibit tumor cell proliferation and metastasis, and are regarded as a significant potential treatment tool for solid tumors (Ciavarella et al., 2011; Yuan et al., 2018). hUCMSCs possess the general characteristics of MSCs, such as self-renewal and multi-directional differentiation ability, and have stronger expansion capability and lower risk of virus contamination during the process of cell-based therapy than BMSCs. Subramanian et al. have discovered that hUCMSCs do not differentiate into tumor-associated fibroblasts during the interaction with the tumor microenvironment, indicating that hUCMSCs are safer than BMSCs (Subramanian et al., 2012).

To date, plenty of studies have focused on the cross-talk interaction between hUCMSCs and tumor cells, and it has been shown that hUCMSCs could either promote or inhibit tumor development (Yuan et al., 2018). For instance, Shen et al. have revealed that IFNβ gene-transfected hUCMSCs could significantly suppress the growth of human triple negative breast carcinoma cell lines MDA-MB-231 and Hs578T (Shen et al., 2016). It has also been reported that hUCMSCs could attenuate proliferation and induce apoptosis of glioma cells through regulating cell cycle progression, downregulating the expression of anti-apoptotic genes, β – catenin, c-Myc, as well as upregulating the level of apoptotic genes such as caspase-3 and caspase-9 (Yang et al., 2014; Yuan et al., 2018). By contrast, Yang et al. have discovered that hUCMSCs could promote tumor proliferation and metastasis, which have been observed in a lymph node, carcinoma, gastric cancer, and esophageal carcinoma (Yuan et al., 2018). To sum up, the effects of hUCMSCs on tumors are different, depending on the types of cancer. In the following part, the relationship between hUCMSCs and tumor dormancy is discussed.

In terms of tumor dormancy, one study has shown that hUCMSCs could support tumor dormancy via induction of cell cycle arrest in specific phases, leading to microscopic tumor cell clusters enter a balance between proliferation and apoptosis (Yuan et al., 2018). However, different types of tumor cells might be arrested in distinct cell cycle phases. For example, hUCMSCs can drive lung cancer cells to be arrested in the G0/G1 phase, while hepatocellular cancer cells are arrested in the S phase (Yuan et al., 2018). In addition to hUCMSCs, Lee et al. have discovered that BMSCs could also suppress the proliferation of hematologic malignancy by inducing tumor cell cycle arrest (Lee et al., 2019).

In addition, another study has indicated that hUCMSCs could not only inhibit the proliferation of human lung cancer cells and human hepatocellular carcinoma cells but also induce these tumor cells into dormancy by downregulating the expression of β-catenin and c-Myc, two key players in the Wnt signaling pathway (Yuan et al., 2018). In conclusion, these studies suggest that the detailed mechanisms of hUCMSCs on tumor dormancy may include inducing cell cycle arrest, promoting tumor cell apoptosis, as well as inhibiting the migration of cancer cells. Moreover, for a better understanding of the effect of hUCMSCs on tumor dormancy, the expression of dormancy biomarkers has been further analyzed. It is found that the level of ephrin receptor, a common tumor dormancy marker, obviously increases, indicating that tumor cells might have entered dormancy (Yuan et al., 2018). There is still a long way in studying the effects of hUCMSCs on tumor dormancy, and studies have shown that the specific mechanism of hUCMSCs on tumor dormancy is different in terms of the types of tumor cells.

Tumor-Associated Mesenchymal Stem Cells

Tumor-associated mesenchymal stem cells, derived from normal MSCs in the tumor microenvironment, are distinct from other organ-derived MSCs such as BMSCs and AMSCs (Trivanovic et al., 2016). TA-MSCs, which actively regulate tumor growth and metastasis during cancer progression, have been discovered in various types of tumors such as gastric, liver, breast, prostatic, and ovarian cancer (Shi et al., 2017). Besides, it has been found that TA-MSCs influence tumor progression through secreting cytokines and chemokines (Shi et al., 2017). These factors are contributing to tumor metastasis and drug resistance. Likewise, TA-MSCs can be differentiated into myofibroblasts and support the survival of CSCs and angiogenesis. All in all, TA-MSCs often play an important role in the progression of tumor growth and metastasis. However, it has been observed that TA-MSCs could also suppress the proliferation of epithelial tumor cells and drive them to enter dormancy during the tissue remodeling stage (Li et al., 2019). Next studies have found that TA-MSCs could

not only inhibit angiogenesis by expressing inhibitory factors such as TNF-related apoptosis-inducing ligand, CXCL12, TGFβ, matrix metalloproteinases, and microRNAs but also initiate a cell cannibalism behavior (Lee et al., 2012; Li et al., 2019). The above effects are both involved in the regulation of tumor dormancy. At present, there is no clear definition and classification between MSCs and TA-MSCs, which, in most studies are discussed together. Earlier in this review, how MSCs influence tumor dormancy is analyzed and these mechanisms could also be discovered during the process of interaction between TA-MSCs and the tumor microenvironment.

Adipose Tissue-Derived Mesenchymal Stem Cells

In recent years, AMSCs have been seen as one of the most promising anticancer treatments in cancer-targeted therapy (Ramdasi et al., 2015). For example, it has been proposed that the AMSCs can regulate the tumor dormancy of breast cancer via secreting multiple circulating miRNAs (Mohd Ali et al., 2020) with the stromal AMSCs and BCCs being co-cultured in a non-contact microenvironment model. After 48h of co-culture, the proliferation of BCCs is significantly inhibited, which may be due to these tumor cells are driven to the dormant state by the AMSCs. Further studies have proposed that this inhibitory effect is correlated with the distribution of the cell cycle and most dormant BCCs were arrested in G0/G1 phase and S phase. In contrast, when returning to the original culture condition, these co-cultured dormant cells can reenter cell regulation. To be more specific, the AMSCs are capable of transporting the exosomes carrying miRNAs to BCCs, which can target the Wnt and TGF-β signaling pathways, thus regulating tumor dormancy.

CLINICAL APPLICATION POTENTIALS

Stem cell-based therapies have been used in anticancer researches for many years and MSCs are considered the most potent therapy tools. The reasons are summarized below. Firstly, MSCs can directly restrain the growth of some tumors by inhibiting the vasculature or arresting the cell cycle. Secondly, the engineered or modified MSCs have also been applied as targeted anticancer carriers for gene therapy (Li et al., 2020), which are more efficient and safer than naive MSCs. Moreover, MSCs can cross-talk with CSCs/DTCs via paracrine mechanisms, and regulate the biological activity of tumors (Lin et al., 2019), for example, AMSCs could suppress the growth of lung carcinoma cells by secreting cytokines such as interferon (IFN)-β and tumor necrosis factor-related apoptosis-inducing ligand (Cortes-Dericks and Galetta, 2019). In this review, the influence of MSCs on tumor dormancy is summarized and the ways of MSCs inducing CSCs and DTCs into dormancy is shown, helping to develop more promising treatment therapies.

During the past decade, researchers have realized that dormant tumor cells might be the primary reasons for therapy failure as the drug-resistant cells and dormant cancer cells share many similar biological features such as heterogeneity and plasticity (Luga et al., 2012), According to current clinical studies, there are two alternative strategies based on tumor dormancy. One way is to reawaken dormant tumor cells before treatment to make these cells susceptible to the therapies such as chemotherapy, immunotherapy, targeted therapy, and radiotherapy. For instance, one study has suggested that DTCs can be eliminated by manipulating their surrounding microenvironment to influence their communication with tumor stromal cells (Peinado et al., 2012). The other way is to drive dormant tumor cells into a perpetual dormant state. Because disseminated cancer cells could enter into the perivascular niche and remain dormant for decades, previous therapies can be applied to prolong the dormancy periods to prevent tumor metastasis and recurrence. For example, one clinical study has discovered the survival of patients with glioblastoma multiforme was significantly reduced, which may be caused by losing the control of dormant tumor cells. However, maintaining a perpetual tumor dormant state or preventing tumor cells from establishing dormancy can prolong the life of these patients (Ono et al., 2014).

More specifically, with the rapid development of stem cell-based therapies and molecular biology, great progress has been achieved in MSCs-based antitumor studies. It has shown that MSCs can be used to regulate tumor dormancy, which will provide more effective clinical strategies to prevent or delay cancer recurrence. Based on tumor dormancy, the potential clinical applications of MSCs on anticancer studies are summarized below.

Firstly, the MSCs and their exosomes can be used as carriers to target cancers. It is acknowledged that MSCs are capable of homing to sites of tissue injury and tumor microenvironment by reducing immune-inflammatory responses (Eltoukhy et al., 2018b). Based on this feature, researchers have proposed that MSCs could be used as carriers to deliver nanoparticles, anti-tumor drugs, proteins, lipids, DNAs, mRNAs and miRNA (Eltoukhy et al., 2018b; Kwon et al., 2019). For example, one study reported that internalization of paclitaxel-loaded nanoparticles to MSCs can achieve promising antitumor efficacy, which is caused by sustained release of the encapsulated paclitaxel to the tumor microenvironment (Wang et al., 2018). Another study proposed that BM-MSCs can induce breast CSCs into dormancy through releasing inhibitory miRNA such as antagomiR-222/223 (Ono et al., 2014; Eltoukhy et al., 2018b). Moreover, Lai et al. have conducted more detailed studies and discovered that the exosomes derived from MSCs are better candidates for drug delivery due to natural features such as easy isolation, strong tolerability, as well as the ability to bind with the plasma membranes (Vallabhaneni et al., 2017). It is believed that MSCs–derived exosomes can be used to prevent the recurrence of cancer. Although in the experimental investigation stage, these studies have developed a new therapeutic strategy for cancer.

Secondly, cannibalism of MSCs can be used to assess the prognosis of cancer. Current studies have suggested that the cannibalism of MSCs can promote tumor dormancy, and happen in highly aggressive tumors most frequently (Bartosh et al., 2016). Therefore, Thomas et al. have proposed that there is a logical cause-and-effect relationship between cell cannibalism and dormancy (Bartosh et al., 2016). To be more specific, cannibalistic

cells can be used to identify cancer phenotype, which will provide new windows for cancer therapeutic intervention and prognostic evaluation. Furthermore, with the improvement of the 3D coculture model, more mechanisms underlying the mediation of cell cannibalism by MSCs will be unraveled and more antitumor tools can be used in clinical application.

Third, the formation of tumor dormancy can be intervened by targeting the key players of interaction between MSCs and tumor cells. Until now, with the discovery of more and more key players regulating organ-specific metastasis and dormancy (Neophytou et al., 2019), targeted therapy has been frequently used in clinical applications. Directly targeting tumor cells is highly effective cancer therapy. However, the MSCs can interact with CSCs and drive them into a state of cycling dormancy through secreting proteins, exosomes, and cytokines, which will make these CSCs resist targeted therapy (Lim et al., 2011; Patel et al., 2012; Ono et al., 2014). Therefore, to make these CSCs drug-sensitive, the interaction between the CSCs and MSCs needs to be disrupted (Eltoukhy et al., 2018a). One of the most promising strategies for reducing bone metastasis is to target dormancy-associated niches. In clinical practice, bone metastasis is reckoned as an indicator of poor prognosis in most cases. With the description of the "bone metastatic niche" concept, which consists of connective tissues, bone stromal cells, and signaling molecules, some researchers have proposed that bone metastatic niche can be targeted to prevent or delay the progression of bone metastasis (Ren et al., 2015). For example, BMSCs, one subtype of bone stromal cells, can either promote or impede the progression of tumor dormancy by secreting BMP7, TGFβ2, GAS6 (Kaplan et al., 2006; Decker et al., 2017; Hu et al., 2020; Li et al., 2020). Targeting the bone metastatic niche can disrupt the interaction between MSCs and tumor cells, thus restraining the formation of tumor dormancy. Consistent with these discoveries, another study has suggested that dormant tumor cells could become sensitive to normal cytotoxic therapies by targeting at DTC dormant niche (Saito et al., 2010). All in all, directly targeting the mechanisms underlying tumor dormancy is a new clinical cancer strategy.

Finally, some researchers have proposed that immune therapy combined with dormancy therapy would develop a new cancer therapy (Linde et al., 2016). For example, immunotherapy has been successfully used in maintaining the dormancy of HIV, which has many similarities to tumor cells (Boire et al., 2019). Simultaneously, MSCs can either suppress or enhance the immune function of cancer cells as influencing tumor proliferation (Roccaro et al., 2013). In conclusion, a better understanding of the immunobiological pathways of dormant tumor cells will help study the mechanisms of how MSCs mediate tumor dormancy (Kleffel and Schatton, 2013).

CHALLENGES AND PROSPECTS

Tumor dormancy has attracted more and more attention from researchers. However, related investigations are limited by the lack of classical models that can represent the human condition. The essential effects of MSCs on tumor dormancy have been discussed. The influence of bone marrow microenvironment such as fibroblasts, endothelial cells, and immune cells can also not be ignored (Reddy et al., 2012). However, it is difficult to recreate the structure of the bone marrow, limiting the studies of other cells within the bone marrow. In the past several years, many researchers have used the 2D model of the bone marrow to replicate the interaction between cellular and soluble factors (Chong Seow Khoon, 2015). However, as time goes on, traditional 2D co-cultures platforms have shown deficiencies (Widner et al., 2018). For instance, 2D co-cultures could not adequately mirror the natural bone marrow microenvironment and physiological conditions of cells, which greatly limits the therapeutic testing and system modeling *in vitro* (Bartosh et al., 2016).

To better study the interactions between bone marrow and tumor dormancy, it is essential to model a platform microenvironment where different types of cells can be co-cultured and cell phenotype can be analyzed (Eltoukhy et al., 2018b). Fortunately, in recent years, with the emergence of 3D bioprinting and engineered organotypic models, an increasing number of fundamental problems of tumor biology have been addressed. For example, an engineered organotypic microvascular niche was utilized to study the interaction between endothelial cells and breast cancer cells. The results showed that endothelial-derived thrombospondin-1 could directly induce sustained breast cancer cells quiescence (Ghajar et al., 2013). Similarly, a 3D system can be created to recapitulate the bone microenvironment (Gungor-Ozkerim et al., 2018). This system can be used to better understand the biological properties of MSCs and mirror natural conditions *in vivo* (Clark et al., 2018). Besides, the model can be used to create an MSCs-tumor stem cell co-culture system, which greatly helps us investigate the interaction between the tumor cells and their niches (Sart et al., 2014; Moore et al., 2018). Likewise, we will easily select cell types and figure out which cells and niches support tumor dormancy (Eltoukhy et al., 2018b). However, though these models have made a great contribution, the whole picture of dormancy-related cellular processes is still far from clear (Cole, 2009). Undoubtedly, only when the data collected from different dormancy models are analyzed together, may the stealth mechanisms of tumor dormancy start to be clarified.

In recent years, a plenty of mouse models have been established to reveal the mechanisms of various diseases. However, it is still difficult to validate the molecular mechanisms of dormancy in mouse models. First of all, due to the fact that tumor dormancy is a phenomenon that occurs at low frequency, developing a well-established mouse model of tumor dormancy is probably impractical. In addition, each mechanism of tumor dormancy has different complex characteristics, and current mouse models cannot precisely monitor and manipulate single or small clusters of tumor cells. On the other hand, even there are applicable conceptual models of dormancy, the result of these experimental studies is still difficult to be validated in patients. Therefore, it is very important to establish a sophisticated experimental dormant model and to isolate CSCs and DTCs from the tumor microenvironment, and then simulate the interaction between dormant tumor cells and the surrounding niches. Currently, since the dormant tumor cells are undetectable via

traditional whole-body imaging tools (Linde et al., 2016), it is better to develop new diagnostic tools that can detect dormant tumor cells in patients as soon as possible. In the long run, if more biomarkers of dormant tumor cells can be identified before tumorigenesis or more quantitative methods and calculable tools can be used in clinical diagnosis, the recurrence and metastasis of cancer will be efficiently prevented.

CONCLUSION

Whether MSCs promote or suppress tumor dormancy is still controversial. From an individual point of view, based on their sources, MSCs can be divided into two types: tumor-derived MSCs and other sources-derived MSCs. The tumor-derived MSCs are progeny of tumor cells, which mainly play a pro-tumor role during the tumor progression but other sources-derived MSCs play an anti-tumor role. However, this rule is not absolute and depends on the types of tumors. Aparts from being influenced by the sources of MSCs and the types of cancer, the effects of MSCs on tumor progression can also be influenced by the experimental models. Therefore, the interpretation of some experimental results was somewhat inconsistent, even the opposite. In the future, with the development of the 3D bioprinting technique, the modeling methods will be greatly improved.

In recent years, MSC-based therapies have been frequently used by clinical researchers. During these processes, MSCs are modified through the transfection of therapy genes and transportation of target agents, but the inherent characteristics and biological properties of MSCs are not changed. Combining with the studies of tumor dormancy, researchers can develop more modified MSCs to intervene in the progression of tumor dormancy. Although more detailed mechanisms underlying the interaction between MSCs and tumor cells needs further study, current studies are trying to apply engineered MSCs as treatment carriers to regulate tumor dormancy to prevent or delay tumor progression. All in all, the treatment potential of MSCs on tumors is infinite. Although it is difficult to apply these strategies clinically due to the limitations of current technical merit, there is no doubt that a huge step forward has been taken in the fight against cancer.

AUTHOR CONTRIBUTIONS

LZ and KZ drafted the review. HH generated the graphs. YY guided the construction of the manuscript. WL edited the review. JL and TL provided input on the scope and content of the review. All authors contributed to the article and approved the submitted version.

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REVIEW
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Therapeutic Mesenchymal Stem/Stromal Cells: Value, Challenges and Optimization

Mehdi Najar[1,2†], Rahma Melki[3†], Ferial Khalife[4], Laurence Lagneaux[1], Fatima Bouhtit[3,5], Douaa Moussa Agha[3,5], Hassan Fahmi[2], Philippe Lewalle[5], Mohammad Fayyad-Kazan[6,7‡] and Makram Merimi[3,5‡]*

[1]*Laboratory of Clinical Cell Therapy, Institut Jules Bordet, Université Libre de Bruxelles (ULB), Brussels, Belgium,* [2]*Osteoarthritis Research Unit, University of Montreal Hospital Research Center (CRCHUM), Montreal, QC, Canada,* [3]*Genetics and Immune-Cell Therapy Unit, LBBES Laboratory, Faculty of Sciences, University Mohammed Premier, Oujda, Morocco,* [4]*Laboratory of Cancer Biology and Molecular Immunology, Faculty of Sciences I, Hadath, Lebanon,* [5]*Laboratory of Experimental Hematology, Institut Jules Bordet, Université Libre de Bruxelles (ULB), Bruxelles, Belgium,* [6]*Department of Natural Sciences, School of Arts and Sciences, Lebanese American University, Hadath, Lebanon,* [7]*Laboratory of Cancer Biology and Molecular Immunology, Faculty of Sciences-I, Lebanese University, Hadath, Lebanon*

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Simone Pacini,
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***Correspondence:**
Mehdi Najar
mnajar@ulb.ac.be

†These authors share first authorship

‡These authors share senior authorship

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Cellular therapy aims to replace damaged resident cells by restoring cellular and molecular environments suitable for tissue repair and regeneration. Among several candidates, mesenchymal stem/stromal cells (MSCs) represent a critical component of stromal niches known to be involved in tissue homeostasis. *In vitro*, MSCs appear as fibroblast-like plastic adherent cells regardless of the tissue source. The therapeutic value of MSCs is being explored in several conditions, including immunological, inflammatory and degenerative diseases, as well as cancer. An improved understanding of their origin and function would facilitate their clinical use. The stemness of MSCs is still debated and requires further study. Several terms have been used to designate MSCs, although consensual nomenclature has yet to be determined. The presence of distinct markers may facilitate the identification and isolation of specific subpopulations of MSCs. Regarding their therapeutic properties, the mechanisms underlying their immune and trophic effects imply the secretion of various mediators rather than direct cellular contact. These mediators can be packaged in extracellular vesicles, thus paving the way to exploit therapeutic cell-free products derived from MSCs. Of importance, the function of MSCs and their secretome are significantly sensitive to their environment. Several features, such as culture conditions, delivery method, therapeutic dose and the immunobiology of MSCs, may influence their clinical outcomes. In this review, we will summarize recent findings related to MSC properties. We will also discuss the main preclinical and clinical challenges that may influence the therapeutic value of MSCs and discuss some optimization strategies.

Keywords: mesenchymal stem/stromal cells, therapeutic features, clinical value, challenges, optimization

GRAPHICAL ABSTRACT | The road map for the use of MSCs.

1 INTRODUCTION

Mesenchymal stem/stromal cells (MSCs) and their secretome have been investigated for the treatment of several medical indications. Establishing a clear definition and characterization of MSCs (including their origin, terminology and identity), identifying the major preclinical and clinical challenges linked to their application and finally proposing suitable therapeutic optimization strategies may help highlight the value of MSCs and therefore develop stem cell-based therapy.

1.1 Definition and Origin

MSCs represent a heterologous subset of nonhematopoietic precursors that are broadly distributed throughout the body. The ontogenic origin of MSCs is still controversial. In addition to their most widely accepted mesodermal origin, MSCs may originate in part from neuroepithelial tissue or perivascular tissue (Huang et al., 2011). Currently, it has been established that MSCs can be isolated from all vascularized tissues since MSCs reside in the walls of blood vessels, forming part of the endothelium (Caplan, 2009). MSCs can be derived from a variety of tissues, including the bone marrow, adipose tissue, peripheral blood, umbilical cords, Wharton's jelly, dental pulp and other tissues (Kumar et al., 2019). MSCs have been investigated in the field of cellular therapy and regenerative medicine to treat a variety of diseases and disorders (Kabat et al., 2020). This research interest is due to the relatively easy and minimally invasive access to MSCs as well as their several properties. MSCs harbor immunomodulatory, anti-inflammatory, angiogenic, antioxidative and antiapoptotic capacities (Wei et al., 2013; Kaukua et al., 2014). As will be discussed, these effects mainly involve paracrine pathways rather than direct cell differentiation (Wu et al., 2020; Xunian and Kalluri, 2020). MSCs present a high self-renewing capacity that enables their *ex vivo* expansion to obtain a sufficient number of cells for clinical purposes (Berebichez-Fridman and Montero-Olvera, 2018). Because MSCs generate most of the stromal cells present in the bone marrow (BM), form part of the hematopoietic stem cell (Klein et al., 2018) niche, and produce various molecules regulating hematopoiesis, their hematopoiesis-supporting capacity has been demonstrated (Fajardo-Orduna et al., 2015). These features allow the use of MSCs for the *in vitro* expansion of HSCs before their transplantation (Sensebe et al., 1995; Yamaguchi et al., 2001).

1.2 Terminology

The terminology of MSCs has considerably evolved over time (**Figure 1**). Since their initial characterization, these cells were referred to as MSCs, an acronym that was used to indicate "Mesenchymal Stromal Cells," "Mesenchymal Stem Cells," "Multipotent Stromal Cells," "Mesodermal Stem Cells" (Caplan, 2017), "skeletal stem/progenitor cells" (Bianco, 2011), "mesenchymal progenitor cells" and "pericytes mesenchymal stem cells" (Caplan, 2017). Initially, Friedenstein termed those cells "mechanocytes" or osteogenic stem cells" and then "marrow stromal cells". In subsequent work, those cells were designated "marrow fibroblasts" (Friedenstein et al., 1970). In the 1980s, the term "marrow stromal cells" was adopted to distinguish mesenchymal stromal cells that were able to maintain hematopoiesis for many weeks *in vitro* from other adherent

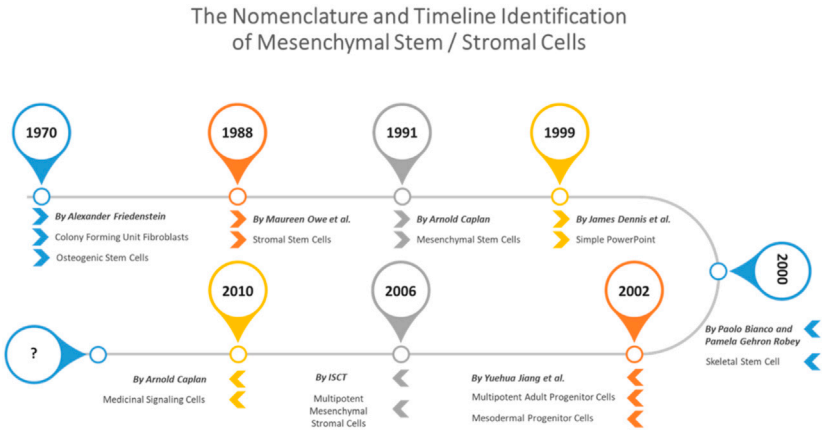

FIGURE 1 | The nomenclature of MSCs over the time.

hematopoietic cells, such as macrophages and marrow fibroblasts (Keating et al., 1984). In 1991, Caplan proposed the term "mesenchymal stem cells" due to their multilineage differentiation potential. Although the term became popular and was widely used, the mesenchymal stem cell nomenclature proved to be problematic when it became obvious that not all plastic adherent stromal cells have comparable self-renewal and *in vivo* differentiation ability into multiple lineages. More recently, Caplan recommended designating these cells "medicinal signaling cells" to highlight the mechanism underlying their therapeutic effects after transplantation, which is believed to be based mainly on the secretion of a plethora of anti-inflammatory, antiapoptotic, proangiogenic and immunosuppressive factors facilitating regenerative processes (Caplan, 2017).

Given that MSCs are now being derived from different tissue sources and exhibit distinct phenotypes and functions, a consortium of international MSC investigators held a series of workshops to address the challenges facing the field, including a reassessment of MSC nomenclature. The consensus of the Nomenclature Working Group has recommended the following terminology for mesenchymal stromal cells: donor type (autologous or allogeneic), species of origin (e.g., human, mouse), tissue source (e.g., bone marrow, adipose tissue, cord blood), and mesenchymal cell type (stem cell, stromal cell or progenitor cell) (Bourin et al., 2013). As proposed by Bhartiya D et al., it is better to revisit the definition of MSCs based on their functional attributes (Bhartiya, 2018).

1.3 Characterization of Mesenchymal Stem/ Stromal Cells

Many research groups have used distinct tissue sources and developed different protocols for MSC isolation, cultivation and expansion, which have resulted in heterogeneous populations of cells and difficulties in comparing experimental outcomes. This difficulty is, in part, due to the lack of a definitive marker of MSCs (Viswanathan et al., 2019).

Due to the growing controversy regarding the nomenclature, degree of stemness and characteristics of MSCs, the International Society for Cellular Therapy (ISCT) published two important reports to address these limitations. The first report clarified the terminology, emphasizing that while most mesenchymal stem or stromal cells are not stem cells, the bulk population represents a multipotent mesenchymal stromal cell population (Horwitz et al., 2005). In the second report, ISCT recommended the usage of "multipotent mesenchymal stromal cells" (MSCs) to refer to the plastic-adherent fraction of stromal tissues regardless of their origin capable of *in vitro* differentiation into specific, multiple cell lineages (Dominici et al., 2006).

To standardize the isolation and characterization of MSCs *in vitro*, ISCT has specified the minimum inclusion criteria for defining MSCs. These criteria include:

1) The ability to adhere to plastic under standard culture conditions;
2) The phenotypic expression of surface markers such as CD73, CD105 and CD90. The absence of a series of surface markers, including CD14, CD19, CD34, and CD45.
3) The ability to differentiate into osteoblasts, chondrocytes and adipocytes *in vitro* under the effect of specific culture media (Dominici et al., 2006).

Later, ISCT proposed criteria for the immunological characterization of MSCs. These include 1) MSC response to interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α); 2) indoleamine 2,3-dioxygenase (IDO) response in cytokine licensing assays; 3) assessment of the functionality of the expanded cell product; 4) usage of purified immune responders in functional assays; 5) analysis of mechanistic and efficacy studies of human MSCs in xenotransplantation models; 6) immune reaction to infused MSCs; and 7) analysis of the lymphocyte populations of patients treated with MSCs (Krampera et al., 2013).

1.4 Therapeutical Potential of Mesenchymal Stem/Stromal Cells

Over the past decades, a large number of studies have emerged using MSC-based therapies in preclinical studies to treat many different pathologies, including cardiovascular diseases, bone and cartilage diseases and immune-/inflammation-mediated diseases (Damasceno et al., 2020). However, the therapeutic potential of MSCs in cancer treatment is still controversial. Depending on several parameters, MSCs have been shown to either promote or suppress tumor development (Hmadcha et al., 2020). Because of this reality, a sustained effort to understand such duality before planning an MSC-based therapy for cancer is required. Herein, we provide an overview of some preclinical and clinical studies that may highlight the value of MSCs.

1.4.1 Preclinical Models

MSCs represent a primary choice for treating immunological disorders such as acute graft-versus-host disease (GvHD) and Crohn's disease (Galipeau and Sensebe, 2018; Carvello et al., 2019). A meta-analysis of 50 studies involving 1,848 animals showed that MSCs significantly prevented mortality and alleviated the clinical manifestations of GvHD (Wang et al., 2019). MSCs represent optimistic hope in treating rheumatoid arthritis (RA), as there is currently no effective treatment for this chronic inflammatory autoimmune disorder (Liu et al., 2020). Intravenous injections of MSCs derived from the human umbilical cord in a mouse model of RA showed a promising therapeutic effect (Yu et al., 2019). A preclinical study using MSCs derived from human adipose tissue reported that several mechanisms were involved in the therapeutic benefit of MSCs for treating RA (Zhou et al., 2011). Using BM-MSCs with induced colony-stimulating factor-1 in a mouse CCL4 (C-C Motif Chemokine Ligand 4) model of cirrhosis synergistically improved reduced liver fibrosis and improved hepatocyte proliferation (Watanabe et al., 2019).

A study performed on a guinea pig model using human bone marrow-derived MSCs (BM-MSCs) combined with hydroxyapatite scaffolds to treat temporal bone defects showed promising results, as the treatment was safe and effective and improved the repair of bone defects (Skoloudik et al., 2018). Another study using human umbilical cord-derived MSCs showed optimistic results in treating vertebral bone defects in weaned rabbits (Cui et al., 2019). Mouse BM-MSCs were shown to attenuate ischemia-reperfusion brain injury and inhibit microglial apoptosis (Cheng et al., 2021). Human umbilical cord MSCs (UC-MSCs) effectively improved renal function and inhibited inflammation and fibrosis in a rat model of diabetic nephropathy (DN) (Xiang et al., 2020). BM-MSCs have been shown to be protective in a rat model of renal ischemia reperfusion injury (Quirici et al., 2002) by inhibiting cell apoptosis and inflammatory responses (Li et al., 2019).

As MSCs are regulators of tissue homeostasis, they are also a promising material for the restoration of skeletal muscles after injury. Although skeletal muscle recovery is mainly provided by muscle stem cells, namely, satellite cells (MuSCs), MSCs may also participate in such regeneration. Thus, MSCs appear to be a promising approach for the restoration of skeletal muscle structure and function. Different studies have reported the positive effects of MSCs on the repair and regeneration of injured skeletal muscle tissue. It was observed that rat BM-MSCs do not show potency for myogenic differentiation *in vitro* under the influence of appropriate inducers and rarely fuse with myoblasts when cocultured. However, the BM-MSCs stimulate the differentiation of muscle tissue cells through paracrine secretion (Sheveleva et al., 2020). As reviewed by Qazi et al. (2019) MSCs from different sources have been shown to improve muscle contractility and structure and reduce inflammation in various muscle injury models. Both BM-MSCs and AT-MSCs improved the regeneration of skeletal muscle laceration injury at short- and long-term durations (rat models). Effective reinnervation of injured muscles occurred only in the long term. However, AT-MSCs showed better regenerative effects, evidenced by a significant increase in the number of myotubes and a significant decrease in collagen deposition (Moussa et al., 2020). MSCs can promote angiogenesis, cell recruitment, migration, proliferation and differentiation within the site of injury. They can also modulate the immune cell population surrounding the injured muscle (Qazi et al., 2019). In particular, MSCs are able to induce the proliferation and differentiation of resident MuSCs and are also able to act on other cellular components of the muscle cell niche by reducing inflammation and infiltration (Sandona et al., 2021). Several studies have demonstrated that the efficacy of MSCs in supporting skeletal muscle regeneration is linked to their secretome. The production of many biologically active factors with a wide spectrum of action may explain the effects of MSCs. Within the site of damage, these factors can exert a bioactive effect either by acting directly on muscle cell populations or by modulating the local environment. As indicated by Wang et al., several signaling pathways may participate in skeletal muscle regeneration (Wang et al., 2020). Using MALDI imaging mass spectrometry, a study revealed the early molecular processes of muscle healing upon treatment with MSCs and highlighted the critical role of trauma-adjacent tissue during the therapeutic response (Klein et al., 2018). Proteomic profiling highlighted that enriched pathways related to extracellular matrix organization, axon guidance, antigen processing, metabolic processes, immunomodulation and positive regulation of nitric oxide are also involved in muscle regeneration by MSCs (Sandona et al., 2021). *In vitro*, *in vivo* and bioinformatic results showed that MSCs promote skeletal muscle regeneration through the synergistic action of EVs and the soluble fraction of the secretome (Mitchell et al., 2019). Thus, several regulators of muscle regeneration, such as ectodysplasin-A2, thrombospondin-1, IL-6, monocyte chemoattractant protein-1 (MCP-1), dickkopf-related protein 1 (DKK1), HGF, VEGF, FGF7, tissue inhibitor of metalloproteinase 1 (TIMP-1), SMAD family member 4 (SMAD4), macrophage inflammatory protein 2 (MIP-2), activin A, insulin-like growth factor-binding protein (IGFBP)-related protein 1 and MMP-1, have been identified (Kim et al., 2016). Recently, a study provided compelling experimental evidence of the ability of the MSC secretome to exert a protective effect against eccentric

contraction (EC)-induced skeletal myofiber damage (murine model). The secretome was able to modulate the behavior of SCs, which are key players in muscle tissue regeneration (Squecco et al., 2021). The long-term effectiveness of BM-MSCs for skeletal muscle regeneration was clearly established after 1 year of treatment. Using a pig model of severe radiation burn, local injection of BM-MSCs improved recovery skeletal muscle damage by acting on muscle regenerative capacity, muscle fibrosis and angiogenesis (Linard et al., 2018). Moreover, combination therapy between MSCs and muscle progenitor cells also enhanced skeletal muscle regeneration in muscular dystrophies. In this case, BM-MSCs create an appropriate muscle pro-regenerative environment by secreting trophic factors that regulate the proliferation and differentiation of muscle progenitor cells as well as immunomodulatory factors to manage local inflammation (Klimczak et al., 2018). EVs produced by MSCs of different tissue origins can influence myogenesis and fibrosis, the main processes that accompany skeletal muscle regeneration. Novokreshchenova et al. (2020) found that EVs derived from rat MSCs of distinct sources (BM, AT, intact muscle) significantly increased the number of newly formed myotubes in myoblast culture *in vitro* and reduced the number and size of fibrotic nodules in muscle fibroblast culture *in vitro*. In a previous report, exosomes from BM-MSCs were shown to promote myogenesis and angiogenesis *in vitro* and muscle regeneration in a mouse model of cardiotoxin-induced muscle injury. Although these exosomes had low concentrations of muscle repair-related cytokines, a number of repair-related miRNAs were identified (Nakamura et al., 2015). A system of MSC-encapsulated fibrin microbeads demonstrated effectiveness in shortening the regeneration period of volumetric muscle loss injury in a rat model (Lalegul-Ulker et al., 2019). We see forward to future studies developing new strategies allowing a full characterization of the profile of the factors contained in the MSC secretome and, therefore, a clear identification of the mechanisms underpinning its protective action.

Stem cell therapies are among the most promising regenerative approaches for cardiovascular diseases. Several animal studies have shown that MSCs may improve cardiac functions via mechanisms of immunomodulation, neovascularization, endogenous repair, inhibition of fibrosis, and proliferation of existing cardiomyocytes (Bagno et al., 2018). The majority of these studies demonstrate that the level of direct MSC contribution to cardiomyocyte replacement is low and, therefore, unlikely to represent a therapeutically meaningful MSC mechanism of action. The secretome of MSCs modulates several key cell processes that contribute to cardiovascular protection and/or repair under different pathological conditions. Genetically modified MSCs overexpressing VEGF (Locatelli et al., 2015), hepatocyte growth factor (HGF) (Zhao et al., 2016) and interleukin 10 (IL-10) (Meng et al., 2018) have been shown to alleviate cardiac injury and therefore promote cardioprotection. To address in detail the behavior of MSCs implanted in preclinical models and their impact on the site of application, labeling and tracking methods are required (Vaegler et al., 2014).

The therapeutic efficiency of human MSC-extracellular vesicles (MSC-EVs) has been observed in preclinical animal models and across many diseases and injuries (Gowen et al., 2020). CD39-expressing $CD4^+$ Th1 cells initiated adenosine-related apoptosis after internalizing BM-MSC-derived exosomes (Exos) in an animal GvHD model (Amarnath et al., 2015). MSC-EVs can reduce clinical symptoms in murine models of osteoarthritis and rheumatoid arthritis. Both MSC-derived exosomes and microparticles (Wilson et al., 2019a) exerted an anti-inflammatory role on lymphocytes independent of MSC priming. In delayed-type hypersensitivity (DTH), a dose-dependent anti-inflammatory effect of MPs and Exos was observed, while in collagen-induced arthritis (CIA) models, Exos efficiently decreased clinical signs of inflammation (Cosenza et al., 2018). hUC-MSC-derived EVs may protect cardiac tissue from ischemic injury, partly by promoting angiogenesis, in a rat model of myocardial infarction (Hossein-Khannazer et al., 2021). In atopic dermatitis mouse models, intravenous administration of EV from human umbilical cord-derived MSCs (hUC-MSCs) has shown anti-atopic effects (Cho et al., 2018). In an *in vitro* AD mouse model, AT-MSC (adipose tissue-MSC)-derived EVs were shown to ameliorate the progression of beta-amyloid-induced neuronal death (Lee et al., 2018).

1.4.2 Clinical Trials

MSCs were first tested as a cellular pharmaceutical agent in human subjects in 1995 by (Lazarus et al., 1995) and have since become the most clinically studied experimental cell therapy platform worldwide. After 20 years of clinical trials, MSCs have earned an excellent safety record but are still only approved for use in Canada, New Zealand, Japan, South Korea and Europe due to a lack of consistent efficacy outcomes. The obligation to register clinical studies before the start of recruitment, requested by the International Committee of Medical Journal Editors (ICJME), provides up-to-date data on ongoing clinical studies. Currently, while preparing this review, there were 1,228 clinical applications for several diseases that have been registered within the database of http://clinicaltrials.gov. Among 1,228 recorded trials, more than 485 were identified as ongoing trials. There were 370 completed clinical trials, of which 272 were in early phase I, phase I or phase II, and 28 studies advanced to phase III and IV. At the same time, 392 studies were classified as suspended, withdrawn, completed or of unknown status. Almost the majority of MSC-based clinical trials are still in phase I and II, where only a small number of trials are in phase III. In general, many of the completed clinical trials showed the efficacy of MSC-based therapies in several conditions, especially in heart diseases/failure, ischemia, arthritis, collagen diseases, infarction, joint diseases, osteoarthritis and many others, in addition to their safe administration. However, many aspects regarding MSC therapy should be deeply characterized, on the one hand, because of their broad spectrum of therapeutic potentials and, on the other hand, because their long-term follow-up safety with outcomes is not yet determined. Of all clinical trials using MSCs, the main indications are musculoskeletal diseases, with 203 registered studies, 146 trials

TABLE 1 | Summary of the clinical trials evaluating MSCs.

NCT Number	Condition	Phases	Type of product	Match	Route of administration	Dose (10^6 cells)	Cell expansion passage	Preconditioning	Status	Gender	Age (years)	Enrollment	Start date	Location	Funded bys	Study results
NCT04919135	Frailty	Phase 1| Phase 2	UC-MSCs	Allogenic	Intravenous	Not provided	–	–	Not yet recruiting	All	60–85	44	July 1, 2021	Not provided	Other	Not available
NCT04453111	Osteoarthritis	Phase 1| Phase 2	BM-MSCs, P-MSCs	Autologous, Allogenic	intra-articular injection	Not provided	–	–	Recruiting	All	18–75	45	January 2, 2020	Ukraine	Industry| Other	Not available
NCT03840343	Diabetic Kidney Disease	Phase 1	AD-MSCs	Autologous	intra-arterial	Not provided	–	–	Recruiting	All	45–75	30	October 23, 2019	United States	Other	Not available
NCT04433104	Chronic Obstructive Pulmonary Disease	Phase 1| Phase 2	UC-MSCs	Allogenic	Intravenous	1/kg	–	–	Recruiting	All	40–75	40	June 9, 2020	Vietnam	Other	Not available
NCT04759105	Chronic Low Back Pain	Phase 2	BM-MSCs	Autologous	intradiscal injection	15/disc	–	–	Recruiting	All	18–65	52	November 17, 2020	Italy	Other	Not available
NCT03874572	Dry Mouth Syndrome	Phase 1	AD-MSCs	allogeneic	Intraglandular injection	Not provided	–	–	Active, not recruiting	All	18–75	10	March 18, 2019	Denmark	Other	Not available
NCT03478215	Kidney Transplantation	Phase 2	MSCs	Autologous	Intravenous	1 to 3/kg	–	–	Recruiting	All	18–65	24	February 2016	United States	Other	Not available
NCT04356287	Systemic Sclerosis	Phase 1| Phase 2	UC-MSCs	Allogenic	Intravenous	1/kg	–	–	Not yet recruiting	All	18 and older	18	July 2021	Not provided	Other	Not available
NCT03876197	Radiation-induced Hyposalivation and Xerostomia	Phase 1| Phase 2	AD-MSCs	Autologous	Intraglandular injection	Not provided	–	–	Enrolling by invitation	All	18–99	30	August 1, 2020	Denmark	Other	Not available
NCT01586312	Osteoarthritis	Phase 1| Phase 2	BM-MSCs	Allogenic	intra-articular injection	40	2	–	Completed	All	18–75	30	April 2012	Spain	Industry| Other	Allogeneic MSC therapy may be a valid alternative for the treatment of chronic knee osteoarthritis. doi: 10.1097/TP.0000000000000678
NCT01860417	Degenerative Disc Disease	Phase 1| Phase 2	BM-MSCs	Allogenic	intradiscal injection	25/disc	expanded *ex vivo* for 3–4 weeks	–	Completed	All	18–75	25	April 2013	Spain	Industry| Other	Quick and significant improvement in algofunctional indices versus the controls. doi: 10.1097/TP.0000000000001484
NCT03389919	Graft Failure	Phase 3	MSCs	Not provided	Intraosseous	Not provided	–	–	Recruiting	All	18 and older	20	January 1, 2017	Russian Federation	Other	Not available
NCT02195323	Chronic Kidney Disease	Phase 1	BM-MSCs	Autologous	Intravenous	Not provided	–	–	Completed	All	25–60	7	April 2014	Iran	Other	Not available
NCT04325594	Chronic Heart Failure	Phase 2	UC-MSCs	Allogenic	intracoronary injection	10	–	–	Enrolling by invitation	All	18 and older	60	March 1, 2020	Kazakhstan	Other	Not available
NCT02495766	Multiple Sclerosis	Phase 1| Phase 2	BM-MSCs	Autologous	Intravenous	Not provided	–	–	Completed	All	18–60	8	May 11, 2015	Spain	Other	Not available
NCT02384018	Islet Autograft	Phase 1	BM-MSCs	Autologous	Intravenous	20 or 50 or 100	–	–	Active, not recruiting	All	18–69	42	December 2014	United States	Other|NIH	Not available
NCT02408432	Heart Failure	Phase 1	BM-MSCs	Allogenic	Intravenous	Not provided	–	–	Recruiting	All	18–80	45	January 11, 2016	United States	Other|NIH	Not available
NCT03795974	Cerebral Palsy	Phase 2	UC-MSCs	Allogenic	intrathecal injection	Not provided	–	–	Unknown status	All	avr-14	108	July 23, 2017	Iran	Other	Not available
NCT02962661	Heart Failure	Phase 1	BM-MSCs	Allogenic	Intravenous	Not provided	–	–	Recruiting	All	18–80	72	July 18, 2020	United States	Other|NIH	Not available
NCT03745417	Psoriasis	Phase 1| Phase 2	UC-MSCs	Allogenic	Intravenous	2/kg	–	–	Not yet recruiting	All	18–65	5	August 31, 2021	China	Other	Not available
NCT02801890	Peritoneal Fibrosis	Phase 1| Phase 2	AD-MSCs	Autologous	Intravenous	1/kg	–	–	Completed	All	18–70	10	August 2015	Iran	Other	Not available
NCT01275612	Solid Organ Cancers	Phase 1	MSCs	Not provided	Intravenous	1/kg	–	–	Withdrawn	All	18–80	0	November 2010	Italy	Other	Not available
NCT00781872	Multiple Sclerosis	Phase 1| Phase 2	BM-MSCs	Autologous	IV, Intrathecal	1/kg	40–60 days	–	Completed	All	35–65	24	October 2006	Not provided	Other	Transplantation of MSCs in patients with MS and ALS is a clinically feasible and relatively safe procedure and induces immediate immunomodulatory effects. doi:10.1001/archneurol.2010.248
NCT04869761	Chronic Kidney Diseases	Phase 1	AD-MSCs	Allogenic	Intravenous	150	–	–	Not yet recruiting	All	40–80	40	May 2021	United States	Other	Not available
NCT03265613	Psoriasis	Phase 1| Phase 2	AD-MSCs	Allogenic	Intravenous	0,5/kg	–	–	Active, not recruiting	All	18–65	7	September 24, 2017	China	Other	Not available
NCT03392311	Psoriasis	Phase 1| Phase 2	AD-MSCs	Allogenic	Intravenous	2/kg	–	–	Enrolling by invitation	All	18–65	8	August 17, 2019	China	Other	Not available
NCT02646007	Kienböck's Disease	Phase 1	BM-MSCs	Autologous	Injection	Not provided	–	–	Unknown status	All	18–65	30	November 2015	Iran	Other	Not available

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TABLE 1 | (*Continued*) Summary of the clinical trials evaluating MSCs.

NCT Number	Condition	Phases	Type of product	Match	Route of administration	Dose (10^6 cells)	Cell expansion passage	Preconditioning	Status	Gender	Age (years)	Enrollment	Start date	Location	Funded bys	Study results
NCT03042143	Acute Respiratory Distress Syndrome	Phase 1| Phase 2	UC-CD362 enriched MSCs	Not provided	Infusion	100, 200, 400	—	—	Recruiting	All	16 and older	75	January 7, 2019	United Kingdom	Other	Not available
NCT00644410	Hart Failure	Phase 1| Phase 2	BM-MSCs	Autologous	intramyocardial injection	77,5	2	—	Completed	All	30–80	59	September 2008	Denmark	Other	Expanded MSCs were safe and improved myocardial function in patients with severe ischaemic heart failure. doi:10.1093/eurheartj/ehv136
NCT04519671	Crohn's Disease	Phase 1| Phase 2	BM-MSCs	Allogenic	Direct injection	75	—	—	Recruiting	All	18–75	40	November 19, 2020	United States	Other	Not available
NCT04785027	Psoriasis	Phase 1| Phase 2	AD-MSCs	Allogenic	Intravenous	2/kg	—	—	Recruiting	All	18–65	16	March 17, 2021	China	Other	Not available
NCT04371393	Covid -19 Acute Respiratory Distress Syndrome	Phase 3	remestemcel-L	Not provided	Intravenous	2/kg	—	—	Active, not recruiting	All	18 and older	223	April 30, 2020	United States	Other| Industry|NIH	Not available
NCT03325322	Chronic Kidney Disease	Phase 2	Not provided	Not provided	Not provided	Not provided	—	—	Recruiting	All	40–80	30	January 2, 2018	United States	Other	Not available
NCT02940418	Diabetes	Phase 1	AD-MSCs	Allogenic	Intravenous	1/kg, 10/kg	5	—	Recruiting	All	18–35	20	February 19, 2017	Jordan	Other	Not available
NCT00395200	Multiple Sclerosis	Phase 1| Phase 2	BM-MSCs	Autologous	Intravenous	2/kg	—	—	Completed	All	18–65	10	July 2008	United Kingdom	Other	The evidence of structural, functional, and physiological improvement after treatment in some visual endpoints is suggestive of neuroprotection. doi: 10.1016/S1474-4422(1170305-2)
NCT03042572	No-option Severe Limb Ischemia	Phase 2| Phase 3	BM-MSCs	Allogenic	Intramuscular injection	150	—	—	Not yet recruiting	All	18 and older	60	December 2018	Netherlands	Other	Not available
NCT02685098	Amputation	Phase 1	BM-MSCs	Allogenic	—	Not provided	—	—	Recruiting	All	40–90	16	January 2017	United States	Other	Not available
NCT04447833	Covid -19 Acute Respiratory Distress Syndrome	Phase 1	BM-MSCs	Allogenic	Intravenous	1/kg, 2/kg	—	—	Active, not recruiting	All	18–65	7	June 17, 2020	Sweden	Other	Not available
NCT01468064	Ischemic Stroke	Phase 1| Phase 2	BM-MSCs and EPCs	Autologous	Intravenous	2,5/kg	33.75 days	—	Completed	All	18–80	20	November 2011	China	Other| Industry	Autologous transplantation of EPCs appears to improve long-term safety in acute cerebral infarct patients. doi: 10.1002/sctm.18-0012
NCT02379442	Graft-Versus-Host-Disease	Phase 1| Phase 2	BM-MSCs	Allogenic	Intravenous	2 × 1/kg, 12 doses	—	—	Terminated	All	4–99	1	February 23, 2015	United States	NIH	Not available
NCT02893306	Diabetes	Phase 2	BM-MSCs	Allogenic	Intravenous	2–3/kg	—	—	Unknown status	All	18 and older	10	March 2012	Chile	Other	Not available
NCT02017912	Amyotrophic Lateral Sclerosis	Phase 2	BM-MSC-NTF	Autologous	Intramuscular and intrathecal	Not provided	—	induced to secrete NTFs by a medium based approach	Completed	All	18–75	48	May 2014	United States	Industry	Not available
NCT03164083	Osteoarthritis	Phase 2	BM-MSCs, SVF	Autologous	intra-articular injection	Not provided	—	—	Withdrawn	All	25–65	0	November 10, 2019	Iran	Other	Not available
NCT03015623	Acute Kidney Injury	Phase 1| Phase 2	MSCs	Allogenic	integrated into the renal replacement circuit	250–750	—	—	Active, not recruiting	All	18 and older	24	June 20, 2017	United States	Industry	Not available
NCT03211793	Ulcers of Systemic Sclerosis	Phase 1| Phase 2	BM-MSCs	Allogenic	Intramuscular	50	—	—	Unknown status	All	18 and older	20	November 2018	Netherlands	Other	Not available
NCT04466098	Covid -19Acute Respiratory Distress Syndrome	Phase 2	MSCs	Not provided	Intravenous	300	—	—	Active, not recruiting	All	18–80	9	July 30, 2020	United States	Other	Not available
NCT02274428	Premature Infants	Phase 1	UC-MSCs	Not provided	—	Not provided	—	—	Completed	All	23–34	9	October 2014	Korea	Other	Not available
NCT00927355	Effect of Thiazolidinediones on Human Bone	Not Applicable	Pioglitazone	Not provided	—	Not provided	—	—	Completed	All	18–80	10	April 2009	United States	Other	Insights into the deleterious effects of TZDs on bone quality in diabetics and support a model in which TZD-induced adipogenesis may be a significant influencing factor on osteoblast differentiation and function. doi: 10.1016/j.trsl.2012.08.006

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TABLE 1 | (*Continued*) Summary of the clinical trials evaluating MSCs.

NCT Number	Condition	Phases	Type of product	Match	Route of administration	Dose (10^6 cells)	Cell expansion passage	Preconditioning	Status	Gender	Age (years)	Enrollment	Start date	Location	Funded bys	Study results
NCT04247945	Allogeneic Hematopoietic Stem Cell Transplantation	Phase 2| Phase 3	MSCs	Not provided	—	Not provided	—	—	Recruiting	All	up to 65 Years	120	February 1, 2020	China	Other	Not available
NCT01144962	Crohn's Disease	Phase 1| Phase 2	BM-MSCs	Allogenic	Local injection	10–30-90	2	—	Completed	All	18 and older	21	June 2010	Netherlands	Other	Local administration of allogeneic MSCs was not associated with severe adverse events in patients with perianal fistulizing Crohn's disease. Injection of 3×10^7 MSCs appeared to promote healing of perianal fistulas.doi: 10.1053/j.gastro.2015.06.014
NCT03102879	Periapical Periodontitis	Not Applicable	Encapsulated UC-MSCs in a biological scaffold	Allogenic	Conventional Root Canal Treatment	Not provided	—	—	Completed	All	16–58	36	September 23, 2016	Chile	Other	Safety and efficacy evidence of the endodontic use of allogenic umbilical cord mesenchymal stem cells encapsulated in a plasma-derived biomaterial. doi: 10.1177/0022034520913242
NCT01739504	Osteoarthritis	Not Applicable	AD-MSCs	Autologous	Intra articular	—	—	—	Terminated	All	18–80	10	March 1, 2014	United States	Industry	Not available
NCT03298399	Sickle Cell Disease	Phase 1	BM-MSCs	Autologous	Intravenous	2/kg	28 days	—	Withdrawn	All	déc-40	0	December 21, 2017	Not provided	Other|NIH	Not available
NCT02421484	Septic Shock	Phase 1	BM-MSCs	Allogenic	Intravenous	0,3/kg, 1/kg, 3/kg	—	—	Completed	All	18 and older	9	May 2015	Canada	Other	The infusion of freshly cultured allogenic bone marrow-derived MSCs, up to a dose of 3 million cells/kg (250 million cells), into participants with septic shock seems safe. doi: 10.1164/rccm.201705-1006OC
NCT02145923	Neutropenic Enterocolitis	Phase 1| Phase 2	BM-MSCs	Allogenic	Intravenous	1,5–2/kg	—	—	Unknown status	All	18–65	16	May 2014	Russian Federation	Other	Not available
NCT03818737	Osteoarthritis	Phase 3	BM-MSCs, AD-MSCs, UC-MSCs	Autologous	Orthobiologic injection	Not provided	—	—	Active, not recruiting	All	40–70	480	March 28, 2019	United States	Other	Not available
NCT02033525	Degenerative Meniscus Injury	Phase 1| Phase 2	XCEL-M-ALPHA	Autologous	—	Not provided	—	—	Completed	All	40–60	20	January 31, 2014	Spain	Other	Not available
NCT02209311	Maxilla Alveolar Process Reconstruction	Phase 1| Phase 2	Oral mucosa-MSCs-tissue engineered construction	Autologous	Implantation	Not provided	3–4 weeks	—	Unknown status	All	20–60	12	September 2014	Russian Federation	Other	Not available
NCT04445220	Acute Kidney Injury	Phase 1| Phase 2	MSCs (SBI-101)	Allogenic	Integration	250–750	—	—	Recruiting	All	18 and older	22	November 19, 2020	United States	Industry	Not available
NCT01849159	Pulmonary Emphysema	Phase 1| Phase 2	BM-MSCs	Allogenic	Intravenous	200	—	Hypoxia (1% pxygen)	Withdrawn	All	35–75	0	March 2014	Russian Federation	Other	Not available

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TABLE 1 | (*Continued*) Summary of the clinical trials evaluating MSCs.

NCT Number	Condition	Phases	Type of product	Match	Route of administration	Dose (10^6 cells)	Cell expansion passage	Preconditioning	Status	Gender	Age (years)	Enrollment	Start date	Location	Funded bys	Study results
NCT04629833	Steroid-Refractory Acute Graft-Versus-Host Disease	Phase 3	MSCs-MC0518	Not provided	Intravenous	1–2/kg	–	–	Not yet recruiting	All	12 and older	210	June 30, 2021	France, Germany, Poland, Spain	Industry	Not available
NCT04007081	XRT Induced Xerostomia	Not Applicable	BM-MSCs	Autologous	Salivary Gland Autotransplantation	50	2 weeks	–	Completed	All	18–89	12	October 18, 2019	United States	Other	Not available
NCT03643614	Post-Irradiation Vaginal-Rectal Fistula	Phase 1	AD-MSCs	Autologous	Direct injection	Not provided	–	–	Completed	Female	20–75	16	August 1, 2017	Russian Federation	Other	Not available
NCT02630836	Hip Fracture	Phase 1| Phase 2	BM-MSCs	Allogenic	Surgical treatment	Not provided	–	–	Withdrawn	All	70–85	0	December 2015	Not provided	Other	Not available
NCT02323477	Myocardial Infarction	Phase 1| Phase 2	UC-MSCs, BM-MSCs	Allogenic; Autologous	peri-infarct areas	UC-MSCs (23), BM-MSCs (700)	–	–	Terminated	Male	30–80	46	February 2, 2015	Turkey	Other| Industry	Intramyocardial administration of HUC-MSCs in combination with CABG displayed higher scores in reducing the scar tissue and restoration of ventricular wall functions compared with autologous BM-MNCs. doi: 10.1007/s12015-015-9601-0
NCT04173650	Dystrophic Epidermolysis Bullosa	Phase 1| Phase 2	Exosomes from BM-MSCs (AGLE-102)	Allogenic	–	2 doses	–	–	Not yet recruiting	All	6 and older	10	January 2022	–	Industry	Not available
NCT04366063	Covid-19 Related Acute Respiratory Distress Syndrome	Phase 2| Phase 3	MSCs + Evs	–	Intravenous	2 MSC infusions (100) + 2 EVs infusions	–	–	Recruiting	All	18–65	60	June 6, 2020	Iran	Other	Not available

for central nervous system diseases, 146 trials for immune system diseases, 139 for wounds and injuries, 130 for collagen diseases, 130 for rheumatic diseases, 128 for joint diseases, 127 for arthritis, 127 for vascular diseases, 123 for ischemia, 118 for respiratory tract diseases, 112 for digestive system diseases and 112 for gastrointestinal diseases.

A small part of the MSC clinical trials started using MSC-derived exosomes instead of MSCs themselves. Clinical trial number NCT: NCT04276987 used MSC-Exos for their advantages over MSCs for treating severe patients hospitalized with novel coronavirus pneumonia (NCP). Twenty-four patients were enrolled in this study (phase I); the patients received MSC-Exos derived from allogenic adipose tissue by aerosol inhalation. Based on this study, another clinical trial was performed; the patients were divided into three groups: Group 1 received Exo type I, Group 2 received Exo type II and Group 3 received a placebo. This is a combination of phase I and phase II, enrolling 30 patients (10 for each group), under the number NCT: NCT04491240. Patients also received the drug by aerosol inhalation. The results for these trials were promising, and safe administration was observed with no side effects.

Coronavirus disease (*COVID-19*), the ongoing pandemic, is a disease caused by the coronavirus. One of the studied treatments for severe cases is the use of exosomes derived from bone marrow MSCs (ExoFlo™). A single dose of 15 ml of ExoFlo was intravenously (IV) administered to 24 COVID-19-positive patients, and the results were promising (Sengupta et al., 2020). Several other studies suggested the beneficial and safe use of MSCs in treating severe conditions of COVID-19 patients (Golchin et al., 2020). Recently, a study evaluated the clinical outcomes of severe/critically severe COVID-19 patients (210) being treated with UC-MSCs ($1–2 \times 10^6$ per kilogram) from October 15, 2020 until April 25, 2021 in Turkey. UC-MSCs demonstrated safety with high potential when used as an added therapeutic treatment for severe COVID-19 patients (Ercelen et al., 2021).

Because of their therapeutic properties, the number of clinical trials using MSCs will significantly increase. **Table 1** and **Table 2**, summarizing the clinical trials evaluating MSCs or their secretome, have been included. Data were extracted on June 18, 2021 from www.clinicalTrials.gov, using the terms "mesenchymal stem/stromal cells" or "MSC EVs," and downloaded into an XML file. The data include the identifier for each trial (NCT number), clinical phase, recruitment status, location, start date, sponsor, gender, age and enrollment. We manually extracted additional information on disease, cell source, match (autologous vs. allogenic), route of administration, dose, cell expansion passage, conditioning and study results that could not be downloaded directly from ClinicalTrials.gov from individual trial records. Data for all of these categories were not found in many cases but were collected when possible. Doses in ClinicalTrials.gov are not reported systematically and were found either as the total numbers of cells/patient or as the number of cells/kg. While many clinical studies are in the recruitment and active phases, many of these terminate without producing a significant publication.

TABLE 2 | Summary of the clinical trials evaluating the secretome of MSCs.

NCT Number	Condition	Phases	Type of product	Match	Route of administration	Dose (10^6 cells) or MV or Exo	Cell expansion passage	Preconditioning	Status	Gender	Age (years)	Enrollment	Start date	Location	Funded bys	Study results
NCT02138331	Type 1 Diabetes	Phase 2| Phase 3	Cell free [UC-MSCs-MV and Exo]	NA	Intravenous	Exo or MV: from SN of 1.2–1.5/kg (MSCs)	NA	NA	Unknown status	All	18–60	20	April 2014	Egypt	Other	NA
NCT04798716	Covid -19 Acute Respiratory Distress Syndrome	Phase 1| Phase 2	Cell free [Perinatal MSCs-Exo]	NA	Intravenous	Tree groups: Escalating Dose: 2/4/8; 8/4/8; 8/8/8 ($x10^3$/mL)	NA	NA	Not yet recruiting	All	18 and older	55	April 2021	United States	Industry	NA
NCT03437759	Macular Holes	Early Phase 1	Cell free [UC-MSCs-Exo]	NA	Intravitreal	20–50 µg MSC-Exo	NA	NA	Active, not recruiting	All	Up to 80	44	March 1, 2017	China	Other	NA
NCT04276987	Covid -19 Pneumonia	Phase 1	Cell free [AD-MSCs-Exo]	Allogenic	Aerosol inhalation	5 x (2 x 10^8 nv/mL)	NA	NA	Completed	All	18–75	24	February 15, 2020	China	Other| Industry	NA
NCT04356300	Multiple Organ Dysfuntion Syndrome	Not Applicable	Cell free [UC-MSCs-Exo]	NA	Intravenous	(14x) 150 mg/day	NA	NA	Not yet recruiting	All	20–80	60	September 1, 2020	NA	Other	NA
NCT04388982	Alzheimer Disease	Phase 1| Phase 2	Cell free [AD-MSCs-Exo]	Allogenic	Nasal drip	5–10–20 µg (2x/W) for 12 W	NA	NA	Recruiting	All	50 and older	9	July 1, 2020	China	Other| Industry	NA
NCT03384433	Ischemic Stroke	Phase 1| Phase 2	Cell free [MSCs-Exo]	Allogenic	Stereotaxis/ Intraparanchymal	NA	NA	Exosome enriched by miR-124	Recruiting	All	40–80	5	April 17, 2019	Iran	Other	NA
NCT04313647	Healthy Volunteers: Tolerance test	Phase 1	Cell free [AD-MSCs-Exo]	Allogenic	Aerosol inhalation	2-4-8–12–16–20 ($x10^8$ nv/3 ml)	NA	NA	Recruiting	All	18–45	27	March 16, 2020	China	Other| Industry	NA
NCT04850469	Severely Infected Children	NA	NA	NA	NA	NA	NA	NA	Not yet recruiting	All	Up to 18	200	January 1, 2022	China	Other	NA
NCT04173650	Dystrophic Epidermolysis Bullosa	Phase 1| Phase 2	Cell free [BM-MSCs-Exo]	Allogenic	Local	6 ascending doses	NA	NA	Not yet recruiting	All	6 and older	10	April 2021	NA	Industry	NA
NCT03608631	Metastatic Pancreas Cancer	Phase 1	Cell free [MSCs-Exo with KRAS G12D siRNA]	NA	Intravenous	(15–20 min on days 1, 4, and 10); 3 courses/ 14 days	NA	KrasG12D siRNA-loaded MSCs-Exo	Recruiting	All	18 and older	28	January 27, 2021	United States	Other	NA
NCT04602442	Covid--19 Pneumonia	Phase 2	Cell free [MSCs-Exo]	NA	Aerosol inhalation	2x (0,5–2 $x10^{10}$ nanoparticles)/10 days	NA	NA	Enrolling by invitation	All	18–65	90	October 1, 2020	Russian Federation	Other	NA
NCT04491240	Covid--19 Pneumonia	Phase 1| Phase 2	Exosomes	NA	Aerosol inhalation	2x (0,5–2 $x10^{10}$ nanoparticles)/10 days	NA	NA	Completed	All	18–65	30	July 20, 2020	Russian Federation	Other	NA
NCT03857841	Bronchopulmonary Dysplasia	Phase 1	Cell free [BM-MSCs-EVs] UNEX-42	NA	Intravenous	20–60–200 pmol EV/kg	NA	NA	Terminated	All	Up to 14 days	3	June 20, 2019	United States	Industry	NA
NCT04213248	Dry Eye	Phase 1| Phase 2	Cell free [UC-MSCs-Exo]	NA	Artificial tears	10ug/drop, (4x)/day (14 days)	NA	NA	Recruiting	All	18–70	27	February 18, 2020	China	Other	NA
Kordelas et al., 2014	Graft-Versus-Host-Disease	NA	Cell free [BM-MSCs-Exo]	Allogenic	Intravenous	1.3–3.5 x 10^{10} particles/ unit; 0.5–1.6 mg/unit)	3	NA	Completed	Female	22	1	NA	Germany	—	The clinical GvHD symptoms improved significantly shortly after the start of the MSC-exosome therapy. Kordelas et al., 2014. doi:10.1038/leu.2014.41
Nassar et al., 2016	Chronic Kidney Diseases	Phase 2| Phase 3	Cell free [UC-MSCs-EVs]	Allogenic	Intravenous/ Intra-arterial	100 µg EV/kg/dose (2 doses)	6	NA	Completed	All	26–44	40	NA	Egypt	Other	Administration of cell-free cord-blood mesenchymal stem cells derived extracellular vesicles (CF-CBMSCs-EVs) is safe and can ameliorate the inflammatory immune reaction and improve the overall kidney function in grade III-IV CKD patients. Nassar et al., 2016. doi 10.1186/s40824-016-0068-0
NCT04270006	Periodontitis	Early Phase 1	Cell free [AD-MSCs-Exo]	Autologous	Local	NA	NA	NA	Recruiting	All	18–50	10	February 12, 2020	Egypt	Other	NA

(Continued on following page)

TABLE 2 | (*Continued*) Summary of the clinical trials evaluating the secretome of MSCs.

NCT Number	Condition	Phases	Type of product	Match	Route of administration	Dose (10^6 cells) or MV or Exo	Cell expansion passage	Preconditioning	Status	Gender	Age (years)	Enrollment	Start date	Location	Funded bys	Study results
NCT04202783	Craniofacial Neuralgia	Not Applicable	Neonatal stem cell -Exo	—	Focused ultrasound epineural injection and Intravenous	2 x (5 ml concentrated Exo) + 45 mg Exo +15 mg Exo	NA	NA	Suspended	All	18 and older	100	December 1, 2021	United States	Other	NA
NCT04202770	Depression, Anxiety, and Dementias	Not Applicable	Cell free (amniotic fluid-MSC-Exo)	Allogenic	Focused ultrasound Intravenous	Eq 20 stem cells	NA	NA	Suspended	All	18 and older	300	December 1, 2019	United States	Other	NA
NCT04384445	COVID-19 Acute Respiratory Distress Syndrome	Phase 1| Phase 2	Amniotic fluid stem cell organicell Flow)	—	Intravenous	2–5 x 10^{11} particles/mL (3 times)	NA	NA	Recruiting	All	18 and older	20	September 8, 2020	United States	Industry	NA
NCT04223622	Osteoarthritis	—	Cell free [AD-MSCs-CM or Evs]	—	ex vivo Osteoarthritis model	NA	NA	NA	Not yet recruiting	All	18 and older	24	February 2020	—	Other	NA
NCT04270006	Periodontitis	Early Phase 1	Cell free [AD-MSCs-Exo]	Autologous	Local	NA	NA	NA	Recruiting	All	18–50	10	February 12, 2020	Egypt	Other	NA
NCT04134676	Chronic Ulcer Wounds	Phase 1	Cell free [WJ-MSCs-CM)	NA	Local	CM gel (for 2 w)	NA	NA	Completed	All	18–80	38	June 1, 2019	Indonesia	Other| Industry	NA
NCT04544215	Pulmonary Infection	Phase 1| Phase 2	Cell free [AD-MSCs-Exo]	Allogenic	Aerosol inhalation	8 × 10^8 or 16 x 108 nano vesicles/3 ml	NA	NA	Recruiting	All	18–75	60	July 1, 2020	China	Other| Industry	NA
Sengupta et al	Severe COVID-19	NA	Cell free [BM-MSCs-Exo]	Allogenic	Intravenous	15 ml of ExoFlo	NA	NA	Completed	NA	18–85	27	April 2020	United States	Other	Owing to its safety profile, capacity to restore oxygenation, downregulate cytokine storm, and reconstitute immunity, ExoFlo is a promising therapeutic candidate for severe COVID-19. Sengupta et al.(2020), doi: 10.1089/scd.2020.0080

1.4.2.1 Primed Mesenchymal Stem/Stromal Cells

To enhance the beneficial properties of MSCs, several priming strategies have been proposed. However, few clinical trials have reported the use of primed MSCs for better therapeutic efficacy, and inflammatory priming has not yet been clinically investigated. Using a medium-based approach, MSCs can be induced to secrete elevated levels of neurotropic factors, which have been shown to have protective effects (Gothelf et al., 2014). These cells, designated MSC-NTF cells (neurotrophic factor-secreting MSCs, also known as NurOwn™) derived from patients' own bone marrow, have been recently used for phase I/II and phase IIa of clinical studies in patients with amyotrophic lateral sclerosis (ALS). In these studies, ALS patients were subjected to a single administration of autologous MSC-NTF cells. The data from these studies indicate that the single administration of MSC-NTF cells is safe, well tolerated and demonstrated early promising signs of efficacy (Abdul Wahid et al., 2019; Berry et al., 2019). Another option involved the culture of allogeneic BM-MSCs in hypoxic conditions (1% oxygen) to potentiate their efficacy (NCT01849159) (https://clinicaltrials.gov/ct2/show/NCT01849159) (Oh et al., 2017). A clinical study addressed the use of autologous platelet lysate (PL) to expand MSCs as a treatment for knee osteoarthrosis (KOA). Thirteen patients were enrolled in this study (phase II). The patients were divided into two groups, with one receiving autologous bone marrow-derived MSCs alone and the other receiving autologous bone marrow MSCs primed with platelet lysate and with both infused by intraarticular injections (https://clinicaltrials.gov/ct2/show/NCT02118519). Preliminary data concluded the safety of injections of MSCs for knee osteoarthritis patients; efficacy was also established for more than 2 years of follow-ups (Al-Najar et al., 2017). Umbilical cord-derived MSCs (UC-MSCs) have also demonstrated safety and efficacy in clinical trials of several diseases and conditions, such as RA (Wang et al., 2019). Clinical trials, such as clinical trial number NCT01547091, infused umbilical cord MSCs (UC-MSCs) intravenously (IV) several times for an interval of time. In that clinical trial (phase I/II), 200 patients were included, where some received MSC treatment, others received disease-modified antirheumatic drugs (DMARDs) and others received a combination of MSCs with DMARDs.

1.4.2.2 Approved Mesenchymal Stem/Stromal Cells Products

Several companies have or are in the process of commercializing MSC-based therapies. Despite the amount of research that has been conducted and the number of clinical trials, there are few approved products (**Table 3**). The European Medicines Agency (EMA) is responsible for the scientific evaluation of centralized marketing authorization applications (MAAs). Once granted by the European Commission, the centralized marketing authorization is valid in all European Union (EU) member states, Iceland, Norway and Liechtenstein (https://www.ema.europa.eu). The US Food and Drug Administration (FDA) has the authority to regulate regenerative medicine products, including stem cell products and exosome products (https://www.fda.gov/). Currently, the only stem cell products that are FDA-approved for use in the United States consist of blood-forming stem cells (also known as hematopoietic progenitor cells) that are derived from umbilical cord blood. In the United States,

stem cell products and exosome products should be carefully verified before use by considering FDA approval or being studied under an Investigational New Drug Application (Berry et al., 2019), which is a clinical investigation plan submitted and allowed to proceed by the FDA. Delivering a safe and effective product is key, and effective guidance from organizations such as the FDA and the ARM (Alliance for Regenerative Medicine) will ease and accelerate the translation of MSC technologies from the bench top to the bedside (Olsen et al., 2018).

Across the world, there are 10 approved MSC-based therapies, including Alofistel for Crohn's disease (approved in Europe); Prochymal for GvHD (approved in Canada and New Zealand); Temcell HS Inj for GvHD (approved in Japan); Queencell for subcutaneous tissue defects; Cupistem for Crohn's fistula, Neuronata-R for amytrophic lateral sclerosis and Cartistem for knee articular cartilage defects (all approved in South Korea); Stemirac for spinal cord injury (approved in Japan); Stempeucel for critical limb ischemia (approved in India); and Cellgram-AMI for acute myocardial infarction (approved in South Korea).

BM-MSCs from a healthy adult donor were used to produce TEMCELL, the first world therapeutic product using MSCs that was approved in Japan in September 2015 for the treatment of acute GvHD (Okada et al., 2017). One of the rare clinical trials in phase III is the use of allogeneic adipose tissue-derived MSCs for complex perianal fistulas in Crohn's disease (clinical trial number NCT: NCT01541579). The TiGenix/Takeda phase III clinical trial that evaluates the use of MSCs for complex perianal fistulas in Crohn's disease (CD) is arguably the most successful late-stage MSC trial to date (NCT01541579). In 2018, MSCs received European approval to be used to treat patients with Crohn's-related enterocutaneous fistular disease (Panes et al., 2018). The approved pharmaceutical drug, Alofisel, is derived from adipose allogeneic MSCs. According to the indicated study, adult CD patients with treatment-refractory, draining, complex perianal fistulas treated with allogeneic AT-MSCs (Alofistel) showed good remission, demonstrating the potential of MSCs to substantially improve the standard of care in chronic illnesses such as CD. The study is one of the clinical trials performed to test Darvadstrocel (Alofisel), which is still called Cx601 (suspension of adipose-derived MSCs). This randomized and double-blind study enrolled 212 patients who received a single dose of 120 million MSCs (Cx601) or 24 ml saline solution (placebo) by intralesional injection. The results were promising, and it was concluded that Cx601 was an effective and safe treatment for perianal fistulas in patients with Crohn's disease (Panes et al., 2016). Another clinical trial, active at present, is testing Darvadstrocel and is registered under the number NCT: NCT03706456. The study is supposed to be completed on January 31, 2023; it enrolled 22 participants, and it will include a follow-up period of 52 weeks after study product administration and a long-term follow-up period from week 52 to week 156.

2 PRE-CLINICAL CHALLENGES

Several preclinical challenges may influence the therapeutic use of MSCs and should be well identified and characterized (**Figure 2**).

2.1 Tissue Sources of Mesenchymal Stem/Stromal Cells

MSCs are virtually present in all tissues and share some characteristics, such as similar shape, phenotype and functions (Song et al., 2020a). Thus, several sources are reported to allow the isolation of MSCs. Even when the expansion step is successful and a high number of cells are transplanted during the procedure, the cells frequently have very reduced viability and low engraftment in the recipient tissue (Haque et al., 2015). These alterations have led to distinct biological properties of MSC populations, which may partly explain the differences in the outcomes of clinical trials with distinct MSCs. It is well established that over culture passages, MSCs enter a state of replicative senescence after 20–30 cell divisions (Martin et al., 2016). During this process, MSC morphology changes from relatively small spindle-shaped cells to larger and flattened cells, with typically more pronounced actin cytoskeleton fibers. Thus, over the passages, MSCs isolated *in vitro* more often resemble a cellular mixture with variable properties, resulting from intrinsic and extrinsic influences in addition to inherent disparities related to different sources and donors (Naji et al., 2019).

2.1.1 Bone Marrow

MSCs have traditionally been derived from bone marrow for clinical trials and *in vitro* research. The isolation and expansion of bone marrow-derived MSCs involves the aspiration of the iliac crest followed by the isolation of the mononuclear cell fraction by density-gradient centrifugation and plating for expansion (Macrin et al., 2017). A number of studies have shown their capacity to differentiate into mesodermal cell lineages (including myocytes, chondrocytes, osteoblasts and adipocytes), ectodermal cell lineages (such as neurons) and endodermal cell lineages (including hepatocytes). BM-MSCs also showed the capacity to be differentiated into airway epithelial cells, renal tubules, osteocytes and myocardial cells (Marolt Presen et al., 2019).

However, the frequency of MSCs in the bone marrow is very low (between 0.0001 and 0.01%) and decreases with age (Yang et al., 2018). In addition, their *ex vivo* expansion can only result in 30–50 population doublings, and long-term cell expansion may lead to chromosomal aberrations (Ahmadi and Rezaie, 2021). Additionally, bone marrow aspiration is a painful procedure that requires local anesthesia. Therefore, the use of BM has drawbacks, prompting the search for alternative sources of MSCs that are easily accessible, generally less invasive and contain larger amounts of MSCs.

2.1.2 Adipose Tissue

Adipose tissue represents a very promising source for cell therapy purposes in terms of safety, collection and culture. Adipose MSCs are easy to obtain since isolation is performed under local anesthesia and presents little risk of morbidity (Seo et al., 2019). Adipose tissue therefore constitutes a source of MSCs in abundant quantities, and AT-MSCs have shown greater proliferation capacities than BM-MSCs. For the same amount of tissue aspirated, adipose tissue contains 550 × more MSCs than

TABLE 3 | MSC and MSC progenitors- based products with marketing approval for clinical application worldwide.

MSC-product	Indication	MSC type	Company	Country (Marketing approval year)	Regulatory agency
Alofisel	Complex perianal fistulas in Crohn's disease	Allogeneic AD-MSCs	Takeda Pharma	Europe (2018)	EMA
Allostem	Bone regeneration	Allogeneic AD-MSCs	AlloSource	United States (2010)	Regulated under CFR 1,270, 1,271 as a human tissue. Do not require pre-market approval from the FDA. (Ref. 1; Ref. 2)
Cartistem	Osteoarthritis	Allogeneic UC-MSCs	Medipost Co., Ltd	South Korea (2012)	MFDS
Grafix	Acute/chronic wounds	Allogeneic placental membrane, incuding MSCs	Osiris Therapeutics	United States (2011)	Products marketed as human cells, tissues, and cellular and tissue-based products ("HCT/Ps"), as defined by the US FDA, that are regulated solely under Section 361 of the Public Health Service Act ("361 HCT/Ps"), and consequently, do not require pre-market approval from the FDA. https://fintel.io/doc/sec-osir-osiris-therapeutics-10k-2019-march-15-17970 other sources: http://www.osiris.com/grafix/certifications/
Prochymal (remestemcel-L)	GvHD	Allogeneic BM-MSCs	Osiris Therapeutics Inc./Mesoblast	Canada (2012) New Zealand (2012)	Health Canada (expired date protection 2020) MEDSAFE (Approval lapsed)
OsteoCel	Orthopaedic repair	Allogeneic BM-MSCs	NuVasive	United States (2005)	Regulated under CFR 1270, 1271 as a human tissue. Do not require pre-market approval from the FDA. (Ref. 1, Ref. 2)
Bio4 (formerly OvationOS)	Bone repair and regeneration	Bone-forming osteoblasts, osteoprogenitor cells and MSCs	Osiris Therapeutics Stryker	United States (2014)	Do not require pre-market approval from the FDA: US FDA regulations for tissue management. US FDA 21 CFR 1271 https://fintel.io/doc/sec-osir-osiris-therapeutics-10k-2019-march-15-17970
Temcell HS	GvHD	Allogeneic BM-MSCs	JCR Pharmaceuticals	Japan (2015)	PMDA
Trinity Evolution	Orthopaedic repair	Allogeneic BM-MSCs	Orthofix	United States (2019)	Do not require pre-market approval from the FDA: US FDA regulations for tissue management. US FDA 21 CFR 1271 (Ref 2)
Trinity Elite	Orthopaedic repair	Allogeneic BM-MSCs	Orthofix	United States (2013)	Regulated under CFR 1270, 127cer1 as a human tissue (Ref1)
QueenCell	Subcutaneous tissue defects	Autologous AD-MSCs	Anterogen Co., Ltd.	South Korea (2010)	MFDS
Ossron	Bone regeneration	Autologous BM-MSCs	Sewon Cellontech CO., Ltd.	South Korea (2009)	MFDS
Obnitix	GvHD	Allogeneic BM-MSCs	Medac	Germany	NA
Stempeucel	Critical limb ischemia	allogeneic BM-MSCs	Stempeutics Research	India (2016)	DCGI
Neuronata-R	Amyotrophic lateral sclerosis	autologous BM-MSCs	Corestem, Inc.	South Korea (2014)	MFDS
Cellgram-AMI	Myocardial infarction	autologous BM-MSCs	Pharmicell Co., Ltd.	South Korea (2011)	MFDS
Cupistem	Crohn's fistula	autologous AD-MSCs	Anterogen Co., Ltd.	South Korea (2012)	MFDS
Stemirac	Spinal cord injury	Autologous BM-MSCs	Nipro Corp	Japan (2018)	PMDA
Cellentra VCBM	Orthopaedic repair	Allogeneic BM-MSCs	Biomet Inc	United States (2012)	US FDA regulations for tissue management. US FDA 21 CFR 1271 (Ref. 1; Ref. 3)

(Continued on following page)

TABLE 3 | (*Continued*) MSC and MSC progenitors- based products with marketing approval for clinical application worldwide.

MSC-product	Indication	MSC type	Company	Country (Marketing approval year)	Regulatory agency
HiQCell	Osteoarthritis/tendonitis	Autologous adipose stromal vascular fraction	Regeneus Ltd. (ASX:RGS)	Australia (2013)	NA
LiquidGen	Bone repair	Allogeneic BM-MSCs	Skye Orthobiologics LLC	United States	NA
CardioRel	Myocardial infarction	Autologous MSCs	Reliance life sciences	India (2010)	NA
Adipocel	Crohn's disease	Autologous AD-MSCs	Anterogen Co., Ltd.	South Korea (2007)	NA
Autostem	Subcutaneous fat loss area	Autologous AD-MSCs	Cha biotech	South Korea (2010)	NA
MesestroCell	Osteoarthritis and knee joint arthritis	Autologous BM-MSCs	Cell Tech Pharmed	Iran (2018)	NA

BM. The primary culture of adipose tissue-derived stem cells proceeds through the mincing and enzymatic digestion of subcutaneous adipose tissue followed by its culture and expansion in culture medium. These cells harbor several interesting characteristics and properties.

2.1.3 Newborn Tissue

It has been reported that cord blood, placental amniotic membrane and fluid as well as the umbilical cord matrix (called Wharton's jelly) contain MSCs (Liu et al., 2021). Perinatal tissues are of great interest due to their accessibility and ease of collection. The MSCs in these tissues are found at a high frequency and show an increased rate of proliferation and differentiation. Moreover, their use does not conflict with ethical issues raised by the use of embryonic stem cells (Aung et al., 2019). However, the cryopreservation step is essential, which can pose long-term storage problems (Peltzer et al., 2015).

2.1.4 Peripheral Blood

MSCs have been shown to circulate at a low frequency in peripheral blood (Chen et al., 2019a). The origin of the presence of MSCs in this source is still under debate. This disparity could be linked to the diversity of isolation, culture and characterization methods used in different studies. The presence of MSCs in peripheral blood has been observed in patients with acute burns, suggesting the potential role of these cells in the regeneration of damaged tissue.

2.1.5 Other Tissue Sources

Although MSCs from other sources, such as dental tissues, periodontal ligament, synovium, dermis, salivary gland, skin, and skeletal muscles, may share many biological characteristics, they also present differences regarding some properties (Mizukami and Swiech, 2018). Some of these differences, such as cell surface phenotype, transcriptome/proteome characteristics and immunotrophic activity, represent specific features of MSCs from a specific tissue source (Park et al., 2007), while others reflect the heterogeneity of MSC populations from different organs. Other differences may simply be attributed to the different isolation and culture protocols (Pelekanos et al., 2012). Other studies suggest that MSCs from different tissue sources retain an epigenetic memory of their original tissue. Thus, it has been shown that the expression profile of homeotic genes can vary from one source to another (Ackema and Charite, 2008). Additional studies have shown that the transcriptional expression of certain genes involved in the immunomodulatory function of MSCs could vary significantly depending on the cell source, even by changing the environmental conditions of culture (Cho et al., 2017; Fayyad-Kazan et al., 2017). Multiple comprehensive transcriptomic and proteomic analyses of human MSCs should help in identifying distinct populations of MSCs with distinct properties and specific clinical indications.

2.2 Phenotype: *In Vitro* Versus *In Vivo* Identity

Despite advances in MSC characterization and their wide use in regenerative medicine, their *in vivo* identity is still poorly understood. The isolation and purification of MSCs was achieved *via in vitro* phenotypic assays assessing the expression of specific cell markers (**Table 4**). Such analysis serves as an important quality control step that can save significant time and reduce experimental variability. The expression profile of several immunological molecules may influence the local immune-inflammatory response and, therefore, modulate the tissue healing process. By analyzing 27 relevant molecules, immunocomparative screening demonstrated that liver-derived stromal cells present a nonimmunogenic profile suitable to promote graft acceptance by the recipient (Merimi et al., 2021b).

However, several artificial conditions during culture may introduce experimental artifacts and hide or impair the native identity of MSCs (Wilson et al., 2019a). Moreover, it is important that MSCs, after being cultured *in vitro*, retain all their receptors (to sense the tissue environment) and adhesion molecules (for migration, homing and cell-to-cell interaction) (Naji et al., 2019). MSC isolation methods, culture conditions and expansion may alter the expression profile of several markers. In addition to ISCT markers, other cell surface antigens, including nestin, CD29,

CD44, CD49b, CD130, CD146, CD166, CD271, CD200, and αV/β5 integrin, have been reported. However, they are not specific to a tissue source of MSCs (da Silva Meirelles et al., 2008; Kuci et al., 2010). Other markers are expressed by MSCs, such as CD71, CD106, CD54, SUSD2, MSCA-1, and STRO-1 (Busser et al., 2015). However, accumulating evidence suggests that marker expression of MSCs is not stable in culture conditions, which renders MSC characterization based on their markers a challenge (Lv et al., 2014). Under the authority of the International Federation of Adipose Therapeutics (IFAT) and ISCT, a joint statement established minimal criteria for the definition of stromal cells from the adipose tissue-derived stromal vascular fraction (SVF) and culture-expanded adipose tissue-derived stromal/stem cells (Bourin et al., 2013). Evidence for CD34 as a common marker for diverse progenitors from adipose tissue, including MSCs, was thus reported. However, current literature has reported that the phenotype of MSCs can change during *ex vivo* expansion, which may represent alterations in the biological features of the MSC population involved in the response to environmental change. Today, no specific and unique marker can be used for isolating or identifying MSCs. Only markers of native mesenchymal stromal cells have been evaluated to enrich the population (Simmons and Torok-Storb, 1991). Positive selection for the CD140b (STRO-1) antigen increases the frequency of colony-forming unit fibroblasts (CFU-Fs) by 100-fold relative to the total cell population. Cells selected for the CD271 antigen have a better potential for proliferation and differentiation than the unselected population. Likewise, the CD200 and CD49a antigens allow significant enrichment of the population of mesenchymal stromal cells derived from the bone marrow by selecting the most multipotent cells (Rider et al., 2007; Delorme et al., 2008).

It has been shown that the MSC profile of cell surface antigens changes during cell culture. A previous study indicated that CD13, CD29, CD44, CD73, CD90, CD105, and CD106 in MSCs are downregulated during culture expansion compared to MSCs in the stromal fraction (Cao et al., 2020). As such, uncultured BM-MSCs isolated from both humans and mice do not express CD44 but express the surface protein (90% positive cells) after being plated in culture (Qian et al., 2012). In contrast to an increase in CD44, the expression of CD106 and CD271 on MSCs is decreased after culture (Jung et al., 2011). Typical markers of cultured MSCs, such as CD73 and CD105, appear to be expressed by the majority of freshly isolated MSCs and are maintained during culture. Currently, a critical marker, STRO-1, which has a high specificity for early passage bone marrow-derived MSCs, is not included in the ISCT criteria. This marker helps to identify, isolate, and characterize stromal progenitor cells. However, the expression of Stro-1 is lost from MSCs during *ex vivo* expansion, and it cannot be considered a valuable marker of MSCs. The selectivity of STRO-1 for cells that are not MSCs is not yet clear (Zhang et al., 2020). To identify relevant markers for the enrichment of MSCs from heterogeneous cultures, the expression of neuron-glial antigen 2 (NG2) and melanoma cell adhesion molecule (CD146) was investigated. The results showed that the expression of CD146 and NG2 was inversely correlated with doubling time during the serial passage of single-cell-derived human BM-MSC cultures. The fraction of MSCs with high expression of NG2 and low scatter properties is more clonogenic than the parental MSC culture from which it was derived (O'Connor, 2019). However, the expression of CD146 during *in vitro* culture showed discrepancies between studies, probably due to various factors, including donor variation, different culture conditions, immunostaining protocols and flow cytometry analysis. CD142 is another surface marker that may represent concern for the systemic administration of MSCs, as it is linked to thrombosis. BM-derived MSCs displayed less expression of CD142 than AT-MSCs. BM-derived MSCs are likely more suitable for intravenous delivery and decrease the risk of thrombosis (Christy et al., 2017; Le Blanc and Davies, 2018).

To date, the lack of specific markers to define MSCs poses an additional challenge in the field, and the use of more advanced molecular criteria has been proposed. Further, several research groups have attempted to develop novel markers, such as transcriptomic, epigenetic and proteomic markers (Wagner et al., 2016; Wiese et al., 2019; Wiese and Braid, 2020).

2.3 Tissue Repair Properties: Multilineage Potential Versus Paracrine Immunotrophic Actions

MSCs are able to migrate to inflamed areas and damaged sites where they promote tissue repair by different functions (Kim et al., 2021). The action of MSCs can be associated not only with a direct mechanism, through their differentiation and replacement of damaged cells, but also primarily with their paracrine properties that reduce the inflammatory response and stimulate (cell empowerment) the proliferation and differentiation of different local progenitor cells (Wang et al., 2014b; Dabrowska et al., 2020; Qiu et al., 2020; Chae et al., 2021). Hereafter, we present an overview of the current findings on the tissue repair properties of MSCs and their consequences for clinical application.

2.3.1 Multilineage Potential

It was initially believed that MSCs mainly repair damaged tissues by cell-for-cell replacement driven by direct differentiation (Neshati et al., 2018; Smaida et al., 2020; Pan et al., 2021). Currently, there are no *in vivo* data demonstrating that MSCs differentiate into resident cells to repair injured tissue. Furthermore, permanent engraftment of MSCs into diseased tissues does not seem to occur. Therefore, the multipotency function of MSCs is likely an *in vitro* characteristic established to define MSCs a few years ago (Caplan, 2017). A number of culture protocols have been developed to induce MSC differentiation into several cell lineages in response to well-defined stimulation (Ullah et al., 2015) (Fitzsimmons et al., 2018). Although initially considered by Caplan to be stem cells, Sacchetti et al. (2007) revealed in 2007 that MSCs represent a rare and heterogeneous population of progenitors involved. The evidence supporting the *in vivo* differentiation of MSCs is relatively sparse and controversial compared to the abundance of data documenting *in vitro* multipotency. Most of the studies claiming *in vivo* differentiation, particularly

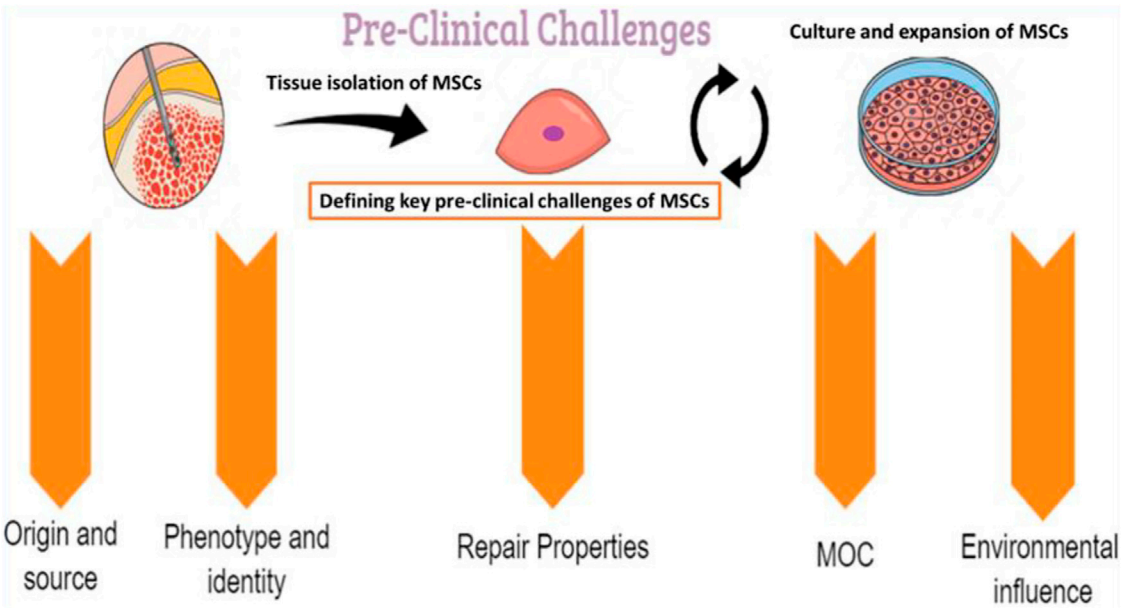

FIGURE 2 | The pre-clinical challenges linked to MSCs. MOC: mechanisms of action.

beyond mesodermal-derived tissue types, have been methodologically flawed or have documented only extremely low frequency events. Relatively few studies have clearly demonstrated MSC engraftment with differentiation and functional incorporation into recipient tissues when subjected to critical review. This discrepancy between *in vitro* and *in vivo* evidence of MSC multipotency highlights the need for *in vivo* data supporting functional incorporation or tissue-specific gene expression of engrafted MSCs, where models of robust engraftment, incorporation and differentiation do not exist.

2.3.2 Paracrine Immuno-Trophic Action

MSCs may act as immunomodulatory and trophic mediators in tissue regeneration and cell therapy. These actions imply interactions and interplay with local tissue cell progenitors as well as immune cells.

2.3.2.1 The Trophic Process

Trophic function appears to have a critical role in mediating the beneficial effect of MSC therapy for degenerative and/or inflammatory diseases. In response to injury, homing receptors and chemokines are released, which subsequently activate MSCs. Activated MSCs are then mobilized into the peripheral blood circulation, where an adhesion step is achieved by the specific interaction between chemokines and homing receptors such as stromal cell-derived factor (SDF-1), CXC chemokine receptor (CXCR) 4, hepatocyte growth factor (HGF), c-Met, hyaluronic acid (HA), CD44, monocyte chemoattractant proteins (MCPs) and C-C chemokine receptor type 2—CCR2/CD19. The transendothelial migration of MSCs to the local site of injury occurs via the degradation of extracellular matrix (ECM) by matrix metalloproteinases (MMPs) (Lin et al., 2017). Through a plethora of molecules (including HGF, IGF, VEGF, TGF-β1, and FGF-2), MSCs may regulate tissue homeostasis within the stromal niches by supporting the maintenance, expansion and/or differentiation of local resident cells (Park et al., 2018). MSCs can produce large amounts of growth factors, which subsequently stimulate endothelial cells, fibroblasts and, most importantly, tissue progenitor cells or stem cells *in situ*. The concerted action of these factors and cells facilitates tissue repair through angiogenesis, remodeling of the extracellular matrix (ECM) and the differentiation of tissue progenitor cells (Wang et al., 2014b).

2.3.2.2 The Immunomodulatory Process

MSCs are able to suppress the activity of the immune system and help resolve inflammation. However, MSCs are also able to stimulate the response of the immune system. This ability has therefore led some authors to hypothesize that MSCs could adopt, depending on the context, a pro- or anti-inflammatory phenotype (Bernardo and Fibbe, 2013; Betancourt, 2013).

MSCs modulate both inflammatory and immune responses by regulating innate and adaptive immunity that favor tissue repair (Chen et al., 2019b; Song et al., 2020b) Their effects are not HLA (human leukocyte antigen)-restricted. MSCs act on all effectors of innate and adaptive immunity and alter cell proliferation and other functions of immune cells. In addition, MSCs can inhibit the proliferation, cytotoxicity and production of IFN-γ in T lymphocytes and NK cells (Prigione et al., 2009). Blocking G0/G1 phases of the cell cycle, inducing apoptotic pathways and impairing the T cell subset ratio and inhibiting dendritic cells are among the mechanisms to inhibit lymphocyte proliferation (You et al., 2019). MSCs may also promote the polarization of macrophages from a proinflammatory phenotype to an anti-inflammatory phenotype, promoting tissue regeneration (Sica and Mantovani, 2012). MSCs also inhibit B lymphocyte proliferation and alter their differentiation into plasma cells. In

TABLE 4 | Markers differentially expressed by MSCs.

CD (cluster of differentiation)	MSC expression
CD3	–
CD9	+
CD10	+
CD11a	–
CD11b	–
CD13	+/–
CD14	–
CD15	+
CD16	–
CD19	–
CD29	+
CD31	+/–
CD34	+/–
CD35	–
CD36	+/–
CD38	–
CD40	+/–
CD44	+/–
CD45	+/–
CD49a	–
CD49b	+
CD49c	+
CD49d	+/–
CD49e	+
CD50	-
CD51	+
CD54	+/–
CD58	+/–
CD55	+
CD56	–
CD58	+
CD61	+/–
CD62e	–
CD62L	+/–
CD68	–
CD71	+
CD73	+
CD79	–
CD80	–
CD86	–
CD90	+
CD91	+
CD102	+/–
CD104	+/–
CD105	+
CD106	+/–
CD117	–
CD120a	+
CD120b	+
CD121a	+
CD124	+
CD133	–
CD134	–
CD140a	+
CD140b	+
CD144	+
CD146	+
CD164	+
CD166	+
CD200	+/–
CD252	–
CD221	+
CD271	+
CD274	+/–
SSEA-4	+
STRO-1	+
MSCA-1	+
HLA-ABC	+
HLA-DR	+/–
HLA-G	+/–

(Continued in next column)

(*Continued*) Markers differentially expressed by MSCs.

addition, MSCs may induce the differentiation of regulatory T lymphocytes (Saeedi et al., 2019).

Several mechanisms have been proposed to explain the immunomodulation carried out by MSCs, among which are (Najar et al., 2016):

- Indoleamine 2,3-dioxygenase (IDO), which catabolizes tryptophan to kynurenine. IDO is crucial for the inhibition of effector T lymphocyte proliferation by human MSCs and acts by depleting the medium of an essential amino acid, tryptophan, and by producing kynurenine, which is toxic to T lymphocytes.
- Prostaglandin E2 (PGE2), in combination with IDO, participates in the inhibition of NK cell proliferation.
- TNF-α stimulates "gene/protein 6" (TSG-6), which acts by a negative feedback control on macrophages by reducing their synthesis of proinflammatory factors, which in particular decreases the recruitment of polymorphonuclear neutrophils within the damaged tissues.

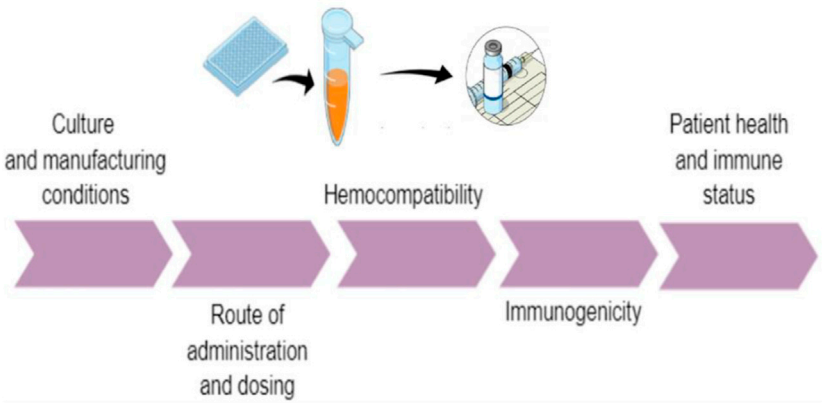

FIGURE 3 | The clinical challenges linked to MSCs.

- HLA-G5, which is thought to be responsible for the production of regulatory T lymphocytes; —"Transforming growth factor"-β (TGF-β), galectins, adenosine and FAS pathways, "programmed cell death protein 1" (PD-1), IL-1RA, IL-10 and Notch, but none of these mechanisms alone summarizes the immunomodulatory activity of MSCs.

Among the proven elements, MSCs are not constitutively immunosuppressive but acquire these properties after stimulation by inflammatory signals from the microenvironment, such as the inflammatory cytokines IFN-γ or TNF-α. MSCs are able to secrete a set of proinflammatory molecules, including IL-6, IL-8, GM-CSF (granulocyte-macrophage colony-stimulating factor) and MIF (macrophage migration inhibitory factor), thus promoting the recruitment and survival of neutrophils. Under the effect of these same signals, it has been shown that MSCs were able to block the synthesis of IL-10 from B lymphocytes, thus promoting a proinflammatory response. Mechanistically, MSCs likely contribute to immunomodulation through cell-to-cell contact or paracrine effects (Burnham et al., 2020). The secretome of MSCs comprises various cytokines and regulatory factors (e.g., TSG-6, TGF-b, hepatocyte growth factor, IFN-γ, prostaglandin E2, PGE2 and IDO pathways), insulin-like growth factor binding proteins, heme oxygenase-1 (HO), human histocompatibility antigen-G5 (HLA-G5), chemokine (C-C motif) ligand 2 (CCL2), IL-10, galectin-1 and galectin-3 (Madrigal et al., 2014; Burnham et al., 2020). The inflammatory context could also lead MSCs to synthesize a set of chemokines, such as CCL2, CCL3 or CCL12, helping the recruitment of macrophages, or like CXCL9, CXCL10 or CXCL11, promoting the chemotaxis of T lymphocytes (Andrzejewska et al., 2019b; O'Connor, 2019). These paracrine pathways may explain the therapeutic effect of MSCs despite their low engraftment, homing and survival after transplantation (Cruz et al., 2017).

2.3.2.3 Extracellular Vesicles

Extracellular vesicles (EVs) are membrane-enclosed heterogeneous structures including exosomes, microvesicles, ectosomes, microparticular membrane particles, exosome-like vesicles and apoptotic bodies that are released into the extracellular space. However, the defining parameters for each of these different classes are not definitive, and the use of the terms exosomes, microvesicles, and microparticles is often ambiguous and not rigorously qualified. These structures have been shown to participate in a wide variety of biological processes and are currently under intense investigation in many different fields of biomedicine (Buzas et al., 2018). EVs may be secreted by multiple types of cells and have been demonstrated to mediate intercellular communication in both physiological and pathological conditions. Generally, EVs can be formed by either inward budding of endolysosomal vesicles followed by exocytosis (e.g., exosomes) or shedding from the plasma membrane (e.g., microvesicles) (Klyachko et al., 2020). Due to their ability to carry key molecules, EVs affect the physiological and pathological functions of recipient cells. Generally, EVs carry a cargo of proteins and nucleic acids that reflect their cell of origin. They represent a sophisticated intercellular communication system and potential healing agents or delivery vehicles of therapeutic agents. Studies have confirmed that a major portion of the beneficial proprieties of MSCs arises from their paracrine activities (Varderidou-Minasian and Lorenowicz, 2020). EVs derived from MSCs may deliver a variety of molecules to the surrounding cells, leading to functional changes in the recipient cells (Hong et al., 2019). The regenerative and immunomodulatory capacity of MSC-derived EVs has been evaluated in several animal disease models, including kidney and liver injury, lung disease, cartilage repair, hind limb ischemia, ischemic brain injury, and spinal cord injury (Harrell et al., 2019; Hu et al., 2019; Liau et al., 2019). MSC-derived EVs represent a potential cell-free therapeutic option, as they are a major key for crosstalk communication between cells.

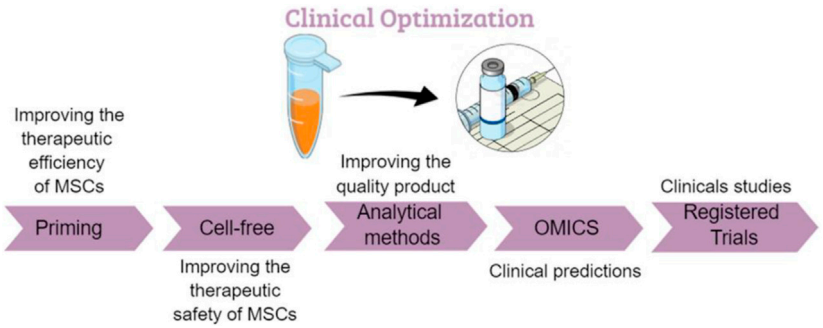

FIGURE 4 | The clinical optimization of MSC therapy.

2.4 Microenvironmental Cues Influencing the Function of Mesenchymal Stem/Stromal Cells

MSCs are considered responsive cells because they are able to sense the tissue environment and adapt their features accordingly. Such capacity to adjust their properties is linked to their functional plasticity. Such plasticity allows MSCs to actively respond to local tissue challenges and therefore display the appropriate therapeutic response (Wang et al., 2014b). Several *in vitro* strategies, including the use of relevant factors/conditions, have been reported to likely modulate the properties of MSCs (Wilson et al., 2019b).

These approaches require appropriate controls before translation for clinical applications because of the risk of immunogenicity, tumorigenicity, epigenetic modifications, loss of viability and efficiency. The production of MSCs using these approaches should be accomplished according to GMP.

2.4.1 Oxygen Saturation

The oxygen (O_2) content under normoxic conditions is 21%, which is of course higher than the concentrations found in the organs of the body (1–10%). For this, the culture of MSCs under hypoxic conditions with an O_2 percentage ranging from 1 to 10% clearly improves the proliferation of MSCs, their survival and the conservation of their multipotent character by keeping them in an undifferentiated state (Elabd et al., 2018). Some studies have also shown an improvement in the paracrine activity of MSCs through increased production of IL-6 and growth factors VEGF, HGF and bFGF (basic fibroblast growth factor). The effect of the hypoxic environment is mainly due to the induction of the transcription factor HIF-1 (hypoxia-inducible Factor 1), which in turn can interfere in different signaling pathways and induce the expression of the target genes involved in angiogenesis, proliferation and metabolism of MSCs (Luo et al., 2019).

2.4.2 Three-Dimensional Culture of Mesenchymal Stem/Stromal Cells

Three-dimensional (3D) culture is another strategy to enhance the potential of MSCs. Culturing MSCs in spheroids creates a hypoxic environment that strengthens their survival and proliferation. In addition, the anti-inflammatory, antifibrotic and proangiogenic activities of these MSCs are improved following an increase in the expression of the immunoregulatory factors TSG6 (TNFα-stimulated gene-6), PGE2 (prostaglandin E2) and IL-6 as well as trophic factors STC-1 (stanniocalcin 1), CXCR4, angiogenin and VEGF (Tsai et al., 2015). The use of specific biomaterials has demonstrated significant improvement in MSC therapy. Antonini et al. (2016) showed that the use of polyethylene terephthalate nanogratings improved the osteogenic differentiation of MSCs.

2.4.3 *In Vitro* Toll-Like Receptors Triggering

The Toll-like receptor (TLR) signaling pathway plays critical roles in the inflammatory response as well as in the regulation of tissue injury and wound healing processes. Depending on their origin and culture condition, MSCs can differentially express several patterns of TLRs. The engagement of these TLRs by their respective ligands results in different biological and immunomodulatory responses by MSCs (Tomchuck et al., 2008). MSCs can adopt pro-inflammatory or anti-inflammatory functions of the MSC1/MSC2 type depending on TLR engagement (Waterman et al., 2010). It was shown that the binding of LPS with TLR4 induces MSC differentiation into a proinflammatory phenotype with high expression of IL-6 and IL-8 and induction of T lymphocyte proliferation cocultured with MSCs (Waterman et al., 2010). On the other hand, the binding of poly (I:C) (polyinosinic-polycytidylic acid) with TLR3 polarizes MSCs toward an anti-inflammatory phenotype with high expression of IL-4, IDO and PGE2 and retains their immunosuppressive effect on T lymphocytes (Waterman et al., 2010). However, contrary to these results, (Liotta et al., 2008) showed an inhibition of the immunosuppressive effect of MSCs independent of TLR3 or TLR4 engagement by the inhibition of the Notch signaling pathway induced by Jagged-1. These contradictory results can be explained by differences in the culture conditions between the distinct studies, the concentrations of ligands used and the duration of treatment. TLRs are therefore important regulators of MSC functions and deserve more in-depth and standardized studies to better understand the influence of their ligands on the potential of MSCs.

2.4.4 *In Vitro* Inflammatory Licensing

Within the injured tissue, a plethora of inflammatory mediators and cytokines are released that may influence the function of MSCs. The immunomodulatory functions of MSCs are regulated by the complexity and intensity of the inflammatory environment. MSCs express several receptors on their surface for inflammatory mediators, such as IFN-γ, TNF-α, IL-1 and IL-6, which makes them capable of perceiving and reacting significantly to inflammatory stimuli. Accordingly, the immunosuppressive properties of MSCs were increased by IFN-γ in *in vitro* and *in vivo* models (Kim et al., 2018). IFN-γ licensing induced IDO expression in MSCs via the JAK/STAT1 signaling pathway. Moreover, it has been shown that MSCs have substantial and beneficial anti-inflammatory effects in the mouse model of GvHD when the latter were injected during the inflammatory peak. This effect was lost when anti-IFN-γ antibody was injected in parallel to WT-MSCs (wild-type MSCs) or when IFNγR1$^{-/-}$ MSCs were used (Ren et al., 2008). In the presence of IFN-γ and TNF-α, the expression of the chemokines CXCL9, CXCL10, RANTES (regulated upon activation, normal T cell expressed and presumably secreted) and CCL3 was considerably induced and involved in the recruitment of immune cells (specifically lymphocytes) to the surroundings of MSCs. They also induced the expression of ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1) molecules that facilitate cell adhesion, and ultimately, they stimulated the production of large amounts of IDO, iNOS and PGE2 involved directly in the immunomodulatory effect of MSCs (Ren et al., 2008; Li et al., 2012; Kim and Cho, 2016). On the other hand, during chronic or controlled inflammation, where the concentrations of IFN-γ and TNF-α are suboptimal, the latter induced the expression of chemokines but was insufficient to induce the production of the soluble mediators IDO and NO in large quantities, which would have a countereffect: the cells will be recruited near the MSCs without being inhibited, and in this case, the inflammatory process is aggravated (Kim and Cho, 2016).

A recent profiling highlighted that following a combination of inflammatory and proliferative signals, the sensitivity and responsive capacity of AT-MSCs were significantly modified (Merimi et al., 2021a). In particular, inflammation leads to an upregulation of IL-6, IL-8, IL-1β, TNF-α and CCL5 cytokine expression. Inflammation and cell passaging increased the expression of HGF, IDO1, PTGS1, PTGS2 and TGFβ. The expression of the TLR pattern was differentially modulated, with TLR 1, 2, 3, 4, 9 and 10 being increased, whereas TLR 5 and 6 were downregulated. Such observations are encouraging and have to be developed as preconditioning strategies to strengthen MSC function and proprieties (Muller et al., 2018).

2.4.5 Orthobiologics

MSCs hold promise for tissue healing, but some criticisms hamper their clinical application, including the need to avoid xenogeneic compound (e.g., animal serum) contamination during *ex vivo* cell expansion and scarce survival after transplantation. Orthobiologics are biological substances used to improve tissue healing and include platelet-rich plasma (PRP) and platelet lysate (PL) (Kruel et al., 2021). Many studies have demonstrated the ability of PRP, a source of many biologically active molecules, particularly growth factors, to positively influence MSC proliferation, survival and functionality, as well as its antifibrotic potential. Previous results suggested that PRP is able to positively affect BM-MSC viability, survival and proliferation, suggesting that it could represent a good serum substitute during *in vitro* cell expansion and could be beneficial toward transplanted cells *in vivo* (Sassoli et al., 2018). In parallel, the proliferation, cell cycle, and migration of umbilical cord-derived MSCs (hUC-MSCs) was significantly promoted in the presence of PL by upregulating relevant genes/proteins (PDGF-AA, IGF-1, TGF-β, EGF and FGF) and activating beclin1-dependent autophagy via the AMPK/mTOR signaling pathway (Yan et al., 2020).

Additionally, PRP may provide a suitable microenvironment that potentiates the enhancement of the functionality of MSCs. In this way, PRP and AT-MSC combined therapy significantly accelerated the healing of diabetic wounds induced experimentally in rats by modulating the Notch pathway, promoting angiogenesis and the proliferation of epidermal stem cells (EPSCs) (Ebrahim et al., 2021).

A study indicated that PRP improved the efficacy of engrafted MSCs to replace lost skin in mice by accelerating the wound healing processes, ameliorating the elasticity of the newly regenerated skin and stimulating their proangiogenic potential through enhanced secretion of soluble factors such as VEGF and SDF-1. These effects were also accompanied by an alteration of MSC energetic metabolism, including the oxygen consumption rate and mitochondrial ATP production (Hersant et al., 2019). Accordingly, there is a need to identify appropriate (regarding safety and efficiency) growth factors acting as preconditioning agents that may improve the cell survival, proliferation and function of MSCs within the host tissue microenvironment.

2.5 Engineering Mesenchymal Stem/Stromal Cells

Genetic engineering has emerged as another challenging yet promising approach to improve the therapeutic properties of MSCs. In fact, MSCs can be genetically engineered to overexpress certain desired elements and soluble factors, such as growth factors, cytokines, chemokines, transcription factors, enzymes and microRNAs (Fricova et al., 2020; Miceli et al., 2021). Distinct strategies have been applied to induce genetic modifications to further enhance the therapeutic potential of MSCs by improving various cellular properties, such as survival, homing and immunomodulatory effects. Several studies have demonstrated the use of genetic engineering (Baldari et al., 2017). Several studies using engineered MSCs have investigated the role of pancreatic duodenal homeobox-1 (PDX-1) and VEGF to produce functional insulin-producing cells as cellular therapy for diabetes; β-glucuronidase (GUSB) gene to improve genetic enzyme deficiency mucopolysaccharidosis type VII (MPSVII); IFN-α and INF-β in cancer therapy; Bcl-xL to stimulate angiogenesis; Bcl-2, heme-oxygenase-1 and Akt1 to improve the cell survival helping heart tissue repair in myocardial infarction; BMPs, to induce osteogenic differentiation; Neurogenin1 (Ngn1) to induce neuronal

differentiation and lipocalin2 (Lcn2) to restore the renewal potential of MSCs (Noronha et al., 2019; Saeedi et al., 2019). Viral vector–based genetic engineering typically has more efficient and durable gene expression but has some safety concerns because genes are integrated into the target cell genome. Nonviral vectors are safer, but the transfection efficiency is typically lower and gene expression is less durable. MSCs can also be engineered with drug-loaded particles. These particles are intracellularly loaded into MSCs to sustain their immunosuppressive profile for an extended period, regardless of the source of MSCs, but particle preparation can increase the cost and complexity when compared to the use of free small molecules. Oncolytic virus (OV) engineering has also been used to engineer MSCs. MSCs function by shielding viruses to avoid immunogenicity and by releasing the virus in tumor tissue to kill tumor cells. One limitation is that regular OVs have only moderate infectivity, although this can be overcome by using certain viral variants with higher infectious capacity (Levy et al., 2020).

2.6 Aging and Senescence

Although data on the functionality of MSCs isolated from aged subjects versus young individuals are still under debate in the literature, some consensual evidence appears. With increasing donor age, MSCs from bone marrow are reported to show a decrease in proliferative and clonogenic/self-renewal capacities, characterized by a number of CFU-Fs, but no phenotypic change is correlated with age (Charif et al., 2017). On the other hand, other studies reported the absence of substantial differences between cells from adult and elderly cohorts; therefore, aging rather than *in vivo* donor aging influences MSC characteristics. Indeed, (Andrzejewska et al., 2019a) compared MSCs from cohorts of young and old donors by analyzing their phenotypic and functional performance, using multiple assays typically employed as minimal criteria for defining MSCs. They found that MSCs from both cohorts met the standard criteria for MSCs, exhibiting similar morphology, growth kinetics, gene expression profiles, proangiogenic and immunosuppressive potential and the capacity to differentiate toward adipogenic, chondrogenic and osteogenic lineages.

The number of population doublings required for obtaining sufficient numbers of MSCs for therapy would be dependent on the initial number of viable MSCs. Therefore, attaining sufficient numbers could be subject to a large number of population doublings with the attendant possibility of stemness attenuation and cellular senescence (Liau et al., 2019). It has been reported that prolonged MSC expansion is accompanied by phenotypical and morphological changes, such as enlarged and irregular cell shapes and shortened telomere lengths, as well as gene, miRNA and protein expression alterations in cells, which ultimately lead to a state of senescence. Cellular senescence is generally defined as an arrest of cell proliferation. Replicative senescence refers to irreversible growth arrest of human diploid cell strains after extensive serial passaging in culture (Zhai et al., 2019). The presence of senescent cells in therapeutic MSC batches is undesirable, as it reduces their viability, differentiation potential and trophic capabilities. It is well documented that human MSCs (hMSCs) lose their differentiation potential after prolonged culture expansion *in vitro* and that cells from late, presenescent passages may not be able to differentiate at all. Additionally, their presence in MSC culture negatively influenced immunomodulatory and homing properties (Turinetto et al., 2016; Robb et al., 2019). Additionally, senescent cells acquire a senescence-activated secretory phenotype, which may not only induce apoptosis in neighboring host cells following MSC transplantation but also trigger an age-related disease phenotype such as osteoarthritis. Current methods for MSC senescence analysis in culture have been developed and were comprehensibly described previously (Zhai et al., 2019).

3 THE CLINICAL CHALLENGES

Several clinical challenges may influence the therapeutic use of MSCs and should be well identified and characterized (**Figure 3**).

3.1 Culturing and Manufacturing Conditions

As the frequency of MSCs is low after isolation, there is a need to expand the cells *ex vivo* to a high number before their use. Thus, the culture and manufacturing conditions may influence the properties of MSCs and should be well identified. On a large scale, and in accordance with good manufacturing practices (GMPs), MSCs are expanded with bioreactors. A bioreactor is a culture system where all conditions, including pH, temperature, and oxygen level, can be managed and controlled for proper cell expansion. Different types of bioreactors are used in MSC expansion, such as stirred tank bioreactors, rocking bioreactors, hollow fiber bioreactors and fixed-bed bioreactors (Mizukami and Swiech, 2018). It should be noted that these different culturing protocols and systems directly affect the therapeutic potential of MSCs; thus, each trial follows restricted rules to obtain the desired final product.

Additionally, the identification of optimal culture conditions is a prerequisite for MSC clinical applications. Animal-derived growth supplements, such as fetal bovine serum (FBS), have been predominantly used for MSC expansion. However, utilization of animal-derived products bears critical limitations and safety concerns. In particular, the risk of contamination and transmission of infectious agents, the potential to activate xenogeneic immune responses and animal welfare should not be neglected. Moreover, the exact composition of FBS remains unclear, and there are often significant variations between lots. Hence, it is necessary to determine suitable alternatives to animal serum that comply with all the relevant clinical requirements and that provide the appropriate quantity of high-quality cells while preserving the required properties. Alternative animal product-free formulations, including human AB serum (HABS), human platelet lysate (HPL) and chemically defined media (CDM), have been developed (Oikonomopoulos et al., 2015; Yin et al., 2019). Despite their batch-to-batch variability, these alternatives resolve most of the basic problems associated with the application of FBS. Although they represent promising supplements due to their native and human origin, further detailed analysis and studies will be required, and guidelines will have to be set to fully guarantee the safety and efficiency of these alternatives. The different

manipulation and storage procedures (freeze-thawed or freshly harvested) may also affect the quality of the product. Most of the allogenic MSCs used in clinical trials are cryopreserved. However, studies have shown that the cryopreservation of MSCs reduces their immunomodulatory and blood regulating properties (Moll et al., 2014a; Moll et al., 2016). Further research is imperative for the optimization of culturing and manufacturing conditions to ensure a better cellular "fitness" of MSCs.

3.2 Route of Application and Dosing

The route of MSC administration is highly dependent on the desired curative ability. Systemic (intravenous IV, intra-arterial IA, inhalation) and local (topical, direct tissue injection, intramuscular, *trans*-epi, *trans*-endocardial, intra-articular) delivery are common routes for the administration of MSCs (Caplan et al., 2019). In addition to the route of administration, the effective and accurate number of MSCs, the number of doses (single or repeated doses) and the interval of time between each dose are among the challenges influencing the safety and efficacy of the therapy (Galipeau and Sensebe, 2018; Kabat et al., 2020).

3.3 Hemocompatibility

MSCs are ABO neutral, and research has demonstrated that they do not inherently express ABO blood group antigens. However, the use of human AB plasma (ABP) while working with MSCs led to an adsorption of ABO antigens proportional to antigen concentration in the serum and adsorption time. Thus, particularly when treating immunocompetent patients or patients with blood type O, it is recommended to wash and infuse MSCs with nonimmunogenic human serum albumin (Moll et al., 2014b; Olsen et al., 2018). In some cases, MSCs initiate instant blood-mediated inflammatory reactions (IBMIRs). This later significantly causes the failure of allogenic graft survival and function. MSC hemocompatibility is mainly determined by procoagulant tissue factor (TF), which is highly correlated with the initiation of IBMIR. BM-MSCs show a lower expression of TF than MSCs from other sources (adipose tissue, perinatal tissue). Thus, MSCs are largely used in clinical trials with intravenous administration to minimize the rate of IBMIR and prolong engraftment survival. Nevertheless, many studies have investigated the effect of many conditions (such as culture media, freeze-thawing and cell expansion) on the ability of MSCs to trigger IBMIR (Moll et al., 2019). However, for clinical applications, it is suggested to add anticoagulant factors with MSC transplantation (Oeller et al., 2018).

3.4 Complement

Understanding the behavior of MSCs after infusion is still the focus of many studies. Culture-expanded human MSCs may elicit an innate immune attack, termed IBMIR. This reaction is characterized by the activation of the complement cascades. This deleterious reaction can compromise the survival, engraftment, and function of these therapeutic cells (Moll et al., 2012). MSCs have a short lifespan after *in vivo* administration and rapidly disappear from tissues. Such an observation does not rule out a beneficial effect of MSCs. It has been reported that the phagocytosis of MSCs may induce the generation of regulatory monocytes. It is also possible that a small proportion of MSCs escape this clean-up process and are responsible for the therapeutic effects (Eggenhofer et al., 2014). In line with this, some circulating MSCs, present at a very low level in healthy individuals, may greatly increase under specific conditions. After being mobilized, these local MSCs are recruited to the site of injury where they participate in the healing process (Xu and Li, 2014). Another hypothesis supports that the very rare presence of MSCs is likely linked to biophysical microdamage rather than the fact that specific molecular cues to a circulatory pool of MSCs are capable of repairing remote organs or tissues (Churchman et al., 2020). Several groups have revealed that MSCs, after infusion, activate complement by unknown mechanisms, leading to their damage and disappearance. The complement system, a part of the innate immune response, helps to remove microbes and damaged cells in parallel to promoting inflammation. Despite its importance, there are few studies investigating the interaction between complement and MSCs. A major role of the complement system during the interaction of MSCs with immune cells as well as in modulating their therapeutic activity was previously described (Moll et al., 2011). The complement-activating properties of MSCs were correlated with their potency to inhibit peripheral blood mononuclear cell proliferation *in vitro*. It was suggested that MSCs could be phagocytosed and removed by monocytes, which participate in their immunomodulatory properties (de Witte et al., 2018). It is proposed that complement opsonization induces phagocytosis of MSCs by monocytes after their intravenous infusion. Indeed, despite the expression of complement inhibitors, including CD46, CD55 and CD59, MSCs are injured after complement binding. Such phagocytosis may induce anti-inflammatory and pro-regenerative M2 monocyte polarization that could explain the therapeutic functions of MSCs (Gavin et al., 2019b). In contrast, some results indicated that complement activation is integrally involved in recognizing and injuring MSCs after their infusion (Li and Lin, 2012). The inhibition of complement activation could be a novel strategy to improve the efficiency of MSC-based therapies. The cell-surface engineering of MSCs with heparin has improved the viability and functions of MSCs after infusion by directly inhibiting complement and by recruiting Factor H, another potent complement inhibitor (Li et al., 2016). As an alternative to other sources of MSCs, placenta-derived decidual stromal cells (DSCs) were shown to be therapeutically efficient. Although complement activation was observed, this effect was particularly decreased when DSCs were supplemented with low-dose heparin (Sadeghi et al., 2019). A previous study found that incubation with autologous serum damaged BM-MSCs, probably following the formation of the complement membrane attack complex (MAC) induced by complement activation. Membrane complement regulatory proteins (mCRPs) can inhibit the activation of complement and thus prevent tissues from being damaged. It was thus suggested that the clinical use of mCRPs during the transplantation of MSCs can decrease the cytotoxicity induced by complement activation and therefore guarantee the survival

and function of these therapeutic cells (Xiao et al., 2017). Deep investigation of the interplay between MSCs and complement activation might be a straightforward and effective step for improving the outcome of current MSC-based therapies.

3.5 Immunogenicity

Because of the lack of consistent assays to measure their specific immunogenicity, MSCs have long been reported to be hypoimmunogenic or "immune privileged." MSCs should be considered immunoevasive cells with low immunogenicity. Depending on the conditions, MSCs do not express HLA class II, and their expression for HLA class I is low, preventing the activation of allorecognition pathways. The expression of HLA, CD40, CD80 and CD86 costimulatory molecules can be influenced by the inflammatory status within the surroundings of MSCs (Dominici et al., 2006). Indeed, the generation of antibodies against MSCs and the possible immune rejection in an allogeneic donor suggest that these cells may not be immune privileged (Ankrum et al., 2014). They can be recognized by the immune system and predisposed to be destroyed by cytotoxic immune cells such as natural killer (NK) cells or cytotoxic T lymphocytes (CTLs)In some "off-the-shelf" allogenic cases, cellular and humoral immune responses were observed. Allogenic BM-MSC injection with MHC mismatch in animal models initiates an immune reaction and therefore leads to transplant rejection. Research studies have explained that the expression of MHC/HLA is altered due to several factors, such as culturing conditions and epigenetic modification (Kot et al., 2019). Moreover, MSC differentiation leads to the upregulation of immunogenic molecules on the cell surface and thus an increase in MSC immunogenicity (Lohan et al., 2017). In addition, a high number of passages for MSCs increases inflammatory reactions after systemic administration. Within immunocompetent mice, allogeneic MSCs provoked an immunogenic response, with the infiltration of inflammatory cells at the transplant site and full graft rejection. Allogeneic islets cotransplanted with preactivated MSCs prolonged graft survival by approximately 6 days compared with islets alone. Such an observation corroborates the hypothesis that allogeneic MSCs are not immune-privileged and that after playing their therapeutic role, they are rejected (Oliveira et al., 2017). To resolve the immunogenicity challenges, two features must be investigated. First, modern assays to appropriately identify and measure immune responses to MHC-mismatched MSCs should be developed (Berglund et al., 2017). Second, new engineering approaches should be applied to overcome the rejection of allo-MSCs, avoid the generation of alloreactive antibodies in parallel to prolong their *in vivo* survival and engraftment and enhance their immunoregulatory paracrine activity.

3.6 Patient Health and Immune Status

Although the biological characteristics of the injected donor cells are inarguably one of the most important factors that determine the efficacy of MSCs, the recipient environment where immunomodulation is supposed to take place should not be neglected. For example, age, skin involvement, lower acute GvHD grade, and the number of infusions are the main prognostic factors affecting the efficacy of MSC therapy for steroid-refractory acute GvHD (Chen et al., 2015). The recipient immune environment can influence the therapeutic outcome following the use of MSCs. As shown by Gavin et al. (2019a), Gavin et al. (2019b) a proinflammatory immune profile within the gut at the point of MSC treatment may impede their therapeutic potential for GvHD. The recipient immune environment can also vary according to the age of the patient. Physiological aging is accompanied by a decline in immune system function. Age-related changes from infants through adults revealed progressive declines in the percentage of total lymphocytes and absolute numbers of T and B cells. The proinflammatory cytokines TNF-α and IL-6 were higher in elderly people than in adults (Valiathan et al., 2016). It is now generally recognized that the immunomodulatory properties of MSCs are not constitutive but are induced by various mediators present in the inflammatory environment. Different inflammatory stimuli are able to polarize MSCs with distinct phenotypes and functions. Inflammatory status changes throughout the course of an immune response and is affected by time, activators of the immune system and many other factors. Therefore, it is likely that the types and amounts of inflammatory cytokines present in the stromal niche will dictate the migration and function of MSCs (Wang et al., 2014b). Thus, adipose-derived MSCs significantly reduced the severity of experimental autoimmune encephalomyelitis (Ebrahim et al., 2021) by suppressing the autoimmune response in early phases of disease and not during disease remission (Constantin et al., 2009). Compared with adults, children generally showed a trend toward better complete responses (Introna et al., 2014; Chen et al., 2015). A multicenter nonrandomized phase II study addressing the infusion of MSCs in patients with severe steroid refractory showed that children responded consistently better than adults, with more complete remissions and less progressive disease (Le Blanc et al., 2008). In addition, multiple infusions of MSCs were more effective for children with steroid-refractory acute disease, especially when employed early in the disease course (Ball et al., 2013). MSCs from pooled bone marrow mononuclear cells of several healthy third-party donors were more effective in the treatment of severe acute GvHD (Kuci et al., 2016). As mentioned previously, preparation of the patient's body with anticoagulants is also necessary in some cases to prevent the initiation of IBMIR and hopefully lead to a better outcome (Moll et al., 2019). In another report, it was assumed that a part of the therapeutic effect of MSCs was mediated by host/patient phagocytic cells. The latter help to remove MSCs administered to the patient and thus modulate MSC activity (Hoogduijn and Lombardo, 2019). Such observations indicate the need to further establish the immune cell profile of patients who may segregate responders from nonresponders to MSC therapy. It is recommended to explore and monitor the inflammatory and immunological status of patients at the time MSCs are infused to help optimize MSC-based therapy. Moreover, discussions about the relevance of preconditioning MSCs before transplantation and the

identification of biomarkers to predict patient responsiveness to MSC therapy are ongoing.

3.7 Clinical Optimization

The clinical optimization of MSCs is required to achieve safe and efficient therapeutic indications (**Figure 4**).

3.7.1 Cell-Free Therapeutic

As previously discussed, several benefits and advantages are linked to the use of EVs isolated from MSC-conditioned media as a cell-free therapy. EVs have many advantages over MSCs; they are easy to dose, prepare, store and administer at the time of choice, cost less, are small and have no risk of vascular obstruction (Phinney and Pittenger, 2017). Based on these data, researchers are leaning toward their use as potential therapies for several diseases. EVs are likely heterogeneous and differ depending on the type of MSCs from which they are derived. Thus, well-defined and characterized EVs are recommended. Moreover, their metabolomic and lipidomic profiles have not yet been well characterized. Other limitations of EV isolation and purification involve the procedure itself, which includes variability in the quality of EV preparations, the yield of EVs, and the potential for non-EV contaminants in the preparation. Likewise, the production and packaging methods for the vesicles produced by MSCs are currently being validated.

In this context, cell-free therapies involving the secretome of MSCs have, in theory, lower safety risks than cellular products. Indeed, these therapies cannot replicate as cells, but this estimated safety risk cannot ignore the risk of influencing tumorigenesis. In line with these findings, several studies have reported that EVs shed by cancer stem cells (CSCs) may significantly contribute to tumor progression. CSC-derived EVs are involved in tumor resistance, metastasis, angiogenesis, maintenance of the stemness phenotype and tumor immunosuppression microenvironment (Su et al., 2021). As stated by the Cell Products Working Party and the Committee for Advanced Therapies of the ISCT, the risk of potential tumorigenicity related to MSC-based therapies should not be minimized, and working on the quality and safety of such products should be increased (Barkholt et al., 2013). Several problems interfere with the clinical application of EVs from adult stem cells (SCs) in cancer treatment, such as safety issues, unpredictable pro-tumor effects, and tissue entrapment (Parfejevs et al., 2020). The risk of tumorigenesis by EVs remains a concern because of the systemic and diverse effects of their cargo. The influence of MSCs on tumor progression is subject to contradictory debate with tumor growth acting as a double-edged sword (Liang et al., 2021). Through several mechanisms and depending on many factors, MSCs may either suppress or promote tumor growth. Similar to MSCs, EVs can be either associated with tumor progression, tumorigenesis, angiogenesis, and metastasis or associated with tumor suppression, exhibiting tumor-suppressor effects (Vakhshiteh et al., 2019). In fact, the tumor microenvironment (TME) is highly affected by EVs from both tumor cells and nonmalignant cells, as they function as carriers for various molecules in the TME (Tao and Guo, 2020). Different studies have reported that MSC-EVs may exert various effects on the growth, metastasis, and drug response of different tumor cells by transferring proteins, messenger RNA, and microRNA to recipient cells (Zhang et al., 2017). Changes in the composition and secretion rate could contribute to the oncogenic effects of EVs by creating a tumor-supportive microenvironment. The cargo of MSC-derived EVs may contain factors involved in cancer metastasis and promote epithelial-mesenchymal transition (EMT). Cancer-derived EVs can thus "educate" nearby MSCs to secrete large amounts of IL-8 and other immunosuppressive cytokines. Interestingly, this inflammatory microenvironment is prone to promote the formation of new blood vessels toward the tumor (Xavier et al., 2020). Within the tumor microenvironment, stromal cells secrete EVs that will support a drug resistance phenotype in otherwise drug-sensitive cancer cells. Breast cancer cells may thus prime BM-MSCs to release exosomes containing distinct miRNA contents, such as miR-222/223, which in turn promotes quiescence in a subset of cancer cells and confers drug resistance (Bliss et al., 2016). Several studies demonstrated that treatment with MSC culture medium or MSC coculture promoted EMT in breast or gastric cancer cells (Kletukhina et al., 2019). Gastric cancer cells acquire an "activated" carcinoma-associated fibroblast (CAF) phenotype and enhance tumor metastasis and growth *in vivo* after being in close contact with MSCs. Paracrine signals induce EMT and promote transwell and transendothelial migration, and the changes are dependent on β-catenin, MMP-16, snail and twist (Xue et al., 2015). EVs derived from adipose tissue-derived MSCs promoted the migration and proliferation of breast cancer cells *via* the activation of the Wnt signaling pathway (Lin et al., 2013). Human umbilical cord mesenchymal stem cell-derived EVs (hUC-MSC-EVs) have been shown to significantly enhance the proliferation, migration and invasion of human breast cancer cells through the activation of the ERK pathway. hUC-MSC-EVs reduced E-cadherin expression and increased N-cadherin expression, thus promoting EMT in breast cancer cells and leading to malignant tumor progression and metastasis (Zhou et al., 2019). In addition, Zhao et al. (2018) found that the EMT-promoting effect in lung cancer was mediated by EVs secreted from hUC-MSCs through the secretion of TGF-β. MSC-EVs may promote the growth and metastasis of tumor cells by different secreted factors. Exosomes derived from BM-MSCs increase tumor growth in a BALB/c nu/nu mouse xenograft model by enhancing VEGF expression through the activation of extracellular signal regulated kinase 1/2 (ERK1/2) and the p38 MAPK pathway (Zhu et al., 2012). Bone marrow stromal cell-derived exosomes were shown to promote the proliferation, survival, and metastasis of myeloma cells by modulating the p38, p53, c-Jun N-terminal kinase, and Akt pathways (Wang et al., 2014a). Surprisingly, human Wharton's jelly mesenchymal stem cell-derived extracellular vesicles (hWJ-MSC-EVs) were reported to promote the growth and migration of human renal cell carcinoma (RCC) by inducing HGF expression and activation of the Akt and ERK1/2 signaling pathways (Du et al., 2014), although antiproliferative and proapoptotic effects of these hWJ-MSC-EVs were described on bladder cancer cells through downregulation of Akt phosphorylation and upregulation of Caspase 3 cleavage

(Wu et al., 2013). Exosomes derived from the MSCs of multiple myeloma patients expressed higher levels of oncogenic proteins, cytokines (IL-6, CCL2) and adhesion molecules (γ-catenin, fibronectin) and lower expression levels of the tumor suppressor miRNA-15a than exosomes derived from normal MSCs. Moreover, the latter inhibited the growth of multiple myeloma cells, whereas exosomes derived from MSCs of multiple myeloma patients promoted tumor growth (Roccaro et al., 2013). Collectively, these observations on the impact of EVs derived from MSCs on tumor biology should be well monitored and clarified to ensure the safety of the cell-free strategy.

As shown, the translation of MSC-EVs to the clinical stage is still at the initial phase. A number of concerns still have to be solved regarding their safety, particularly regarding tumors, their mechanisms of action, the possible alteration of their properties because of isolation/purification methods, and/or the best approach for large-scale clinical production (Massa et al., 2020).

3.7.2 Quality Control of Mesenchymal Stem/Stromal Cells

Before their application in clinical trials or cryopreservation and throughout their production, MSCs need to undergo quality-control determination. Quality-control criteria include the determination of many characteristics, including surface markers, morphology, differentiation potential, senescence status, secretome, immunophenotype and others (Torre et al., 2015). Functional assessment before and after cryobanking is crucial, because MSCs are susceptible to alteration in their function and characteristics under freeze-thawing conditions. Furthermore, at the level of clinical grade production, culturing and manufacturing conditions are able to highly influence MSCs; thus, they must be in compliance with the principles of good manufacturing practices (GMPs) to ensure their safety and efficacy (Sensebe et al., 2013). Several techniques and assays had to be performed to assess safety and efficacy; tests for contamination, including endotoxin assays, sterility tests and Gram staining, and detection of *mycoplasma*, had to be performed to ensure safe production (Galvez et al., 2014). In addition, genome stability is pivotal to prevent oncogenic risks and should be assessed by performing tests such as comparative genome hybridization (CGH) or fluorescence *in situ* hybridization (FISH) (Sensebe et al., 2013). Finally, each study, according to its needs and goals, had to perform several tests and assays and had to follow specific rules to benefit from a final product with high quality and potential.

3.7.3 Clinical Prediction Tools Including OMICS

Far away from the traditional techniques that measure cell surface markers and cellular morphology, omics-based biomarkers such as proteomics, genomics, epigenomics, metabolomics and transcriptomics are new revolutionary methods for distinguishing MSCs with different features that may lead to the failure or success of the treatment at a clinical level. In other words, the characterization of omics in different culture conditions (monolayer cell culture versus aggregate cell culture) explained the therapeutic potential of MSCs and suggested that some of the failed clinical trials were due to the different abilities of MSCs in monolayer cultures versus *in vivo*. The results demonstrated that the aggregate culture enhanced the secretory capacity of MSCs and altered the metabolism of several proteins and lipids (Doron et al., 2020). Furthermore, transcriptome analysis is key for understanding the functional and differentiation potency of MSCs. Studies have shown that transcriptional profiling could be a predictive tool for stem cells (Wells and Choi, 2019). Clinical prediction tools assist in clinical outcome prediction, and omics approaches have recently served in many studies to identify the targets of several treatments based on MSCs.

4 CONCLUSION

MSCs have been investigated as a therapeutic strategy for several medical indications. The fate and behavior of MSCs are regulated by their environment, which may consequently influence their repair potential. The mechanisms of action of MSCs are mainly linked to their secretome, including chemokines, cytokines, growth factors and nucleic acids. These regulatory elements can be secreted separately or packaged into extracellular vesicles. As MSCs are able to sense and respond appropriately to local tissue challenges, such plasticity raises the possibility of preconditioning (licensing or priming) MSCs to adopt a distinct fate and function while targeting specific diseases. Currently, applied MSCs should be handled with precaution, as minor unknown or less characterized effects may hamper their therapeutic effect. Discussing new insights into the biological properties of MSCs, as well as the different preclinical and clinical challenges, will help to develop and optimize a safe and efficient therapeutic strategy.

AUTHOR CONTRIBUTIONS

MM and MN conceived and designed the review. All authors listed have made a substantial, direct and intellectual contribution to the work. All authors listed contributed to manuscript writing, revision, reading, and approved the submitted version.

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GLOSSARY

MSCs Mesenchymal stem/stromal cells
ISCT International Society for Cellular Therapy
IFN Interferon
TNF Tumor Necrosis Factor
IDO Indoleamine 2,3-dioxygenase
GvHD Graft-versus-host disease
CVD Cardiovascular disease
VEGF Vascular endothelial growth factor
HGF Hepatocyte growth factor
IL Interleukin
CXCR4 C-X-C Motif Chemokine Receptor 4
RA Rheumatoid arthritis
MSC-EVs MSC-Extracellular vesicles
EVs Extracellular vesicles
BM-MSC Bone Marrow derived MSCs
MSC-Exos MSC-derived exosomes
Exos Exosomes
MPs Microparticles
hUC-MSCs Human umbilical cord-derived MSCs
AT-MSCs Adipose tissue-derived MSCs
MSC-NTF Neurotrophic factor-secreting MSC
ALS Amyotrophic Lateral Sclerosis
IV Intravenously
DMARDs disease-modified antirheumatic drugs
FDA Food and Drug Administration
CD Crohn disease
CFU-F Colony-forming unit-fibroblasts
NG2 Neuron-glial antigen 2
IGF-1 Insulin-like growth factor 1
EPO Erythropoietin
GDNF Glial Cell Derived Neurotrophic Factor
MMPs Matrix Metalloproteinases
FGF-2 Fibroblast growth factor-2
IGFBP-6 Insulin-like growth factor-binding protein 6
MHC-I Major histocompatibility complex-class1
HLA Human leukocyte antigen
FAS Fas Cell Surface Death Receptor
GM-CSF Granulocyte-macrophage colony-stimulating factor
MIF Macrophage migration inhibitory factor
bFGF Basic fibroblast growth factor
PGE2 Prostaglandin E2
COX-2 Cyclooxygénase-2
OA Osteoarthritis
RCTs Randomized clinical trials
TLRs Toll-like receptors
RANTES Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
ICAM InterCellular Adhesion Molecule
VCAM-1 Vascular cell adhesion molecule
IBMIR Instant-blood mediated inflammatory reaction
TF Tissue factor
DSCs Decidua stromal cells
mCRPs Membrane complement regulatory proteins
HSA Human serum albumin
ECM Extracellular matrix
MSCs-CM MSCs-conditioned medium
TME Tumor microenvironment
EMT Epithelial-mesenchymal transition
hWJ-MSC-EVs Human Wharton's jelly mesenchymal stem cells derived extracellular vesicles

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Single-Cell Transcriptome Integration Analysis Reveals the Correlation Between Mesenchymal Stromal Cells and Fibroblasts

Chuiqin Fan[1†], Maochuan Liao[1†], Lichun Xie[2†], Liangping Huang[1], Siyu Lv[3], Siyu Cai[1], Xing Su[1], Yue Wang[3], Hongwu Wang[3], Manna Wang[3], Yulin Liu[1], Yu Wang[3], Huijie Guo[3], Hanhua Yang[2], Yufeng Liu[4]*, Tianyou Wang[5]* and Lian Ma[3,1,2]**

[1]Department of Pediatrics, The Second Affiliated Hospital of Shantou University Medical College, Shantou, China, [2]Department of Pediatrics, The Third Affiliated Hospital of Guangzhou Medical University (The Women and Children's Medical Center of Guangzhou Medical University), Guangzhou, China, [3]Department of Hematology and Oncology, Shenzhen Children's Hospital of China Medical University, Shenzhen, China, [4]Department of Pediatrics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [5]Department of Hematology and Oncology, Beijing Children's Hospital, Capital Medical University, Beijing, China

Background: Mesenchymal stromal cells (MSCs) and fibroblasts show similar morphology, surface marker expression, and proliferation, differentiation, and immunomodulatory capacities. These similarities not only blur their cell identities but also limit their application.

Methods: We performed single-cell transcriptome sequencing of the human umbilical cord and foreskin MSCs (HuMSCs and FSMSCs) and extracted the single-cell transcriptome data of the bone marrow and adipose MSCs (BMSCs and ADMSCs) from the Gene Expression Omnibus (GEO) database. Then, we performed quality control, batch effect correction, integration, and clustering analysis of the integrated single-cell transcriptome data from the HuMSCs, FMSCs, BMSCs, and ADMSCs. The cell subsets were annotated based on the surface marker phenotypes for the MSCs (*CD105*$^+$, *CD90*$^+$, *CD73*$^+$, *CD45*$^-$, *CD34*$^-$, *CD19*$^-$, *HLA-DRA*$^-$, and *CD11b*$^-$), fibroblasts (*VIM*$^+$, *PECAM1*$^-$, *CD34*$^-$, *CD45*$^-$, *EPCAM*$^-$, and *MYH11*$^-$), and pericytes (*CD146*$^+$, *PDGFRB*$^+$, *PECAM1*$^-$, *CD34*$^-$, and *CD45*$^-$). The expression levels of common fibroblast markers (*ACTA2*, *FAP*, *PDGFRA*, *PDGFRB*, *S100A4*, *FN1*, *COL1A1*, *POSTN*, *DCN*, *COL1A2*, *FBLN2*, *COL1A2*, *DES*, and *CDH11*) were also analyzed in all cell subsets. Finally, the gene expression profiles, differentiation status, and the enrichment status of various gene sets and regulons were compared between the cell subsets.

Results: We demonstrated 15 distinct cell subsets in the integrated single-cell transcriptome sequencing data. Surface marker annotation demonstrated the MSC

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***Correspondence:**
Hanhua Yang
Drsam@126.com
Yufeng Liu
lyf6012@163.com
Tianyou Wang
Wangty999@sohu.com
Lian Ma
malian8965@sina.com

†These authors have contributed equally to this work

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Abbreviations: ADMSCs, adipose mesenchymal stromal cells; BMSCs, bone marrow mesenchymal stromal cells; FSMSCs, foreskin mesenchymal stromal cells; GEO, gene expression omnibus; HuMSCs, human umbilical cord mesenchymal stromal cells; ISCT, International Association for Cell Therapy; Log2FC, log2 fold change; MSCs, mesenchymal stromal cells; PCA, principal component analysis; PCs, principal components; RAS, regulon activity score; RSS, regulon-specific score; TSNE, *t*-distributed stochastic neighbor embedding; VIM, vimentin.

phenotype in 12 of the 15 cell subsets. C10 and C14 subsets demonstrated both the MSC and pericyte phenotypes. All 15 cell subsets demonstrated the fibroblast phenotype. C8, C12, and C13 subsets exclusively demonstrated the fibroblast phenotype. We identified 3,275 differentially expressed genes, 305 enriched gene sets, and 34 enriched regulons between the 15 cell subsets. The cell subsets that exclusively demonstrated the fibroblast phenotype represented less primitive and more differentiated cell types.

Conclusion: Cell subsets with the MSC phenotype also demonstrated the fibroblast phenotype, but cell subsets with the fibroblast phenotype did not necessarily demonstrate the MSC phenotype, suggesting that MSCs represented a subclass of fibroblasts. We also demonstrated that the MSCs and fibroblasts represented highly heterogeneous populations with distinct cell subsets, which could be identified based on the differentially enriched gene sets and regulons that specify proliferating, differentiating, metabolic, and/or immunomodulatory functions.

Keywords: fibroblast, mesenchymal stromal cells, integration analysis, pericytes, single-cell transcriptome sequencing

INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent adult stromal cells that are used for tissue repair (Maqsood et al., 2020) and immunomodulation (Mo et al., 2016). MSCs have been applied to the treatment of a variety of diseases, such as steroid-refractory graft-versus-host disease (Le Blanc et al., 2004), diabetes (Bhansali et al., 2017), multiple sclerosis (Cohen et al., 2018), cardiac ischemic injury (Qayyum et al., 2017), systemic lupus erythematosus (Wang et al., 2014), inflammatory bowel disease (Dhere et al., 2016), osteoarthritis (Lamo-Espinosa et al., 2018), and sepsis (Simonson et al., 2015). MSCs were first isolated from the bone marrow (Friedenstein et al., 1974) and subsequently from other sources, such as the umbilical cord Wharton's jelly (Mitchell et al., 2003), adipose tissue (Zuk et al., 2001), foreskin (Najar et al., 2016), synovial fluid (De Bari et al., 2001), dental pulp (Gronthos et al., 2000), and endometrium (Schwab et al., 2008). MSCs derived from different tissues consist of heterogenous cellular populations with distinct biological properties (Mattar and Bieback, 2015). Fibroblasts are the most common type of stromal cells found abundantly in the connective tissues; they secrete proteins that constitute the extracellular matrix and play an essential role in wound repair, tissue development, and fibrosis (Muhl et al., 2020). Fibroblasts have also been isolated from various tissues, such as the cornea, skin, adipose tissue, heart, skeletal muscle, intestine, and bladder (Chang et al., 2002; Muhl et al., 2020).

MSCs are isolated based on the International Association for Cell Therapy (ISCT) criteria (Dominici et al., 2006), which includes (1) adherent growth in plastic Petri dishes; (2) high expression of surface markers such as CD73, CD90, and CD105 and low expression or absence of HLA-DR, CD11b or CD14, CD19 or CD79a, CD34, and CD45 expression; and (3) the ability to differentiate into osteoblasts, chondrocytes, and adipocytes *in vitro*. The fibroblasts demonstrate adherent growth in plastic Petri dishes with a spindle or wide flat shape (Alt et al., 2011). Fibroblasts also express CD73, CD90, and CD105 and lack the expression of HLA-DR, CD11b or CD14, CD19 or CD79a, CD34, and CD45 (Chen et al., 2007; Haniffa et al., 2007; Alt et al., 2011; Blasi et al., 2011; Soundararajan and Kannan, 2018). Moreover, fibroblasts demonstrate the potential to differentiate into osteoblasts (Chen et al., 2007; Blasi et al., 2011), chondroblasts (Chen et al., 2007; Alt et al., 2011), and adipocytes (Chen et al., 2007; Blasi et al., 2011) under specialized growth conditions. Therefore, fibroblasts show significant similarity with the MSCs based on the ISCT criteria. Furthermore, both MSCs and fibroblasts exert immunosuppressive effects through cell–cell interactions and paracrine mechanisms (Soundararajan and Kannan, 2018; Ugurlu and Karaoz, 2020). MSCs and fibroblasts also show similarities in cell morphology (Alt et al., 2011), multipotent differentiation and replicative ability (Alt et al., 2011), gene expression profile (Bae et al., 2009), and immunosuppressive functions (Haniffa et al., 2007). Besides, MSCs and fibroblasts also show similar plasticity and stemness as they can be induced to differentiate into hepatocytes, cardiomyoblasts, islet cells, muscle cells, and germ cells (Chen et al., 2015; Maldonado et al., 2017; Zhang et al., 2019; Ugurlu and Karaoz, 2020) and can be reprogrammed into induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006; Buccini et al., 2012). It is also postulated that the differences in methylation patterns between the MSCs and fibroblasts (de Almeida et al., 2016) are related to the donor's age and long-term culturing (Koch et al., 2011). Furthermore, fibroblast surface markers have been reported in senescent MSCs (Soundararajan and Kannan, 2018). This suggests that both cell types are essentially the same type of cells in different life cycle stages; that is, fibroblasts may represent aging MSCs, and MSCs may represent naive fibroblasts (Soundararajan and Kannan, 2018). In contrast to this view, some studies suggested that fibroblasts do not represent aging MSCs and were differentiated from the MSCs under certain conditions (Mishra et al., 2009; Chan et al., 2019). Regardless of these

conflicting views, it is generally accepted that fibroblasts are closely related to MSCs. However, it is important to clearly distinguish the MSCs from the fibroblasts because MSCs are used for several clinical applications. Therefore, in this study, we performed comprehensive bioinformatics analysis of integrated single-cell transcriptome sequencing data from the human umbilical cord mesenchymal stromal cells (HuMSCs), foreskin mesenchymal stromal cells (FSMSCs), bone marrow mesenchymal stromal cells (BMSCs), and adipose mesenchymal stromal cells (ADMSCs) to identify distinct cell subsets that show similarities or differences in gene expression patterns, biological functions, and transcriptional regulatory networks between the MSCs and fibroblasts.

METHODS

Data Collection and Ethical Approval

The single-cell transcriptome data of the BMSCs and ADMSCs were extracted from the GEO database [BMSCs: GSE115149 (Jitschin et al., 2019) and GSE162692 (Ruoss et al., 2021); ADMSCs: SRP148833 (Liu et al., 2019)]. These data were available for the public and did not require ethical approval. Furthermore, the collection of umbilical cords and foreskin tissues was approved by The Second Affiliated Hospital of Shantou University Medical College of China (Institutional Review Board approval numbers: 2020-11 and 2021-89). The white connective tissues of the umbilical cord and the dermal tissues of the foreskin were cut into small pieces and cultured with Dulbecco's Modified Eagle Media/Nutrient Mixture F-12 (Gibco, United States) containing 10% fetal bovine serum (Gibco, United States) to generate the primary HuMSCs and FSMSCs, respectively. MSCs from the third passage were subjected to single-cell transcriptome sequencing using the 10× Genomics Chromium sequencing platform. The sequencing data of the HuMSCs and FSMSCs were processed with the 10× Genomics Cell Ranger software (version 3.1.0) (Zheng et al., 2017) and registered at the Mendeley database (https://data.mendeley.com/datasets/f4b2ykfv56/1).

Quality Control and Integration

The gene expression data of the BMSCs from the control and unprocessed groups were obtained from the GSE115149 (platform: 10× Genomics Chromium, passage: 3) and GSE162692 (platform: 10 × Genomics Chromium, passage: unknown) datasets. The gene expression data of the ADMSCs were obtained from the SRP148833 dataset (platform: 10 × Genomics Chromium, passage: 3) using the 10× Genomics Cell Ranger software (Zheng et al., 2017). Then, the Seurat objects for the BMSCs, ADMSCs, HuMSCs, and FSMSCs were created using the Seurat R package (version 4.0.0) (Hao et al., 2020). The quality control screening included retaining high-quality cells after excluding data for cells that contained less than 200 genes or more than 20,000 genes and/or greater than 20% mitochondrial genes. Finally, we merged all the filtered Seurat objects from the high-quality cells.

Data Processing

The merged data were normalized and standardized to correct the sequencing depth of each cell. The principal component analysis (PCA) was performed based on the 2,000 genes with high variability to acquire the first 50 principal components (PCs), which were then subjected to batch effect correction using the R package harmony (version 1.0) (Korsunsky et al., 2019). The first 50 harmony dimensions were used for *t*-distributed stochastic neighbor embedding (TSNE) with a resolution of 0.5. The cluster tree for the cell subsets of cells was constructed using the BuildClusterTree function of Seurat, and the cell subsets were annotated.

Analysis of the Cell Subsets

The expression levels of the marker genes representing the MSC, fibroblast, and pericyte phenotypes were calculated for the different cell subsets using the Findallmarker function of the R package Seurat with an adjusted p-value <0.05 and an absolute value of log2 fold change (Log2FC) ≥1. Then, gene set enrichment analysis was performed to evaluate the potential biological functions of the cellular subsets by calculating the enrichment scores and p-value of the hallmark gene sets in different cell subsets using the R package msigdbr (version 7.2.1) (Dolgalev, 2020) and singleseqgset (version 0.1.2.9000) (Cillo, 2021). The gene sets with a p-value <0.5 were regarded as statistically significant. Finally, the differentiation degree of each cell subset was calculated using the R package CytoTRACE (version 1.8.0) (Gulati et al., 2020).

Analysis of Gene Regulatory Networks

The co-expression modules of different transcription factors and their target genes were analyzed from the gene expression data using the python package pySCENIC (version 0.10.3) (Aibar et al., 2017). Then, the significant motifs in the co-expression module were analyzed by the motif enrichment analysis after deleting the target genes with low scores. The target genes and their corresponding transcription factors in the co-expression module were considered as a regulon. The regulon activity score (RAS) of each regulon in a cell was calculated. Then, the RAS threshold of each regulon was estimated. If RAS was greater than the threshold, the regulon in the cell was considered as activated. Otherwise, the regulon was considered as silent. The RAS matrix was transformed into a binary matrix based on the "0/1" scoring according to the threshold value to eliminate technical bias and identify the differences. Finally, the regulon-specific scores (RSS) of different regulons in the cellular subsets were calculated using the R package philentropy (version 0.4.0) (HG, 2018). The first five regulons (highly expressed) in different cell subsets were then filtered and analyzed.

RESULTS

Quality Control

Seurat objects were created for BMSCs, ADMSCs, HuMSCs, and FSMSCs, and quality control parameters were set to retain cells with expression readouts between 200 and 2,000 genes and ≤20%

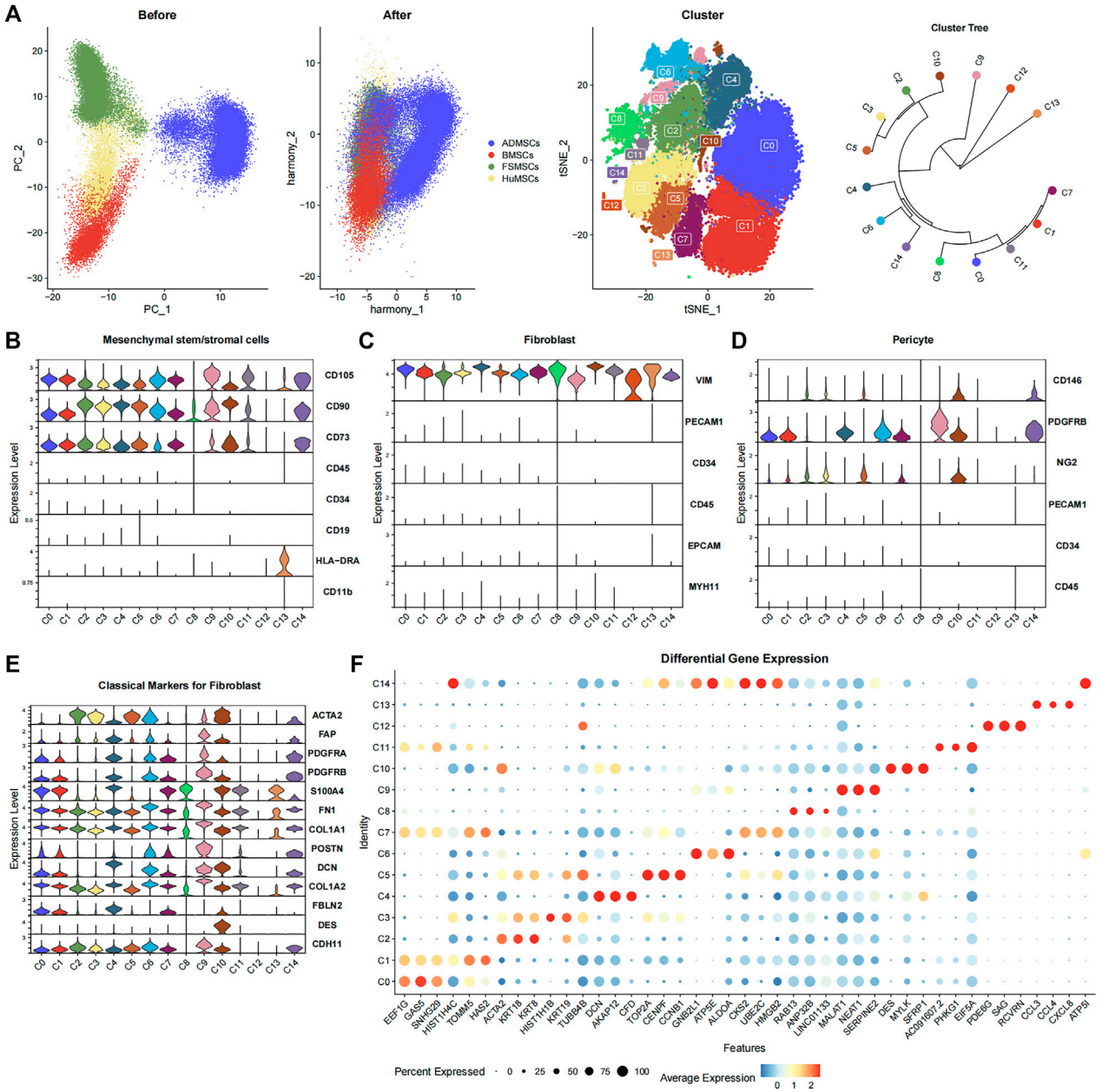

FIGURE 1 | Characterization of the 15 cell subsets derived from ADMSCs, BMSCs, FSMSCs, and HuMSCs using various bioinformatics analysis methods. **(A)** The PCA plots show the distribution of ADMSCs (blue), BMSCs (red), FSMSCs (green), and HuMSCs (yellow), which are derived from different tissues before and after correcting the batch effects. The TSNE plot shows distribution of the 15 cell subsets (C0–C14). The clustering tree shows similarity between the 15 cell subsets. **(B–D)** The violin plot shows the expression status of **(B)** MSC-related markers, **(C)** fibroblast-related markers, and **(D)** pericyte-related markers in the 15 different cell subsets. **(E)** The violin plot shows the expression of classical fibroblast markers in the 15 different cell subsets. **(F)** The bubble plot shows the differential expression of the top three highly expressed genes in each cellular subset among all the 15 cellular subsets. The size of the bubble is inversely proportional to the *p*-value; that is, the larger the bubble, the smaller the *p*-value. The expression level is color coded, with dark red representing high expression and blue representing low expression.

mitochondrial genes per cell. Subsequently, we identified 5,180 high-quality BMSCs (median UMI: 12,340; median genes: 3,058), 29,178 high-quality ADMSCs (median UMI: 40,208; median genes: 5,602), 13,386 high-quality HuMSCs (median UMI: 14,854; median genes: 3,797), and 7,432 high-quality FSMSCs (median UMI: 33,022; median genes: 5,463).

Integration Analysis

The merged data were normalized and standardized to correct the sequencing depth of each cell. We identified the first 50 PCs using PCA based on 2,000 genes with high variance. Then, the 50 PCs were used to correct the batch effect, and the first 50 harmony dimensions were identified. The reduction plot between PCA

dimensionality and harmony dimensionality showed a significant overlap between MSCs derived from the same tissue and partial overlap between MSCs derived from different tissues (**Figure 1A**). This suggested an effective correction of the batch effects from different datasets and resulted in identification of biological differences between the MSCs derived from different tissues. The first 50 harmony dimensions were also used for TSNE plots with a clustering resolution of 0.5, and 15 major cell subsets were identified (**Figure 1A**). The cluster tree was constructed by the BuildClusterTree function of the R package Seurat to analyze the correlations between the 15 cell subsets, and the tree plot was displayed using the R package ggtree (version 2.4.1) (Yu, 2020) (**Figure 1A**).

Biological Annotations

The 15 cell subsets were annotated based on the surface marker expression profiles of MSCs (*CD105*$^+$, *CD90*$^+$, *CD73*$^+$, *CD45*$^-$, *CD34*$^-$, *CD19*$^-$, *HLA-DRA*$^-$, and *CD11b*$^-$) (Dominici et al., 2006) (**Figure 1B**). The expression of *CD105*, *CD90*, and *CD73* was significantly high, and the expression of *CD45*, *CD34*, *CD19*, *HLA-DRA*, and *CD11b* was significantly low or absent in 12 cell subsets, namely, C0–C7, C9–C11, and C14. This suggested that these 12 cell subsets were the MSCs. C8 and C13 subsets showed lower expression of *CD90* and *CD105*, respectively. Therefore, C8 and C13 did not show the classical MSC phenotype. The 12 cell subsets expressed *VIM* but did not express *PECAM1*, *CD34*, *CD45*, *EPCAM*, and *MYH11* (**Figure 1C**). This suggested that all the 12 cell subsets also showed the fibroblast phenotype. Furthermore, we observed the expression of fibroblast markers such as *ACTA2* (Muhl et al., 2020), *FAP* (Sunami et al., 2020), *PDGFRA* (Muhl et al., 2020), *PDGFRB* (Muhl et al., 2020), *S100A4* (Meng et al., 2020), *FN1* (Meng et al., 2020), *COL1A1* (Meng et al., 2020), *POSTN* (Meng et al., 2020), *DCN* (Guerrero-Juarez et al., 2019), *COL1A2* (Meng et al., 2020), *FBLN2* (Guerrero-Juarez et al., 2019), *DES* (Sunami et al., 2020), and *CDH11* (Tarbit et al., 2019) in one or more cellular subsets (**Figure 1E**). This demonstrated that the cell subsets could not be distinguished using a single classical fibroblast marker. The pericyte surface markers such as *CD146*$^+$, *PDGFRB*$^+$, *PECAM1*$^-$, *CD34*$^-$, and *CD45*$^-$ (Crisan et al., 2008; Crisan et al., 2012) were expressed in some cell subsets. C10 and C14 highly expressed *CD146* and *PDGFRB* but did not express *PECAM1*, *CD34*, and *CD45*, suggesting that both of them met the pericyte phenotype (**Figure 1D**). This suggested that some cell subsets represented overlapping pericyte and MSC phenotypes.

Biological Function Analysis

The Findallmarker function of R package Seurat filtered out 3,275 statistically significant genes using the adjusted p-value < 0.05 and the absolute value of Log2FC ≥ 1 (**Supplementary Table S1**). This included 80, 65, 91, 127, 146, 139, 215, 39, 35, 554, 80, 44, 931, 502, and 227 genes in the C0, C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, and C14 subsets, respectively. The bubble plot shows the top three highly expressed differential genes from each cell subset (**Figure 1F**).

Then, the hallmark gene set enrichment analysis was performed using the R package msigdbr and singleseqgset. The enrichment scores and p-values of all hallmark gene set were compared between the 15 cell subsets. We identified 305 differentially expressed gene sets using an adjusted p-value of less than 0.05 as the cutoff (**Figure 2**; **Supplementary Table S2**). The C0, C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, and C14 cell subsets showed significant differences in the expression of genes belonging to 41, 33, 8, 41, 29, 13, 18, 3, 16, 15, 16, 10, 29, 16, and 17 gene sets, respectively. The biological functions of different cell subsets are shown in **Figure 2**. The gene set analysis showed that the C3, C4, C5, C7, C10, and C14 cell subsets demonstrated both MSC and fibroblast phenotypes. Proliferation-related gene sets such as "E2F Target genes" and "G2M Checkpoint genes" were enriched in the C3, C5, C7, and C14 subsets. The metabolism-related gene sets belonging to "Oxidative Phosphorylation," "Bile Acid Metabolism," "Reactive Oxygen Species Pathway," "Fatty Acid Metabolism," "Protein Secretion," "Hypoxia," "Glycolysis," and "Heme Metabolism" were downregulated in the C0, C2, C1, and C11 subsets. The "Protein Secretion" gene set was upregulated in the C3 and C5 subsets. Immune-related gene sets belonging to "Interferon Alpha Response" and "Interferon Gamma Response" were enriched in the C4, C10, and C13 subsets. The C13 subset demonstrated only fibroblast phenotype with a high expression of HLA-DRA and absence of *CD80* and *CD86* expression (**Supplementary Figure S1**).

The differentiation status of the cell subsets was evaluated using the R package CytoTRACE. The batch effect correction was performed using the iCytoTRACE function. The CytoTRACE scores of different cell subsets are shown in the TSNE plots (**Figures 3A,B**) and box plots (**Figure 3C**). The differentiation status was considered lower when the CytoTRACE score was closer to 1.0. C8, C12, and C13 cell subsets showed CytoTRACE scores closer to 0.0 and were considered to belong to a higher differentiation state. C8, C12, and C13 demonstrated only the fibroblast phenotype and did not show characteristics of the MSC phenotype. This suggested that MSC characteristics are lost as they undergo differentiation. Furthermore, the differentiation status of the C14 and C10 cell subsets was not low despite showing the pericyte phenotype.

Gene Regulatory Networks Analysis

We identified 384 regulons by integrating the single-cell transcriptomic data using the pySCENIC python package. Each regulon contained a transcription factor, a significant motif, and the corresponding target genes (**Supplementary Table S3**). Regulon activity scores (RASs) of different regulons in each cell were evaluated. The RAS threshold of each regulon was also calculated separately. The regulon was considered as activated when the RAS value of the regulon was greater than its RAS threshold. Otherwise, the regulon was considered as silent. The RAS matrix was transformed into a binary matrix to highlight the differences between different cell subsets and eliminate the technical bias based on the threshold value. Furthermore, the regulon specificity score (RSS) of each regulon was calculated in different subsets using the

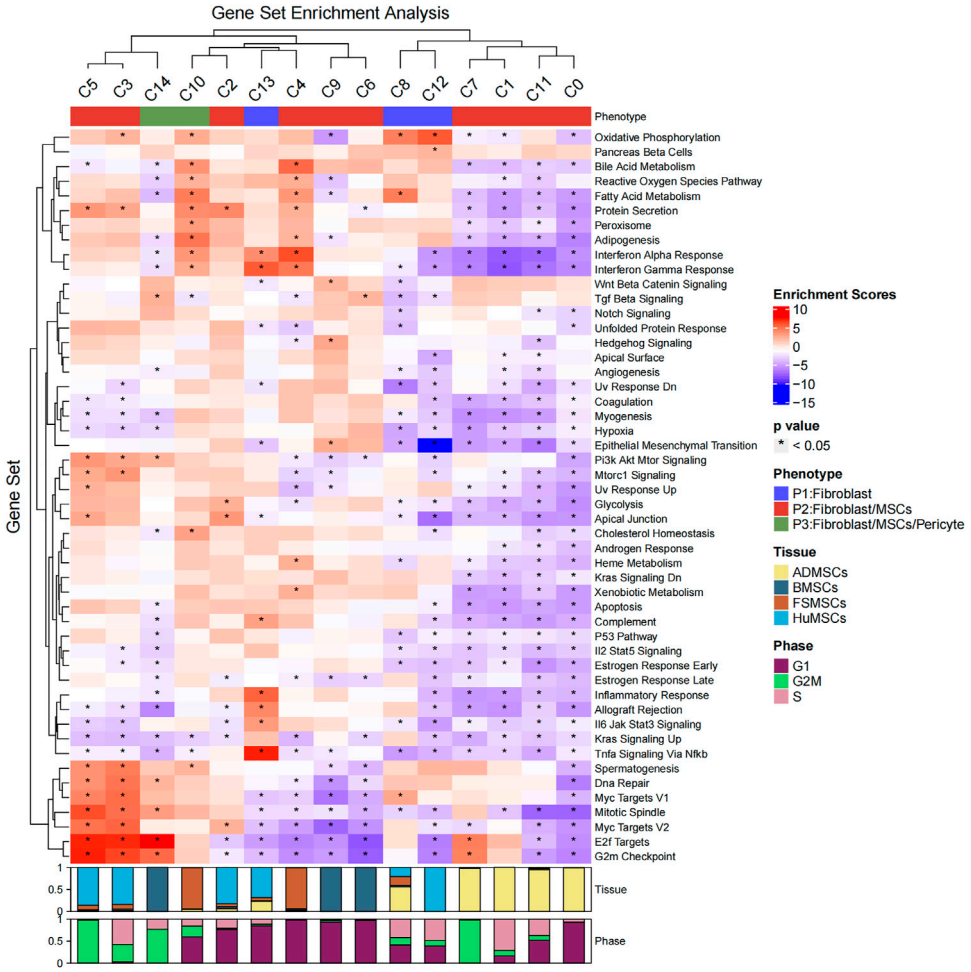

FIGURE 2 | Hallmark gene set enrichment analysis of the 15 cell subsets. The heatmap plot shows the enrichment scores and *p*-values of different hallmark gene sets among the 15 different cellular subsets. The color of the grids denotes the enrichment scores of the gene set, with red representing high enrichment and blue representing low enrichment. The bar graphs (top) represent different molecular phenotypes (fibroblasts, MSCs, and/or pericytes) represented by the 15 different cell subsets and is shown with different colors. The asterisk indicates that the *p*-value was less than 0.05. The bar charts (below) represent the cell cycle phases of different proportions of cells in the 15 cell subsets.

philentropy R package (**Figure 4A**). The heatmap shows the top five regulons in each cell subset based on the regulon specificity scores (**Figure 4B**). The C3 subset showed high expression of DNA replication regulators such as *SMC3* (Gregson et al., 2001) and *E2F2* (Lukas et al., 1996; Delgado et al., 2011) (**Figure 4C**). The cells in the C3 subtype were mainly derived from the umbilical cord tissue. The C4 subset showed high expression of *IRF2* (**Figure 4C**), which exerts anti-inflammatory effects through inhibition of pro-inflammatory factors that are induced by lipopolysaccharide (Cui et al., 2018). The cells in the C4 subset were mainly derived from the foreskin tissue.

DISCUSSION

Traditional bulk transcriptome sequencing detects differences in gene expression between groups of cells, whereas single-cell transcriptome sequencing detects differences in gene expression between individual cells (Yan et al., 2021). Therefore, single-cell transcriptome sequencing technique demonstrates the composition of cells with differential functions in heterogeneous cell groups such as tumor cells, MSCs, and fibroblasts. In this study, we comprehensively analyzed the integrated single-cell transcriptome data of MSCs derived from four different tissues, namely, BMSCs, ADMSCs, HuMSCs, and FSMSCs and identified 15 cell subsets. Then, we performed cluster annotation of these 15 cell subsets based on the surface expression characteristics of MSCs (*CD105*$^+$, *CD90*$^+$, *CD73*$^+$, *CD45*$^-$, *CD34*$^-$, *CD19*$^-$, *HLA-DRA*$^-$, and *CD11b*$^-$), fibroblasts (*VIM*$^+$, *PECAM1*$^-$, *CD34*$^-$, *CD45*$^-$, *EPCAM*$^-$, and *MYH11*$^-$), and pericytes (*CD146*$^+$, *PDGFRB*$^+$, *PECAM1*$^-$, *CD34*$^-$, and *CD45*$^-$).

We identified MSCs based on the ISCT criteria, which is commonly used in most studies (Dominici et al., 2006). However,

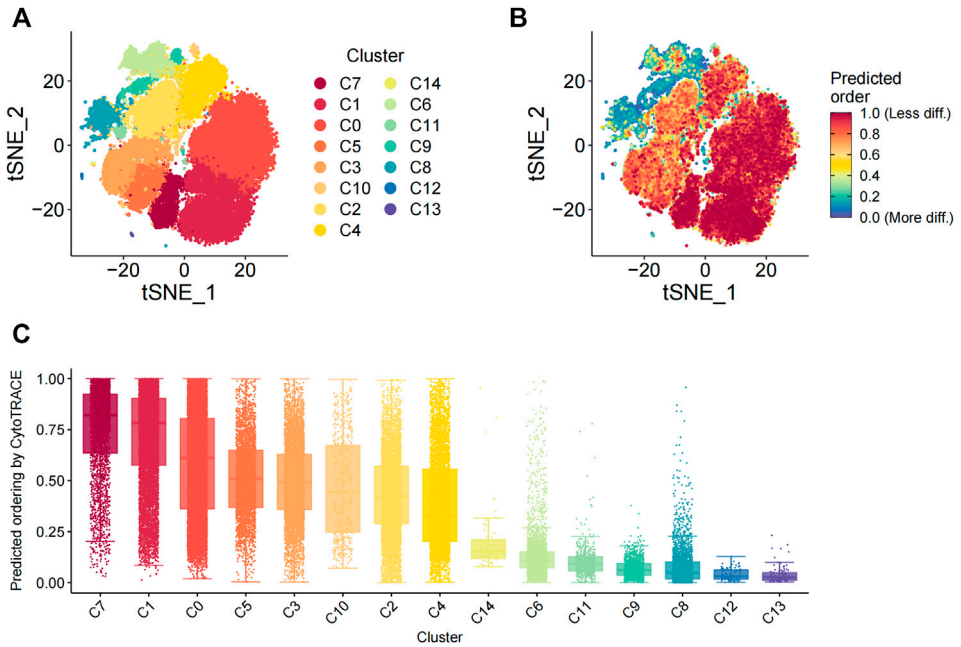

FIGURE 3 | Evaluation of the differentiation status of cells in the 15 cellular subsets. **(A)** TSNE_plot shows the distribution of the 15 different cell subsets with different color codes in the low-dimensional space. **(B)** TSNE plot shows the distribution of the 15 different cell subsets based on the degree of differentiation. The degree of differentiation is denoted by the color code, with red representing low differentiation status and blue denoting high differentiation status. **(C)** The box plot shows the differentiation status of all cells in the 15 different cell subsets. The cells are ordered from low to high degrees of differentiation.

CD34 expression is observed sometimes in the early passages of freshly isolated ADMSCs (Baer et al., 2013). This suggested that the CD34 expression was highly variable between the donors. ADMSCs from some donors were $CD34^+$, whereas those from other donors were $CD34^-$. Moreover, CD34 expression decreased with the increasing number of passages of ADMSC cultures *in vitro*. Another single-cell RNA-sequencing study showed that 0.008% of cultured human ADMSCs were $CD34^+$ (Liu et al., 2019). Furthermore, one review hypothesized that *in vitro* cultured MSCs were $CD34^-$, whereas tissue-resident MSCs were $CD34^+$ (Lin et al., 2012). The minimal ISCT criteria suggest that CD34 positivity in the MSCs must be ≤2% based on the flow cytometry analysis. This suggests that the proportion of $CD34^+$ cells in the *in vitro* cultured MSCs is low. In our study, MSCs of all the datasets were cultured *in vitro*. Therefore, CD34 was used as a negative marker for determining the MSC phenotype.

Standardized identification criteria do not exist for fibroblasts despite being isolated, cultured, and characterized prior to the MSCs. Few reports suggest that fibroblasts can be identified based on vimentin (VIM) expression (Chang et al., 2002; Tarbit et al., 2019). VIM is the main structural component of the intermediate filaments in cells and is responsible for biological functions such as cell contraction, migration, and proliferation. However, *VIM* is also expressed in the endothelial cells, epithelial cells, and immune cells. Therefore, markers for endothelial cells (*PECAM1*), hematopoietic cells (*CD34*), immune cells (*CD45*), epithelial cells (*EPCAM*), and smooth muscle cells (*MYH11*) need to be tested alongside VIM to classify fibroblasts. A report also showed that fibroblasts express HLA-DR (Olsson et al., 1994). However, a comparative study showed that unstimulated fibroblasts and MSCs were both negative for HLA-DR (Denu et al., 2016). This suggested that HLA-DR expression was heterogeneous in the fibroblasts and was not necessary for identifying fibroblasts.

In our study, cluster annotation demonstrated that all the 15 cell subsets expressed fibroblast-related markers. However, only 12 cell subsets (C0, C1, C2, C3, C4, C5, C6, C7, C9, C10, C11, and C14) expressed MSC-specific markers. The remaining three cell subsets, namely, C8, C12, and C13, exclusively expressed fibroblast-specific markers. This suggested that MSCs may be derived from the fibroblasts or may represent a subclass of fibroblasts. However, MSCs are not equivalent to the fibroblasts because our single-cell transcriptome analysis demonstrates subtle differences in gene expression between the MSCs and the fibroblasts. Furthermore, the expression of classic fibroblast markers (ACTA2, FAP, PDGFRA, PDGFRB, S100A4, FN1, COL1A1, POSTN, DCN, COL1A2, FBLN2, COL1A2, DES, and CDH11) was not uniform among the 15 cell subsets. Hence, a single classic fibroblast marker was not sufficient to sort out all the cell subsets that show the fibroblast phenotype. Therefore, multiple classic fibroblast-specific markers are required to sort out all the fibroblast subsets because individual markers may not be expressed in some fibroblast subsets.

The C10 and C14 cell subsets with the MSC phenotype also demonstrated the pericyte phenotype. The expression of *NG2* was positive in C10 and negative in C14. This was consistent with a previous study that reported NG2 expression in only few pericyte

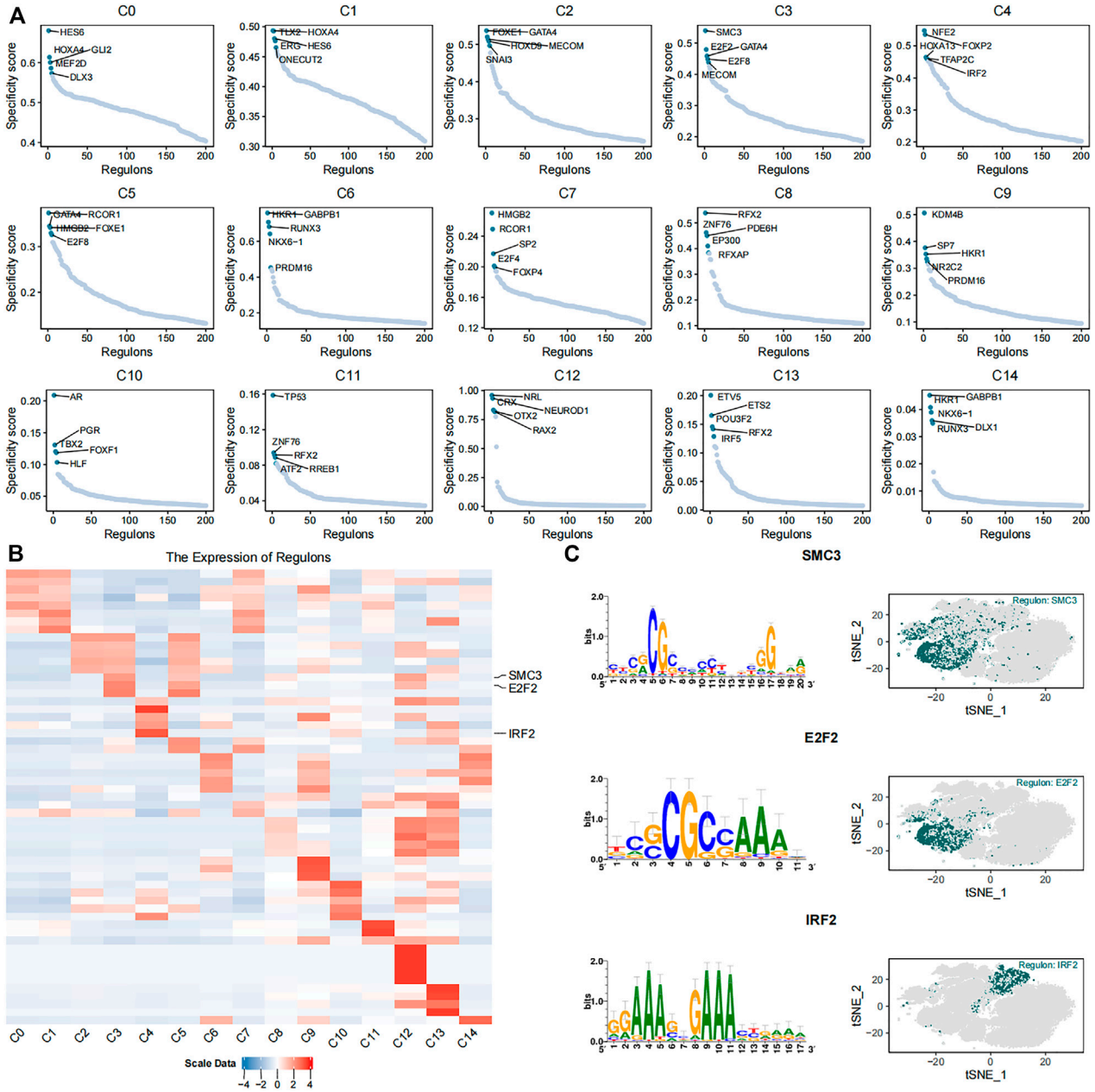

FIGURE 4 | Identification of key gene regulatory networks in the 15 cell subsets. **(A)** The scatter plots show expression of the top five highly expressed regulons in each of the 15 cell subsets. **(B)** The heatmap plot shows expression levels of the top five highly expressed regulons in the 15 different cell subsets. *SMC3*, *E2F2*, and *IRF2* regulons are labeled. **(C)** The plots show the putative binding motifs of *SMC3*, *E2F2*, and *IRF2*, as well as their corresponding expression levels in different cells among the 15 cell subsets.

subsets (Crisan et al., 2012). *NG2* is mainly distributed on the surface of vascular pericytes, MSCs, hematopoietic stem cells, and other pluripotent stem cells, which participates in angiogenesis process and regulates stem cell differentiation, stemness maintenance, and self-renewal. Our study demonstrated some overlap between the pericytes and MSCs.

We also analyzed the differences in the status of differentiation between the 15 cell subsets. C8, C12, and C13 subsets that demonstrated only the fibroblast phenotype were more differentiated than the other 12 cell subsets, which demonstrated both fibroblast and MSC phenotypes. This implied that MSCs represented a more primitive cellular stage that gradually disappeared as the cells underwent differentiation. Furthermore, C10 and C14 cell subsets with the pericyte phenotype did not represent the least differentiated cells. This finding was not consistent with a previous study, which suggested that MSCs were derived from the pericytes (Crisan et al., 2008). This suggested that MSCs may be derived from multiple lineages.

We then analyzed the differences in the biological functions between the 15 cell subsets. Our results showed that although the C3, C4, C5, C7, C10, and C14 subsets demonstrated both MSC and fibroblast phenotypes, the proliferation-related gene sets such as "E2F Targets" and "G2M Checkpoint" were enriched only in the C3, C5, C7, and C14 subsets. This suggested that cells in the C3, C5, C7, and C14 subsets were proliferating and in the G2M or S phase of the cell cycle. The C5 and C3, C14, and C7 subsets were mainly derived from the umbilical cord, bone marrow, and adipose tissues, respectively. Our data suggested that the proliferation activity of the HuMSCs, BMSCs, and ADMSCs was significantly higher than the FSMSCs. The C2 subset was mainly derived from the umbilical cord; C9 and C6 subsets were mainly derived from the bone marrow; the C10 subset was mainly derived from the adipose tissue. However, proliferation-related "E2F Target" and "G2M Checkpoint" gene sets were not enriched in these four cell subsets. This suggested that MSCs and fibroblasts consisted of heterogeneous cell populations with different biological functions. These findings were consistent with previous reports (Dunn et al., 2021; Plikus et al., 2021; Wruck et al., 2021; Zou et al., 2021). In the C0, C1, C2, and C11 subsets, metabolism-related genes belonging to "Oxidative Phosphorylation," "Bile Acid Metabolism," "Reactive Oxygen Species Pathway," "Fatty Acid Metabolism," "Protein Secretion," "Hypoxia," "Glycolysis," and "Heme Metabolism" gene sets were downregulated. These four cell subsets were mainly derived from the adipose tissues. This suggested that ADMSCs were metabolically inactive. The "Protein Secretion" gene set was upregulated in the C3 and C5 subsets, which were mainly derived from the umbilical cord. This suggested that the exocrine functions were activated in the HuMSCs. The immune-related "Interferon Alpha Response" and "Interferon Gamma Response" gene sets were enriched in the C4 and C10 subsets, which are derived from the foreskin tissues. The interferon alpha and interferon gamma response genes are essential components of the immune response to viral infections. This suggested that the foreskin-derived MSCs may play an important role in response to inflammation (González-Navajas et al., 2012). Furthermore, foreskin is a source of immunotherapeutic MSCs (Najar et al., 2016). MSCs derived from the foreskin tissue significantly promote the increase of the proportion of Th17 cells (Najar et al., 2021). Moreover, in our unpublished manuscript (**Supplementary Material**), we demonstrated through *in vitro* experiments that the immunomodulatory capacity of the FSMSCs was significantly higher than the HuMSCs. This suggested the potential clinical significance of FSMSCs in treating diseases related to immune regulation. The C13 cell subset that demonstrated only the fibroblast phenotype was enriched with both "Interferon Alpha Response" and "Interferon Gamma Response" gene sets. Furthermore, the C13 subset showed high expression of *HLA-DRA*, thereby indicating stronger immunogenicity. However, the expression of co-stimulatory factors, *CD80* and *CD86*, was not detected in the C13 subtype. This suggested that the *HLA-DRA*$^+$ phenotype of the fibroblasts does not directly correlate with immunogenicity. *HLA-DRA* seemed to indicate that C13 might possess stronger immunogenicity.

The plasticity of MSCs and fibroblasts may be a likely source of heterogeneity (Lindsay and Barnett., 2021; Plikus et al., 2021; Rauch and Mandrup, 2021). The gene regulatory networks play a vital role in maintaining the plasticity of the MSCs and fibroblasts. The C3 subset showed high expression of the cell-cycle-related regulons, *SMC3* and *E2F2*, whereas anti-inflammatory-related regulon *IRF2* was highly expressed in the C4 cell subset. This suggested that cells in the C3 subset were proliferative, whereas cells in the C4 subset may play an essential role in the inflammatory responses. We speculate that the differences in the transcriptional regulatory networks may be responsible for the diversity of biological functions observed in distinct cell subsets derived from the same source or within cells demonstrating either MSC or fibroblast phenotypes.

Both MSCs and fibroblasts are multipotent stromal cell populations that can be induced to differentiate into various kinds of cells under different microenvironments or culture conditions. Besides, MSCs and fibroblasts demonstrate different gene expression patterns under subtle changes in oxygen concentrations and culture conditions. Therefore, it is challenging to distinguish MSCs from fibroblasts. However, our single-cell transcriptomic analysis demonstrates distinct differences between different subsets of MSCs and fibroblasts despite the intrinsic heterogeneity of gene expression, biological functions, and transcriptional regulation.

CONCLUSION

Our study showed significant differences and similarities between 15 different cell subsets derived from HuMSCs, BMSCs, FSMSCs, and ADMSCs based on comprehensive analysis of integrated single-cell transcriptome data. Our study also demonstrated that several molecular markers were shared by distinct cell subsets and may be linked to their biological functions. Therefore, the single-cell transcriptome sequencing technique shows great promise in detecting the heterogeneity between cellular populations, classification of different cellular subsets, and elucidating the differences in biological functions between different subsets in a heterogenous population of cells. However, the single-cell technology is not perfect and needs further development. For example, it is limited for detecting low-expression genes that may have significant biological relevance. Moreover, it cannot postulate interactions between different cell subsets. Nevertheless, single-cell technology has enabled significant progress in cellular research and shows significant potential in broader clinical applications.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. The data can be found here: BMSCs (GEO database: GSE115149, GSE162692); ADMSCs (GEO database: SRP148833); HuMSCs and FSMSCs (Mendeley database: https://data.mendeley.com/datasets/f4b2ykfv56/1. The treatment of two samples is detailed in an unpublished article. This unpublished article is another relevant work and submitted to another journal. Meanwhile, the copy of the unpublished article is available in **Supplementary Material**).

AUTHOR CONTRIBUTIONS

CF, ML, and LX performed the main bioinformatics analysis; LH, SL, SC, XS, and YW were responsible for project administration and language editing; HW, MW, YL, YW, and HG wrote the original draft of the manuscript; and HY, YL, TW, and LM guided, reviewed, and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.798331/full#supplementary-material

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Senescence State in Mesenchymal Stem Cells at Low Passages: Implications in Clinical Use

*Raquel M. Alves-Paiva[1], Sabrina do Nascimento[2], Denise De Oliveira[1], Larissa Coa[1], Kelen Alvarez[1], Nelson Hamerschlak[1], Oswaldo Keith Okamoto[1,3], Luciana C. Marti[2], Andrea T. Kondo[1], Jose Mauro Kutner[1], Maria Augusta Tezelli Bortolini[4], Rodrigo Castro[4] and Juliana A. Preto de Godoy[1]**

[1]Department of Hemotherapy and Cellular Therapy, Hospital Israelita Albert Einstein, São Paulo, Brazil, [2]Experimental Research Laboratory, Hospital Israelita Albert Einstein, São Paulo, Brazil, [3]Human Genome and Stem Cell Research Center, Department of Genetics and Evolutionary Biology, Biosciences Institute, University of São Paulo (USP), Sao Paulo, Brazil, [4]Paulista School of Medicine, Federal University of São Paulo, São Paulo, Brazil

Mesenchymal stem cells (MSCs) are multipotent cells found in various tissues and are easily cultivated. For use in clinical protocols, MSCs must be expanded to obtain an adequate number of cells, but a senescence state may be instituted after some passages, reducing their replicative potential. In this study, we report a case where MSC derived from an elderly donor acquired a senescence state after three passages. The bone marrow was aspirated from a female patient submitted to a cell therapy for the incontinency urinary protocol; MSCs were cultivated with DMEM low glucose, supplemented with 10% autologous serum (AS) plus 1% L-glutamine and 1% antibiotic/antimycotic. Senescence analysis was performed by β-galactosidase staining after 24 and 48 h. Controls were established using BM-MSC from healthy donors and used for senescence and gene expression assays. Gene expression was performed using RT-PCR for pluripotency genes, such as *SOX2*, *POU5F1*, *NANOG*, and *KLF4.* MSC telomere length was measured by the Southern blotting technique, and MSCs were also analyzed for their capacity to differentiate into adipocytes, chondrocytes, and osteocytes. The patient's MSC expansion using AS displayed an early senescence state. In order to understand the role of AS in senescence, MSCs were then submitted to two different culture conditions: 1) with AS or 2) with FBS supplementation. Senescence state was assessed after 24 h, and no statistical differences were observed between the two conditions. However, patients' cells cultured with AS displayed a higher number of senescence cells than FBS medium after 48 h ($p = 0.0018$). Gene expression was performed in both conditions; increased expression of *KLF4* was observed in the patient's cells in comparison to healthy controls ($p = 0.0016$); reduced gene expression was observed for *NANOG* ($p = 0.0016$) and *SOX2* ($p = 0.0014$) genes. Telomere length of the patient's cells was shorter than that of a healthy donor and that of a patient of similar age. Osteocyte differentiation seemed to be more diffuse than that of the healthy donor and that of the patient of similar age. MSCs could enter a senescence state during expansion in early passages and can impact MSC quality for clinical applications, reducing their efficacy when administered.

Keywords: mesenchymal stem cells, senescence, telomere, differentiation, clinical use

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Gerlinde R. Van De Walle,
Cornell University, United States

****Correspondence:***
Juliana A. Preto de Godoy
juliana.godoy@einstein.br

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1 INTRODUCTION

Mesenchymal stem cells (MSCs) are non-hematopoietic cells capable of self-renewal and differentiation into cells of mesodermal origin. They are easily isolated from several tissues/organs and can be largely expanded under culture conditions. According to the International Society for Cell and Gene Therapy (ISCT), three minimal criteria were proposed for defining MSCs: 1) adherence to plastic; 2) surface antigen expression where MSC must express CD90, CD73, and CD105 and no expression for CD45, CD34, CD14 or CD11b, CD19 or CD79α, and HLA-DR; 3) multipotent differentiation potential where MSCs should be able to differentiate *in vitro* into osteoblasts, adipocytes, and chondroblasts (Dominici et al., 2006).

MSCs are being widely used as a source of adult stem cells for therapy of several diseases such as graft versus host disease (Le Blanc et al., 2004), Crohn's disease (García-Olmo et al., 2005), multiple sclerosis (Connick et al., 2012), and COVID-19 (Shi et al., 2021). Most clinical protocols use high cell doses per patient which has an impact on MSC culture due to the requirement of a high replicative rate and numerous cell passages to achieve the desired dose.

MSCs have a limited lifespan *in vitro* and, after some cell divisions, they enter a state of senescence that is characterized by irregular cell morphology and a decrease in proliferation rate (Wagner et al., 2008). However, a senescence state could also be achieved in initial passages; senescence in dividing cells after a period of normal growth is followed by a proliferation cease. This phenomenon is accompanied by significant cellular changes such as the typical Hayflick phenomenon, a decrease in proliferation, telomere shortening, and impairment of functional properties (Bonab et al., 2006). The typical Hayflick phenomenon is a concept that helps explain some events that lead to aging of normal cells (Shay and Wright, 2000).

This study discusses a case where bone marrow-derived MSCs from a patient enrolled in a clinical protocol decreased their proliferation rate in an initial passage and the impact on the quality and number of cells intended to be delivered to the patient.

2 MATERIALS AND METHODS

2.1 Patients

MSCs were isolated from the bone marrow from the iliac crest of three patients (56, 52, and 60 years old) initially enrolled in a cell therapy protocol approved by the National Ethics Research Commission (Comissão Nacional de Ética em Pesquisa/CAAE: 18150613.7.3001.0071). MSCs from a healthy donor (34 years old) were used in differentiation, senescence assay, and gene expression; the telomere length was performed using cells from a patient that presented senescence, from the healthy donor, and from two other participants in the cell therapy protocol (52 and 60 years old). No data from this cell therapy trial were presented in this article; we presented a case report related to a senescence state that could impact the clinical use of these cells. Cells from four different patients were used for performing assays and are summarized in **Table 1**, and the experiments will be identified according to this table from now on.

2.2 Mesenchymal Stem Cell Culture

Bone marrow mononuclear cells were separated by Ficoll-Paque Plus gradient density (GE Healthcare) and MSCs were cultivated in DMEM low glucose (DMEM-LG) supplemented with 1% antibiotic–antimycotic solution (10,000 units/mL of penicillin, 10,000 μg/ml of streptomycin, and 25 μg/ml of amphotericin B), 1% L-glutamine 200 mM, and 10% fetal bovine serum (FBS) or 10% autologous serum (AS) in T75 flasks at 37°C in a humidified 5% CO_2 atmosphere. Non-adherent cells were discarded after 48 h; the adherent layer was washed twice with DMEM-LG and maintained in culture until the 3rd passage. The medium was changed every other day, and cell harvesting was performed using TrypLE™ Express (Gibco) for 5 min at 37°C and inactivated by complete medium; centrifugation was performed, and cells were counted. Cell viability was assessed by trypan blue staining (Sigma-Aldrich, St. Louis, MO, United States).

2.3 Immunophenotyping

As the release criteria, MSCs must exhibit specific cell surface expression profile: positive for CD105 (FITC or PE mouse anti-human CD105—endoglin, BD Pharmingen, clone 266), CD73 (PE mouse anti-human CD73, BD Pharmingen, clone AD2), and CD90 (PE mouse anti-human CD90, BD Pharmingen, clone 5E10), and negative for hematopoietic markers CD14 (APC mouse anti-human CD14, BD Pharmingen, clone M5E2), CD34 (PE mouse anti-human CD34, BD Pharmingen, clone 4H11), CD45 (FITC mouse anti-human CD45, BD Pharmingen, clone HI30), CD19 (PE-Cy7 mouse anti-human CD19, BD Pharmingen, SJ25C1), and HLA-DR (PerCP-Cy5.5 mouse anti-human HLA-DR, BD Pharmingen, clone LN3) surface molecule (Dominici et al., 2006). Cells in passage 3 were resuspended in a staining solution (PBS supplemented with 1% FBS and 0.05% azide). Staining was performed for 30 min at room temperature in the dark. The data were acquired using a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA) and analyzed by Kaluza software (Beckman-Coulter). At least 10,000 events were acquired for each sample.

2.4 Cell Lineage Differentiation

After the establishment of MSC cultures on the 3rd passage, the cells were differentiated into adipocytes, osteoblasts, and chondrocytes.

2.4.1 Adipocyte Differentiation

MSCs were differentiated into adipocyte lineage, for that cells at P3 were plated in 12-well culture plates (Corning, St. Louis, MO, United States) in triplicates, one as a negative control, at a density of 4 × 104 cells/well for 48 h before changing to a specific medium for adipogenic induction (StemPro® Adipogenesis Differentiation Kit). The medium was changed every three days, and the negative control was kept in complete DMEM-LG. After 21 days, the cells were fixed in 4% paraformaldehyde, washed with PBS, and stained. Intracellular lipid granules were visualized after

TABLE 1 | Patients' cells used in different assays in the study.

Patient	Age (years)	Assays	Observations
#1	56	Differentiation, doubling time, cytometry, Southern blotting, beta-galactosidase, and gene expression	Senescent
#2	60	Differentiation, doubling time, cytometry, and Southern blotting	No senescent
#3	34	Differentiation, doubling time, cytometry, Southern blotting, beta-galactosidase, and gene expression	No senescent
#4	52	Southern blotting	No senescent

staining with 0.3% Oil Red O stain (Fisher Scientific, New Hampshire, United States). The plates were analyzed using an inverted microscope (EVOS M5000, Invitrogen).

2.4.2 Osteocyte Differentiation

MSCs were differentiated into osteogenic lineages, for which cells (P3) were seeded in triplicate, one as a negative control, onto 12-well plates at a density of 2 × 104 cells/well. After 48 h, the medium was switched to an inducing medium (StemPro Osteogenesis Differentiation Kit) or maintained in regular growth medium for a negative control sample. After 21 days, cells were fixed in 4% paraformaldehyde and Ca^{2+} deposits stained with 1% Alizarin Red S (Acros Organics, New Jersey, United States). The plates were analyzed using an inverted microscope (EVOS M5000, Invitrogen).

2.4.3 Chondrocyte Differentiation

MSC cells were differentiated into chondrogenic lineage; for that, cells (P3) were seeded in triplicate, one well as a negative control, onto 12-well plates at a cell density of 3,75 × 106 cells/mL to generate a micromass seeding of 5-μL droplets of cell solution in the center of each well. After 2 h, the medium was switched to an inducing medium (StemPro Condrogenesis Differentiation Kit) or maintained in regular growth medium for a negative control sample. After 21 days, cells were fixed in 4% paraformaldehyde and proteoglycan deposits stained with Alcian blue stain (Fisher Scientific, New Hampshire, United States). The plates were analyzed using an inverted microscope (EVOS M5000, Invitrogen).

2.5 Cumulative Population Doublings

Population doublings were calculated for each MSC culture (#1, #2, and #3) using the following equation:

$$\log 10\left(\frac{N}{No}\right) x\ 3.33,$$

where N is the number of cells at harvest and N_0 is the number of cells plated. Cumulative population doublings were calculated for each passage as the sum of the current and all the previous population doubling values.

2.6 Cellular Senescence Assay

MSCs from patients 1 and 3 were seeded at a density of 2 × 104 cells/well and cultured in complete DMEM-LG for 24 and 48 h; some wells were cultivated with 10% FBS and others with 10% autologous serum. The activity associated with β-galactosidase was analyzed by the Senescence β-Galactosidase Staining Kit (Cell Signaling, Danver, MA, United States) following the manual instructions.

2.7 Gene Expression by qRT-PCR

2.7.1 Total RNA Extraction and cDNA Synthesis

Total RNA was isolated using the RNeasy® Mini kit (Qiagen) following the manufacturers' instructions. The purified total RNA quality was assessed by spectrophotometry using Nanodrop (Thermo Scientific). A total volume of 1 μg of the extracted RNA was reverse transcribed into cDNA using the Quantitec Reverse Transcription Kit (Qiagen).

2.7.2 Quantitative Real-Time Polymerase Chain Reaction

PCR amplifications were performed on the ABI Prism 7500 (Applied Biosystems). The amplification program consisted of initial denaturation at 95°C for 1 min, followed by 40 cycles at 95°C for 10 s, and an annealing and extension phase at 60°C for 30 s. The qRT-PCR assays were performed in technical triplicates, the relative expression levels of each gene were normalized to GAPDH using the 2-ΔΔ Ct method. The gene-specific primers used for amplification using the QuantiFast® SYBR® Green PCR Kit and their sequences are described in **Table 2**.

2.8 Telomere Length

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, United States). Mean telomere length was measured using MSC DNA by Southern blotting [TeloTAGGG Telomere Length Assay (Roche)], as previously described by Gutierrez-Rodrigues (2014). Briefly, 800 ng genomic DNA was digested by FastDigest HinfI and RsaI (Thermo Scientific, Waltham, MA, United States) at 37°C for 2 h. Following digestion, DNA fragments were electrophoresed for 5 h on a 0.8% agarose gel, denatured, neutralized, and transferred to a nylon membrane for Southern blot analysis with proprietary digoxigenin (DIG)-labeled probes and chemiluminescent substrates. We calculated the mean TRF length using the equation Σ(ODi)/Σ(ODi/Li), where ODi represents the chemiluminescent signal and Li is the fragment length at a given position. For the experiment, a reference sample was included. This assay was performed by an external service (University of Sao Paulo—Ribeirao Preto).

2.9 Statistical Analysis

Data were presented as mean ± SEM. The Mann–Whitney non-parametric test or Student's t test with or without Welch's correction was used, when appropriate, for comparison

TABLE 2 | Sequence of gene-specific primers used for qRT-PCR.

Primer	Sequence	Concentration (nM)
GAPDH	Forward: GAGAAGGCTGGGGCTCA Reverse: GTCCTTCCACGATACCAAA	400
SOX2	Forward: TGGGAGGGGTGCAAAAGAGG Reverse: GAGTGTGGATGGGATTGGTG	250
POU5F1	Forward: AGAAGTGGGTGGAGGAAGCTGACAA Reverse: TGGGTTTCGGGCACTGCAGG	800
NANOG	Forward: CCTATGCCTGTGATTTGTGG Reverse: CTGGGACCTTGTCTTCCTTT	250
KLF4	Forward: ACCTACACAAAGAGTTCCCATC Reverse: ATCTGAGCGGGCGAATTT	400

TABLE 3 | Quality control of MSCs.

Assay	Expected	Patient
Endotoxin	<5 EU/mL	<5 EU/mL
Mycoplasma	Negative	Negative
Bacterial culture	Negative	Negative
Karyotyping	46, XX 46, XY	46, XX
MSC differentiation	Adipocyte, chondrocyte, and osteocyte	Adipocyte, chondrocyte, and osteocyte
Immunophenotyping	CD73, CD90, and CD105 (positive) CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR (negative)	CD73, CD90, and CD105 (positive) CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR (negative)

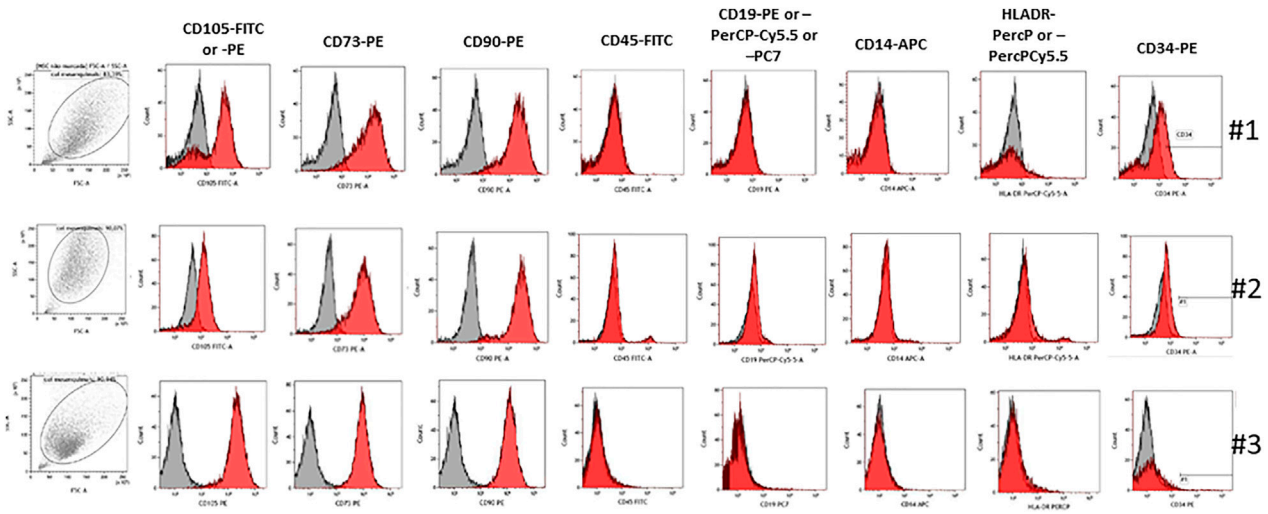

FIGURE 1 | Immunophenotyping of MSCs from the healthy donor and senescence patient. Representative histograms of expression for MSCs derived from bone marrow from the healthy donor and senescence patient. Cells stained positive for CD90, CD105, and CD73, and they were negative for CD14, CD19, CD45, CD34, and HLA-DR.

between groups, using GraphPad Prism 5.00 (GraphPad Software). $p \leq 0.05$ was considered statistically significant.

3 RESULTS

The patient's bone marrow-derived MSCs were grown as described in **Section 2.2**. The patient, female, 60 years old, was included in the clinical trial due to urinary stress incontinence. In the protocol, it was established that MSCs should be cultivated with AS up to passage five in order to achieve 10 million cells to be infused after releasing tests were performed according to local guidelines (**Table 3**). According to the National Health Surveillance Agency (ANVISA) guidelines (RDC 508/2021), some guidelines should be followed in order to

TABLE 4 | Marker expression profile of MSCs.

Marker	#1	#2	#3
CD105	71,34	80,83	99,35
CD73	93,23	94,31	99,56
CD90	96,9	95,87	99,62
CD45	0,18	4,61	0,44
CD19	0,36	1,02	1,16
CD14	0,18	3	0,43
HLA-DR	5,14	4,44	1,39
CD34	15,84	0,61	1,05

release cells for clinical use: microbiological examinations, endotoxin assay, mycoplasma detection by RT-PCR, karyotyping, immunophenotyping, and potency assay (differentiation).

Cells from patients 1, 2, and 3 stained positively for canonical MSC surface markers CD90, CD105, and CD73, whereas they were negative for CD45, CD19, CD14, and HLA-DR (**Figure 1**). However, #1 cells presented a decrease in CD105 expression where there was a negative population for this marker; 71.34% of CD105 expression for #1 patient when compared to #3 patient who presented 99.35%. Patient #2 also presented a decrease in CD105 expression (80.83 *vs.* 99.35%) (**Table 4**). The expression of CD34 was increased in #1 cells (15.84%) in relation to #2 (0.61%) and #3 (1.05%) (**Table 4**).

In this case report, MSC culture supplemented with 10% AS presented a senescence state at passage 3. According to Hayflick and Moorhead (1961), cells enter a state called replicative senescence after some passages, being they stop dividing the main characteristic. As observed in our case, the cumulative population doubling level (CPDL) was calculated according to the following formula: $\log_{10}(N/N_0) \times 3.33$, where N is the number of cells at the harvesting and N_0 is the number of cells plated (**Table 5**). MSCs from the patients #1, #2, and #3 were cultivated according to a standard operation procedure and CPDL calculated. As observed in **Figure 2**, #1 (**Figure 2A**), #2

TABLE 5 | Cell density ($x10^6$) in different passages.

Patient	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5	Observations
#1	2.95	7.68	11.52	1.87	31.68	26 days of culture
#2	5	0.77	1.1	12.75		34 days of culture
#3	14	37	128	257.6		19 days of culture

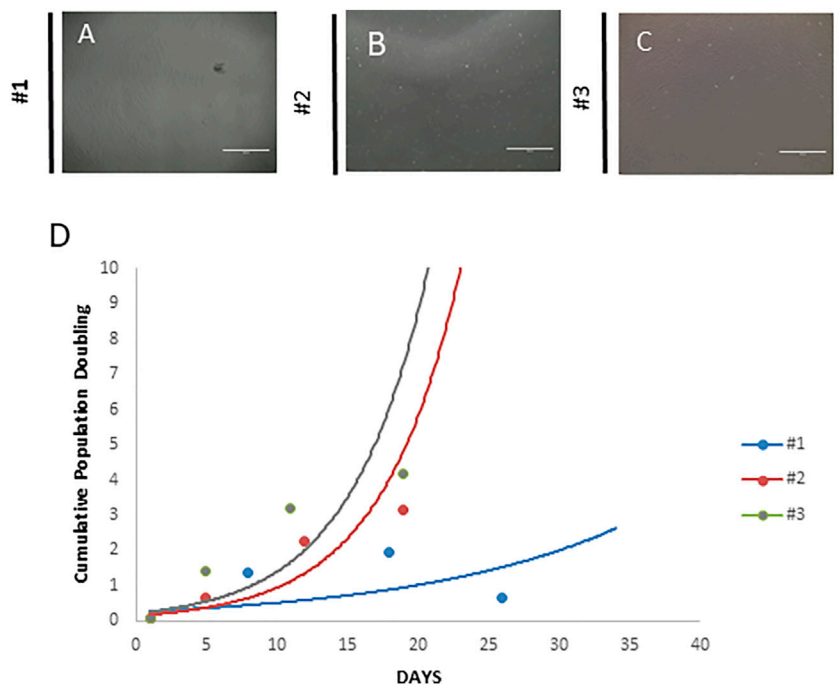

FIGURE 2 | Morphology of the mesenchymal stem cell cultures. **(A)** Mesenchymal stem cells from the patient (senescence). The cells show fibroblast morphology, but in higher passages, this pattern seems to be less evident, suggesting a senescence state. **(B)** Mesenchymal stem cells from the healthy donor. These cells exhibited a typical fibroblastoid morphology, and it is not lost even in high passages. **(C)** Mesenchymal stem cells from the patient (no senescence). These cells exhibited a typical fibroblastoid morphology, and it is not lost even in high passages. **(D)** Cumulative cell population doubling. Both cultures were analyzed using the following formula: $\log_{10}(N/N_0) \times 3.33$, where N is the number of cells at the harvesting and N_0 is the number of cells plated. Scale bar = 1,000 μm.

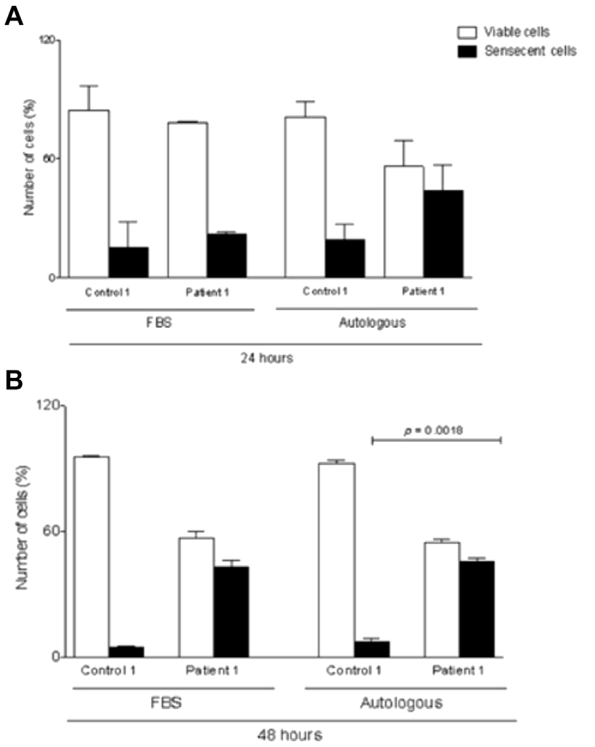

FIGURE 3 | β-galactosidase staining of BM–MSCs from healthy control and patient maintained in culture for 24 and 48 h. **(A)** Staining of senescent cells from hMSC culture supplemented with 10% FBS and/or 10% autologous serum. **(B)** Quantification of senescent cells from hMSC culture supplemented with 10% FBS and/or 10% autologous serum. Data are mean ± SD. Statistical significance is shown using Student's *t*-test analysis ($n = 3$); $^{*}p = 0.0019$.

(**Figure 2B**), and #3 (**Figure 2C**), patients' MSCs showed the same morphology; however, senescence changed the cell proliferation rate as seen on graphic (**Figure 2D**). The CPDL showed a logarithmic rate in #2 and #3 patient's cells and at the same time, patient #1 showed a more linear rate. **Table 5** shows the cell density plated in the different cell cultures and in the different passages.

To find out the explanation about cell culture low rate growth, MSCs from patient #1 were also cultivated with 10% fetal bovine serum (FBS) used as standard. Additionally, MSCs from #3 patient were also cultured with 10% patient's AS and 10% FBS up to passage 3. MSCs were then submitted to senescence assay, using a senescence β-galactosidase staining kit, and data were analyzed after 24 and 48 h. **Figure 3** represents the senescent cells from both patients under 10% FBS and/or 10% AS conditions. The upper panel shows the MSC from #3 patient with a reduced number of senescent cells in comparison to the lower panel (#1 MSCs). The senescent profile was more pronounced under AS supplementation after 48 h in #1 patient's cells in comparison to #3 MSCs ($p = 0.0019$) (**Figure 3**). However, #1 patient's MSCs cultivated with FBS also presented senescence cells, suggesting that this event is more related to a cell factor instead to some soluble molecule.

Surprisingly, #1 MSC differentiation into adipocytes and chondrocytes seemed to be normal (**Figure 4A**), but when analyzing the osteocyte differentiation, the staining seemed to be more diffuse when than that of #2 or #3 patient (**Figure 4A**). In the osteogenic differentiation, there is a non-specific staining and background in #1 patient; this may indicate that the cells are still at the beginning of osteogenic differentiation or the differentiation capacity is decreased. A better resolution image was obtained for the osteogenic differentiation from patients #1 and #3; as observed in **Figure 4B**, the osteogenic differentiation and staining with Alizarin red S showed more localized calcium deposits in patient #3 and also formed structures similar to lines; #1 cells showed calcium deposits more disorganized, without defined structures.

To quantitatively compare the cells from patients #1 and #3, qRT-PCR was used to detect the expression of genes related to pluripotency. The attenuated cell proliferation from patient #1 cells was further evidenced by increased expression of *KLF4* ($p = 0.0016$) and reduced expression of *SOX2* ($p = 0.0014$), *OCT3.4*, and *NANOG* ($p = 0.0016$) (**Figure 5**).

Southern blot was performed to measure telomere length in the patient's MSCs #1, #3, #5, and #7. Patients #1, #2, and #4 were enrolled in the same clinical trial for urinary incontinence. In this assay, the patients were nominated as patient 1 (#1), patient 2 (#3), patient 3 (#4), and healthy donor.

It is well known that MSCs have longer telomeres than leukocytes (Baxter et al., 2004; Fathi et al., 2019). Although we do not have a control curve for MSC, we purposely plotted the MSC data on the leukocyte curve of healthy donors (**Figure 6**). We found that the healthy MSC donor had a longer telomere (9.2 Kb; 90th percentile) than the telomere of patients 1 and 2 (7.1 Kb and 7.4 Kb, respectively; 50th percentile), and patient 3 presented a telomere length comparable to the healthy donor (8.7 Kb; 90th percentile) (**Table 6**). It is worth noting that the patients were close in age and the control was younger. Therefore, we found that MSC from patients 1 and 2 have shorter telomeres than patient 3 and healthy donor.

4 DISCUSSION

Senescence is a complex process that may require different approaches to alleviate or even prevent this profile and improve the clinical application of MSCs. In this study, we presented data from a single patient with uncommon MSC senescence at passage 3. Banfi et al. (2002) showed that bone marrow stromal cells display a decrease in telomere length when in culture, and despite their inability to maintain the telomere length (no telomerase activity was detected), these cells were able to undergo around 40 population doublings. Telomere shortening is observed over a lifetime in MSCs. The average telomere length in early-passage MSCs depends on the age of the donor, and it has been proposed that it ranges from 10 to 11 kb in cells from fetal to 7 kb in cells from postnatal (Guillot et al., 2007). Telomere-dependent MSC senescence has been confirmed; the

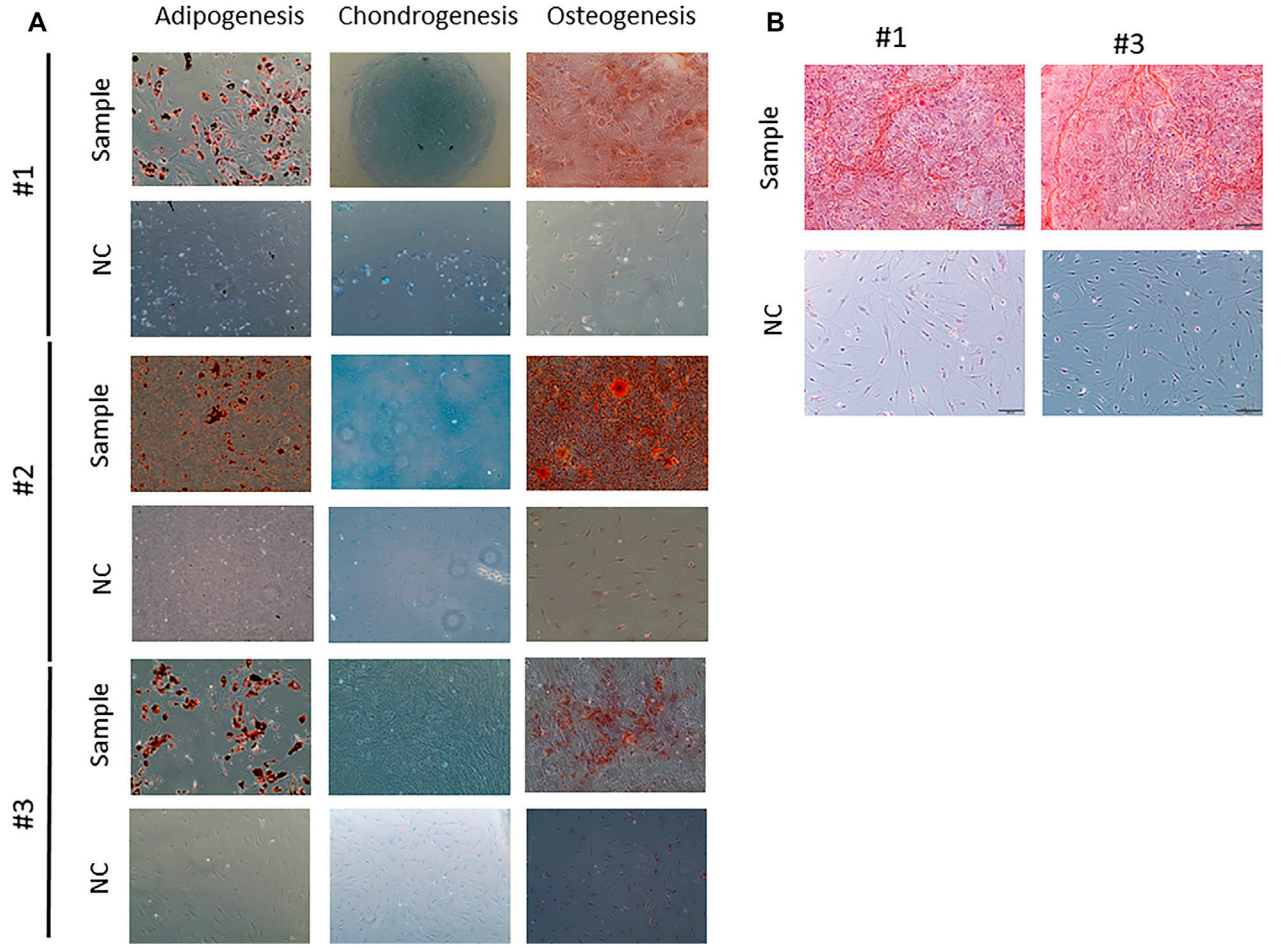

FIGURE 4 | Multilineage Mesenchymal stem cell differentiation. **(A)** Adipogenesis differentiation. In negative control group (without adipogenic differentiation), no lipid droplets were observed. Lipid droplets are given in red. Chondrogenesis differentiation. In the control, no blue reaction was observed there is a slightly positivity showing the presence of proteoglicans. Osteogenesis differentiation. In the negative groups, no calcium deposits were observed. The healthy donor cells showed a strong presence of calcium deposits, and the patient cells exhibited a weak staining. 20-fold magnification. **(B)** Images of osteogenic differentiation from patients #1 and #3.

growth stops when telomeres reach a length of 5.8–10.5 kb (Baxter et al., 2004). Although telomere shortening is a marker of cell senescence, as we have shown here, the length of telomeres varies from donor to donor, making telomere length an inconstant measure of MSC senescence (Li et al., 2017).

The differentiation capacity of stem cells is an important characteristic for the repair and regeneration of tissues/organs; however, the patient's MSC differentiation into adipocytes and chondrocytes seemed to be normal, but the osteocyte differentiation may be reduced. Studies have been reported that the osteogenic activity of MSC is progressively reduced as a function of increasing lifespan (Banfi et al., 2000; Turinetto et al., 2016).

According to Martin et al. (1997), bone marrow stromal cells when cultivated using fibroblast growth factor-2 (FGF-2) were able to maintain their osteogenic potential after the *in vitro* expansion. However, Hagmann et al. (2013) showed that while maintaining its differentiation potential, the use of FGF-2 preselects some MSC phenotypes. Regarding the use of MSC in clinical protocols, the use of growth factors may not be the best option; some MSC subtypes can also account for its efficacy.

CD105 is involved in multiple functions of MSCs such as differentiation, angiogenesis, and regenerative potential (Aslan et al., 2006; Mark et al., 2013). Na et al. (2015) showed that the marker CD105 seems to be more sensitive than the others, CD73 and CD90, to viability-associated events, and CD105 may be used as an indicator of cell death. In addition, it can be an important marker to understand the mechanisms during MSC senescence. Corroborating our study, this marker was evaluated in the senescent MSC-derived microvesicles, and the results showed that senescent MSCs secreted microvesicles of smaller size, and the level of $CD105^{+}$ also decreased (Lei et al., 2017). The senescence biomarker SA-β-gal is used to investigate the functional implications of aging in MSCs (Dimri et al., 1995). In this study, patients' MSCs showed that a higher number of cells stained positive for SA-β-gal than healthy donors' MSCs,

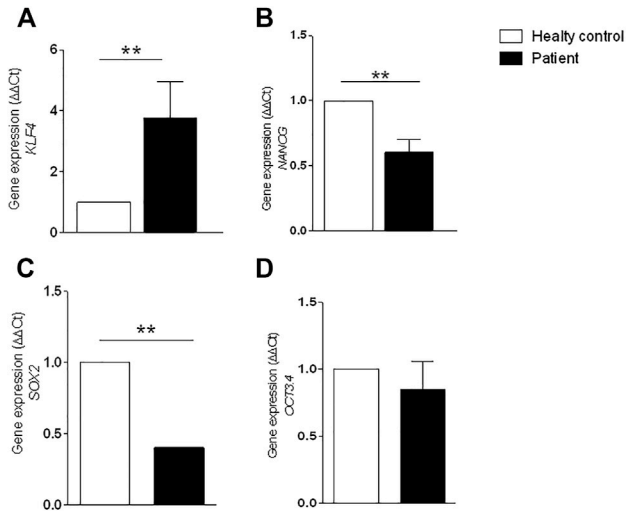

FIGURE 5 | Gene expression by RT-PCR. Expression was analyzed using GAPDH reference gene and results were normalized through the control samples. **(A)** Increased expression of *KLF4* gene (** $p = 0.0016$); **(B)** reduced expression of *NANOG* gene (** $p = 0.0016$); **(C)** reduced expression of *SOX2* gene (** $p = 0.0014$); **(D)** reduced expression of *POU5F1 (OCT3.4)* gene. Error bars represent SD. Statistical significance was determined by Student's t test. Data represent triplicate of 2 independent experiments.

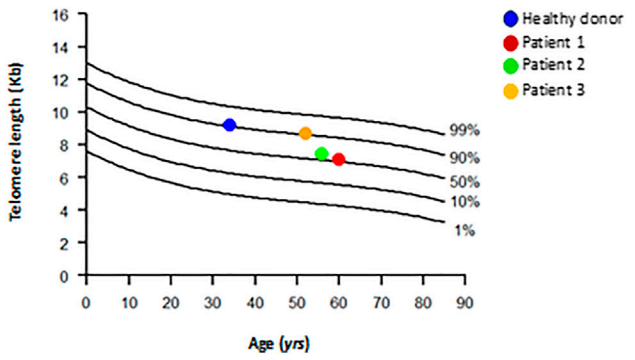

FIGURE 6 | Distribution of age-matched telomere length in DNA from healthy subjects leukocytes and DNA from healthy subject and patients' MSCs. Healthy subjects ($n = 261$) are represented as line; MSCs from patients and one healthy control are represented as colored circles. Telomere length is given as kilobases (kb) by Southern blot.

TABLE 6 | Telomere length by Southern blot analysis.

#ID	Age (years)	MSC telomere length (Kb)
Healthy donor	34	9.2
Patient 1[a]	60	7.1
Patient 2	56	7.4
Patient 3	52	8.7

[a]*Patient related to this study (senescent cells).*

especially after using AS as a supplement. Several studies have shown the importance of stem cells in repair and in regenerating tissue and organs. Differentiation capacity of young MSCs is superior to old cells, and old MSCs proliferated more slowly and became senescent due to telomere attrition (Kretlow et al., 2008; Li et al., 2017).

The molecule CD34 is a hematopoietic progenitor marker, and its expression is lost when the maturation process takes part (Hughes et al., 2020). The expression of CD34 is higher *in vivo* than in cultured MSCs, and some growth factors in culture medium could impact in its expression *in vitro* (Bellagamba et al., 2018; Viswanathan et al., 2019). An study showed a decrease in the proliferation rate in adventitial stromal cell-like cells, and it was related to the accumulation of $CD105^+/CD146^+/CD271^+$ and also $CD34^+$, which could represent a population that entered the cell cycle but did not complete the cell division (Braun et al., 2013). Patient #1 displayed a higher expression of CD34, which is not expected and could be related to an accumulation of non-proliferating cells, leading to the senescence state.

To evaluate the potential impairment of self-renewal and differentiation of MSC due to early senescence, we investigated the expression of genes related to pluripotency (*KLF4*, *OCT 3.4*, *NANOG*, and *SOX2*). An increased expression of *KLF4* was observed in the patient's MSC culture in comparison to healthy controls.

The zinc finger protein Krüppel-like factor 4 (KLF4) is responsible for regulating gene transcription and cell fate, promoting malignant transformation, cell differentiation, tumor suppression, and stem cell properties (Kaczynski et al., 2003; Rowland and Peeper, 2006; Lin et al., 2011).

KLF4 is known to act in different ways by reducing proliferation rates by pathways such as $p21^{Waf1/Cip1}$ and may be present in promoting malignant properties by suppressing p53 or by upregulation of NOTCH1 (Kaczynski et al., 2003; Lin et al., 2011). Direct blockage of KLF4 expression has been demonstrated to promote cell differentiation and reduce cell aging in MSCs (Rowland and Peeper, 2006). KLF4, a downstream mediator, can either repress or activate transcription and participate in cell cycle regulation and differentiation (Rowland et al., 2005; Li et al., 2013; Lu et al., 2019).

A decreased expression of *NANOG*, *SOX2*, and *OCT3.4* was also observed in the patient's MSC in comparison to healthy controls. The reduced expression of these genes may indicate a reduced proliferation rate in the patient's MSCs in comparison to healthy control MSCs.

NANOG is a transcription factor that is involved in the self-renewal of embryonic stem cells. The knockdown of *NANOG* and/or *OCT4* in MSCs has been shown to not maintain MSCs in an undifferentiated state. In contrast, the overexpression of OCT4 and NANOG in MSCs has been shown to increase the cell proliferation rate and differentiation potential and inhibit spontaneous differentiation, becoming essential markers for maintaining MSC properties (Shields et al., 1996).

POU5F1 (also known as OCT4 and OCT3.4) and SOX2 are essential transcription factors for pluripotency and self-renewal. Both genes are expressed in MSCs at low levels in early passages,

and their levels gradually decrease as the passage number increases. The overexpression of OCT4/SOX2 in MSC has been shown to improve cell proliferation and differentiation. Many researchers have investigated the effects of these pluripotent genes on MSCs, but the results are still debatable. Forced expression of pluripotent cell-specific factors (Oct4, Sox2, Nanog, and cMyc) or combinations of these genes for reprogramming somatic or adult stem cells has been used to improve the pluripotency of MSCs (Zhang et al., 2000; Seo et al., 2009; Lin et al., 2011; Yoon et al., 2011; Han et al., 2014; Gawlik-Rzemieniewska et al., 2016). SOX2, implicated in the maintaining the stem cell potency of BM-MSCs (Otsubo et al., 2011), recently was reported in a senescence context; the co-culture of senescent endothelial cells with BM-MSCs was associated with an increased expression of miR-126a-3p, in association with a significant decrease of SOX2, targeted by miR-126a-3p (Lazzarini et al., 2019).

A previous study with BM-MSCs from different healthy young donors identified some markers that could predict the expansion capacity of MSCs before reaching senescence. In this study, for each early passage, the gene expression levels of pluripotency markers OCT4, NANOG, and SOX2 were correlated with the final population doubling (PD) number and, in agreement with our results, revealed that a high OCT4 gene expression might be a potential hallmark and predictor of MSCs' lifespan *in vitro* (Piccinato et al., 2015). A recent study also showed that OCT4 maintains the self-renewal ability of MSCs and may reverse the senescence phenotype (Lu et al., 2019).

Low-passage cultures are recommended for clinical expansion of cultures (Lechanteur et al., 2016). Therefore, there is no explicit passage number provided for the 15 MSC products in clinical use, as resumed by Liu and contributors (2020). However, for MSCs to be clinically functional, it is essential to monitor the senescence of MSC aging. Recent studies have discussed and summarized some strategies for monitoring senescence, and the molecular mechanisms involved (Li et al., 2017; Liu and Chen, 2020). Although the topic has much clinical relevance, the current knowledge of senescence is mainly based on classical events, and sometimes, there are difficulties not previously described.

Further research is required to determine the approaches for safety and effectiveness in MSC-based cell therapy. MSC culture is quite simple; however, it is necessary to carefully monitor the expansion process to detect any potential changes that may interfere with clinical management. Improving the conditions for *ex vivo* expansion and monitoring of aging markers for MSCs would help suppress and monitor *in vitro* aging.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comissão Nacional de Ética em Pesquisa/CAAE: 18150613.7.3001.0071. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conception and design of the study: RA-P, SN, and JG; acquisition of data: RA-P, SN, DO, LC, LM, KA, and JG; draft or revise the manuscript: RA-P, SN, DO, LC, KA, NH, OO, LM, AK, JK, MMB, RC, and JG. RA-P and SN equally contributed to the paper. All authors have approved the final manuscript.

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MINI REVIEW
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From Vial to Vein: Crucial Gaps in Mesenchymal Stromal Cell Clinical Trial Reporting

Danielle M. Wiese[1], *Catherine A. Wood*[1] *and Lorena R. Braid*[1,2]*

[1]Aurora BioSolutions Inc., Medicine Hat, AB, Canada, [2]Simon Fraser University, Burnaby, BC, Canada

Retrospective analysis of clinical trial outcomes is a vital exercise to facilitate efficient translation of cellular therapies. These analyses are particularly important for mesenchymal stem/stromal cell (MSC) products. The exquisite responsiveness of MSCs, which makes them attractive candidates for immunotherapies, is a double-edged sword; MSC clinical trials result in inconsistent outcomes that may correlate with underlying patient biology or procedural differences at trial sites. Here we review 45 North American MSC clinical trial results published between 2015 and 2021 to assess whether these reports provide sufficient information for retrospective analysis. Trial reports routinely specify the MSC tissue source, autologous or allogeneic origin and administration route. However, most methodological aspects related to cell preparation and handling immediately prior to administration are under-reported. Clinical trial reports inconsistently provide information about cryopreservation media composition, delivery vehicle, post-thaw time and storage until administration, duration of infusion, and pre-administration viability or potency assessments. In addition, there appears to be significant variability in how cell products are formulated, handled or assessed between trials. The apparent gaps in reporting, combined with high process variability, are not sufficient for retrospective analyses that could potentially identify optimal cell preparation and handling protocols that correlate with successful intra- and inter-trial outcomes. The substantial preclinical data demonstrating that cell handling affects MSC potency highlights the need for more comprehensive clinical trial reporting of MSC conditions from expansion through delivery to support development of globally standardized protocols to efficiently advance MSCs as commercial products.

Keywords: mesenchymal stromal (stem) cell (MSC), ATMP, clinical trial, retrospective analysis, cell therapy (CT), regulatory approval, cell fitness, cell potency

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Mayasari Lim,
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Hudson Institute of Medical Research, Australia

***Correspondence:**
Lorena R. Braid
lorena@aurorabiosolutions.com
lrbraid@sfu.ca

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Abbreviations: CFU, colony forming units; cryo, cryopreservation; ELISA, enzyme linked immunosorbent assay; FBS, fetal bovine serum; h, hour; HLA, human leukocyte antigen; IDO, indoleamine 2,3-deoxygenase; IFN, interferon; IL, interleukin; NTF, neurotrophic factor; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; QC, quality control; TNF, tumor necrosis factor.

INTRODUCTION

Mesenchymal stromal cell (MSC) products are rapidly advancing as clinical treatments for a range of inflammatory diseases and regenerative medicine applications (Davies et al., 2017; Martin et al., 2019; Levy et al., 2020; Wright et al., 2021). MSC therapies have consistently proven safe (Levy et al., 2020; Krampera and le Blanc, 2021), but clinical outcomes from both autologous and allogeneic MSC trials have been variable and often less beneficial than in preclinical studies (Galipeau and Sensébé, 2018; Martin et al., 2019; Levy et al., 2020; Krampera and le Blanc, 2021). The inconsistent performance of MSC products has been attributed to numerous factors, most of which remain poorly understood or controlled. These have been comprehensively reviewed by others and include MSC heterogeneity between donors, tissues of origin and expansion level (Martin et al., 2019; le Blanc and Davies, 2018; Wiese et al., 2019a; Galipeau et al., 2021), preparation/manufacturing protocols (de Wolf et al., 2017; Mennan et al., 2019; Yin et al., 2019; Levy et al., 2020), administration route (Braid et al., 2018; Giri and Galipeau, 2020; Levy et al., 2020; Moll et al., 2020; Galipeau et al., 2021) and the underlying biological differences between patient recipients (Martin et al., 2019; Levy et al., 2020; Moll et al., 2020; Galipeau et al., 2021).

The realization of MSCs as advanced therapy medicinal products/advanced medicinal products (ATMP/AMP) requires global standardization of MSC manufacturing protocols, critical quality attributes, release criteria, and product preparation and delivery protocols at treatment sites (Mendicino et al., 2014; de Wolf et al., 2017; Viswanathan et al., 2019; Galipeau et al., 2021; Wilson et al., 2021; Wright et al., 2021). Retrospective analysis of clinical trial outcomes is a vital exercise to identify the practices that correlate with successful outcomes and those that result in variable outcomes or unsatisfactory efficacy. Statistically powered comparisons of trial procedures and outcomes are limited, however, by the degree to which clinical trial data are recorded and reported.

In this review, we analyze the product and procedural information provided in peer-reviewed clinical trial reports published since 2015. Our analysis focuses on reporting of cell handling procedures from dose preparation–either fresh or thawed–through completion of cell transfer. Surprisingly, we discovered that few clinical trials specify and/or report the handling of MSC products during this window in which the cells are vulnerable to insult and may experience uncontrolled conditions. This lack of information precludes retrospective analysis of the influence of product handling and delivery with clinical outcomes.

METHODS

Search Strategy

The search terms *mesenchymal stromal cell clinical trial* and *mesenchymal stem cell clinical trial* were searched in PubMed and Google Scholar with filters to include the clinical trial article type, published from 2015 to 2021 inclusive, with an available abstract and full text. These queries returned 471 articles effective 21 January 2022.

Report Selection and Data Extraction

The reports were filtered to include only trials using human-derived live MSC products for human use. Because reporting standards can vary by region, we further limited the scope of our analysis to clinical trials performed in North America. Rationale and Design articles were excluded. These refinements produced 45 peer-reviewed clinical trial reports for analysis.

Data was extracted verbatim from the curated reports according to four categories:

1) Trial and report particulars: Authors, article doi, trial location, publication year, trial phase, product name, affiliate company and clinical trial identifier
2) Study design: Disease or injury indication, administration route, MSC tissue of origin, selected MSC population (if any), MSC state (fresh, cryopreserved or culture-rescued after thaw) and donor relationship (allogeneic or autologous)
3) Dose preparation and handling: MSC dose (per kg and/or mean number), MSC concentration, delivery buffer, rate and duration of cell transfer, dose scheme, storage conditions and duration between dose preparation and administration, and miscellaneous handling details as listed
   Where applicable: cryopreservation mode (aliquot or bag), cryomedia formulation, thaw procedures and cell recovery protocols.
4) MSC product characterization: culture media formulation, MSC population doubling level or passage, and quality control attributes including safety (sterility, endotoxin, *mycoplasma*, viral pathogens, karyotyping, residual FBS, tumorigenesis and others as listed), identity (morphology, surface marker profiles, multilineage potential, HLA profiling, clonogenicity and others as listed), functional attributes (PMBC suppression, cytokine expression, IDO-1 expression, T-cell proliferation, others as listed) and viability including post-thaw viability for cryopreserved products.

RESULTS

Clinical Trial Parameters

The reports predominantly described Phase 1 clinical trials (44%) performed in the United States (90%). The therapeutic indication and clinical trial identifier associated with each publication are listed in **Supplementary Table S1**. The trials spanned a range of indications, including Graft versus Host Disease (GVHD), autoimmune diseases, cardiovascular injury and disease, sepsis, cancer and others (**Supplementary Table S1**). The majority of trials used bone marrow-derived (BM) MSCs (71%) delivered intravenously (IV; 40%).

All the clinical trial reports specified the MSC tissue of origin, whether the cell source was autologous or allogeneic, and the administration route (**Supplementary Table S1**; **Tables 1**, **2**). Most of the trials (93%) reported the dose of MSCs in units of

TABLE 1 | Clinical trial publications inconsistently report details relevant to MSC dose preparation and bedside handling. Dashed lines represent unreported data.

Author	Administration Route	Cell dose		Cell delivery buffer	Rate and/or duration of administration	Dose and/or delivery detail	Prep-to-admin storage and timing
		# per kg	Mean #				
Amirdelfan et al. (2021)	Intradiscal	—	6 or 18 M	Hyaluronic acid (HA) carrier	—	2 ml (1 ml of 30 or 90 M cells/5 ml + 1 ml 1% HA)	Thawed and combined with HA carrier at time of administration
Lanzoni et al. (2021)	IV	—	100 ± 20 M	Plasma-Lyte, HSA, Heparin	10 ± 5 min	2 × 50 ml dose Plasma-Lyte, HSA, Heparin (D0, D3)	Thaw quickly, less than 3 h from thaw to administration
Bolli etal. (2018); Bolli et al. (2021)	Endocardial injections	—	75–150 M	Plasma-Lyte	—	6 ml	—
Soder et al. (2020)	IV	—	—	—	—	—	Thawed immediately on day of administration
Kurtzberg et al. (2020)	IV	2 M	50 M	Plasma-Lyte A	1 h	50 ml dose	Thawed and resuspended immediately before administration
Kebriaei et al. (2020)	IV	2 M	—	Plasma-Lyte, 50 g/L (5%) HSA, 10% DMSO	4–6 ml/min	—	Thawed and immediately infused
Chahal et al. (2019)	Intraarticular	—	1, 10 or 50 M	2.5% patient serum in Plasma-Lyte A	—	Dose in 6.5 ml +/- 1.5 ml	15–25°C for 8 h in Plasma-Lyte A then 2–10°C for 24 h
Schlosser et al. (2019)	IV	0.3, 1 or 3 M (total ≤300 M)	—	80% Plasma-Lyte A, 20% Alburex-25 human albumin	20 min (10 ml), 40 min (35 ml) or 60 min (100 ml) by dose cohort	—	—
Berry et al. (2019)	IT and IM injection (bicep and tricep)	—	125 M IT, 48 M IM	Culture media (DMEM)	—	5 ml IT and 1 ml × 24 IM; DMEM placebo	Validated shipping system at controlled temperature 2–8°C
Dozois et al. (2019)	Fistula plug	—	20 M/plug	Maintained in Lactated Ringer's solution until delivery	—	—	—
Yau et al. (2019)	Intramyocardial	—	150 M	Cryoprotective medium as sham	15 min	16–20 injections of 0.2 ml	Thawed longer than 90 min discarded
Levy et al. (2019)	IV	0.5, 1, or 1.5 M	—	Lactated Ringer's solution	2 ml/min	1 M cells/ml in 1–3 × 60 ml syringes; 0.1 ml intradermal for patient reactivity prior	Stored at 2 to 8°C and infused within 8 h
Singer et al. (2019)	IT	—	10 M, 2 × 50 M or 2 × 100 M	Lactated Ringer's solution	1–2 min	Dose followed by 1 ml flush	Used within 12 h of preparation
Myerson et al. (2019)	Arthrodesis surgery	N/A (device)	—	—	—	—	—
Schweizer et al. (2019)	IV	1 M or 2 M (max 100 M or 200 M total)	—	6% hetastarch in 0.9% NaCl injection, 2% HSA, 5% DMSO	—	—	—
Powell and Silvestri (2019)	Intratracheal	10 M (2 ml/kg in 2 aliquots) or 20 M (4 ml/kg in 4 aliquots)	—	Normal saline	5–10 min	5 M/ml	Administered within 3 h of thawing and resuspension
Chan et al. (2020)	Intramyocardial	—	Targeted 150 M, minimum 15 M	0.9% NaCl	—	3 ml in 30 × 100 µl	—

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TABLE 1 | (*Continued*) Clinical trial publications inconsistently report details relevant to MSC dose preparation and bedside handling. Dashed lines represent unreported data.

Author	Administration Route	Cell dose		Cell delivery buffer	Rate and/or duration of administration	Dose and/or delivery detail	Prep-to-admin storage and timing
		# per kg	Mean #				
Harris et al. (2018)	IT	—	5.3–10 M (3 doses 3 months apart)	Saline	—	—	—
McIntyre et al. (2018)	IV	0.3, 1 or 3 M to max of 300 M	—	80% Plasma-Lyte A, 20% Alburex-25 human albumin	20 min (10 ml), 40 min (35 ml) or 60 min (100 ml) by dose cohort	—	—
Matthay et al. (2019)	IV	10 M	—	Plasma-Lyte A	60–80 min	100 ml dose	—
Swaminathan et al. (2018)	Intraaortic	2 M	—	10% DMSO, 5% HSA in Plasma-Lyte A, pH 7.4[a]	1–3 min	100 ml dose	On refrigerated gel packs and administration within 8 h preparation
Keller et al. (2018)	IV	1, 2 or 4 M	5 M	Plasma-Lyte, 0.5% DMSO	2–3 ml/min during the first 15 min, with the option to be adjusted up to 5 ml/min if tolerated	Cells diluted 5-fold in 100 ml	—
Tompkins et al. (2017)	IV	—	100 or 200 M	0.9% saline[a]	2 ml/min	100 ml; squeeze infusion bag every 15 min, 25 ml flush at end	—
Glassberg et al. (2017)	IV	—	20, 100 or 200 M	PBS, 1% HSA[a]	—	—	Cryo: thaw in 37°C water bath, wash, resuspended; Fresh: resuspended[a]
Dietz et al. (2017)	Fistula plug	—	20 M per plug	Lactated Ringer's solution	—	—	—
Golpanian et al. (2017)	IV	—	20, 100 or 20 M	0.9% saline[a]	2 ml/min	100 ml; squeeze infusion bag every 15 min, 25 ml flush at end	—
Florea et al. (2017)	Transendocardial	—	20 or 100 M	PBS +1% HSA or Plasma-Lyte A+ 1% HSA[a]	—	20 M/ml; 0.5 cc per injection × 10	Thaw at 37°C in water bath, pellet resuspended[a]
Saad et al. (2017)	Intraarterial	0.1 or 0.25 M	—	Lactated Ringer's solution	5 min	10 ml	—
Butler et al. (2017)	IV	1.5 M	—	Lactated Ringer's solution	—	1M/ml, 1 ml/kg	Thawed within pharmacy, infusion within 8 h
Bajestan et al. (2017)	Alveolar graft	—	15–44 M/ml, 2–5 ml/ patient	Isolyte +0.5% HSA mixed with b-TCP carrier	—	10 ml ixmyelocel-t in Isolyte +0.5% HSA mixed with b-TCP carrier; 2.5 ml/patient	At 4°C for up to 40 h
Hare et al. (2017)	Transendocardial	—	100 M (≥80 M autologous)	PBS +1% HSA or Plasma-Lyte A+ 1% HSA[a]	0.4 ml/min, 10 × 0.5 ml each	20 M/ml	Thaw at 37°C in water bath, pellet resuspended[a]
Harris et al. (2016)	IT	—	—	Saline with CSF	—	Saline with 3 ml CSF then 2 ml CSF flush	—
Steinberg et al. (2016)	Post-craniostomy implant	—	2.5, 5 or 10 M	—	10 µl per minute, 15 min per track × 3 tracks	—	—
Dhere et al. (2016)	IV	2, 5 or 10 M	—	Plasma-Lyte A with 0.05% HSA	Roughly 60 min	4 M cells/ml	—

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TABLE 1 | (*Continued*) Clinical trial publications inconsistently report details relevant to MSC dose preparation and bedside handling. Dashed lines represent unreported data.

Author	Administration Route	Cell dose		Cell delivery buffer	Rate and/or duration of administration	Dose and/or delivery detail	Prep-to-admin storage and timing
		# per kg	Mean #				
Staff et al. (2016)	IT	—	10, 50, 50 M × 2, 100 M	Lactated Ringer's solution	1-2 min	2 or 10 ml	Administered post-thaw or post-thaw + 4 days
Castillo-Cardiel et al. (2017)	To mandibular fracture line pre-open reduction and internal fixation (ORIF)	—	10–600 M from 50cc adipose tissue	—	—	—	—
Coetzee et al. (2016)	Arthrodesis surgery	N/A (device)	—	—	—	—	—
Patel et al. (2016)	Transendocardial	—	35–295 M[a]	—	—	5.8–8.4 ml was delivered as a series of 12–17 injections of 0.4 ml each[a]	—
Levy et al. (2016)	Corpora cavernosum base injection	—	1 ml product (# not quantified)	Isotonic saline	—	1.5 ml of 3 ml dilution	—
Perin et al. (2015)	Transendocardial	—	25, 75 or 150 M	Cryoprotective medium as sham[a]	—	16–20 injections of 0.2 ml	—
Levy et al. (2015)	Peyronie plaques, corpora injection	—	—	Isotonic saline	—	Up to 2 ml of 3 ml dilution	—
Skyler et al. (2015)	IV	0.3, 1 or 2 M	—	Normal saline	45 min	100 ml	Thawed immediately before use
Wilson et al. (2015)	IV	1, 5 or 10 M	—	Plasma-Lyte A	60–80 min	100 ml	2 h of stability, then 60–80 min gravity feed
Maziarz et al. (2015)	IV	1, 5 or 10 M (repeat 1 or 5M × 3/week or 5M × 5/week	—	Plasma-Lyte A, 5% DMSO	5–10 ml/min	23–61 ml or 100–143 ml or 133–294 ml (diluted based on body weight)	Infused within 6 h after thaw
Pettine et al. (2015)	Intradiscal	—	~726 M (121 ± 11 M/ml × 6)	Non-expanded BM concentrate	—	6 ml	—

[a]*Denotes publications which have information referenced in external references or supplemental material. Abbreviations: BM, bone marrow; D, day; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; HSA, human serum albumin; IM, intramuscular; IT, intrathecal; IV, intravenous; M, million; MEM, modified eagle's media; min, minute; N/A, not applicable; NaCl, sodium chloride; NEAA, non-essential amino acids; P, passage; PBS, phosphate buffered saline; PDL, population doubling level.*

cells/kg patient weight, or mean cells per patient (**Table 1**). Three trials (7%) did not disclose or even quantify the number of cells per dose (**Table 1**). Twenty-three trials (51%) included dose-escalation schemes. Twenty-six trials (58%) used fixed doses rather than a dose/kg scheme (**Table 1**).

Reported MSC Product Characterization

Some form of cell product characterization was usually reported (89%), although the assessment criteria used was mixed (**Supplementary Table S2**). Viability was the most commonly reported metric, but the acceptable threshold ranged from 50 to 98% between trials (**Supplementary Table S2**). Studies using frozen cells stipulate whether viability assessments were made before cryopreservation, on a sample thawed lot, or per vial/bag at the time of use. Safety criteria, including tests for bacterial, fungal and viral contamination, chromosomal stability and residual FBS, were reported in 32 studies (71%; **Supplementary Table S2**). Thirty-three reports (73%) listed cell identity tests, including surface marker profiling, multi-lineage differentiation, and clonogenicity (**Supplementary Table S2**). Functional assessments were only reported for 12 clinical trials (27%) and included peripheral blood mononuclear cell (PBMC) and T-cell suppression, IDO-1 expression after IFN-γ stimulation, or secretion of other relevant proteins (**Supplementary Table S2**).

Details related to product formulation and handling were poorly documented. Twenty-five publications (55%) failed to fully define the medium in which the MSCs were expanded or administered, and 23 reports (51%) provided no information about the population doubling level (culture age) of the cells (**Table 2**). Of the 21 reports (47%) that provided some description of MSC expansion level, 10 (22%) only provided number of days in culture. Three (7%) reports provided discrete population doubling levels; the remaining studies reported passage number.

Reported MSC Product Handling

Most trials (62%) used previously frozen MSCs, while six publications (13%) did not stipulate whether their MSC

TABLE 2 | Clinical trial publications underreport MSC manufacturing details. Dashed lines represent unreported data.

Author	Donor	Manufacturing information		Other preparation details	MSC state	Cryopreservation mode	Cryomedia formulation
		Culture media	MSC culture age				
Amirdelfan et al. (2021)	Allogeneic	—	—	—	Frozen	—	—
Lanzoni et al. (2021)	Allogeneic	DMEM Low Glucose, 10% platelet gold, 1 × GlutaMAX, 1 × MEM-NEAA	—	—	Frozen	—	—
Bolli etal. (2018); Bolli et al. (2021)	Autologous	Lymphocyte cell separation media	—	—	Frozen	—	—
Soder et al. (2020)	Allogeneic	—	P5	—	Frozen	Aliquot	Plasma-Lyte A, DMSO, HSA
Kurtzberg et al. (2020)	Allogeneic	—	P5	—	Frozen	Aliquot	Plasma-Lyte A, DMSO, HSA
Kebriaei et al. (2020)	Allogeneic	Supplemented with 10% FBS[a]	P5	—	Frozen	Bag	Plasma-Lyte, 50 g/L (5%) HSA, 10% DMSO
Chahal et al. (2019)	Autologous	DMEM low glucose, 1% Glutamax, 10% FBS	P3 (day 30) or P4 (day 37)	Washed 2x in Plasma-Lyte A, 1x in Plasma-Lyte A+ 2.5% patient serum (excipient)	Fresh	N/A	—
Schlosser et al. (2019)	Allogeneic	NutriStem XF	PDL ≤12	Culture 5–12 days after thaw (PDL≤18)	Culture-rescued after thaw	—	—
Berry et al. (2019)	Autologous	—	—	3–4 weeks culture for neurotrophic factor secretion	Fresh	N/A	10% DMSO in growth medium, controlled rate, pre-MSC-NTF generation[a]
Dozois et al. (2019)	Autologous	—	—	Thawed to adhere to fistula plug (proprietary)	Frozen	—	—
Yau et al. (2019)	Allogeneic	—	—	—	Frozen	Aliquot 4 × 1 ml	7.5% DMSO, 50% α-MEM, 42.5% ProFreeze[a]
Levy et al. (2019)	Allogeneic	—	P4	5% O2; washed in Lactate Ringer's solution	Frozen	Aliquot	Cryostor CS10
Singer et al. (2019)	Autologous	—	—	Thaw from cryo, culture in PLTMax for 3–5 days	Culture-rescued after thaw	—	—
Myerson et al. (2019)	Allogeneic	—	—	—	—	—	—
Schweizer et al. (2019)	Allogeneic	α-MEM, 2 mM L-glutamine, 10% FBS, no antibiotics	—	—	Frozen	Bag 20 ml	6% hetastarch in 0.9% NaCl injection, 2% HSA, 5% DMSO
Powell and Silvestri (2019)	Allogeneic	—	—	—	Frozen	—	—
Chan et al. (2020)	Autologous	α-MEM, 20% FBS, gentamicin	To P3 in 21 days	N/A	Fresh	N/A	N/A
Harris et al. (2018)	Autologous	Lonza NPMM	2–3 weeks after thaw at P2-3		Culture-rescued after thaw	—	—
McIntyre et al. (2018)	Allogeneic	NutriStem XF	PDL ≤12	Culture 5–12 days after thaw (PDL≤18)	Culture-rescued after thaw	—	—
Matthay et al. (2019)	Allogeneic	—	—	Wash to remove DMSO before resuspension	Frozen	Aliquot	Contains DMSO
Swaminathan et al. (2018)	Allogeneic	—	—	—	Frozen	Bag 20 ml	20 ml (120 M cells) PlasmaLyte A w/ 10% DMSO, 5% HSA, pH 7.4[a]

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TABLE 2 | (*Continued*) Clinical trial publications underreport MSC manufacturing details. Dashed lines represent unreported data.

Author	Donor	Manufacturing information		Other preparation details	MSC state	Cryopreservation mode	Cryomedia formulation
		Culture media	MSC culture age				
Keller et al. (2018)	Allogeneic	α-MEM, 9.8% HyClone Characterized FBS	—	—	Frozen	—	20 ml, 2.5% DMSO
Tompkins et al. (2017)	Allogeneic	α-MEM, 20% FBS	P1 (21–24 days)[a]	Wash with Plasma-Lyte A+ 1% HSA[a]	Fresh	N/A	N/A
Glassberg et al. (2017)	Allogeneic	α-MEM, 20% FBS	P1 (21–24 days)[a]	Washed[a]	Fresh and frozen	—	Pentaspan (10% pentastarch in 0.9% NaCl), 2% HSA, 5% DMSO[a]
Dietz et al. (2017)	Autologous	—	—	Thaw from cryo, bioreactor 3–6 days for plug adherence	Culture-rescued after thaw	—	—
Golpanian et al. (2017)	Allogeneic	α-MEM, 20% FBS	P1 (21–24 days)[a]	Wash with Plasma-Lyte A+ 1% HSA[a]	Fresh	N/A	N/A
Florea et al. (2017)	Allogeneic	α-MEM, 20% FBS	P1 (21–24 days)[a]	—	Frozen	—	Pentaspan (10% pentastarch in 0.9% NaCl), 2% HSA, 5% DMSO[a]
Saad et al. (2017)	Autologous	Isolated 6 weeks prior, 2 weeks in Advanced MEM with PLTMax (5% platelet lysate, 100 U/ml penicillin, 100 g/ml streptomycin, 2 mM L-glutamine)	—	N/A	Fresh	N/A	N/A
Butler et al. (2017)	Allogeneic	—	—	Hypoxia	Frozen	—	Cryostor CS10
Bajestan et al. (2017)	Autologous	IMDM, 10% FBS, 10% horse serum, 5 mM hydrocortisone	12 days in bioreactor	N/A	Fresh	N/A	N/A
Hare et al. (2017)	Autologous and Allogeneic	α-MEM, 20% FBS	P1 (21–24 days)[a]	—	Frozen	—	Pentaspan (10% pentastarch in 0.9% NaCl), 2% HSA, 5% DMSO[a]
Harris et al. (2016)	Autologous	2–3 passages/7–54 days in Lonza MSCGM +10% patient serum, plus 7–24 days in Lonza NPMM		N/A	Fresh	N/A	N/A
Steinberg et al. (2016)	Allogeneic	—	—	—	—	—	—
Dhere et al. (2016)	Autologous	α-MEM, 10% HSA	P1	N/A	Fresh	N/A	N/A
Staff et al. (2016)	Autologous	Advanced MEM, 5% hPL	<P5	—	Frozen	Aliquot	—
Castillo-Cardiel et al. (2017)	Autologous	DMEM, 10% FBS, antibiotics	24 h	N/A	Fresh	N/A	N/A
Coetzee et al. (2016)	Allogeneic	—	—	—	—	—	—
Patel et al. (2016)	Autologous	—	12 days in bioreactor	N/A	Fresh	N/A	N/A
Levy et al. (2016)	Allogeneic	—	—	—	—	—	—
Perin et al. (2015)	Allogeneic	—	P5 or <20 PDL	—	Frozen	Aliquot 4 × 1 ml	4% DMSO, 50% α-MEM, 42.5% ProFreeze
Levy et al. (2015)	Allogeneic	—	—	—	—	—	—
Skyler et al. (2015)	Allogeneic	Media (unspecified), FBS	—	—	Frozen	—	4% DMSO, 50% α-MEM, 42.5% ProFreeze
Wilson et al. (2015)	Allogeneic	—	—		Frozen	—	Contains DMSO
Maziarz et al. (2015)	Allogeneic	FBS	—	Wash in HSA before cryo	Frozen	—	Contains DMSO
Pettine et al. (2015)	Autologous	—	—	—	—	—	—

[a]*Denotes publications which have information referenced in external references or supplemental material. Abbreviations: cryo, cryopreservation; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; HSA, human serum albumin; IM, intramuscular; IV, intravenous; M, million; MEM, modified eagle medium (D, Dulbecco's); MSC, mesenchymal stromal cell; N/A, not applicable; NaCl, sodium chloride; NEAA, non-essential amino acids; NTF, neurotrophic factor-secreting; P, passage; PDL, population doubling level.*

products were derived from fresh cultures or had been thawed (**Table 2**). Of the 28 publications that used previously frozen MSC products, nearly half did not list the cryopreservation media (**Table 2**). Cryo-rescue procedures were essentially unreported, even though all but four trials administered MSCs directly following thaw without a recovery period or transfer of cells from cryopreservation media to delivery buffer/vehicle. Only seven papers stated that a wash step was performed, but no further details of the wash procedures were provided (**Table 1**).

Injection/infusion buffers were fairly well reported (91%) and predominately consisted of Plasma-Lyte, Plasma-Lyte A, Lactated Ringer's solution, and saline with or without human serum albumin (HSA) or dimethylsulfoxide (DMSO) at varying concentrations (**Table 1**). Buffer solution was not used in an AD MSC bone allograft device in arthrodesis surgery (Coetzee et al., 2016; Myerson et al., 2019). One publication reported intradiscal injection of non-expanded BM concentrate (Pettine et al., 2015).

Duration of cell transfer was reported for the majority (78%) of trials that used IV infusion, either in minutes or ml/min (**Table 1**). Infusion time ranged from 5 min to 1 h. Of the trials using other administration routes, 28% reported the duration or rate of administration (**Table 1**). Most reports (84%) provided no information about the elapsed time from when the dose was prepared until cell transfer was complete (**Table 1**). Seven (16%) reports specified a maximum elapsed time from dose prep or thaw to administration, which ranged from 90 min to 12 h (**Table 1**). The three studies that included product handling protocols each used different methods; prepared doses were held in refrigeration, on cold packs or at room temperature (**Table 1**).

DISCUSSION

MSCs are fundamentally responsive to subtle changes in their environment. MSCs respond to changes in atmospheric gases (Lin et al., 2014; Gorgun et al., 2021; Roemeling-Van Rhijn et al., 2013; Ejtehadifar et al., 2015; Kang et al., 2019; von Bahr et al., 2019), temperature (Stolzing et al., 2006; Kubrova et al., 2020; Shimoni et al., 2020), hydrostatic pressure (Steward et al., 2012; Becquart et al., 2016; Pattappa et al., 2019) and aggregation (Robb et al., 2019; Yuan et al., 2019; Burand et al., 2020; Xie et al., 2021). It is surprising then, that the steps and duration between dose preparation and delivery of MSC therapies are ill-defined and under-reported. We predict that bedside handling of MSC products may contribute substantially to the variability and reduced efficacy documented in clinical trials. Retrospective analysis to test this hypothesis, however, is currently impossible due to the absence of relevant information (Sart et al., 2014).

As example, MSCs have a natural tendency to self-assemble and form aggregates [reviewed in (Myerson et al., 2019)]. It has been reported that spontaneous aggregation can alter the immunosuppressive properties of MSCs, rendering them incapable of T cell suppression (Lanzoni et al, 2021). Thus, steps must be taken to control MSC aggregation between dose preparation and the completion of cell transfer. Even though cell doses were held for up to 12 h in the reviewed clinical trials, almost no measures to manage cell aggregation were described. Two studies reported squeezing the bag every 15 min during infusion, but no other reports described strategies to mitigate spontaneous aggregation. If the reports had documented the steps taken (if any) to prevent MSC aggregation during administration, retrospective analysis could potentially reveal whether implementing these strategies improves clinical outcomes.

Retrospective analysis could similarly be used to determine whether wash number, wash duration, centrifugation speed and buffer composition correlates with clinical outcomes. Thawed cells are fragile so thaw temperatures, duration and subsequent wash steps likely impact MSC fitness. The steps used to reconstitute frozen MSCs thawed immediately prior to administration were never reported. Moreover, few trials that thawed frozen MSCs immediately prior to administration stated the density at which the cells were cryopreserved, composition of the cryopreservation media, how the cells were thawed, whether or not they were washed, frequency of washing and the wash buffer used.

Currently, any changes in MSC fitness and performance in the hours between dose preparation and completion of infusion or injection is a black box devoid of data. To our knowledge, few studies have formally tested potential loss of function through sampling of MSC products during this window, or by recapitulating these conditions in laboratory tests (Pal et al., 2008; Chen et al., 2013; Niu et al., 2013). Intermittent bedside product testing admittedly is a logistical challenge. Thus, we suggest that clinical trial design include laboratory development of defined bedside procedures to ensure that the patient receives the same quality of MSC product that was prepared earlier and was subject to quality testing. Establishing and reporting these cell handling procedures, as well as any deviations from these protocols, may provide invaluable insight for retrospective analysis and ultimately ensure that patients consistently receive high quality MSC treatments.

There is a global movement towards standardization of MSC products. Such standardization includes development of tests to establish minimum cell performance criteria (Chinnadurai et al., 2018; Galipeau and Sensébé, 2018; Wiese et al., 2019b; Martin et al., 2019; Wiese and Braid, 2020a; Wiese and Braid, 2020b; Moll et al., 2020; Galipeau et al., 2021; Krampera and le Blanc, 2021), which are a critical to obtain regulatory approval for commercialization (Mendicino et al., 2014; Galipeau et al., 2015; de Wolf et al., 2017; Galipeau and Sensébé, 2018). Consistent with this movement, we found that most clinical trials reported some type of cell characterization. Viability and cell identity, based on accepted MSC cell surface profiles, were the most commonly reported tests. Consistent with a recent review of MSC characterization in clinical trials (Wilson et al., 2021), cell performance in functional assays or surrogate potency assays was documented infrequently, and performance thresholds were not disclosed. Post-thaw viability was also reported far less frequently than expected, especially since most of the trials used cryo-rescued cells.

We propose that ongoing global efforts to define the critical quality attributes of MSC ATMPs and subsequent release criteria be mindful of the need to identify markers and tests that can rapidly report MSC fitness and potency. These rapid-response markers will enable future development of in-process and bedside testing of MSC products, an important advancement in the realization of MSCs as commercially viable cell therapies.

Finally, retrospective analysis would be better enabled by establishing formal guidelines for clinical trial reporting. A recent clinical trial design by Baker et al. (2021) provides an excellent model to establish reproducible and transparent bedside cell handling procedures. We propose that clinical trial reports include all available cell characterization data and carefully document bedside handling of MSC products. Making this information readily available in the main report rather than citing other publications would facilitate accessibility for statistical analysis of large data sets and improve confidence that the data correlates with actual events and cell doses used in the trial.

CONCLUSION

We urge the MSC community to incorporate and report bedside MSC handling protocols and best practices in clinical trial design and reporting. The notable lack of information and data surrounding how these exquisitely responsive cells are treated when the cells are most vulnerable is not likely an issue of propriety. Rather, this aspect of the cell therapy journey from vial to vein appears to have been designated as arbitrary, a classification that we argue is flawed. Documenting and reporting bedside cell processing and handling procedures will aid effective retrospective analysis of clinical trial outcomes and expedite the commercialization of MSC products.

AUTHOR CONTRIBUTIONS

LB conceived the manuscript. DW and CW contributed to literature search and analysis. DW and LB prepared the manuscript with assistance from CW. LB generated financial support for the research. All authors approved the final manuscript submitted for consideration.

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SUPPLEMENTARY MATERIAL

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Posology and Serum-/Xeno-Free Engineered Adipose Stromal Cells Cell Sheets

*Jun Ochiai, Larakaye Villanueva, Hope Niihara, Yutaka Niihara and Joan Oliva**

Emmaus Life Sciences, Inc., Torrance, CA, United States

Well-characterized adipose stem cells and chemically defined culture media are important factors that control the production of the cell sheet, used in translational medicine. In this study, we have developed and engineered multilayer adipose stem cell cell sheets (ASCCSs) using chemically defined/serum-free culture media: undifferentiated or differentiated into osteoblasts and chondrocytes. In addition, using the cell sheet transmittance, we estimated the number of cells per cell sheet. Undifferentiated ASCCSs were engineered in 10 days, using serum-free/xeno-free culture media. They were $CD29^+$, $CD73^+$, $CD90^+$, CD105+, HLA-A+, and HLA-DR-. ASCCSs differentiated into chondrocytes and osteoblasts were also engineered using chemically defined and animal-free culture media, in only 14 days. The addition of an ROCK inhibitor improved the chondrocyte cell sheet engineering. The decrease in the cell sheet transmittance rate was higher for the osteoblast cell sheets due to the intracellular Ca^{2+} accumulation. The estimation of cell number per cell sheet was carried out with the transmittance, which will provide important information for cell sheet posology. In conclusion, three types of ASCCSs were engineered using serum-free, xeno-free culture media, expressing their specific markers. Their transmittance measurement allowed estimating the number of cells per cell sheet, with a non-invasive methodology.

Keywords: mesenchymal stem cells, cell sheets, transmittance, multilayer, serum-/xeno free, posology

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Rita Yen-Hua Huang,
Taipei Medical University, Taiwan

Reviewed by:
Tzu-Wei Wang,
National Tsing Hua University, Taiwan
Ruili Yang,
Peking University Hospital of Stomatology, China

****Correspondence:***
Joan Oliva
joliva@emmauslifesciences.com

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INTRODUCTION

In 2006, a consortium of experts released the minima criteria of mesenchymal stem cells (MSCs): adherence to a plastic surface, expression of specific MSC markers ($CD73^+$, $CD90^+$, $CD105^+$, $CD11b^-$, $CD14^-$, $CD34^-$, $CD45^-$, and $HLA\text{-}DR^-$) and the capacity to differentiate into cells from the three embryonic germ layers: ectoderm, mesoderm, and endoderm (Dominici et al., 2006). The International Society for Stem Cell Research, gathering experts in the field of cell and gene therapy, already released guidelines in 2006 and updated ones in 2018, based on two decades of experience (ISSCR, 2016). The use of MSCs offers different advantages such as unlimited and easy availability and low immunoreactivity. The interest in using mesenchymal stem cells for translational application keeps increasing since the discovery of different sources of MSCs: umbilical cords, adipose tissues, dental pulp, and peripheral blood (Nagamura-Inoue and He, 2014). In January 2021, 1,259 clinical trials were found using mesenchymal stem cells (www.clinicaltrials.gov). Clinical trials involving MSCs are currently targeting a wide range of diseases such as cardiac disease, diabetic nephropathy, autoimmune diseases, and liver failure. Because of the increase of conducted clinical trials and the experience acquired from these trials, federal agencies have modified and improved

their guidelines for cell and gene therapy to ensure the patient's safety and create new regulations to adapt to the new cell and gene therapy clinical trials (Konomi et al., 2015; Administration FDA, 2020b).

Isolated MSCs require not only their characterization but also their quality must be ensured after their expansion and/or differentiation because billions of cells can be required in clinical trials. It is recommended by the FDA that cell-based therapeutics developed in laboratories should be very similar to the products used in translational applications. Results obtained in laboratories using the products for research only can be very different from the cell-based therapeutics developed with United States Pharmacopeia (USP)–grade compounds and with master and working cell banks. Several manufacturers ensure that good quality MSCs are being provided to laboratories for their research and have adapted their production and tests based on FDA guidelines. Animal serum has always been a concern for the FDA because of the potential transmission of bovine spongiform encephalopathy (Administration FDA, 2020a). In addition to the potential infection from animal-contaminated animal serum, the composition of animal serum varies between each lot, making it difficult to obtain reproducible results in laboratories. Many laboratories have explored different sources of serum or supplements for cell culture to use as an alternative for animal serum. For example, epithelial cell sheets are engineered using mice 3T3 NIH feeder cells in the presence of fetal bovine serum (FBS), since the 1970s (Rheinwald and Green, 1977). To address the concerns of regulatory agencies, different laboratories have engineered epithelial cell sheets by eliminating the need for xeno feeder cells and FBS. In 2009, xeno feeder cells were replaced by bone marrow stem cells to engineer epithelial cell sheets, but FBS was still used (Omoto et al., 2009). Okano's group stepped forward and engineered cell sheets by using human autologous serum, in absence of feeder cells and animal serum (Murakami et al., 2006). However, the variability of the serum between the patients could be an influencing factor in the success rate of cell-sheet engineering and will require additional tests to characterize the patient serum (Murakami et al., 2006). Other groups used human platelet lysate to replace the animal or human serum (Becherucci et al., 2018). In terms of ASC proliferation, Gimble's group showed that 0.75% of the platelet lysate is equivalent to 10% FBS, and the platelet lysate maintained the differentiation properties of the ASCs (Cowper et al., 2019). However, another group showed that 5% of platelet lysate is the equivalent of 10% FBS in terms of the proliferation of ASCs (Kakudo et al., 2019). The quality of cells and/or serum substitutes could be the factors explaining the divergences in the results, underlying the importance of controlling the quality of every product used in cell culture. Manufacturers also developed culture media compatible with human trials, based on the changes and requirements in federal agency guidelines. Chemically defined, xeno-/serum-free culture media to maintain MSC properties are an important criterion to reproduce the results and compare the outcomes. Chemically defined serum-free culture media is the best option to control the proliferation and differentiation of the cells and compare the outcome of treated patients. Many publications report the use of serum-free culture media to expand and differentiate ASCs (Chase et al., 2010) and showed better results. The differentiation of ASCs into dopaminergic neurons was higher in serum-free culture media than in culture media with a low animal serum percentage. In addition, differentiated cells, with serum in the culture media, secreted twice the amount of tyrosine hydroxylase (Faghih et al., 2019).

Cell sheets have advantages over the injection of isolated cells such as a higher survival rate of the injected cells, accurately targeting the damaged area, and therefore better healing properties (Gyongyosi et al., 2008; Urban et al., 2008; Hamdi et al., 2011; Katagiri et al., 2013). In the United States, only hematopoietic progenitor cells derived from umbilical cord blood are used to treat patients with blood production disorders, and no MSC product has been approved by the FDA while they are used in clinical trials. In many of the clinical trials, the MSCs are injected in the patients. It is an easy and cheap methodology to treat patients, and the posology can be well-controlled. The healing mechanism of action of the MSC is not yet well-determined. It could be done through the physical cell-to-cell interaction or it could be because of the paracrine effect (Xiang et al., 2009; Spees et al., 2016; Jiang et al., 2017), which is becoming a more popular theory. However, injected cells can migrate ectopically in the organism, mainly in the lungs (Pereira et al., 1998; Gao et al., 2001; Schrepfer et al., 2007; Lee et al., 2009), which could lead to health issues such as thrombosis, impairment of the organs, or tumor formation (Lee and Hong, 2017; Coppin et al., 2019).

Our team has previously reported successful engineering of human adipose stem cell cell sheets (ASCCSs), using culture media complete with serum (Oliva et al., 2019). In this study, we propose to engineer three different types of ASCCSs with chemically defined culture media (xeno- and serum-free culture media): undifferentiated, chondrocyte, and osteoblast multilayered cell sheets. In addition, using a transmittance device developed by our group, the difference in transmittance was studied to establish standard curves of the ASCCSs during their differentiation (Ochiai et al., 2021). We also estimated the number of cells per cell sheet using the transmittance values, which is important information to provide to federal agencies in terms of posology.

MATERIALS AND METHODS

Cell Culture

Human adipose stromal cells (ASCs) were purchased from RoosterBio, Inc (RoosterBio, Inc., Frederick, MD). Human ASCs were used for the following experiments. The hASCs were expanded up to passage 5, in a T75 flask (USAScientific, Ocala, FL), using RoosterNourish™-MSC-XF (RoosterBio, Inc., Frederick, MD).

Engineering of the Adipose Stromal Cell Cell Sheets

ASCs were seeded at 10.2×10^4 ASC per cm^2 in a 35-mm culture dish (Corning, Corning, NY). The ASCs were cultured with

RoosterNourish™-MSC-XF culture media (RoosterBio, Inc., Ballenger Creek, Maryland). The differentiation of the cell sheets started when they reached confluence, on day 4 after initial seeding.

- Undifferentiated ASCCSs: ASCs were cultured with RoosterNourish™-MSC-XF. The culture media were replaced every 2 days, up to 18 days from the initial seeding day.
- Osteoblast cell sheets: ASCs were cultured with Osteomax-XF Differentiation Medium (Millipore-Sigma, Burlington, MA). The culture media were replaced every 3 days, up to 17 days from the initial seeding day.
- Chondrocyte cell sheets: ASCs were cultured with a MesenCult™-ACF Chondrogenic Differentiation Kit (Stem Cell, Vancouver, Canada). The culture media were replaced every 2 days, up to 19 days from the initial seeding, and were also replaced when 10 M of ROCK Inhibitor y-27632 (MedChemExpress, Monmouth Junction, NJ, United States) was used.
- Skeletal muscle differentiation medium (PromoCell, Heidelberg, Germany) was used to show that the cells do not form a multilayer cell sheet and the cell density does not change by measuring the transmittance.

Dyeing of the Adipose Stem Cells Cell Sheets

Multilayer undifferentiated, chondrocyte and osteoblast cell sheets cells were stained with the Alcian Blue Stain kit (Bioquochem, Llanera, Spain) and with the Alizarin Red Stain kit (Millipore-Sigma, Burlington, MA). The protocols from the manufacturer were modified to stain the cross section of the cell sheets.

For Alcian blue, fixed tissues are deparaffinized with xylene treatment (Fisher Scientific, Waltham, MA, United States) and by hydrating them with 100 (two times), 90, and 50% ethanol (Millipore-Sigma, Burlington, MA). The tissues are washed with deionized water and washed 2 × 1 min with 3% glacial acetic acid. Alcian blue is added on top of the tissue for 30 min at room temperature. Alcian blue will be rinsed with 3% glacial acetic acid (Bioquochem, Llanera, Spain). The tissues are washed with tap water (1 min) and deionized water (1 min), and the washing steps are repeated. The nuclei are dyed with nuclear fast red for 5 min. The tissues are washed with tap water (1 min) and deionized water (1 min), and the washing steps are repeated. The tissues are dehydrated with 70, 90, and 100% ethanol. Ethanol is washed three times with xylene. Mounting media (Alban Scientific, St Louis, MO) and coverslips (Fisher Scientific, Waltham, MA, United States) are placed on the tissues.

For Alizarin Red, the fixed tissues are deparaffinized with xylene treatment and by hydrating them with 100 (two times), 90, and 50% ethanol. The tissues are washed with deionized water for 2 × 5 min. Alizarin Red is added on top of the tissue for a few minutes at room temperature. Alizarin red is removed with tap water (1 min) and deionized water (1 min), and the washing steps are repeated. The tissues are dehydrated with 70, 90, and 100% ethanol. The tissues are then rinsed with acetone, acetone: xylene (1:1) (Millipore-Sigma, Burlington, MA) followed by washing thrice with xylene. Mounting media and coverslips are placed on the tissues.

Transmittance of the Differentiated or Not Differentiated ASCCSs

The transmittance is measured only after the culture media are replaced, and it was measured only when the cells reached confluence until they form a multilayer cell sheet. The transmittance of the adipose stromal cells cell sheets was measured using the device described in the reference (Ochiai et al., 2021). In summary, the cell culture dish is placed on the stage, always in the same position. The edge of the cell culture dishes is marked to ensure that the cell culture dishes are always placed in the same position to measure the transmittance of the same nine spots. A light, produced by the light source, will shine through the cell sheet, and the light intensity will be measured by a detector placed under the stage. A single measurement is an average of 100 values per read from the detector with 10 reads per second, for 10 s per point (nine points per cell sheet) and per cell sheet (Ochiai et al., 2021). The reference value was obtained by measuring the intensity of the light coming through a cell culture dish that has the same amount of culture media but with no cell sheet (blank sample). The collected values were converted to percent transmittance with respect to the value of the reference blank sample.

Harvesting of the ASCCSs

To harvest the multilayer cell sheets, we used a CellShifter membrane, with a 30 mm diameter (CellSeed, Inc., Tokyo, Japan) and forceps. The forceps (Roboz, Gaithersburg, MD) were used to cut the edge of the cell sheet from the cell culture dish wall. The CellShifter was placed on top of the cell sheet. The culture media were removed, and the side of the cell sheet was wrapped over the edge of the CellShifter. By using forceps, the cell sheet was lifted and placed in a new cell culture dish. Once placed in a cell culture dish, a few drops of PBS were poured over the CellShifter. Using the forceps, the CellShifter was lifted and separated from the cell sheet, which stayed attached to the cell culture surface. The cell sheets were fixed in 10% neutral buffered formalin for immunohistochemistry staining (Fisher Scientific, Waltham, MA, United States) or entirely used for measuring the DNA content. It is important to report that it was not possible to harvest ASCCSs, cultured with skeletal muscle differentiation culture media, because the cells did not form a multilayer cell sheet.

Estimation of Cell Numbers per Cell Sheet

The number of cells was estimated by isolating the total genomic DNA from the entire cell sheets and compared with the quantity of genomic DNA from a determined number of isolated ASCs. Genomic DNA, from isolated ASCs and multilayer cell sheets, were isolated with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, United States), following the manufacturer's protocol. After harvesting the cell sheets, the

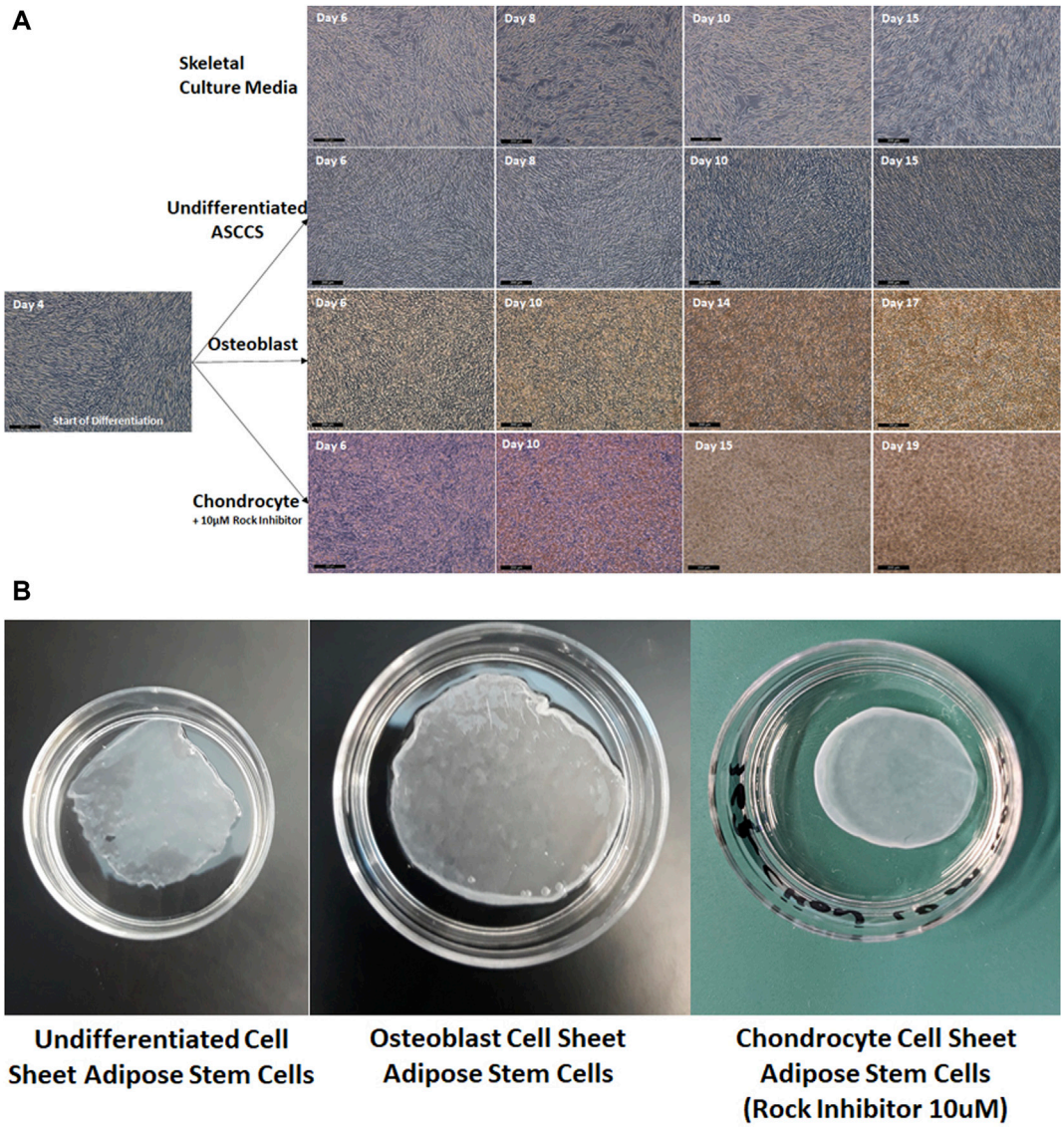

FIGURE 1 | (A) Pictures of the cell sheets, during the differentiation time (the scale bars are 200 μm). **(B)** Pictures of the harvested cell sheets, placed in 35-mm cell culture dishes.

ASCCSs were dissociated by sonication (Fisherbrand™ Model 705 Sonic Dismembrator) at 20 Hz for 5 s (Fisher Scientific, Waltham, MA, United States). The quantity of double-stranded DNA per cell was estimated using the QuantiFluor dsDNA System (Promega, Madison, WI, United States), using an aliquot of the total DNA. The standard curve shown in **Figure 6A** and the dilution factor of the aliquot were used to estimate the number of cells per cell sheet.

Immunocytochemistry Staining

The engineered cell sheets were fixed in 10% neutral buffered formalin and embedded in paraffin. The tissue sections were used for immunofluorescent staining with CD19 (Cat # NBP2-25196), CD73 (Cat # NBP2-25237) (NovusBIo, CO, United States), CD29 (Cat # ab134179), HLA-A (Cat # ab52922), HLA-DR (Cat # ab92511) (Abcam, MA, United States), and Oct3/4 (Cat # NB-100-2379SS) (Novus Biologicals LLC., Littleton, CO, United States). Alexa Fluor 488 donkey anti-rabbit conjugated secondary antibodies and Alexa Fluor 488 donkey anti-mouse conjugated secondary antibodies (Invitrogen, Carlsbad, CA, United States) were used. Propidium iodide (Invitrogen, Carlsbad, CA, United States) was used to stain nuclear DNA. An EVOS M5000 microscope was used to analyze the slides (Invitrogen, Carlsbad, CA, United States).

Statistical Analysis

All results are expressed as the mean ± standard deviation. The data were analyzed using one-way ANOVA. A $p < 0.05$ was considered statistically significant.

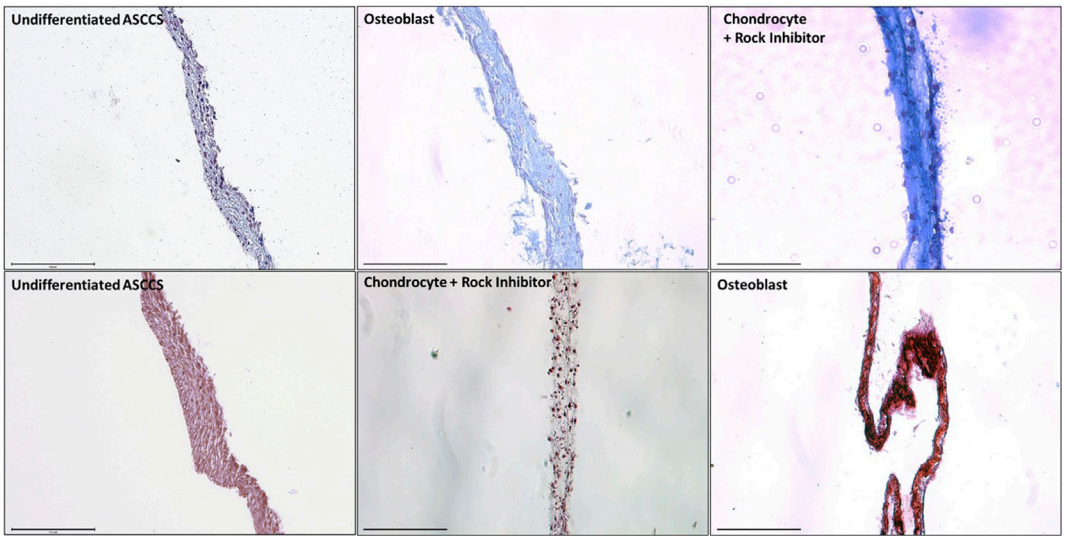

FIGURE 2 | Dye of the undifferentiated ASCCSs, chondrocyte cell sheets, and osteoblast cell sheets with Alcian blue and Alizarin red. Scale bars are 150 μm.

RESULTS

In **Figure 1**, pictures of the cell sheet formation, over time, show the increase of the cell densities. On day 4, when the ASCs reached confluence, the culture media were replaced. As a negative control, the ASCs did not form a multilayer cell sheet when they were cultured with skeletal culture media (used to differentiate iPSCs into myoblast) (**Figure 1A**). The cell density did not increase, which is related to the level of transmittance maintained at around 80%, up to 16 days (**Figure 3**). The ASCs kept growing over time, forming a strong multilayer cell sheet (**Figure 1A**). The transmittance of these undifferentiated ASCCSs decreased at around 60% as reported in a previous publication but using different culture media (Ochiai et al., 2021). Osteoblast cell sheets were engineered in 15 days. The ASCs differentiated very fast, in a few days, and differentiation increased over time. This can be noticed from the orange deposit at the top of the cell sheets (**Figure 1A**).

On day 4, the ASCCSs were treated with the MesenCult™-ACF Chondrogenic Differentiation Kit, but the success rate of the engineered chondrocyte cell sheets was very low. Very few chondrocytes cell sheets were engineered in 25 days. The cell sheets were detaching spontaneously during differentiation (data not shown). We noticed that the cell sheets always detached from the edges due to centripetal forces. To overcome the spontaneous detachment, a ROCK inhibitor (10 μM) was used during the differentiation period. The use of the ROCK inhibitor had two advantages: 1) Cell sheets never detached during the differentiation period and 2) the time for differentiation was shortened by around six days (**Figure 1A**).

In **Figure 1B**, the harvested cell sheets are shown. It is difficult to describe the physical properties of the cell sheets when harvested, but it is important to mention that the chondrocyte cell sheets have a rubber physical property when they were touched with forceps. This physical property plays a role in maintaining the round shape of the cell sheet. Osteoblast cell sheets were more rigid when they were touched with forceps.

In **Figure 2**, the cell sheets were dyed with Alcian blue and Alizarin red to show the level of differentiation. Alcian blue dyes strongly to the glycosaminoglycans, which are present on the cartilage (Ovchinnikov, 2009; Rigueur and Lyons, 2014). Alcian blue stained more differentiated chondrocyte cell sheets than the undifferentiated ASCCSs or the osteoblast cell sheets (**Figure 2** top row). Alizarin red binds to calcium, which is one of the major ions present in the bones (Zhu and Prince, 2012). Alizarin red strongly dyed the osteoblast cell sheets compared with the undifferentiated ASCCSs or the chondrocyte cell sheets (**Figure 2** bottom row).

Immunostaining of the harvested cell sheets was carried out by Alcian blue and Alizarin red dyes. In **Figure 3**, the expression of specific markers of ASCs was confirmed: HLA-A^{+}, CD29^{+}, CD73^{+}, CD105^{+}, Oct3/4^{+}, HLA-DR^{-}, and CD19^{-} (**Figure 3**) (Dominici et al., 2006).

In **Figure 4**, we confirmed the expression of specific markers of chondrocytes and osteoblast cell sheets. In **Figure 4A**, chondrocyte cell sheets expressed HLA-A and secreted protein acidic and rich in cysteine (SPARC) (Aeschlimann et al., 1995), collagen II (Lian et al., 2019), and aggrecan (PMID: 11942407) (Kiani et al., 2002). In **Figure 4B**, osteoblast cell sheets expressed HLA-A and osteocalcin (Manolagas, 2020). For both types of cell sheets, HLA-DR was not detected, which will allow allogeneic transplantation with a lower risk of immune rejection.

Once the protocol to engineer different types of ASCCSs was established, the transmittance of the cell sheets was measured until harvesting (**Figure 5**). ASCCSs cultured with skeletal muscle differentiation culture media did not grow over time (**Figure 1**). The cell density was stable for 16 days, and it is concordant with the stability of the transmittance (**Figure 5**, yellow line). It was not possible to harvest those cells because of the low cell density, which plays a role in the cell–cell connection, observed in the

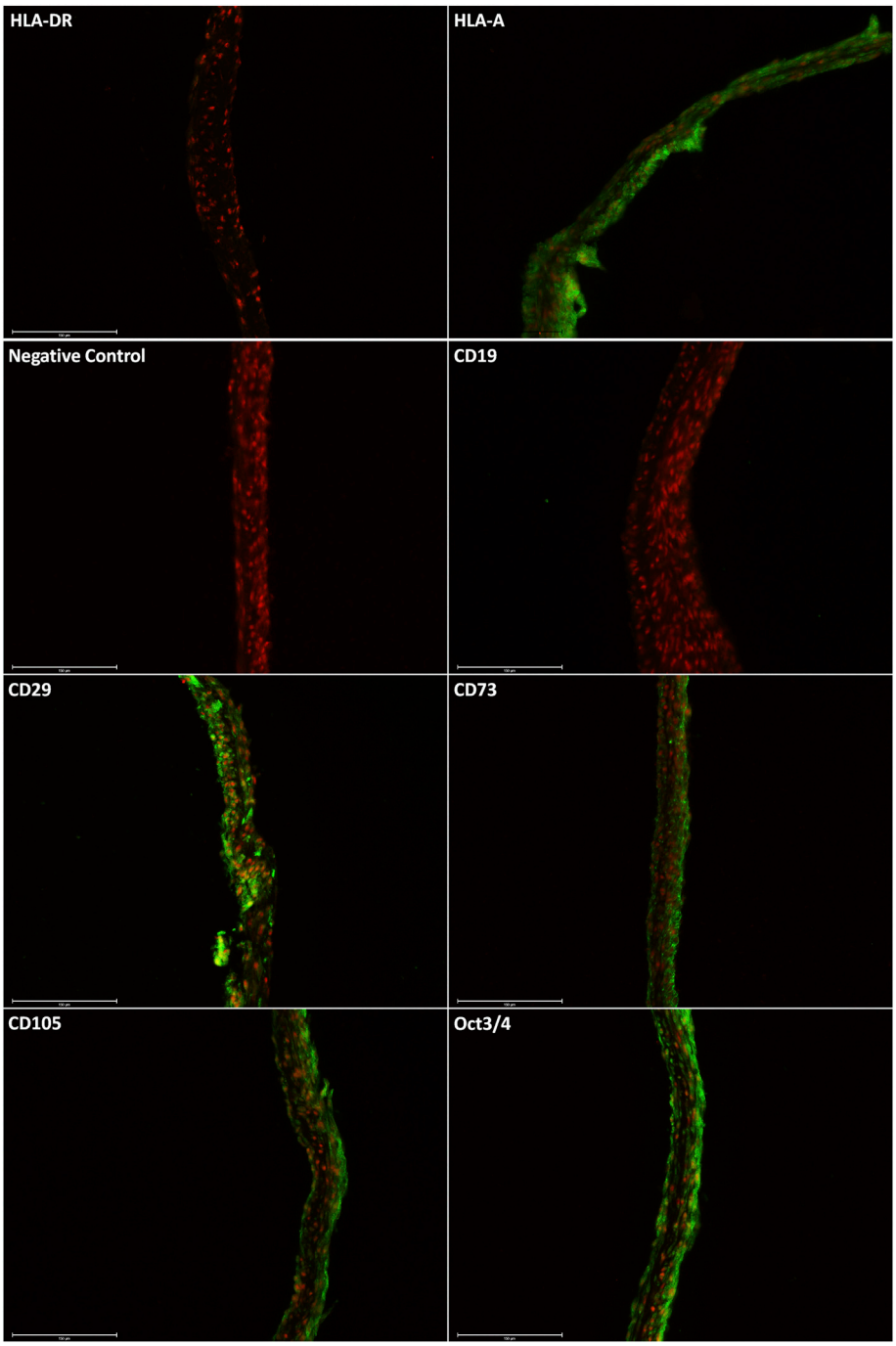

FIGURE 3 | Immunostaining shows the positive expression of HLA-A, CD29, CD73, CD105, and Oct3/4. The proteins HLA-DR and CD19 were not detected. The scale bars are 150 μm.

other cell sheets. The transmittance of undifferentiated cell sheets and chondrocyte cell sheets was similar, even if the chondrocyte cell sheet transmittance was lower (reaching 50%), when undifferentiated ASCCSs reached 53%. One important morphological cell difference between both cell sheets is that the chondrocyte cells are round and their number increases over time, whereas the cells from the undifferentiated cell sheets maintain some of their fibroblastic shape and form harmonious "waves" of cells.

On day 14, the transmittance was similar along with the macro morphology of the cell sheets. Undifferentiated cell sheets can be harvested once the transmittance is lower than 75% (data not shown). However, to compare the transmittance over time, among the four types of cell sheets, undifferentiated ASCCSs

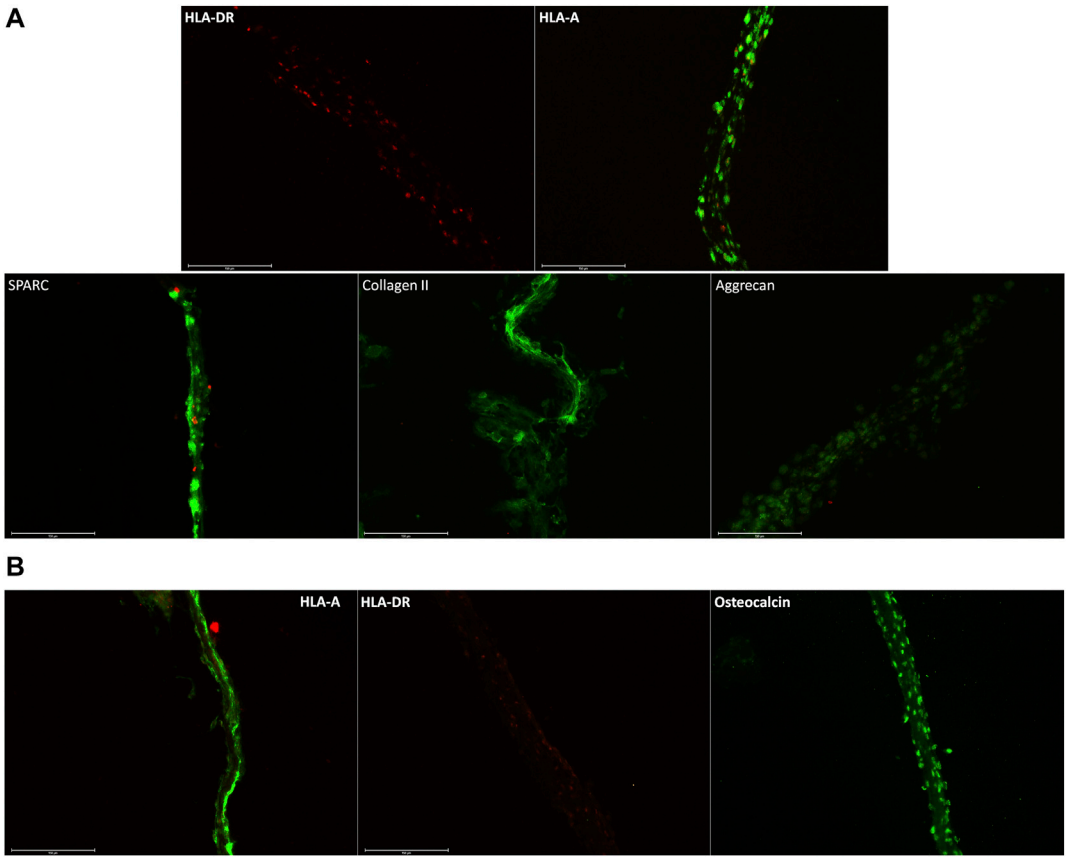

FIGURE 4 | (A) Immunostaining of chondrocyte cell sheets, with HLA-A^{+}, SPARC^{+}, collagen II^{+}, aggrecan^{+}, and HLA-DR^{-}. SPARC, collagen II, and aggrecan being specific markers of cartilage. **(B)** Immunostaining on osteoblast cell sheets with osteocalcin, a specific marker of osteoblast. Scale bars are 150 μm.

were maintained in the culture. The slope of the curves is very similar: -1.9 for the undifferentiated ASCCSs and -2 for the chondrocyte cell sheets (**Figure 5** blue and gray lines). The transmittance of the osteoblast cell sheets was interesting because the transmittance of the cell sheets always increased during the first three days of cell culture. This increase is because the cells are contracting and getting smaller (**Figure 5**, day 3 of differentiation), as a morphological transition (**Figure 5**, orange line). However, soon after this transmittance increase, the transmittance decreased much faster than that of the undifferentiated and chondrocyte cell sheets. The slope of the curve is -3.6, and it could be explained by the accelerated accumulation of calcium in the cells, which is correlated with cell sheet opacity increase (**Figures 2**, **5**). The transmittance reached 38% on the day of harvesting.

Undifferentiated chondrocyte and osteoblast cell sheets were harvested easily, using a CellShifter. These cell sheets can be transplanted onto the patients. One major piece of information to provide to the FDA is the number of cells transplanted onto the patient. To determine the exact number of cells, the whole cell sheets must be dissociated with enzymes resulting in that the cell sheets can no longer be transplanted. Using a relation between the transmittance and the total DNA content, the estimation of cell number per cell sheet is possible. In **Figure 6**, the relation between those two values is shown. Using a standard where the number of ASCs corresponds to the quantity of isolated DNA; the number of cells per cell sheet was determined after the isolation of the total cell sheet DNA. As the transmittance of each cell sheet was recorded independently, we could correlate the number of cells per cell sheet with the transmittance (**Figure 6**). Osteoblast cell sheets had the lowest number of cells, despite having the lowest transmittance. This is correlated with the possibility of harvesting the cell sheets where the osteoblast cell sheets seem thinner than the other two ones. The undifferentiated and chondrocyte cell sheets have a higher number of cells and transmittance values. The center of the circles is calculated based on the transmittance average of the transmittance and the average of the cell number. The objective is that with the measured transmittance of the cell sheets cultured in specific culture media placed in these circles will help estimate the number of cells per cell sheet before transplantation, using a non-invasive and safe methodology.

DISCUSSION

Numerous studies reported the beneficial effects of using MSCs in the treatment of cartilage (Ma et al., 2018), graft-versus-host disease (Zhao et al., 2019), ischemia (Oliva, 2019), myocardial

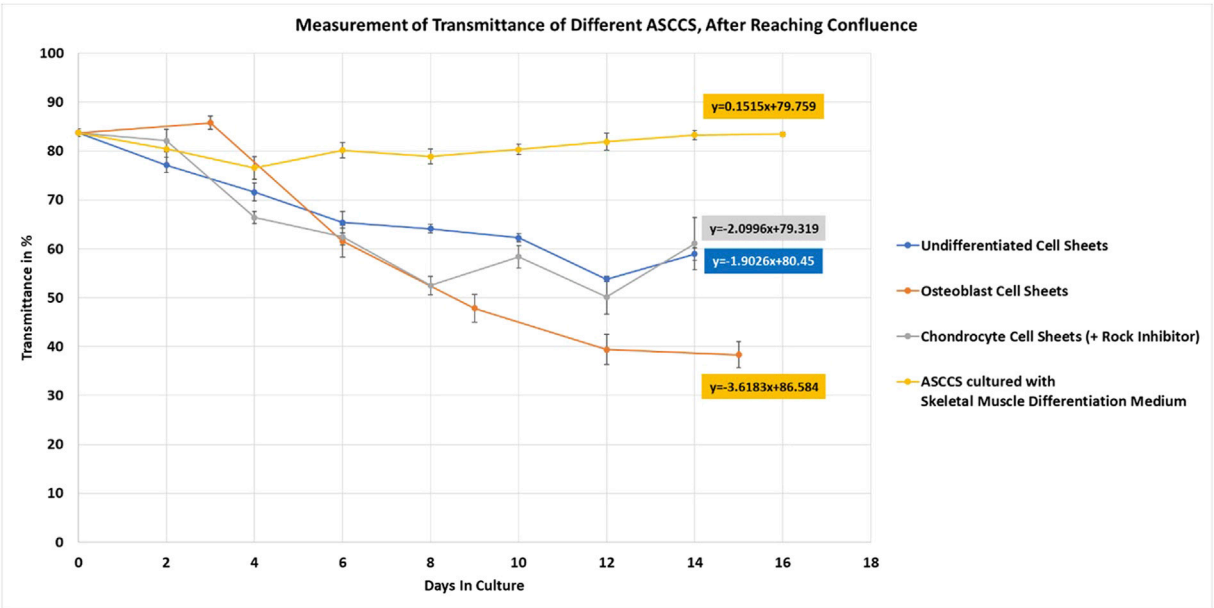

FIGURE 5 | Measurement of undifferentiated, osteoblast, and chondrocyte cell sheets. ASCCSs cultured with skeletal muscle differentiation culture media were used as a control to show the unchanged transmittance over time. Number of days in culture is the number of days after the cells reached confluence (day 4) (number of cell sheets measured per point n = 5, except for days 0, n = 10).

infarction (Luo et al., 2017), and sclerosis (Dudek et al., 2020). In this study, osteoblast and chondrocyte cell sheets were engineered, which can be harvested and transplanted in the required areas (Tuan et al., 2013; Richardson et al., 2016; Oryan et al., 2017; Yorukoglu et al., 2017; Abdel Meguid et al., 2018; Egido-Moreno et al., 2021). The use of chemically defined culture media, in the absence of serum, is strongly recommended by the FDA and can also aid in not having to change the protocols once the product needs to be produced following the FDA guidelines. We decided to focus on chemically defined serum-free culture media that could be used for clinical trials. These two criteria are two major factors that play a role in terms of reproducibility and can facilitate the transition to clinical studies. Undifferentiated osteoblast and chondrocyte cell sheets were engineered with success by overgrowing the cells and by inducing the differentiation once the ASCs reached confluence. Our approach is easy to reproduce. The same culture media can be used throughout the differentiation time, whereas other studies have shown that different culture media must be used to obtain MSC differentiation (Park and Cho, 2010). The ASC cell sheets expressed specific markers (**Figures 2–4**), and the absence of HLA-DR will help build cell sheet banks for allogeneic transplantation. As mentioned, cell sheets have other advantages such as the increase not only in the production of cytokines but also the production of proteins involved in the cell–cell connection. Okano's group reported that compared with a 2D monolayer umbilical cord mesenchymal stem cell, 3D cell sheets produced more β-catenin, connexin 43, integrin β1, and laminin (Bou-Ghannam et al., 2021). The production of these proteins explains why the cell sheets are strong enough to be harvested and lifted mechanically, without using enzymes or a thermo-responsive surface. As reported by our group in this study, the difference in the harvested cell sheet physical properties was noticed, but we were not able to translate them into numbers or graphs. We understand that it is necessary to develop new tools and experiments to understand better the cell sheet physical properties as explained in this excellent review (Efremov et al., 2021). Different physical properties could be studied, such as how the cell sheets can be stretched in one or two directions (Homma et al., 2020; Huang et al., 2021), the nanoindentation (Qian and Zhao, 2018), or optical properties (Ochiai et al., 2021). Shimizu's group reported that detached and stretched cardiomyocyte cell sheets from a temperature-responsive surface were longer, but that the cardiomyocyte cells had a unidirectional alignment. The cells in the control cell sheets were randomly aligned. For such a methodology, the cell sheets must be detached from the support and can be transplanted or seeded again on a cell culture dish for further studies (Homma et al., 2020). Nanoindentation could be used directly on the 3D cell sheets to determine the resistance of the cell sheets to forces and could help determine if the cell sheets are strong enough for harvesting or strong enough for medical purposes. Such techniques could be used to increase our knowledge about 3D cell sheets. Nanoindentation is a non-invasive approach that was not used to the best of our knowledge. We decided to use another non-invasive approach to study the 3D engineered cell sheets. A device, developed by our group, was used to study the changes in cell sheet transmittance over time and during the differentiation (Ochiai et al., 2021). We reported that the transmittance of the three types of cell sheets decreased and that once the transmittance reached a certain value, it was possible to mechanically harvest the undifferentiated cell sheets (Ochiai et al., 2021). However, the

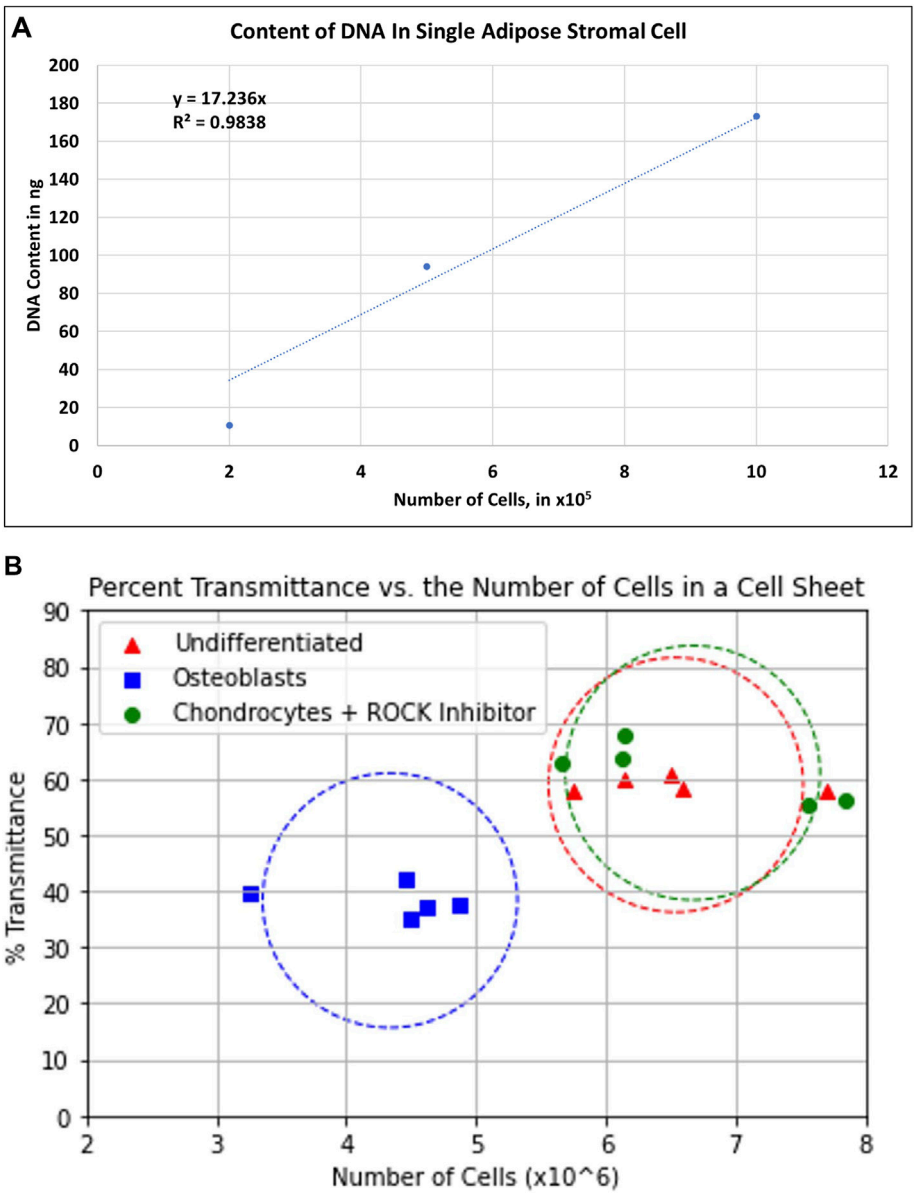

FIGURE 6 | (A) Standard curve of total DNA content per number of single adipose stromal cells. The equation y = 17.236x was used to estimate the number of cells from each cell sheet. **(B)** Graph showing the correlation between the number of cells per type of cell sheet and the transmittance.

value of the transmittance does not reflect the strength of the cell sheets. Chondrocyte and undifferentiated cell sheets were strong and easy to harvest. On the other hand, the transmittance of the osteoblast cell sheets was the lowest, but the harvesting of the cell sheets was more delicate; cell sheets could break easily compared with the undifferentiated and chondrocyte cell sheets. The use of nanoindentation will be a very good tool to determine how resistant the cell sheets are and correlate their strength with the outcome after transplantation. Transmittance values can help in following the differentiation of the cell sheets over time, but they also can help in estimating the number of cells per cell sheet CAR-T cells are approved by the FDA to treat certain cancers. CAR-T are single cells, and it is easy to determine how many cells are injected per patient, but it is impossible to know how many cells in a cell sheet are transplanted (Kochenderfer et al., 2015; Neelapu et al., 2017; Schmidts and Maus, 2018). The only way to know exactly how many cells compose a cell sheet is to digest it with enzyme, but this will make it useless for transplantation. We have developed a device that can translate the level of cell sheet transmittance to estimate the time of cell sheet harvesting (Ochiai et al., 2021). In addition, by measuring the transmittance, the day of harvesting, and the number of cells isolated from the cell sheets, we will be able to estimate the number of cells per type of cell sheet, cultured under specific conditions.

Engineering chondrocyte cell sheets is more difficult than engineering undifferentiated and osteoblast cell sheets. For the chondrocyte cell sheet engineering, using the MesenCult-ACF culture media by itself, the success rate in engineering chondrocyte cell sheets was 11% (3/27). Another group reported, which we also noticed, that during the chondrocyte differentiation, cell sheets were spontaneously detaching from the edge and detaching completely from the bottom of the cell culture dish, at early stages (less than 6–8 days from the starting differentiation day) (data not shown) (Thorp et al., 2020). Not being satisfied by the low rate of success in engineering chondrocyte cell sheets, we noticed that the cause of the complete detachment of the cell sheets was due to forces exercised at the edge of the cell sheets. The cytoskeleton plays a role in this spontaneous detachment. Cytoskeleton proteins are involved in the organization of the cell–cell connection and the cell–extracellular matrix connection. It was reported that cells exert a certain tension over the surface when they are in contact with the extracellular matrix (ECM) (Ganz et al., 2006). In a publication, Schiller et al demonstrated that the pathway involving ROCK mediates the forces at the edges of the cells; accordingly, the same pathway could be the cause of the spontaneous cell sheet detachment. Manually cutting the edge of the cell sheets from the cell culture dishes, once we noticed that the cell sheets were detaching slowly from the edges, improved the production of chondrogenic cell sheets (data not shown). However, cutting the edges is not practical for the GMP cell sheet manufacturing stage and could increase the risk of cell sheet damage and contamination. Based on the cited literature, an ROCK inhibitor, such as Y27632, was used to improve the chondrogenic differentiation of MSCs. The ROCK inhibitor was shown to decrease the cytoskeleton tension of MSCs and increased the chondrogenic differentiation of the MSCs (Wang et al., 2018). When the ASCs reached confluence, they were cultured with the chondrogenic differentiation culture media, complete with Y27632 (10 μM final concentration) from the first to the last day of differentiation. It is important to note that when the ROCK inhibitor was added to MesenCult-ACF at 10 μM, the success rate was 100% (45/45), confirming the idea that the ROCK inhibitor improved the success rate of engineering chondrocyte cell sheets. No additional stimuli were used to produce the chondrocyte cell sheets such as vibration, compression, gravity, magnetic field (Zhang et al., 2015; Uddin et al., 2016; Hou et al., 2020; Li et al., 2020), and stacking (Zhou et al., 2015; Enomoto et al., 2016).

We are aware of the limitations of this study. Many more cell sheets should be engineered to obtain a better estimation of the number of cells per cell sheet, in correlation with the transmittance. In addition, the reported data are based on our cell culture media and the cells we have used, and different data could be obtained if the quality of the cells or culture media is different. However, this device can provide valuable information in terms of physical properties, maturity of the cell sheets, and the cell sheet posology using a non-invasive approach. In addition, animal studies will be required to evaluate the xenotransplantation of the cell sheets in terms of regenerative properties and confirm their hypo-immunogenicity.

CONCLUSION

We succeeded in engineering three types of cell sheets, using a xeno/serum-free culture media, and were able to harvest them. Undifferentiated ASCCSs maintained the expression of ASC markers. Chondrocyte cell sheets expressed specific markers (SPARC, collagen II, and aggrecan) along with the osteoblast cell sheets (osteocalcin). Based on the transmittance measured on the harvesting day and a standard DNA quantification from a single ASC, we can estimate the number of cells per cell sheet and per type of cell sheets. Using an affordable non-invasive method, this device could be used in translational regenerative medicine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization, JOl; methodology, JOc, LV, HN, YN, and JOl; data curation, JOc, LV, HN, YN, and JOl; writing—original draft preparation, JOl; writing—review and editing, JOc, LV, HN, YN, and JOl. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.873603/full#supplementary-material

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