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Perspectives

Biomanufacturing of human mesenchymal stem cells in cell therapy: Influence of microenvironment on scalable expansion in bioreactors

Teng Ma*, Ang-Chen Tsai, Yijun Liu

Department of Chemical and Biomedical Engineering, Florida State University, 2525 Pottsdamer St., Tallahassee, FL 32310, USA

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ABSTRACT

Human mesenchymal stem cells (hMSCs) are the primary candidate in cell therapy and have demonstrated significant potential in a wide range of diseases. The clinical translation of hMSC therapy requires robust and scalable expansion technology for the biomanufacturing of therapeutically competent cells. By nature, hMSCs are highly sensitive and responsive to the microenvironments and their therapeutic potency is significantly influenced by the culture conditions during expansion. Here, we discuss the emerging roles of microenvironments in regulating hMSC fate and the implication of regulating hMSC microenvironment to achieve scalable hMSC expansion in bioreactors.

1. Introduction

Human mesenchymal stem or stromal cells (hMSCs) have gained considerable attention in cell therapy and tissue regeneration for non-hematopoietic tissues with bone marrow, adipose tissue, and umbilical cord as the major sources for hMSC derivation [1]. Clinical trials involving hMSCs are being conducted in a range of human diseases with majority of the trials in the early stages. As the use of hMSC is progressing through clinical development and moving toward cell-based therapies, cell expansion technology for hMSC manufacturing as a cell therapy product that meets safety, regulatory, and potency requirements is critical for the success in clinical translation.

Cell-based therapies are tightly regulated and complex biological products that are sensitive to their environment and processing history. To date, large-scale cultivation of mammalian cells has been primarily used for the production of biologics such as viruses and recombinant proteins, in which cells are not the final product. Apart from the chondrocytes used in autologous chondrocyte transplantation [2], hMSC expansion at clinical scale represents an adventure for the production of anchorage dependent cells that are directly used for clinical indications. To ensure the safety and quality of the cells, cell expansion must significantly increase cell numbers without negatively affecting hMSC's therapeutic potency. From biomanufacturing perspective, variation in hMSC properties comes from the input materials, process conditions, and processing history. hMSCs are known to be sensitive to environmental cues and their therapeutic potency deteriorates with time in conventional planar culture [3]. As defining the biological markers that are predictive of hMSC potency remains a challenge, understanding the impact and effective control of in-process conditions are important to ensure the quality and potency of the final products.

Stem cells have extraordinary proliferative capacity and multilineage differentiation potential, and their fate choice is tightly regulated by the extracellular microenvironment, or niche, that includes signaling molecules, cell–cell interactions, and extracellular matrices (ECM). In addition to the structural and humoral factors, the stem cell microenvironment also includes acellular components such as oxygen tension and localized biomechanical properties such as matrix stiffness and elasticity [4]. Identifying the key components of the stem cell niche and understanding their regulatory roles are important in recreating the specialized microenvironment and culture system capable of preserving hMSC innate properties during in vitro expansion. This perspective highlights the influence of microenvironmental factors on hMSC properties and discusses their role in scaling up hMSC production process.

2. hMSC expansion and culture-induced changes in clinical applications

Originally isolated from bone marrow aspirate, adhesion to plastic surface is a defining property of hMSCs that allowed their

* Corresponding author.
E-mail address: teng@eng.fsu.edu (T. Ma).

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isolation from non-adherent hematopoietic cells [5]. Since then, hMSCs have been commonly expanded in monolayer culture on plastic substrate for in vitro and preclinical studies. In bone marrow, the concentration of hMSC is low and bone marrow aspirate typically contains less than 1 in 10^5 mononucleated cells, and only about 6000 MSCs can be obtained from a single procedure [6]. In preclinical studies, about 10^7–10^8 hMSCs are used whereas clinical doses in the range from 1×10^6 to 5×10^6 cells/kg are needed in clinical application. In addition to quantity, maintaining hMSC's innate properties and therapeutic potency during large-scale expansion is equally important for the translation of hMSC therapy to clinical application. While hMSCs were initially studied for their multi-lineage potentiality, it is now accepted that their therapeutic effects derive from their trophic properties for the secretion of anti-inflammatory, immune modulatory, and pro-angiogenic factors [7]. hMSC can home to the site of tissue injuries and repair damaged tissue through the creation of a milieu that modulates immune responses and enhances the endogenous tissue repair. These intricate tissue repair capacity along with their unique sensing and migratory properties that respond to local stimuli are crucial to their therapeutic potency. For the manufacturing of hMSC-based therapies, preserving these properties is of primary importance in ensuring product potency; this must also be achieved reproducibly in the large-scale cell manufacturing processes at relatively low cost.

Extensive in vitro and pre-clinical studies have shown that culture conditions influence hMSC properties and functional outcome (Table 1). hMSCs are highly sensitive to biological, biomechanical, and physiochemical cues in the culture environment and that genetic and phenotype changes occur immediately after isolation and are continuously acquired during culture expansion on planar surfaces [3]. This is compounded by the fact that hMSCs are being isolated from diverse tissue sources and applied in a variety of clinical indications. Despite effort, defining robust markers that are predictive of hMSC in vivo efficacy remains a challenge. hMSC potency, defined as a quantitative measure of relevant biological functions, needs to be evaluated with respect to the specific medical indication [8]. For example, the potential of hMSCs to undergo osteogenic differentiation is paramount to their application in bone repair [9]. On the other hand, preserving hMSC's secretion of immune-modulating and anti-inflammatory functions is crucial for the outcome of hMSC's application in graft-versus-host disease (GvHD) [10]. In these applications, hMSC expansion is a significant factor that influences hMSC property for clinical application. Clinical trials using hMSC that have undergone extensive expansion for the treatment of steroid refractory GvHD reported negative results in phase III studies, whereas trials using hMSC that underwent substantially less expansion demonstrated positive clinical outcomes and improved patient survival [11,12]. Thus, understanding the key factors of the in vitro culture system and their influence on hMSC properties is important in the development of the hMSC production processes.

3. Influence of microenvironment on large scale hMSC expansion

Increasing evidence suggests that MSCs or "MSC-like" cells reside in the perivascular niche and are broadly distributed in virtually all postnatal organs and tissues. The tissue resident MSCs actively participate in tissue repair and regeneration and are highly responsive to environmental cues. As stromal cells, MSCs have extensive capacity for the secretion of humoral and structural proteins that actively modulate their immediate microenvironment. In vitro studies have also revealed hMSC's mechanosensing and responding capability suggesting the involvement of mechanotransduction processes in response to physical cues. These microenvironmental factors converge via a multitude of cellular signaling pathways and collectively influence hMSC phenotype and cell fate choice.

Exploiting the interactions of hMSC with their microenvironment is crucial in defining optimal conditions for hMSC expansion while preserving their therapeutic potency. To date conventional T-flask culture has been the mainstay to obtain hMSCs in preclinical studies whereas Cell Factory™ are typically used in clinical studies. Currently, there is an intense effort in developing microcarrier bioreactor technology as the main approach to scale up hMSC expansion. Microcarriers in bioreactor provide cell adhesion surfaces in suspension and microcarrier bioreactors are the default manufacturing platform for large-scale expansion of adherent cells. However, its adaption to large-scale hMSC expansion for cell therapy also poses new challenges. The culture environments in microcarrier bioreactor impact not only hMSC expansion kinetics and expression of phenotypic markers but also their properties and therapeutic potency. Indeed, recent studies comparing the effects of flask culture and microcarrier bioreactor on hMSCs from different donor and perinatal sources of umbilical cord and amniotic membrane have shown clear clustering of gene expression profiles in distinct groups solely depending on the expansion process [13]. These results suggest that the physiochemical and physiological properties of the culture environment need to be characterized and considered in developing large-scale hMSC expansion processes. Compared to planar tissue culture plates, few studies have focused on the characteristics and the regulatory roles of microenvironment formed on the microcarriers in the dynamic culture environment. As depicted in Fig. 1, the following sections discuss the unique features of hMSC microenvironment in regulating cell fate and the importance of understanding these microenvironmental factors in scaling up hMSC expansion processes.

3.1. hMSC in vitro microenvironment and clonogenicity

As hMSC's in vivo identity is still being debated, hMSCs have been the subject of tissue culture experiments [14]. As stromal cells, hMSCs have extensive capacity to secrete ECM and regulatory factors in vitro to form an interactive milieu that influence cell fate. In 2D plastic culture, hMSCs are significantly influenced by seeding density and have the ability to undergo clonal expansion, in which discrete colonies arising from single precursor cells exhibit a fibroblast-like morphology, termed colony-forming unit-fibroblastic (CFU-F) [15]. Colony forming efficiency has been used as a simple in vitro measure of the content of multipotent cells in a heterogeneous hMSC population with differing phenotypic properties, and serves as a reliable predictor of hMSC multipotency and the efficacy of hMSC therapies [16]. For example, Zhang et al. showed that a clonally purified hMSC population is more potent to repair infracted myocardium compared to the parent population [17], and Prockop's group showed that rapidly proliferating hMSCs exhibit preferential tissue engraftment relative to more slowly proliferating hMSCs [18].

hMSC clonal culture also revealed hMSC's unique ability to generate their own microenvironment and it reciprocal impact on hMSC fate. Clonal analysis of single-cell derived colonies has shown cellular heterogeneity even at clonal level with varying differentiation capacity among cells derived from a single colony [19]. These studies have also shown that the tri-potent clones have the highest proliferation rate compared to the bi-potent and uni-potent clones, suggesting a hierarchically structured population. Because the content of the clonogenic and multipotent subset in heterogeneous hMSC culture is not only correlated with cell proliferation but also with therapeutic outcome, preservation of hMSC clonogenicity is central to maintaining hMSC therapeutic potency. In standard

Table 1
Influence of planar culture conditions on hMSC in vitro and in vivo properties.

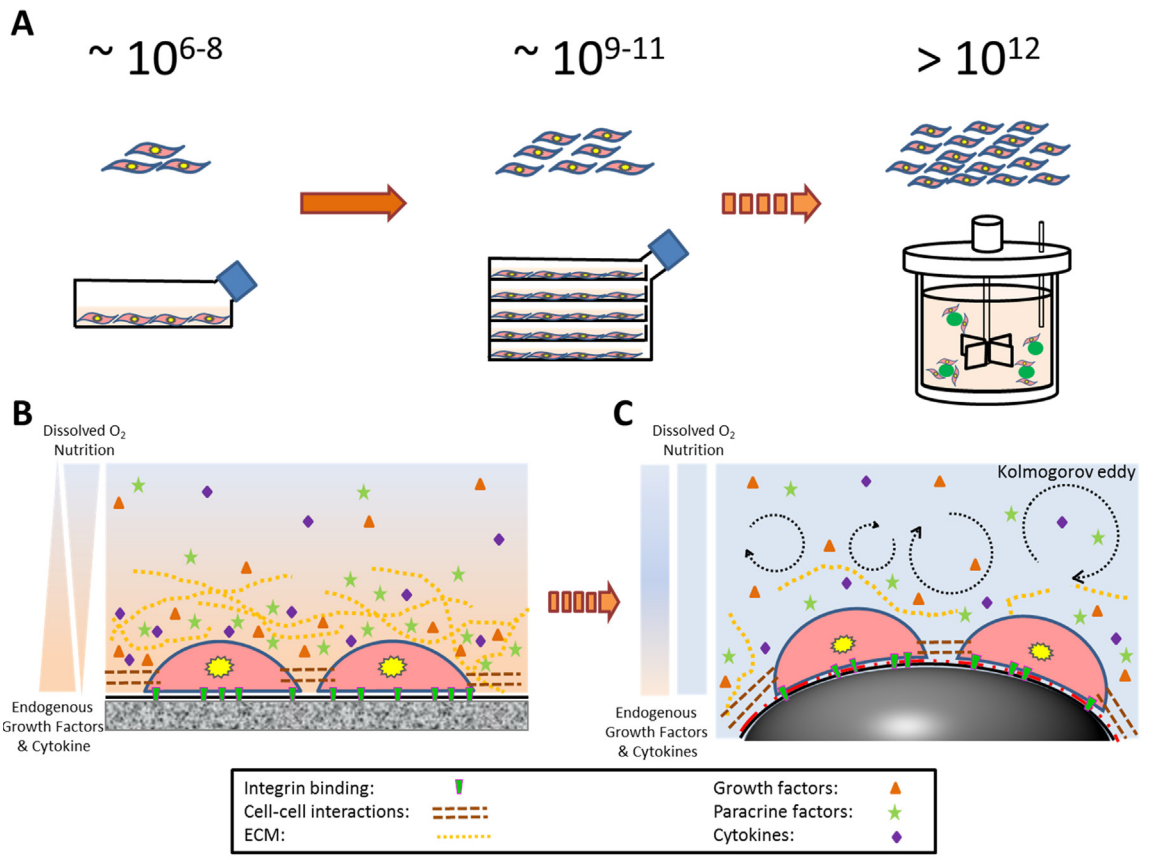
Culture parameters	MSC source	Expansion conditions	In vitro properties	In vivo functions	Reference
Passage	hBMSCs	Passaging up to P5	↓Proliferation and trilineage differentiations	↓Bone forming efficiency in mice	[50]
	hBMSCs	Passaging up to P5	↓Surface antigen expression of CD106, CD146, CD9, integrin alpha 11	n/a	[51]
	hASCs hBMSCs	Passaging up to ~P25 (~60 PD)	↑Population heterogeneity; ↓Proliferation, CFU-F, and differentiations	n/a	[52]
	hBMSCs	Passaging up to P16	↑DNA methylation, genetic modification, and senescence	n/a	[53]
	hBMSCs	Passaging up to P5	↑Cell size, population heterogeneity	n/a	[54]
	hBMSCs	P0 (freshly isolated)	↑CFU-F	↑Osteogenicity in a engineered bone substitutes	[55]
	hBMSCs	Expansion for 2–3 months	↑Senescence, changes of DNA methylation patterns, epigenetic modification; ↓Differentiation	n/a	[56]
Density and confluence	hBMSCs	Clonal expansion	↑Differentiation into cells of all 3 germ layers	↑Engraftment after intra-myocardial transplantation into mice with myocardial infarction; ↑Onset angiogenic and antiapoptotic cytokines secretion	[57]
	hBMSCs	Clonal strains of BMNC	↑Expression of CD146	↑Developing heterotopic bone marrow in vivo by regulating Ang-1 production	[58]
	hBMSCs	Plating density at 100 cells/cm^2	↑Clonogenity and migration capacity associated with surface antigen CD49f and PODXL	↑Retention rate after intravenous infusion into mice with myocardial infarcts	[59]
	hBMSCs	Plating density at 100 cells/cm^2	↑Motility associated with CXCR4 expression	↑Engraftment in hippocampal region in anesthetized mice after intravenous infusion	[60]
	hBMSCshUMSCs	Plating density (100% confluence)	↑Senescence (β-gal expression); ↓Proliferation and CFU-F	n/a	[21]
	hBMSCs	Density (90% confluence)	↑Immunosuppression of activated T cells	n/a	[61]
	hBMSCs	Density (100% confluence)	↓Expression of surface antigen CD146, NG2; ↓CFU-F, differentiation, and proliferation	n/a	[62]
Accumulative oxidative stress	hBMSCs	Atmospheric oxygen (21%)	↓Proliferation and differentiation; ↑Senescence and growth arrest	n/a	[63]
	hBMSCs	Oxidative stress (gamma irradiation)	↑Senescence, altered secretory profile in response to inflammation; ↓Migratory capacity	↓Protective activity in a murine model of LPS-induced lethal endotoxemia	[64]

Abbreviations: Human bone marrow derived mesenchymal stem cells (hBMSCs); human adipose derived stem cells (hASCs); human umbilical cord mesenchymal stem cells (hUMSCs); colony forming unit-fibroblast (CFU-F); lipopolysaccharides (LPS); bone marrow nucleated cells (BMNC); Angiopoietin-1 (Ang-1); increased (↑); decreased (↓).

planar plastic culture, hMSC undergo sharp transition from division with high expression of genes related to cell cycle and DNA repair and replication to the genes regulating cytoskeleton and extracellular matrix [20], highlighting the temporal changes in hMSC properties in culture. Additionally, cell–cell contact in hMSC in culture leads to ROS accumulation and induce senescence that significantly alters their secretory profiles [21]. In planar culture, strategies such as low oxygen tension and addition of metabolic modulators have been used in hMSC expansion to better preserve their clonogenic properties during culture expansion [22,23]. While scalability remains a focus in bioreactor development, understanding the impact of microcarrier surface and physiochemical properties on the development of hMSC microenvironment and its role in large-scale expansion informs the development and optimization of hMSC biomanufacturing process.

3.2. Mechanosensitivity

hMSCs are mechanosensitive and highly responsive to biophysical properties of their immediate environment such as elasticity



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	Components of Culture Environment	Planar Culture	Microcarrier Suspension in Bioreactors
Surface Properties	Stiffness	Rigid	Varied
	Coating / Charge	Standard Tissue Culture Treated / Anionic	ECM-Coated / Cationic or None
	Geometry	Planar	Curvy
Media Environment	Working Volume / Primary Transport	A Thin Layer / Diffusion	Bulk / Convection
	Agitation / O_2 & Nutrition Distribution	None / Gradient	Stirred / Well-mixed
	Fluid Impact / Shear Stress Distribution	None / None	Shear Stress / Heterogeneous
	Cell-Cell Collision	None	Frequent
Endogenous Environment	Cell-Cell Interaction	High	Low
	ECM & GF Accumulation / Paracrine Effect	High / Large	Low / Small

Fig. 1. (A) Planar culture plates routinely used in the laboratory can be expanded to parallelized monolayer culture devices such as CellFactory™ to produce hMSC up to 10^9–10^{11}. Further increase in lot size requires the development of the fully automated bioreactor system such as suspension microcarrier bioreactor. (B, C) Comparison of hMSC microenvironments between the planar culture plastics and the microcarriers in suspension. (D) Summary of the key components of the hMSC microenvironments on planar culture plates and microcarriers.

or topographical features. hMSC sense and response to substrate stiffness through cell adhesion apparatus, cytoskeleton, and signaling pathways such as RhoA-Rock [24]. It has been observed that hMSC spreading on culture substrate with high rigidity enhances osteogenic differentiation, whereas intermediate stiffness promotes muscular lineage and soft substrate leads to neuronal differentiation [25]. On the substrate of high stiffness, hMSCs are able to form mature focal adhesion with more extensive and robust stress fibers that promote osteogenic differentiation. On the soft substrate, MSCs exhibit fewer and less developed focal adhesion with weak stress fibers, which favors the expression of adipogenic genes. Regulation of cytoskeletal tension through geometric cues has similar effects on hMSC fate and guides their lineage specification. In the presence of soluble factors permitting osteogenic and adipogenic differentiation, hMSCs at the perimeter of concave islands, which are under higher mechanical stress, prefer to differentiate into osteogenic lineage. In contrast, hMSCs at the interior of the island region have a low degree of stress and tend to differentiate into adipocytes [26]. In addition to single cells, geometric cues also regulate hMSC fate at the multicellular level with lower cytoskeletal tension in multicellular patterns better maintained the expression of MSC phenotypic makers [27]. Beyond multilineage potential, hMSC also exhibit stiffness dependent secretion of secretion of trophic factors and angiogenic properties with maximum influence on tubulogenesis observed from fibronectin-modified hydrogel at 40 kPa [28]. Significant upregulation of IL-8, uPA, and VEGF has also been reported for hMSC cultured on harder hydrogels of 20 kPa compared to soft ones of 2 kPa [29].

These results highlight the importance of the biomechanical cues on hMSC properties and the need to investigate the impact of microcarrier stiffness and topographical features in large-scale

expansion. Many microcarriers used in hMSC expansion are derived from base materials through polymerization with varying degree of hydration and cross-linking, leading to a large variation in microcarrier biomechanical properties [30,31]. In addition to substrate stiffness, topographical features of the substrate surface at nanometer scale regulate hMSC fate and induce a switch from osteogenic stimulation to the maintenance of multipotent markers [32]. Interestingly, recent study has shown that hMSCs are able to retain mechanical information from past physical environments and that the mechanical dosing influences hMSC fate decisions through the activation of Yes-associated protein (YAP) and its transcriptional co-factors [33]. Taking advantages of hMSC's mechanosensitivity, modulating microcarrier biomechanical properties provides an opportunity to control hMSC fate in the bioreactor to optimize hMSC growth environment in large-scale culture.

3.3. Fluid microenvironment in suspension microcarrier bioreactor

In biomanufacturing, stirred-tank bioreactors provide a well-mixed environment with precisely controlled pH, nutrients, and dissolved oxygen and vessels up to 6000 L have been implemented for production of viral vaccines using transformed adherent cells [34]. In stirred tank bioreactor, mixing by agitation ensures uniform nutrient distribution but the spatial shear stress distribution is far from homogenous with localized "hot spots" near the stirrer and low shear stress near the top. For the transformed cell lines, the negative impact of shear stress exposure on cell growth and protein production and properties has been well characterized. In the case of hMSCs, exposure to sustained shear stress with large variation may significantly alter cell properties and therapeutic potency without apparent changes in cell phenotype markers. Using DNA microarray and quantitative real-time reverse transcription-PCR analysis, studies have revealed consistent and marked up-regulation of IL-1β and mitogen-activated protein kinase (MAPK) signaling pathway within 1–10 dyn/cm^2 [35]. Other studies have shown that exposure to shear stress as low as 0.3 dyn/cm^2 on planar surface and 0.01 dyn/cm^2 in 3D scaffolds induces hMSC osteogenic differentiation [36,37]. These studies illustrate the need for detailed understanding on how the fluid microenvironment influences hMSC properties in order to optimize bioreactor agitation conditions.

In addition to shear stress, convective flow also disrupts accumulation of the cell-secreted regulatory macromolecuels that are crucial for maintaining hMSC phenotype. hMSCs are potent source of bioactive factors including ECM and growth factor and that the microenvironment formed by the endogenous biomacromolecules profoundly influences hMSC phenotype [38]. In 3D fibrous scaffolds, the endogenous microenvironment plays a key role in regulating hMSC proliferation and osteogenic differentiation and that the convective removal of endogenous FGF-2 in 3D fibrous scaffolds significantly reduces hMSC proliferation and clonogenic activities [39]. Thus, development of microcarriers with high affinity to cell-secreted growth factors or ECM may be an avenue to optimize the microcarrier microenvironment to enhance hMSC proliferation.

4. Perspectives

Advanced cell expansion technology is central to the manufacturing of therapeutically competent hMSC in the translation of hMSC therapy to clinical application. In this process, a major challenge is to address the variation in cell properties and reduced therapeutic potency introduced during hMSC expansion [11]. Large-scale hMSC expansion processes inevitably introduce selective pressure that can alter hMSC properties and the therapeutic outcome. It is important to identify these changes in hMSC production because the unwanted cell population not only increase overall production cost but also introduce side effects due to differing phenotypic or secretory profiles. As discussed in the following, innovations in biomaterial, bioreactor design, and process development play important roles in advancing the cell expansion technology.

Innovation in biomaterials has led to the "synthetic niches" that can be incorporated in large-scale cell expansion to regulate hMSC fate. For example, cell detachment is a critical step for subculturing and harvesting in large-scale microcarrier culture of the adherent cells such as hMSCs. To overcome this barrier, thermal responsive microcarriers using poly(N-isopropylacrylamide) and microcarriers coated with a synthetic ECM composed of a disulfide-crosslinked hyaluronan and gelatin hydrogel were developed to allow for enzyme-free cell detachment under mild reductive conditions or reduced temperature [40,41]. Apart from regulating cell–material interface, a recovery of more than 95% of the hMSCs from microcarriers was achieved by using a combination of trypsinization and increased agitation rate to reduce Kolmogorov eddy sizes [42]. These novel and potentially scalable solutions demonstrate the potential of modulating the physiochemical and biomechanical properties of the microenvironments to overcome the barriers in scaling up cell expansion processes.

Microcarrier bioreactor is an established technology in large scale expansion of adherent cells and offers many advantages. However, compared to pluripotent stem cells (PSCs) or the transformed cell lines that form 3D aggregates or grow as multi-layers on the microcarriers, hMSCs typically grow as monolayers and the formation of 3D structure in adjacent carriers significantly reduces cell proliferation. As a result, hMSC have significantly lower expansion fold compared to PSC in the microcarrier bioreactor which results in low bioreactor productivity and lot size [43]. As hMSCs are highly responsive to biomechanical cues such as stiffness and modulus, microcarriers' biomechanical properties should be characterized and optimized to control hMSC fate during expansion. In addition to the improvement in microcarrier design, incorporation of novel 3D scaffolds in scalable bioreactor system may be an alternative that provides optimal cellular microenvironments while maintaining scalability for large-scale expansion. Over the last two decades, research in tissue engineering and biomaterials has provided a wide range of scaffolds with surface, structural, and mechanical properties that recapitulate the bone marrow niche environment by enriching endogenous ECM and growth factors that preserve hMSC properties. On the other hand, packed-bed bioreactors (PBB) using porous carriers or fibrous scaffolds provide high carrier surface-area to volume ratio and support high density culture up to 10^8 cells/mL in a fully controlled and automated environment that is attractive for Good Manufacturing Practice (GMP) facilities [44].

Ensuring sufficient nutrient and oxygen supply has been an important goal for cell expansion in bioreactor. However, sustained stimulation of nutrient sensing and activation pathways induces senescence with reduced therapeutic potency [45]. On the other hand, environmental preconditioning such as hypoxia, low concentration of oxidative stress, and nutrient deprivation, is effective in enhancing hMSC survival and trophic properties [46]. Compared to the gene transfection strategy targeting specific genes for cell survival or secretion, the environmental preconditioning strategy does not have the risk associated with gene transfection and therefore does not require additional regulatory approval. Some of the strategies such as hypoxia and nutrient deprivation can be readily implemented in large-scale cell expansion processes or as a post-expansion treatment step. Taking advantage of hMSC's responsiveness to environmental cues, targeted hMSC

preconditioning by altering their environmental conditions may provide another implementable strategy to enhance hMSC therapeutic potential in hMSC biomanufacturing.

Apart from bioreactor design, innovation in production process has provided fully closed systems that integrate sequential unit operations from expansion and harvesting to cryopreservation [47]. Integrating cell expansion in a continuous perfusion bioreactor with a tangential flow filtration system for concentration and washing shortened hMSC expansion and process time and demonstrated continued cell recovery without changes in cell properties [48]. These studies demonstrate the potential of integrated cell production in an automated and closed system that is important to ensure product quality and GMP-compliance.

Parallel to the advances in culture tools and processes, research has focused on the identification of critical determinants of hMSC that link their in vitro attributes to in vivo tissue repair and regeneration ability in clinically relevant animal models [49]. The combination of pre-clinical animal model with a broad set of in vitro characteristics provides quantifiable parameters that are predicative of hMSC in vivo potency and can serve as benchmarks for the development of cell manufacturing technology. Establishment and subsequent incorporation of these criteria in the development of cell manufacturing technology will significantly advance the commercial realization of hMSC therapy.

hMSC-based therapies hold significant potential in a wide range of diseases and hMSC-based products are undergoing a rapid expansion. In the development of large scale hMSC production processes, the impact of the culture microenvironment and processing conditions on hMSC properties must be considered and addressed in process design. To this end, understanding the regulatory mechanism of hMSC microenvironment and how it regulates hMSC properties is the rational path to the successful development of hMSC manufacturing technology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2015.07.014

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