Functional Heterogeneity of Mesenchymal Stem Cells: Implications for Cell Therapy

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ABSTRACT

The term mesenchymal stem cell (MSCs) was adopted in the 1990s to describe a population of bone-marrow-derived cells that demonstrated the capacity for tri-lineage differentiation at a clonal level. Research conducted during the ensuing decades has demonstrated that MSCs fulfill many functions in addition to connective tissue progenitors including contributing to the HSC niche and regulating the function of immune effector cells of both the innate and adaptive immune system. Despite these advances, fundamental aspects of MSC biology remain indeterminate. For example, the embryonic origin of MSCs and their niche in vivo remains a highly debated topic. More importantly, the mechanisms that regulate self-renewal and lineage specification have also been largely unexplored. The later is significant in that MSC population's exhibit considerable donor-to-donor and intra-population heterogeneity but knowledge regarding how different functional attributes of MSCs are specified at the population level is unknown. This poses significant obstacles in research and in efforts to develop clinical manufacturing protocols that reproducibly generate functionally equivalent MSC populations. Herein, I discuss data demonstrating that MSC populations are intrinsically heterogeneous, elaborate on the molecular basis for this heterogeneity, and discuss how heterogeneity impacts clinical manufacturing and the therapeutic potency of MSCs. J. Cell. Biochem. 113: 2806–2812, 2012. © 2012 Wiley Periodicals, Inc.

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Nearly half a century has passed since Friedenstein and colleagues identified a population of osteogenic progenitors in bone-marrow characterized based on their capacity to grow as colonies in vitro and form hetero-topic osseous tissue in vivo [see Phinney, 2002]. Research based on these seminal discoveries subsequently demonstrated that these osteogenic marrow progenitors were capable of multi-lineage differentiation at both the population and clonal level [Dennis and Caplan, 1996; Pittenger et al., 1999], which led to their re-classification as mesenchymal stem cells (MSCs). In the past decade, MSCs have been shown to exhibit potent tissue reparative properties, which have been largely ascribed to the secretion of paracrine-acting factors that augment endogenous tissue repair programs [Phinney and Prockop, 2007] and that modulate the maturation and function of immune effector cells of the innate and adaptive immune system [English and Mahon, 2011]. Accordingly, much attention has been focused on exploiting these properties to treat a range of human diseases. Indeed, over 100 clinical trials are ongoing or in the planning phase that employ MSC-based therapies [Trounson et al., 2011]. In most cases, clinical administration of MSCs entails large scale expansion of cells in vitro

and a growing number of commercial laboratories and medical research centers routinely manufacture MSCs for human administration.

One critical unresolved issue that impacts the use of MSCs in research and clinical medicine is the paucity of information regarding how their different functional attributes are specified at the population level. In the absence of such knowledge, it is difficult to predict how culture expansion and exposure to extrinsic factors alter population dynamics and function. This problem is exacerbated by the fact that MSC populations exhibit donor-to-donor and intra-population heterogeneity. Intrinsic heterogeneity coupled with large scale clinical manufacturing may explain, in part, why data across MSC-based clinical trials are largely incongruent. Indeed, classification of MSCs as bona fide stem cells dictates that populations are subject to molecular mechanisms that govern fate specification according to established paradigms in stem cell biology. Therefore, while transcriptional regulators that specify osteogenic (RUNX), adipogenic (PPAR-γ) and chondrogenic (SOX9) fates have been extensively studied and extrinsic factors that modulate angiogenic, anti-inflammatory and immune-modulatory

activities are being elucidated, the molecular mechanisms that operate upstream of these factors/pathways that regulate self-renewal and determine progenitor frequency and fate in populations remain largely unexplored. Indeed, most present day research is focused on exploiting the paracrine action of MSCs for a therapeutic intent. In this article, I review evidence demonstrating that MSC populations are intrinsically heterogeneous and provide evidence that cell specification follows a hierarchical program. I also explore questions related to the embryonic origin of MSCs and how this may provide important clues toward unraveling mechanisms driving self-renewal. Finally, I emphasize how a better understanding of the processes regulating self-renewal and lineage specification could be exploited to produce more homogeneous and potent cellular products for clinical therapies.

DONOR–TO–DONOR HETEROGENEITY AND THE VALUE OF CELL SURFACE MARKERS

Numerous studies have demonstrated that MSC populations exhibit donor-to-donor heterogeneity. For example, an analysis of MSCs derived from the bone-marrow of 17 healthy human donors revealed marked disparities in growth rate, alkaline phosphatase levels, and osteogenic potential [Phinney et al., 1999a] and similar differences were also evident in MSCs derived from different inbred strains of mice [Phinney et al., 1999b]. Other studies have confirmed these findings and attributed this donor-to-donor heterogeneity to several factors including sampling bias during marrow aspiration [Muschler et al., 1997; Phinney et al., 1999a], age of the bone-marrow donor [Zhou et al., 2008] and methods used to culture expand populations post-harvest [Lennon et al., 1996; Wagner and Ho, 2007]. The ramifications of these findings with respect to the clinical application of MSCs were immediately evident and spurred efforts to identify unique cell surface markers that could be used to purify cells from marrow to homogeneity. For example, Pittenger et al. [1999] described an isolation procedure that reproducibly yielded human MSC populations that uniformly expressed CD29, CD44, CD71, CD73, CD90, CD105, CD106, CD120a, and CD124 and exhibited tri-lineage differentiation into adipocytes, chondrocytes, and osteoblasts. Additional surface antigens including CD271 [Quirici et al., 2002], CD146 [Sacchetti et al., 2007], SSEA1 [Anjos-Afonso and Bonnet, 2007], SSEA4 [Gang et al., 2007], and nestin [Mendez-Ferrer et al., 2010] have also been described that may be used to prospectively isolate MSCs from bone-marrow. However, antibodies against each of these markers have been shown to yield the entire compliment of colony forming unit-fibroblasts from bone-marrow. Therefore, they all identify MSCs but not uniquely. Consistent with these results, a European consortium recently reported that the MSC phenotype is specified by 113 transcripts and 17 proteins and that these proteins in combination distinguish MSCs from hematopoietic, endothelial, and periosteal cells and synovial fibroblasts [Charbord et al., 2011]. Therefore, the question remains as to whether MSCs express any unique surface epitopes and more importantly whether epitopes described to date have value in predicting MSC function. Initial studies suggested the answer to this question was no. For example, several groups reported that MSCs

loose multi-potency with continued passage without exhibiting discernable changes in surface phenotype [DiGirolamo et al., 1999; Banfi et al., 2000; Pittenger et al., 2001] and Kuznetsov et al. [1997] demonstrated that a significant percentage of MSCs that undergo osteogenic differentiation in vitro failed to form hetero-topic osseous tissue in vivo, indicating that surface phenotype does not provide a rigorous assessment of differentiation potential. More recent studies have shown that changes in plating density results in dramatic changes in expressed levels of some surface antigens including CD146 [Sacchetti et al., 2007] and podocalyxin-like protein [Lee et al., 2009] and that fractionation of populations based on sorting with CD271, W8B2, and CD56 discriminates those with chondrogenic versus adipogenic potential [Battula et al., 2009]. Therefore, these studies indicate that at least a subset of surface epitopes or combinations thereof may be used to monitor growth, potency, and effects of cell density.

INTRA–POPULATION HETEROGENEITY

Our laboratory was one of the first to catalog the transcriptome of human MSCs, which revealed expressed transcripts encoding a diverse repertoire of proteins that regulate angiogenesis, hemato-poiesis, cell motility, neural activities, and immunity and defense [Tremain et al., 2001; Baddoo et al., 2003]. Based on these findings, we argued that single cells were unlikely to posses all properties ascribed to MSC populations and postulated that different functional attributes were relegated to distinct sub-populations, which was initially supported by immuno-staining analysis [Baddoo et al., 2003]. Subsequent studies showing that expression of brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) was clonally restricted in human MSC populations and that only clones expressing these neurotrophins were capable of supporting survival and neurite outgrowth in SH-SY5Y cells and primary dorsal root ganglion explants confirmed that specific MSC functions are relegated to distinct sub-populations. Collectively, these data revealed that the composition of MSC populations is more complex at a biochemical level then previously envisioned and suggested that selective pressures imposed on populations by long-term or large-scale expansion in vitro may greatly impact the composition, function, and therapeutic potency of populations.

Further evidence demonstrating that MSC populations are functionally heterogeneous comes largely from studies evaluating tri-lineage differentiation potential. For example, studies by Muraglia et al. [2000] involving analysis of 185 non-immortalized human MSC clones demonstrated that populations were predominantly comprised of tri-potent, osteo-chondrogenic, and osteogenic progenitors. In this study, clones restricted to the osteo-adipogenic, adipo-chondrogenic, adipogenic, or chondrogenic lineages were not observed, which suggested that fate specification toward connective tissue lineages followed a simple linear progression. A subsequent study evaluating a large panel of hTERT immortalized human MSC clones identified seven of eight possible categories of potency with respect to tri-lineage differentiation potential [Okamoto et al., 2002]. Herein only clones that lacked adipo-chondrogenic potential were

not detected. However, only 5% of clones were found to be tri-potent and 66% failed to exhibit any differentiation potential, which contrasted sharply with results from the previous study. Most recently, Russell et al. [2010] developed culture conditions that were capable of supporting all eight possible categories of tri-lineage progenitors in human MSC populations as determined by clone splitting assays. In these studies the most prevalent clones identified from two separate human MSC donor populations were those exhibiting tri-potent, osteo-chondrogenic and osteogenic potential, which was consistent with previous studies [Pittenger et al., 1999; Banfi et al., 2000; Muraglia et al., 2000]. However, the vast majority (∼50%) of clones were tri-potent and only a small percentage (<5%) lacked any differentiation potential. Subsequent studies demonstrated that tri-potent clones proliferated at a significantly ($P < 0.01$) higher rate than uni-potent clones and possessed a significantly lower ($P < 0.01$) rate of apoptosis [Russell et al., 2011]. Collectively, these studies indicate that tri-lineage differentiation potential is specified hierarchically within populations and this conclusion is supported by computational models showing that variations in the growth rates of secondary colonies established from primary human MSC clones results from the fact that populations are hierarchically structured [Sengers et al., 2010]. In these studies, it is important to remember that only approximately 50% of cells within a population undergo clonal expansion and 50% of clones exhibit tri-lineage differentiation potential. Therefore, one in four cells may be classified as tri-potent progenitors and nearly half of cells within a population are of indeterminate function. Based on these data it appears that tri-potent clones yield more restricted bi-potent progenitors of variable frequencies and that bi-potent progenitors yield slow growing precursors restricted to a single

lineage, which exhibit an increased rate of apoptosis (Fig. 1). Whether bi-potent progenitors can trans-differentiate or de-differentiate, for example, move laterally or backwards within the hierarchy, is not know. It is also unclear to what extent the hierarchy is influenced by stochastic and deterministic events but the fact that intrinsic differences in progenitor frequency exist between donor populations suggest that stochastic events play some role in the process.

Unfortunately, knowledge regarding how functions other than tri-lineage differentiation are specified within MSC populations remains largely unexplored. We have surveyed via RT–PCR transcript levels encoding various transcription factors, signaling molecules, and proteins with immuno-suppressive and anti-inflammatory activity in human MSC clones and this analyses has indicated that most transcripts exhibit some level of clonal restriction. For example, we found that expressed transcripts encoding several natural occurring antagonists of the WNT and BMP signaling pathways including DKK1, DKK3, and Gremlin are significantly more abundant in clones as compared to parental populations, and in general are enriched in tri-potent progenitors (Donald G. Phinney, personal communication). These results suggest that MSCs employ some form of autocrine or paracrine mechanism to inhibit activation of pathways that promote lineage specification. Additionally, our analyses revealed that some transcripts were clonally restricted but not correlated with potency and others were more abundant in bulk populations as compared to clones (Fig. 1). Collectively, these results clearly refute the notion that MSC populations are functionally homogeneous and uniformly multi-potent and indicate that lineage specification is a highly complex process.
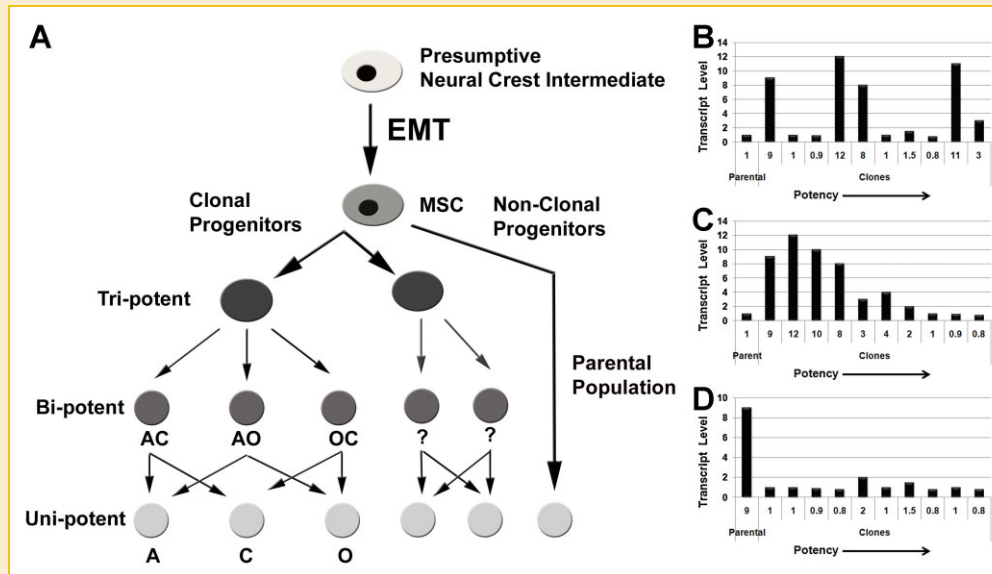

Fig. 1. Lineage specification in MSCs follows a hierarchical program. A: Schematic illustrating proposed hierarchy of MSC lineage specification. Specification of adipogenic (A), chondrogenic (C) and osteogenic (O) progenitors is based on data from Russell et al. [2010]. Other progenitors with distinct function, for example, immuno-modulatory, anti-inflammatory, etc. are presumed to exist based on RT–PCR analysis of clonally derived populations (see B–D). Relationship between progenitors of different potency/function is indeterminate and therefore not indicated. B–D: Graphical illustrations showing trends in mRNA expression data for encoded proteins secreted by MSCs. Transcripts encoding some proteins are clonally restricted (B) and correlated with tri-lineage differentiation potential (C) and others are more abundant in parental population as compared to clones (D).

SPECIFICATION DURING EMBRYOGENESIS

One difficulty in delineating the molecular mechanisms that regulate MSC self-renewal and fate determination is the lack of consensus regarding the origin of the MSC lineage during embryonic development. The widespread tissue distribution of MSCs or MSC-like cells has been interpreted to indicate that the cells reside in the vascular pericyte population in vivo. This concept is consistent with studies showing that MSCs express antigens detected on pericytes, endothelial and pervascular cells and that post-capillary venule pericytes from bone-marrow and peri-vascular cells from most tissues exhibit MSC-like characteristics [Shi and Gronthos, 2003; Crisan et al., 2008]. However, while the surface phenotype and transcriptome of peri-vascular cells, pericytes and fibroblasts resemble that of marrow-derived MSCs [Covas et al., 2008, pp. 7–9], the latter generally lack the contractility of pericytes and all four lineages exhibit distinct differences in differentiation potential. For example, the contribution of post-natal skeletal muscle pericytes to muscle fiber formation is much greater [Dellavalle et al., 2011] and the capacity of placental-derived MSCs to generate bone and cartilage is much weaker compared to marrow-derived MSCs. Moreover, marrow-derived MSCs generate hetero-topic osseous tissue in vivo whereas dental-pulp derived MSCs produced dentin and pulp tissue [Batouli et al., 2003].

Alternatively, several studies have suggested that MSCs are derived from neuro-epithelium via a neural crest intermediate during development. For example, Takashima et al. [2007] demonstrated that culture expansion of embryonic stem cells (ESCs) under conditions that drive mesodermal specification, for example, serum-containing media, yielded a PDGFRα^+ lineage restricted to the adipogenic lineage. In contrast, exposure of ESCs to retinoic acid, which drives differentiation into a SOX1$^+$ neuro-epithelial intermediate, followed by culture in serum-containing media also yielded PDGFRα^+ cells but this population displayed phenotypic and functional characteristics of MSCs, for example, extensive growth and tri-lineage differentiation. Consistent with these findings, Mendez-Ferrer et al. [2010] demonstrated that bone-marrow cells isolated based on expression of the neuro-epithelial marker nestin behave functionally as MSCs and exhibit the capacity to serially regenerate hetero-topic osseous tissue in vivo. Based on these findings, one may assume that specification of the MSC lineage from a neuro-epithelial intermediate occurs via an epithelial-to-mesenchymal transition (EMT), a process that plays a well established role in cellular diversification during development [Thiery et al., 2009]. Moreover, the transcription factor TWIST1 has also been implicated in mesoderm specification and is known to function as a potent inducer of the EMT program. By association, one may assume that TWIST also plays an important role in lineage specification of MSCs and indeed several lines of evidence support this hypothesis. For example, ectopic expression of TWIST1 blocks osteogenic and chondrogenic differentiation of human MSCs [Isenmann et al., 2009] and FGF2-induced expression of TWIST2 blocks tri-lineage differentiation of primary mouse MSCs [Lai et al., 2011]. Moreover, forced expression of TWIST1 in mammary epithelial cells has been shown to generate mesenchymal derivatives with MSC-like properties [Battula et al., 2010]. Collectively, these studies provide strong evidence that MSCs originate from the neural crest via an EMT and that TWIST1 plays an important role in lineage specification of these cells.

DISCRIMINATING BETWEEN SELF–RENEWAL, PROLIFERATION, AND IMMORTALIZATION

Deciphering the molecular mechanisms that regulate MSC self-renewal is also hindered by misconceptions about the process and the paucity of suitable assays to examine it in mammalian cells. For example, self-renewal describes a process whereby a single stem cell divides to generate an identical daughter stem cell(s) and/or a committed progenitor(s). Asymmetry with respect to fate determination in daughter cells may be acquired by polarized segmentation of intrinsic factors such as membrane, cytoplasmic and nuclear proteins or DNA. These processes have been aptly demonstrated in stem cell populations of lower organisms [Knoblich, 2010] and appear to be conserved in mammals [Morin and Bellaiche, 2011]. However, demonstrating asymmetric self-renewing cell divisions in mammalian adult stem cells has proven to be difficult. For example, HSC self-renewal is evaluated almost exclusively by repopulation assays in vivo due to a lack of culture conditions that promote sustained self-renewal of these cells in vitro. Similarly, Friedenstein and Kuralesova [1971] and Mendez-Ferrer et al. [2010] have both demonstrated that MSCs exhibit the capacity to serially regenerate a hetero-topic osseous tissue in vivo and as such undergo self-renewal. However, dissecting the process in vitro is more difficult. To circumvent this difficulty, an emerging trend has been to erroneously equate self-renewal with a sustainable high rate of cell proliferation in vitro. Consequently, a number of researchers have claimed that MSCs undergo self-renewal based on their sustained growth rate and tri-lineage differentiation potential over prolonged passage. However, these properties do not distinguish between progenitor cells and bona fide self-renewing stem cells. It has also led to the concept that stem cells have the capacity for "unlimited" self-renewal, a property not typically evident in vivo.

The problem is most evident when discussing rodent MSCs. Historically, isolation of primary mouse MSCs has proved challenging due to the fact that plastic adherent cultures derived from mouse bone-marrow support sustained granulopoiesis and B-cell lymphopoiesis in vitro. Therefore, attachment to plastic is ineffective in removing hematopoietic cell lineages that persist in these cultures even after serial passage [Baddoo et al., 2003]. Accordingly, a growing number of laboratories have adopted purification schemes that employ long-term culture expansion as a means to select for self-renewing stem cells [Meirelles and Nardi, 2003; Peister et al., 2004; Li et al., 2008]. Herein, culture expansion of the plastic adherent fraction of marrow for weeks to months under standard conditions selects for a sub-population of rapidly growing cells that are isolated by cloning or limiting dilution. However, these populations survive in culture for over 50 passages in vitro and therefore resemble immortalized cell lines. Indeed, immortalization occurs at a much higher frequency in rodent versus human populations due to differences in checkpoint control mechanisms [Wadhwa et al., 2004]. For example, growth restrictive conditions

have been shown to select for cells with inactivating mutations in p53, a protein mutated in the vast majority of immortalized rodent cell lines [Harvey and Levine, 1991]. Recently, we reported that primary mouse MSCs are sensitive to oxygen-induced growth arrest and that oxygen sensitivity is mediated via a p53-dependent mechanism [Boregowda et al., 2012]. Therefore, based on these data it is clear that long-term exposure to atmospheric oxygen selects for sub-populations of rodent MSCs with deficient p53 function, which allows escape from oxygen-induced growth inhibition, and explain why populations isolated via this approach exhibit unlimited growth potential in vitro and inevitably accumulate karyotypic abnormalities following extended passage. Therefore, these findings illustrate the danger associated with equating stem cell self-renewal with sustained or unlimited cell growth.

INFLUENCE OF HETEROGENEITY ON CLINICAL MANUFACTURING AND THERAPEUTIC EFFICACY

MSCs are now being used clinically to treat a broad array of diseases and the number of indicated uses for the cells continues to expand at a rapid pace. Currently, no standardized manufacturing platform exists although most facilities employ standard release criteria to measure sterility, viability, and chromosomal stability to meet FDA regulations. Ironically, while trial design has a significant impact on the clinical manufacturing process the intended use of the product appears to be less important in the overall manufacturing scheme. For example, in small Phase I/II trials that employ multiple donor populations constraints on cell yield are less stringent, which allows for populations to be minimally expanded before infusion into humans. Minimal culture expansion aids in reducing selective pressures that may alter cellular composition and function. However, if the desired functional attribute is restricted to a minor sub-population, minimal expansion may fail to enrich for this sub-population resulting in poor overall potency in downstream applications. In contrast, demands on yield are much greater in trails that restrict the number of donors and/or enroll large numbers of patients. Herein, cells are typically produced in a batch-wise fashion to ensure sufficient cell doses are available to meet demand. However, large-scale expansion of cells introduces bias into the culture process that is difficult to predict and/or control. While selective processes that operate during culture expansion are anticipated to be largely stochastic in nature, they may also be influenced by cell-based mechanisms and/or extrinsic factors introduced into the culture media. How these processes affect intra-population heterogeneity are largely unexplored and more importantly rarely evaluated post-production. Therefore, it is conceivable that large-scale expansion may select for or against a particular sub-population, thereby enhancing or reducing potency, respectively. Moreover, since these outcomes are hard to predict, one may assume that products produced via different manufacturing processes or by the same processes at different times may vary in composition and function. Indeed, a recent study by Seeger et al. [2007] demonstrated post-manufacturing differences in cell yield, colony forming capacity and functional properties of bone-marrow mononuclear cells and MSCs that were manufactured

using to different clinical trial protocols. These results, together with the growing body of work demonstrating that MSC populations are functionally heterogeneous, warrants development and implementation of specific assays that assess biological potency of MSCs prior to release for clinical administration. For example, Rizzo et al. [2011] recently reported that IL-10 stimulated expression of HLA-G in MSCs is significantly ($P < 0.0008$) positively correlated with inhibition of PBMC proliferation stimulated with PHA. Therefore, this assay may be used to evaluate and compare the immune-regulatory function of manufactured populations. While continued development of such assays is important, it should be noted that in vitro potency assays may not reliably predict cell function in vivo. Recall that the ability of MSCs to form hetero-topic osseous tissue in vivo is not well predicted by in vitro osteogenic differentiation assays [Kuznetsov et al., 1997]. More recently, a long-term follow-up of patients with steroid-refractory acute graft versus host disease (GvHD) that were administered MSCs revealed no correlation between the ability of MSCs to suppress T cell proliferation in mixed lymphocyte assays in vitro and their clinical efficacy in vivo [von Bahr et al., 2011]. Therefore, while issues related to trail design and patient selection have largely been used to explain inconsistencies in MSC-based therapies in humans, donor-to-donor and intra-populations heterogeneity and effects of large-scale expansion on cellular composition and function may also be a contributing factor.

FUTURE OUTLOOKS

The use of MSCs in clinical medicine will likely to continue to grow at a rapid pace. However, it is unclear whether knowledge about how clinical manufacturing affects MSC biology will become sufficiently advanced to fulfill the promise of this therapy. A critical limitation in the field is the lack of insight into the molecular mechanisms that drive MSC self-renewal and lineage specification. In the absence of this knowledge, it is difficult to predict how culture expansion alters the cellular composition and function of populations. Moreover, manufacturing platforms cannot be tailored to drive expansion of determined lineages, which may have unique biological properties suited for a specific therapeutic intent. It is anticipated that more homogeneous cell preparations will yield more consistent clinical outcomes that exhibit both dose responsiveness, something currently lacking based on available data, and more reproducible outcomes. A first step toward bridging the gap between basic research and clinical manufacturing is the development of post-production potency assays that can be used to assess cellular function. In addition, effects of cryopreservation and transport should also be carefully evaluated. Once the potency assays are established, cells can be evaluated post-production in animal models to determine if in vitro metrics of potency correlate with observed clinical outcomes. As mechanism regulating fate specification are delineated, this new knowledge can be exploited in the rational design of improved culture conditions to preserve potency and/or select for specific cellular functions tailored to a particular disease. A critical first step is recognizing that donor-to-donor and intra-population heterogeneity is a critically important aspect of

MSC biology. Only then can future studies aimed at understanding heterogeneity by exploited to enhance the clinical efficacy of MSC-based therapies.

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