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Concise Review: When Colonies Are Not Clones: Evidence and Implications of Intracolony Heterogeneity in Mesenchymal Stem Cells

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ABSTRACT

The emergence of heterogeneity in putative mesenchymal stem cell (MSC) populations during in vitro expansion is not appreciated fully by the various communities who study, engineer, and use such stem cells. However, this functional diversity holds direct implications for basic research and therapeutic applications of MSCs that require predictable phenotypic function and efficacy. Despite numerous clinical trials pursuing MSC therapies, the in vitro expansion of homogeneous populations to therapeutically relevant quantities remains an elusive goal. Variation in MSC cultures has been noted not only among donors and within populations expanded from the same donor, but also debatably within single-cell-derived colonies. The potential for even intracolony heterogeneity suggests that any purified subpopulation will inevitably become heterogeneous upon further expansion under current culture conditions. Here, we review the noted or retrospective evidence of intracolony MSC heterogeneity, to facilitate discussion of its possible causes and potential solutions to its mitigation. This analysis suggests that functional diversity within an MSC colony must be considered in design of experiments and trials for even nonclonal stem cell populations, and can be mitigated or even exploited when the mechanisms of onset are better understood. STEM CELLS *2016;34:1135–1141*

SIGNIFICANCE STATEMENT

The emergence of heterogeneity in putative mesenchymal stem cell (MSC) populations during in vitro expansion is not fully appreciated; however, it holds direct implications for basic research and therapeutic applications of such stem cells. In this concise review, data documenting heterogeneity even within single-cell-derived colonies are highlighted, illustrating that any purified subpopulation will become heterogeneous upon further expansion under current culture conditions and that the common, underlying assumption of MSC clonal purity should be reexamined. The impact of recognizing the existence of such inevitable heterogeneity could lead to studies aimed toward elucidating its causes and engineering its mitigation for improved clinical outcomes.

INTRODUCTION

Bone marrow stromal cells, a subset of which can be classified as multipotent mesenchymal stem cells (MSCs), are cells harvested from bone marrow, isolated by plastic adherence, and expanded in specialized media. Cell populations are typically considered MSCs if capable of colony formation (self-replication [1, 2]), trilineage differentiation (along osteo-, chondro-, and adipogenic mesenchymal tissue lineages) in vitro and, when measured and reported, expression of several cell surface markers (the curated list of which is debated continually) [3]. MSCs can be obtained from adults and donated autologously, and they have been shown to facilitate bone repair in vivo [4]. This functional plasticity has motivated much laboratory-scale research to engineer MSC function in vitro, including chemical and physical cues that promote differentiation toward tissue engineering applications [5, 6]. Additionally, MSCs are reported to secrete bioactive molecules with immunomodulatory and anti-inflammatory properties [7, 8]. These attributes have prompted exploration of a broad range of therapeutic potentials, with hundreds of clinical trials currently at various stages of progress (http://www.clinicaltrials.gov) for conditions ranging from bone defects to liver cirrhosis to autism.

The term "MSC" has been used confoundingly to refer to "marrow stromal cell," "multipotent stromal cell," "mesenchymal stromal cell," or "mesenchymal stem cell," all of which have been loosely defined and debated [3, 9–11]. Despite the currently wide use of the term MSC to indicate stem cell populations in both basic studies of differentiation cues and translational applications, it is increasingly appreciated that current methods of stem cell identification and population expansion can result in functional heterogeneity [12–17]. MSCs require large-scale in vitro expansion for many laboratory-scale studies and also for translational in vivo applications such as implanted grafts or systemic injections: the high clearance rate of MSCs in vivo [14] has been addressed by administration of large cell populations to ensure sufficient cell activity and response. As is the case with culture-expansion of most cell populations in vitro, it is intended and tacitly assumed that the manufactured cell populations are functionally similar to the smaller MSC source population from which they are expanded. This assumption has led to the common practice of characterizing small batches of cells at early passage numbers (in vitro population doublings) to screen for subpopulations from given donor(s) that show characteristics of interest to that application (e.g., surface marker expression or proliferation rate). Typically, this batch-check is not followed by extensive additional characterization upon population expansion that precedes in vivo administration. The increasing development and use of large-scale culture systems such as multitiered flasks and bioreactors can potentially exacerbate this issue, as such vessels are less amenable to microscopic observation of cells (and concomitantly to the identification and documentation of heterogeneity at the basic, morphological level). Thus, emergent heterogeneity within putative stem cell populations imposes limitations for expansion, presents a challenge to efficacy of many potential clinical applications, and has been described by many researchers as a barrier in understanding their basic biological properties and potential uses [12, 16]. From a clinical standpoint, heterogeneity onset in MSCs may plausibly reduce the potential maximum effectiveness of this cell type, in contrast to hematopoietic stem cells (HSCs) that engraft in bone marrow to proliferate further (thus requiring fewer true HSCs in a heterogeneous population of hematopoietic stem and progenitor cells) and have unique, established surface markers that correlate to different cellular functions. These HSC characteristics render that cell type relatively easier to purify into subpopulations of predictable outcomes (e.g., as shown by Oguro et al. [18]).

Here, we highlight the reported evidence for, challenges posed by, possible causes of, and implications of functional heterogeneity among MSCs originating from single-cell-derived colonies. The existence of this heterogeneity—even within apparently clonal populations of MSCs—indicates that any isolated subpopulation of MSCs will inevitably become heterogeneous upon further expansion. These findings and implications for basic and translational research indicate that increased understanding of heterogeneity onset is needed for impactful progress.

Heterogeneity at the Cell Population Level

Heterogeneity in the measurable number, physical characteristics, and in vitro multidifferentiation potential of MSCs has been demonstrated in the context of donor-to-donor variability and within single samples expanded from a single donor, typically sourced from bone marrow [19, 20]. Here, we focus more narrowly on heterogeneity of cells within a single population that is obtained and expanded from a single donor, down to the limit of cells derived from a single mother cell. One of the earliest noted characteristics of MSC heterogeneity was the morphology of adherent cells [21]—which was much later linked to functional heterogeneity [14]—within typical culture expansion conditions. Mets and Verdonk [21] observed a mixture of smaller and larger cells at higher population doublings and commented on two distinct subpopulations: "type I" cells described as exhibiting a typical fibroblast-like morphology, and "type II" cells that exhibited a large, flat, epithelial-like morphology. Type I cells divided rapidly, but reduced in proportion of the total cells with successive passages (and attendant population doublings); however, they also gave rise to nondividing type II cells. Prockop et al. later described the type I cells as rapidly self-renewing cells (RSCs) and the type II as mature MSCs [22]; this group subsequently suggested that sparser cultures (lower number of cells per culture surface area) preserved more of the smaller RSCs within the population. More recently, Whitfield et al. tracked cell lineages via time-lapsed optical microscopy of sparsely seeded MSC cultures and showed that the larger cells were daughters of the smaller cells prevalent at low population doublings in vitro, but had exited the cell cycle and increased in relative fraction of the adherent population [12].

These differences in morphology among subpopulations of bone marrow-derived MSCs have been linked to functional heterogeneity of cells in vitro and subsequently in vivo. For example, the proliferation rates of smaller cells are greater [21, 22], but typical cultures are dominated at high population doublings by the larger cells that are considered either senescent or committed osteoprogenitors [12, 13]. Other biophysical distinctions that have been identified recently include relative cell stiffness and extent of nuclear membrane fluctuations. Specifically, Lee and Shi et al. showed that MSC subpopulations meeting three biophysical criteria—sufficiently low cell diameter ($<20\,\mu m$), low mechanical stiffness (<375 Pa), and high fluctuations of the nuclear membrane ($>1.2\%$)—exhibited higher colony formation, proliferation rates, multilineage differentiation potential in vitro, and multitissue repair in vivo, compared to other isolated subpopulations or to the initially heterogeneous MSC populations [13]. Those biophysically distinct subpopulations did not differ detectably in flow cytometry profiles of so-called stem cell markers (surface antigens) but did exhibit distinct gene expression profiles and levels of secreted factors and cytokines [7]. These functional distinctions among MSC subpopulations have been explored further, to demonstrate how in vivo administration of heterogeneous populations can reduce clinical efficacy in at least some specific cases. For example, recovery of lethally irradiated mice was improved significantly upon administration of a sorted subpopulation of high-diameter ($>20\,\mu m$) mesenchymal stromal cells ($>80\%$ survival at 50 days post-irradiation, compared with 20% survival for administration of a heterogeneous MSC population and 0% survival for low-diameter MSC subpopulations) [14]. That study additionally reported that the secreted factors from high-diameter cells on explanted, irradiated bone tissue elicited a pro-angiogenic effect not observed when using the secretome

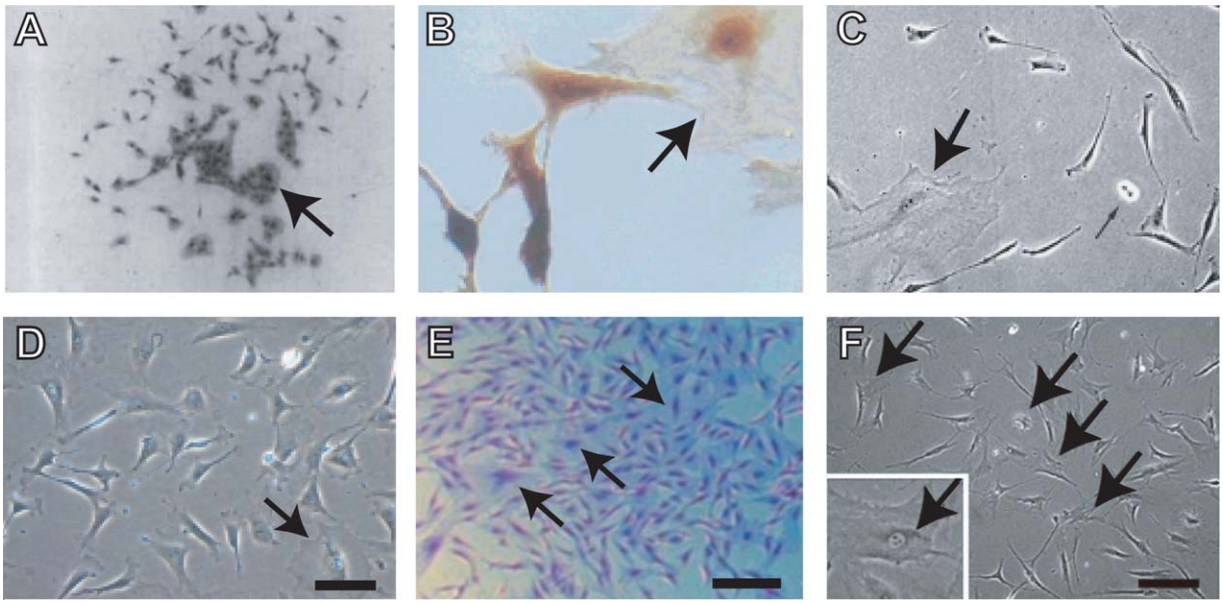

Figure 1. Historical indicators of morphological heterogeneity within colonies and colony-derived populations of mesenchymal stromal cells. Arrows indicate at least one large, flat, type II cell amidst a single-cell-derived colony or colony-derived population containing several small, spindle-shaped, type I cells. **(A)**: A colony of human mesenchymal stem cells (MSCs) at passage 7 and low seeding density, stained with May-Grunwald and Giemsa (×25) (Mets and Verdonk [21]). **(B)**: Cells from a rat MSC colony-derived population (expanded from a single-cell-derived colony initially grown at 10 cells per cm^2), stained brown for neuron-specific enolase (NSE) expression (×350) (Woodbury et al. [25]). **(C)**: A colony of human MSCs at passage 3, initially plated at 3 cells per cm^2 (×180) (Colter et al. [22]). **(D)**: A colony-derived population of adult mouse PDGFRα$^+$Sca1$^+$CD45$^-$TER119$^-$ MSCs. The colony was derived from a single cell initially plated at ∼13–26 cells per cm^2 and underwent several passages before imaging (scale bar = 100 μm) (Morikawa et al. [27]). **(E)**: A colony of adipose-derived MSCs (P1) stained with 0.5% crystal violet, initially plated in 6 cm dishes at a density of 1×10^3 (scale bar = 100 μm) (Vishnubalaji et al. [26]). **(F)**: A colony of LNGFR$^+$THY1$^-$ human MSCs. Colonies were assumed to have grown from single, isolated cells in wells of a 96-well plate. Arrows included from original authors were used to indicate cells with larger apparent cytoplasm (scale bar = 100 μm) (Mabuchi et al. [15]). All images are reprinted with permission.

of a heterogeneous population of cells. Together, these and other findings have demonstrated that in vitro-expanded MSC populations exhibit reduced multipotency and reduced proliferation rates over extended population doublings that can be attributed to the emergence of a replicatively senescent osteoprogenitor subpopulation [12, 13].

Evidence for Intracolony Heterogeneity

There is also increasing evidence of heterogeneity within single-cell-derived colonies [3, 17, 21, 23]. It has been reported that colonies derived from single cells, each of which were selected initially from the same isolated subpopulation, differ in even the most basic characteristics (such as colony size and degree of osteogenic induction [24]). Several researchers have also shown that colonies derived from single cells which each originated from a given heterogeneous population (e.g., a sparsely seeded culture flask) also differed from one another [15, 17, 21]. A more controversial assertion is that cells within a given MSC colony become functionally heterogeneous as the number of cells within the colony increases. Single-cell-derived colonies are considered "clonal" populations [2] and are tacitly assumed to be a homogeneous population, given that all cells originated from the same mother cell and replicated under very similar culture conditions. However, several instances of MSC intracolony heterogeneity have been demonstrated to varying degrees of recognition and conviction, which we summarize below.

Some of the simplest observations of MSC intracolony heterogeneity come from observing multiple morphologies within an apparent colony or colony-derived population. Figure 1 provides six examples of images suggesting such morphological intracolony heterogeneity, published as part of broader studies of MSCs. Four of these studies noted this visually apparent heterogeneity [15, 21, 22, 25], while two did not comment on these image features explicitly [26, 27]. Even the initial accounts of morphological differences by Mets and Verdonk [21] claimed single-cell-derived colonies containing mixed morphologies (Fig. 1A). Large, flat cells touched in small clumps, while small, spindle-shaped cells were observed around part of the colony periphery. Woodbury et al. [25] presented images (Fig. 1B) of several colony-derived rat MSC populations cultured in neuronal induction medium, noting that some of these induced populations contained a large, flat cell unresponsive to induction. Colter et al. [22] also reported and commented on single-cell-derived colonies containing multiple morphologically distinct subpopulations (Fig. 1C; [22]). In 2009, Morikawa et al. [27] presented several images (one of which is shown in Fig. 1D) of colony-derived adult mouse MSC populations and did not note these morphological differences among the colony-derived populations. Morphological heterogeneity was also apparent in adipose-derived MSC colonies (Fig. 1E) [26], although again this feature was not highlighted by the authors. Most recently, as part of a larger study aiming to identify new MSC surface markers, Mabuchi et al. reported that colonies derived from single LNGFR$^+$THY-1$^-$ cells (i.e., cells positive for surface marker CD271 and negative for CD90, but otherwise

considered bone marrow MSCs under standard isolation procedures of plastic adherence and colony formation; see Supporting Information) contained several cells that were not spindle-shaped (Fig. 1F; [15]).

Ylostalo et al. [17] focused directly on MSC intracolony heterogeneity in terms of both morphology and gene expression, comparing cells in inner and outer regions of colonies. The authors commented that cells in the inner region appeared larger than cells at peripheries, although this comparison was not quantified and is difficult to discern from the images provided. Ylostalo et al. also found that cells extracted from the inner regions of colonies were upregulated for genes associated with extracellular matrix production, while cells near the periphery were upregulated for cell cycle genes. Seven years prior, Tremain et al. [23] reported that an MSC colony showed both early and late markers for multiple lineages, using microserial analysis of gene expression (microSAGE). Because a single MSC could not express genes for multiple lineages, they reasoned, different MSCs within the colony must have committed to different lineage paths. Additionally, Tremain et al. calculated the percentage of cells (derived from the same mother colony) that expressed markers selected for validation of microSAGE results via fluorescence-activated cell sorting. Only three of the twelve markers tested were expressed by 100% of the cells, while the other nine markers were expressed by 5.7%–90% of the colony-derived population. Tremain et al. concluded from these data that a typical single cell-derived MSC colony is heterogeneous.

Identifying Possible Causes of Heterogeneity

Experimental designs that can enable accurate and facile identification of single-cell-derived MSC colonies remain an important and outstanding component of well-controlled MSC studies, particularly when studying possible causes of heterogeneity onset. Very few recent studies have demonstrated unequivocally that the colonies analyzed were derived from single cells, although this is often asserted as such. In studies involving presumably isolated single cells, microwells are checked typically for the presence of a single colony days after plating; wells identified as containing zero colonies or multiple colonies are omitted from analysis. However, this process does not verify that these isolated colonies each started from a single cell, and a colony that originated from two cells in close proximity has high likelihood of exhibiting heterogeneity. Supporting Information Figure S1 graphically summarizes the practical challenges of confirming single-cell-derived colonies in vitro, as well as the potential scenarios that could result in apparent intracolony heterogeneity with such microwell formats. To draw conclusions about MSC heterogeneity or responses to stimuli, it is critical to confirm thoroughly that MSC colonies undergoing analysis indeed originated from single cells. Techniques used to acquire such validations are further discussed in Supporting Information.

The significant potential for intracolony heterogeneity prompts renewed consideration of the causes of heterogeneity onset and, when desirable, subsequent engineering of its mitigation. In so doing, it is first helpful to report and describe cell population origins clearly. The emergence of morphological and/or functional heterogeneity in a population expanded from a single cell implies that any purified stem cell population can become heterogeneous upon expansion under standard culture conditions. However, the term "clone" colloquially implies an exact copy and is often used interchangeably with the word "colony." Therefore, consistent use of the term colony to describe an intact group of cells originating from the same mother cell and "intracolony" to describe differences among those progeny can promote clarity in exchange of information across disciplines and fields that use MSCs. (The interested reader is referred to Supporting Information for more detailed discussion of such distinctions.)

Second, it is important to reflect on the factors considered by the field as potential causes of heterogeneity in culture-expanded MSC populations. "Spontaneous differentiation"—that is, commitment toward a lineage without the purposeful addition of chemical or mechanical induction cues—has been mentioned occasionally in MSC literature with speculation of its causes. For example, DiGirolamo et al. [20] reported that a portion of the colonies studied began to deposit mineral (indicative of osteogenic commitment) in the absence of induction. They proposed that perhaps the particular serum they used in that study contained osteogenic-inducing factors. Their speculation relates to a separate and larger issue of culture condition inconsistency among MSC studies: even within a single research group, differing culture media compositions, undefined sera obtained from different lots, MSCs harvested from different tissue sources or by different methods of isolation, and different cell culture systems among studies can each confound interpretation of results regarding heterogeneity. Banfi et al. [28] also commented that levels of osteocalcin expression increased in MSC populations cultured in long-term, unstimulated conditions. They speculated that the osteogenic pathway may be the default tissue lineage for an MSC population, but that the in vitro culture conditions (in which we would include the stiff tissue culture polystyrene as one descriptor) were also possible causes. The wider debate as to the correlational and causal effects of extracellular mechanical cues on MSC differentiation is ongoing and beyond the scope of our present analysis of intracolony heterogeneity under otherwise constant culture conditions.

Another possible contributing factor to heterogeneity onset is high cell confluency. Higher cell densities in noncolony expansions ($>$200 cells per cm^2) are reported to exhibit decreased growth rates and colony formation [29]. This culture density-dependent decrease can be related to Ylostalo et al.'s observation that cells at the center versus the periphery of a colony differed by upregulation of genes associated with extracellular matrix (ECM) production versus proliferation, respectively [17]. These phenomena may be attributable to several possible causes, including localized nutrient and protein gradients, increased cell-cell contact (shown to be correlated with increased chondrogenic differentiation in MSCs [30]), ECM modifications, or cell signaling among the various subpopulations (for instance, through exosome-mediated micro- and mRNA exchange, reported to occur in MSCs by Olson et al. [31], Tomasoni et al. [32], and Chen et al. [33]). However, to our knowledge, these mechanisms

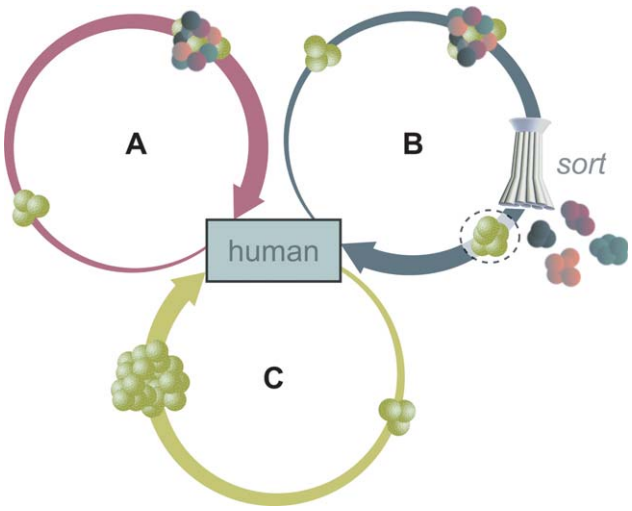

Figure 2. Implications of mesenchymal stem cell (MSC) heterogeneity. **(A)**: Current methods for autologous MSC administration involve harvesting bone marrow stromal cells, expanding cells in vitro to result typically in heterogeneous populations, and delivering this population to the human recipient. **(B)**: Label-free sorting of such culture-expanded subpopulations, for example, via biophysical markers, can enable delivery of a more narrowly defined population with the potential for more predictive outcomes. This method, however, results in a reduction of the number of cells available per source cell population. **(C)**: Alternatively, identification of the cause(s) of MSC heterogeneity onset can enable development of culture protocols that minimize such heterogeneity onset. Such understanding and methods could facilitate harvest and expansion of desired subpopulations to greater cell numbers and potentially improved outcomes than attainable in scenario (A) or (B).

have not been examined thoroughly as explicit possible causes or correlatives of heterogeneity onset in MSCs and warrant further studies.

Toward this aim, a recent time lapse image analysis study by Whitfield et al. [12] was conducted to quantify and determine possible causes of heterogeneity onset in MSC populations. The authors of that study did not identify any one specific metric described as an extrinsic factor, such as the duration or extent of cell-cell contacts, that was a predictive indicator of whether or not a cell would give rise to at least one senescent daughter cell in vitro [12]. However, their retrospective analysis of time-lapsed data and lineages for non-colony, sparse cultures over 1 week indicated that the lifetime (hours to cell division) of a given MSC was best predicted by the lifetime of its mother cell, suggesting intrinsic factors (such as telomere shortening or aneuploidy—both shown in MSCs to be exacerbated by oxidative stress and linked to senescence [34, 35]—or perhaps epigenetic modifications) and/or unidentified extrinsic factors as potential causes or indicators of heterogeneity onset.

Finally, genetic mutation and uneven partitioning of proteins during cell division have been speculated as possible causes of heterogeneity onset in HSCs, although these potential factors were ruled out by those authors as highly unlikely due to the timescales of the experiments in that study (see Supporting Information) [36]. To our knowledge, no reports to date have considered whether heterogeneity onset in MSCs could be linked to such cell events. Thus, the correlations, causes, and detailed mechanisms by which morphological and functional heterogeneity emerges in culture-expanded MSC populations—including intracolony heterogeneity—remain important questions and open challenges.

Moving Forward: Understanding Implications of Intracolony Heterogeneity

We have summarized multiple studies that demonstrate or suggest that MSC morphological and functional heterogeneity are observed even within single colonies. The implications of such intracolony MSC heterogeneity are significant for both fundamental understanding and practical translation of stem cell biology to humans. Together, these motivate further study of correlations and causes of heterogeneity onset, as well as consideration of new ways to manage or exploit such heterogeneity in culture-expanded populations. Figure 2 illustrates how such heterogeneity, when recognized, can be addressed to minimize or to exploit emergent differences among culture-expanded MSCs. Basic research will benefit from the increased appreciation of the potential for heterogeneity in even commercially available "mesenchymal stem cells" as well as the increased clarity in designing and reporting studies of MSC (sub)populations. Clinical translation will generally require large numbers of cells that will elicit a predictable therapeutic response (e.g., hallmark expression profiles of a stem or a progenitor cell). Emergent functional heterogeneity (Fig. 2A) among cells within the same single-cell-derived colony implies that in vitro expansion of any group of MSCs, however pure, will result in a heterogeneous population when performed with current culture protocols. In the case of large-scale culture systems, direct observation of MSCs is further limited; it is plausible that lower-than-expected yields of expanded MSCs in such systems are related in part to an unknown fraction of large, senescent cells.

Sorting by label-free biophysical markers to achieve homogeneous MSC populations with predictable therapeutic efficacy (Fig. 2B) can improve clinical outcomes. It is not anticipated that all clinical indications for which MSCs are considered therapeutic options will exhibit improved response to administration of MSC subpopulations that are sorted by biophysical or other characteristics. However, this potential for increased effectiveness has been demonstrated for the few in vivo studies that report such comparisons. For instance, the development of an inertial microfluidic spiral channel device has enabled high-throughput, size-based sorting of MSCs [37]. As noted above, Poon et al. [14] showed that such enrichment of MSC subpopulations with larger cell diameter promoted improved recovery of lethally irradiated mice, as compared with response to administration of heterogeneous MSCs or small-diameter subpopulations. Thus, this or other means of identification and separation of desired subpopulations [13], including the potential for use of new and more specific biophysical and/or biochemical sorting approaches, can be pursued to manage emergent functional diversity for large cell populations. However, inevitable heterogeneity from expansion implies that any subpopulation isolated from a larger MSC population must be used without further expansion and that such "useful" subpopulations represent only a fraction of a given population from a single-donor sample. This limitation can significantly dilute quantities

of multipotent stem cells within a putative stem cell population and thus diminish outcomes for at least some clinical applications [14].

Nevertheless, such sorting approaches are engineered solutions to a phenomenon that is not yet fully understood, and thus impactful progress requires increased understanding of the conditions that cause and can mitigate functional heterogeneity among MSCs (Fig. 2C). Such understanding can provide the potential to expand cells to large numbers without promoting heterogeneity, for those specific cases that the research community finds to benefit from reduced functional diversity within the cell population. In fact, these challenges and benefits from addressing emergent population heterogeneity are not limited to MSCs, and they can be considered for other tissue-derived stem and induced pluripotent cell populations. The insight gained by quantifying the systematic emergence and incursion of senescent, nonstem stromal cells in colony-derived MSC populations—for example, via single-cell analyses and gene expression regulation—will enable significantly improved approaches to maintain rapid proliferation, multipotency, and predictably modulated functions of this malleable class of stem cells.

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Author Contributions

D.A.R. and K.J.V.V.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing. K.J.V.V.: final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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