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ON THE ORIGIN AND IMPACT OF MESENCHYMAL STEM CELL HETEROGENEITY: NEW INSIGHTS AND EMERGING TOOLS FOR SINGLE CELL ANALYSIS

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Abstract

Mesenchymal stem cells (MSCs) display substantial cell-to-cell variation. This heterogeneity manifests among donors, among tissue sources, and within cell populations. Such pervasive variability complicates the use of MSCs in regenerative applications and may limit their therapeutic efficacy. Most conventional assays measure MSC properties in bulk and, as a consequence, mask this cell-to-cell variation. Recent studies have identified extensive variability amongst and within clonal MSC populations, in dimensions including functional differentiation capacity, molecular state (*e.g.* epigenetic, transcriptomic, and proteomic status), and biophysical properties. While the origins of these variations remain to be elucidated, potential mechanisms include *in vivo* micro-anatomical heterogeneity, epigenetic bistability, and transcriptional fluctuations. Emerging tools for single cell analysis of MSC gene and protein expression may yield further insight into the mechanisms and implications of single cell variation amongst these cells, and ultimately improve the clinical utility of MSCs in tissue engineering and regenerative medicine applications. This review outlines the dimensions across which MSC heterogeneity is present, defines some of the known mechanisms that govern this heterogeneity, and highlights emerging technologies that may further refine our understanding and improve our clinical application of this unique cell type.

Key words: Stem cells - differentiation, tissue engineering/regenerative medicine, cells/tissue – analytical methods, genetics – gene expression, proteomics.

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Introduction

In tissue engineering applications, the structure and function of the extracellular matrix (ECM) are crucial determinants of the success or failure of an engineered construct. The ECM is created and maintained by resident cells, and hence, the choice of cell source strongly influences construct performance. For example, in engineered cartilage, the quality and organisation of the matrix produced differs substantially based on the cell types employed. Chondrocytes, the cells resident in native cartilage, excel at producing robust extracellular matrices *in vitro*, even under nutrient limiting conditions (Johnstone *et al*., 2013; Kock *et al*., 2012). Unfortunately, chondrocytes are difficult to obtain in sufficient number, and chondrogenically induced stem cells are often used as an alternative. Mesenchymal stem cells (MSCs) are readily obtained from adult tissue, expand well in culture, and can undergo chondrogenic differentiation. However, even with the most effective differentiation protocols, MSCs generally fail to fully match the performance of chondrocytes (Huang *et al*., 2010; Johnstone *et al*., 2013). Such gaps in performance between MSCs and differentiated cell types likely exist, in part, as a consequence of marked variation in the ability of individual MSCs to undergo lineage commitment. Some MSCs robustly undergo differentiation while others fail to do so (Huang *et al*., 2010). While these underperforming, alternatively performing or non-responsive subpopulations hinder the maturation of engineered tissues, their poor performance is often masked by bulk assays that pool signal across entire cell populations (Fig. 1). Recently, given the advent of single cell methods and a growing

appreciation that ensemble measurements can mask important variation, new findings have begun to delineate MSC heterogeneity. Here, we review the current understanding of heterogeneity among and within MSC populations, and discuss how single cell techniques may be used to further parse this variability.

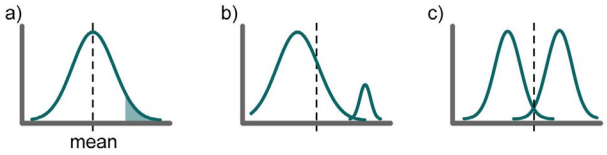


Fig. 1. Bulk observations can mask heterogeneity, including **a**) 'tail' observations, **b**) small subpopulations, and **c**) bimodal behaviour. Inspired by Altschuler and Wu, 2010.

Mesenchymal stem cell heterogeneity

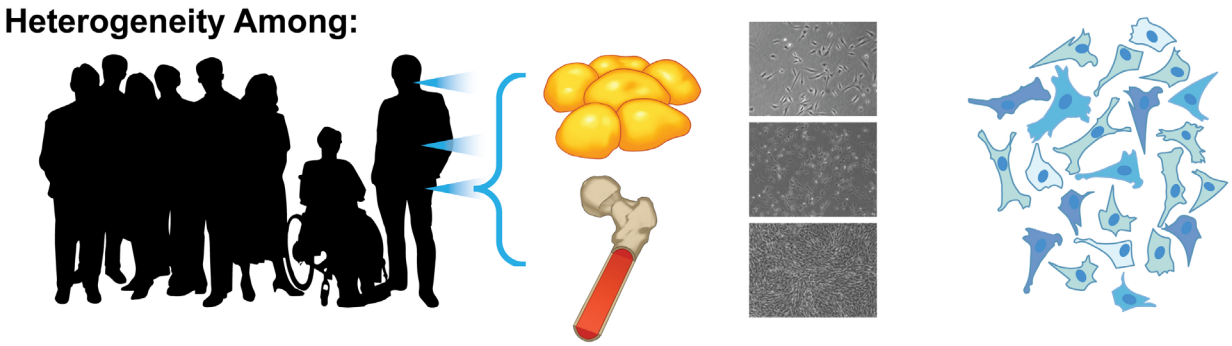
The defining properties of mesenchymal stem cells

As a cell type, MSCs are defined by three criteria. MSCs must: 1) be plastic adherent; 2) express the surface markers CD105, CD73 and CD90, and lack expression of CD45, CD14 or CD11b, CD79 or CD19 and HLA-DR; and 3) be capable of differentiating into osteoblasts, adipocytes, and chondroblasts (Bourin *et al*., 2013; Dominici *et al*., 2006). These criteria are periodically updated, and the reader is referred to the website of the International Society for Cellular Therapy MSC Subcommittee for the most up-to-date information (Web ref.1). Even so, this operational definition does not necessarily define a homogenous population of multipotent progenitors. Instead, it describes a heterogeneous group of cells that demonstrate variability among tissues of origin, among individual donors, amongst clonal subpopulations, and at the single cell level (Fig. 2).

MSCs exhibit heterogeneity on multiple levels

While MSCs were first isolated from bone marrow (Friedenstein, 1976; Johnstone *et al*., 1998; Pittenger *et al*., 1999), they have since been identified in many connective tissues, including adipose tissue, the umbilical cord and dental pulp (Erices *et al*., 2000; Gronthos *et al*., 2000; Zuk *et al*., 2001) to name a few. In standard isolation techniques, adipose or bone marrow aspirates are progressively centrifuged, and filtered before being plated into culture. A small fraction of the cells (the presumed MSCs) will adhere to the tissue culture plastic, and proliferate. Both bone marrow- and adipose-derived MSCs are readily available (Estes *et al*., 2010), yet they originate from stem cell niches that provide distinct biological, chemical and mechanical cues. Tissue-dependent variation in differentiation capacity, surface markers and transcriptional and proteomic profiles is widely studied, and the reader is referred to recent reviews for comparisons of MSCs across tissue sources (Kern *et al*., 2006; Mattar and Bieback, 2015; Strioga *et al*., 2012).

Even when derived from the same tissue of origin, MSCs demonstrate tremendous donor-to-donor variability. Intuitively, donor health may influence the availability and functional potential of MSCs (Kuznetsov *et al*., 2009; Wang *et al*., 2013). Similarly, as donors age, MSC availability, self-renewal capacity



Donors
Phinney *et al*., 1999
D'Ippolito *et al*., 1999
Kuznetsov *et al*., 2009
Mindaye *et al*., 2013

Tissues
Johnstone *et al*., 1998
Pittenger *et al*., 1999
Erices *et al*., 2000
Gronthos *et al*., 2000
Zuk *et al*., 2001

Clones
Muraglia *et al*., 2000
Larsen *et al*., 2010
Russell *et al*., 2011
González-Cruz *et al*., 2012
Selich *et al*., 2016

Single Cells
Lee *et al*., 2014
Marble *et al*., 2014
Freeman *et al*., 2015
Cote *et al*., 2016
Li *et al*., 2016

Fig. 2. MSC heterogeneity exists at multiple levels, including among donors, tissues, clonal subpopulations, and single cells. Selected works are highlighted at each of these levels.

and differentiation potential have been reported to decline (D'Ippolito *et al*., 1999; Katsara *et al*., 2011; Stenderup *et al*., 2003). Surprisingly, however, even MSCs isolated from young, healthy donors exhibit stark differences in their proliferation rate, differentiation capacity, and ultimate clinical utility (Phinney *et al*., 1999). This functional variation extends to the molecular status of these cells (Mindaye *et al*., 2013; Mindaye *et al*., 2015). For example, mass spectroscopy of MSCs isolated from six donors revealed that only 62 % of all identified proteins were found in at least half of the donors, and only 13 % of identified proteins were found in cells from each donor (Mindaye *et al*., 2013). Such donor-to-donor variation complicates use, and motivates a more detailed investigation of MSC variability.

Further study revealed that donor- and tissue-dependent differences are superimposed upon cell-to-cell variation amongst MSCs within a single population. For example, multiple bone marrow aspirates isolated from the same donor over a period of six months, or bilaterally from a donor at a single time point, yield MSC cultures that proliferate at different rates (Phinney *et al*., 1999). Even within a single isolate, cell-to-cell variation in MSC phenotype becomes evident during culture expansion and downstream use (Fig. 3, Fig. 4). This variation is commonly examined by comparing clonal subpopulations (groups of cells that are not only genetically identical, but also recently derived from single parent cells) (Huang, 2009).

MSCs readily form clones, and their clonogenicity can be observed by sparsely plating an initial isolate and monitoring colony formation. Clonal subpopulations can be obtained by sub-culturing these colonies, or by seeding single cells into individual culture wells by limiting dilution or flow cytometry (Smith *et al*., 2004). While variability is

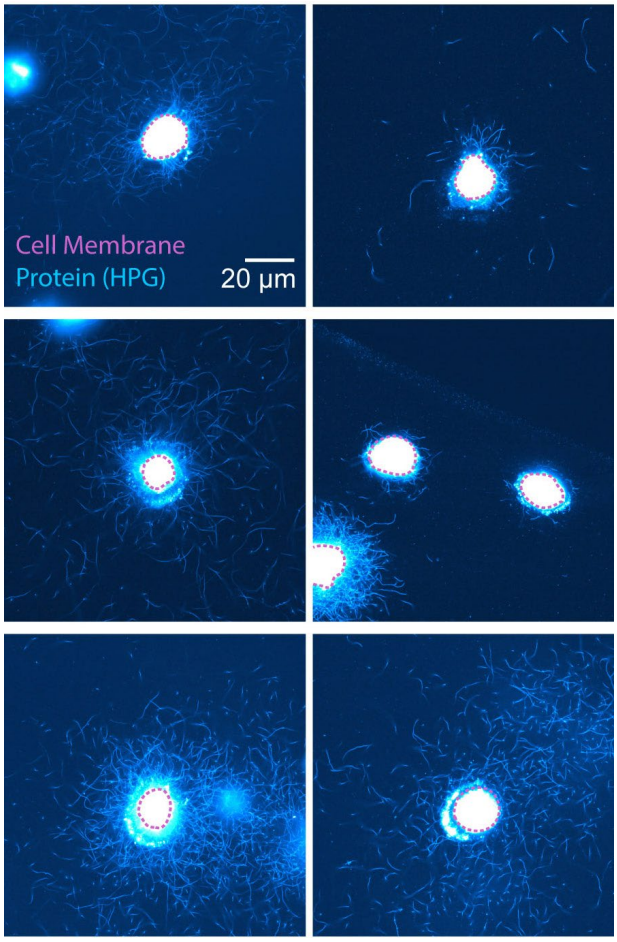


Fig. 3. Example of MSC-to-MSC variation in extracellular matrix production. Confocal cross-sections of individual chondrogenically-induced MSCs, metabolically labelled to tag proteinaceous extracellular matrix components (HPG), demonstrate cell-to-cell variability in matrix amount and organisation. Reproduced under the terms of the Creative Commons Attribution License, from McLeod and Mauck, 2016.

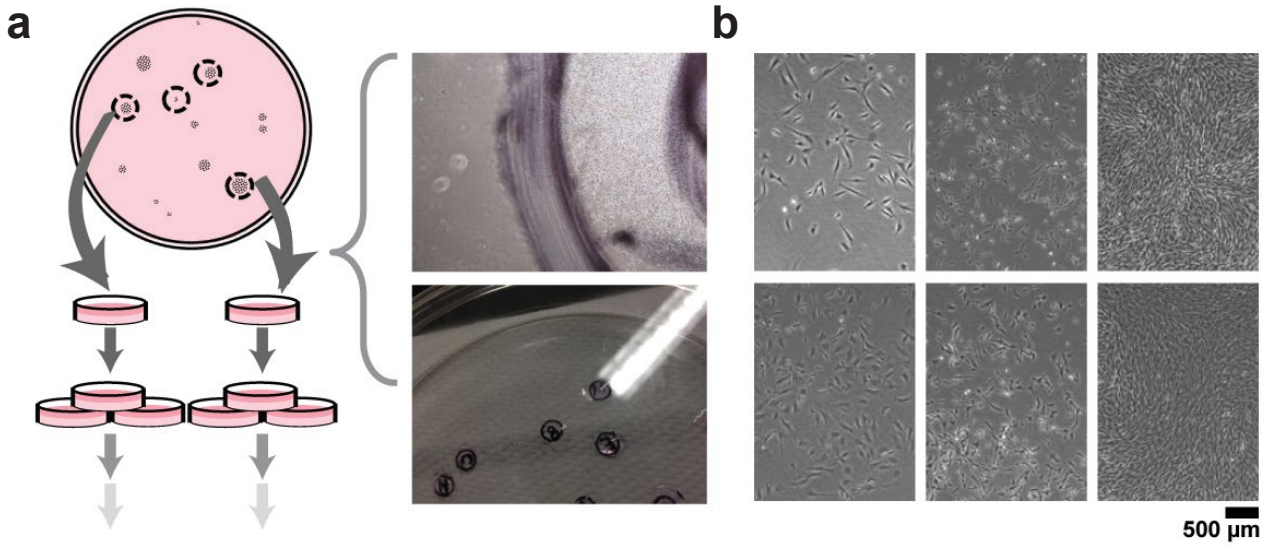


Fig. 4. Isolating MSC colonies. **a**) Schematic and photographs of procedure to isolate MSC colonies from the initial plating. Colonies are identified before selective trypsinisation. **b**) Morphological differences are apparent between MSC colonies in the initial isolate (each image represents a different colony).

inherent amongst the clone-initiating cells, it develops even further during *in vitro* culture and population expansion, where expansion on hard tissue culture surfaces may promote such divergence and/or reduction in potency (Li *et al*., 2017; Yang *et al*., 2014). Inter-clonal heterogeneity (variation among clones) most obviously manifests as morphological and proliferative diversity. Clones differ in morphological phenotype, ranging from elongated spindle-like cells to large flattened cells and highly protrusive cells (Colter *et al*., 2001; Muraglia *et al*., 2000) (Fig. 4). Similarly, individual clones proliferate both more quickly and more slowly than their corresponding polyclonal parent populations and differ in their self-renewal capacity, with select clones reaching early senescence (Russell *et al*., 2011). These apparent and near-immediate differences in clonal behaviour yield *in vitro* conditions that vary between clones (*e.g.* monolayer density) and potentially amplify heterogeneity as the subpopulations further expand.

To summarise, MSC variability is inherent among donors, tissues and clonal subpopulations. Within subpopulations, further variation emerges during culture expansion, and is observable in not only the phenotypic characteristics mentioned above, but also in the functional differentiation capacity, molecular signature and the mechanical state of the cell. The following sections elaborate on these dimensions of emergent heterogeneity.

Inter-clonal functional variation

The differentiation capacity of clonal MSCs was first studied to prove the existence of multipotent cells capable of committing towards adipogenic, osteogenic and chondrogenic fates (Pittenger *et al*., 1999). However, evaluation of MSC multipotency has also identified clonal populations that are unresponsive to current differentiation protocols and appear to have restricted differentiation potential (Muraglia *et al*., 2000; Pittenger *et al*., 1999; Russell *et al*., 2010). While the specific fraction of clones that may successfully undergo differentiation is both protocol- and donor- dependent, estimates suggest that ~50 % of clones are tri-potential, ~30 % are bipotential (either osteo-chondro or osteo-adipo), and ~10 % are unipotential osteoprogenitors (Russell *et al*., 2010).

This functional heterogeneity is also apparent *in vivo*, and influences the utility of MSCs in multiple regenerative contexts. For example, clonal MSC populations implanted subcutaneously in mice demonstrate variable osteogenic capacity, with only approximately half of clonal implants undergoing some degree of osteogenesis (Kuznetsov *et al*., 1997). Similarly, clonal populations screened *in vitro* for above-average chondrogenic capacity result in repair of cartilage defects more robustly than unscreened populations (Jiang *et al*., 2014). Inter-clonal functional heterogeneity also extends to include commitment towards non-canonical fates. Stem cells derived from dental pulp demonstrate heterogeneous myogenic potential, and clones that are highly myogenic *in vitro* also engraft into muscle defects more efficaciously than the polyclonal parent populations (Yang *et al*., 2010). Such functional variability may offer the opportunity to harness clonal identity and prospectively identify MSC subpopulations best suited to drive the functional restoration of a range of tissues. However, the challenge of performing *in vitro* screening of clonal functional capacity at a clinically-useful scale has motivated ongoing work to identify molecular or biophysical markers of MSC differentiation potential, as will be addressed later in this review.

Inter-clonal molecular variation

Inter-clonal functional heterogeneity must derive from underlying molecular variation. While proteomic studies comparing individual clonal populations are challenging due to inherently limited cell number, comparisons between pooled fast- and slow-growing clones suggest broad trends. Fast-growing clones are more likely to be tri-potential than slow-growing clones (Mareddy *et al*., 2007; Russell *et al*., 2010), and rapidly self-renewing MSCs engrafted into tissues more readily than slowly renewing MSCs (Lee *et al*., 2006). Indeed, fast- and slow-growing MSCs differ proteomically, with differential expression of proteins including intermediate filaments (*e.g.* lamin A/C), calcium-binding proteins (*e.g.* calmodulin), and glycolytic proteins (*e.g.* glyceraldehyde-3-phosphate dehydrogenase) (Mareddy *et al*., 2009). Furthermore, surface marker expression across clones suggest that CD200 marks osteogenic subpopulations, while SSEA4 and CD140a are associated with adipogenic capacity (Pontikoglou *et al*., 2016; Rostovskaya and Anastassiadis, 2012).

Clonal heterogeneity also extends to the transcriptome. Certainly, there is great divergence in transcriptional signature between high- and low-potential clones following exposure to differentiation conditions (Mareddy *et al*., 2010). There is also now evidence for clonal variation in basal gene expression in undifferentiated cells. Screens comparing stem cell gene expression between fast- and slow-growing clones identify extensive differences in the expression of genes associated with the cell cycle and cellular division (Mareddy *et al*., 2010; Menicanin *et al*., 2010). Fast-growing clones also express select growth factors (*e.g.* BMP2, FGF2, IGF1), lineage markers (*e.g.* aggrecan, alkaline phosphatase, collagen I, collagen II) and self-renewal markers (*e.g.* SOX2) more highly than slow-growing clones. Conversely, other genes, including CD44, are more highly expressed in slow-growing clones. Separately, direct comparisons of clonal transcriptomes indicate that clones with greater functional potential have enriched basal expression of genes implicated in skeletal and muscular development, including extracellular matrix components and MAP kinase signalling elements (Elsafadi *et al*., 2016; Larsen *et al*., 2010; Sworder *et al*., 2015). Notably, high baseline

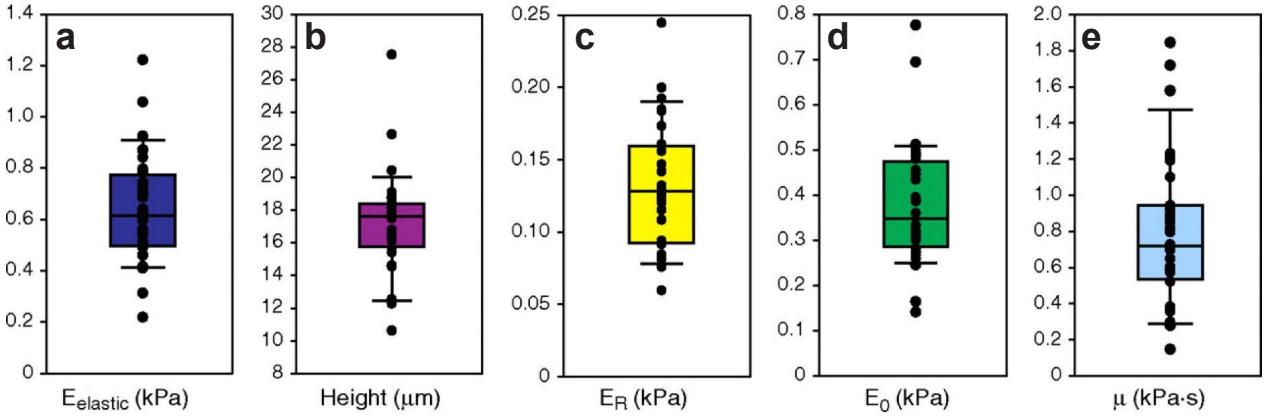


Fig. 5. Heterogeneity in biophysical parameters across 32 MSC clones. **a**) Elastic modulus, **b**) cell height, **c**) relaxed modulus, **d**) instantaneous modulus, and **e**) apparent viscosity are presented as the geometric mean of each clone, overlaid on box-whisker plots. Reproduced from González-Cruz *et al*., 2012.

expression of calponin negatively correlates with clonal multipotency (Sworder *et al*., 2015). Within an osteogenic context, high potential clones also express extracellular matrix genes and genes regulated by osteogenic transcription factors to a greater extent than poorly osteogenic clones (Larsen *et al*., 2010). Strikingly, basal expression of four genes, including decorin and lysyl oxidase, is more predictive of clonal osteogenesis than the expression of traditional osteoblastic markers, including Runx2, collagen type I and osteopontin (Larsen *et al*., 2010). Such findings suggest that transcriptome-wide analysis of undifferentiated MSCs may be key to identifying prospective markers of stem cell fate; however, these predictors must also be validated through mechanistic studies identifying their role in maintenance of multipotency and/or lineage specification.

Transcriptional activity is determined not only by the presence and activity of transcription factors, but also by the epigenetic status of the cell. DNA methylation, one type of epigenetic modification, is generally associated with a loss of gene expression and is crucial in stem cell differentiation. In undifferentiated adult stem cells, lineage-associated promoters are often hypomethylated (Berdasco and Esteller, 2011). Investigation of clonal MSC adipogenesis shows that while adipogenesis-associated promoters are hypomethylated in MSC clones, the specific pattern of methylation varies among clonal subpopulations. Moreover, there is no clear relationship between methylation status and the gene expression pattern or ultimate adipogenic potential for a particular clone (Noer *et al*., 2006; Noer *et al*., 2007).

Collectively, these results indicate clear clonal variability in proteomic, transcriptomic and epigenetic status. Correlative relationships between transcriptional status and functional capacity are established at the clonal level, but the identification of any such relationship between a clones' epigenetic marks and differentiation potential remains elusive and require further study across multiple clonal populations.

Biophysical variation amongst clones and individual cells

The mechanical state of the cell has emerged as another potential biomarker indicative of cellular phenotype. Cellular mechanical properties reflect the underlying structure of the cell, including the cytoskeleton and nucleus. These structures change with differentiation, and also differ among committed cell types. Interestingly, increased nuclear deformability correlates with pluripotency. With differentiation, chromatin condenses within the nucleus and the nuclear envelope is reinforced by increasing lamin A/C content (Heo *et al*., 2016; Pajerowski *et al*., 2007). Indeed, embryonic stem cells are 6-10 fold softer than their differentiated counterparts (Chowdhury *et al*., 2010; Pajerowski *et al*., 2007). Mechanical differences of similar magnitude have been noted among individual, undifferentiated MSCs isolated and passaged together (Maloney *et al*., 2010). This mechanical variability may reflect the high degree of functional heterogeneity observed when these individual cells are tasked with a specific lineage transformation.

Mechanical differences also exist among clonal MSC populations. A study monitoring 32 MSC clones suggests that cellular mechanics can be prospectively used to predict differentiation capacity in individual clones (González-Cruz *et al*., 2012) (Fig. 5). The functional potential of clones correlated with elastic and viscoelastic properties. Clones with the highest adipogenic potential are characterised by taller cells with lower elastic moduli. Conversely, osteogenic capacity correlates with a higher elastic modulus, instantaneous modulus and relaxed modulus, while chondrogenic capacity correlates with elastic modulus and apparent viscosity. Separately, efforts

to biophysically sort MSCs in a high-throughput manner suggest that the cells of tri-potential MSC subpopulations are smaller, less stiff, and exhibit greater nuclear membrane fluctuations than cells with bi-potent (osteo-chondro) differentiation potential (Lee *et al*., 2014).

Cell mechanics not only indicate cell phenotype, but also mediate the physical interaction between a cell and its environment. Many cell types, including MSCs, are able to sense and respond to mechanical cues. Biophysical stimuli including the elasticity of the microenvironment and exogenous forces have been widely examined as determinants of stem cell fate (Cosgrove *et al*., 2016; Engler *et al*., 2006). These cues elicit changes in biochemical signalling, gene expression, and ultimately cell phenotype and function.

A number of studies have begun to probe how individual MSCs respond to biophysical cues. Dual adipogenic/osteogenic media causes polyclonal MSC populations to undergo mixed osteogenic and adipogenic differentiation; the relative balance between these two differentiated states is regulated by the physical stiffness of the cell microenvironment (Fu *et al*., 2010; Guvendiren and Burdick, 2012; Khetan *et al*., 2013). Cell response is most uniform in extremely soft or stiff environments, which favour adipogenesis and osteogenesis respectively. However, in environments of intermediate stiffness, commitment is variable: subpopulations of cells will differentiate towards each fate. Furthermore, in dynamic systems where an initially soft substrate can be stiffened, the ratio of adipogenic to osteogenic commitment is regulated by the timing of the soft-to-stiff transition (Guvendiren and Burdick, 2012). Collectively, these findings suggest that MSC subpopulations may have subtly different mechanical properties that alter the set points at which mechanically-regulated fate commitment occurs.

Any such set point likely relates to the tension sensed and contractility generated by an individual cell. On a population level, osteogenesis is associated with the ability of cells to spread and generate tension, while adipogenesis is promoted by conditions that restrict cell spreading and contractility. Interestingly, the traction force generated by an individual cell after short-term exposure to bipotential media serves as an indicator of its ultimate differentiation propensity: high contractility has been associated with osteogenic potential, while low contractility has been associated with adipogenic capacity (Fu *et al*., 2010). Thus, single cell or clonal heterogeneity in the ability of cells to generate traction may correspond to the functional variability observed. This also offers the intriguing possibility that adherence and traction against a substrate might be a selection tool to enrich for MSC sub populations with varying differentiation potential.

Variation also extends to cellular activity along the pathways responsible for mechanotransduction. For example, calcium signalling is highly mechanosensitive, and regulates processes including differentiation and proliferation (Matta and Zakany, 2013). Investigations of baseline calcium signalling in MSCs have shown that some cells exhibit spontaneous calcium oscillations, while others do not (Kawano *et al*., 2002; Kim *et al*., 2009; Sun *et al*., 2007). The extent of variation is microenvironment dependent: the fraction of MSCs experiencing calcium oscillations increases with substrate stiffness, with 59-98 % of cells oscillating on glass culture surfaces (Kawano *et al*., 2002; Kim *et al*., 2009; Kim *et al*., 2014; Sun *et al*., 2007). Furthermore, this variation extends to the cellular response to active mechanical stimulation. Subpopulations of undifferentiated MSCs encapsulated in hydrogels differentially respond to compression: calcium signalling was upregulated in one subset of cells, while it was downregulated in another (Campbell *et al*., 2008). It remains to be seen if similar variation extends to other mechano-sensitive pathways, including MAPK and RhoA/ROCK signalling.

To summarise the above points, clonal and individual MSCs differ not only in their inherent mechanical properties, but also in their response to passive and active physical stimuli. These biophysical characteristics correlate with differentiation outcomes, and collectively form an additional dimension of heterogeneity (beyond the molecular characteristics described in the preceding section) and another potential handle by which subpopulation may be selected to increase homogeneity of response for a given application.

Intra-clonal heterogeneity and cell-to-cell variation

Studies investigating inter-clonal heterogeneity often imply that clonal subpopulations are relatively homogeneous. However, there is growing appreciation that even within a clone, cellular phenotype can be highly variable (Rennerfeldt and Van Vliet, 2016). Cells within a clone can differ in their morphology and ability to differentiate. When intact colonies are exposed to adipogenic or osteogenic differentiation cues, differentiation initiates in the dense, inner portion of the colony (Ylöstalo *et al*., 2008). Colony micro-dissection and subsequent analysis reveal spatial differences in gene and protein expression. Cells located in the colony interior expressed extracellular matrix genes to a greater extent than cells located in the outer periphery, while "outer" cells expressed higher levels of genes associated with cell proliferation and mitosis (Ylöstalo *et al*., 2008). Building on this analysis, technological advances have enabled the interrogation of single cell gene expression amongst clonal and polyclonal populations. Single molecule RNA FISH, to assess single cell gene expression within spatially intact MSC clones, suggests that transcriptional variability is pervasive even amongst individual clones (Fig. 6) (Cote *et al*., 2016). In parallel, RNA FISH and single

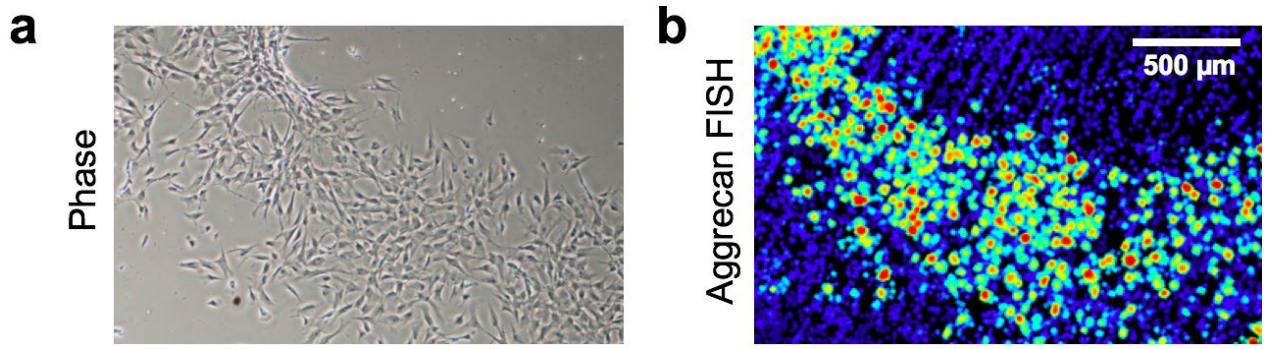


Fig. 6. RNA FISH reveals single cell transcriptional heterogeneity within an individual clone. **a**) Corresponding phase-contrast and **b**) FISH images, pseudo-coloured such that red indicates high RNA content and blue indicates low RNA content, indicate high intra-clonal variation in gene expression of the chondrogenic marker aggrecan.

cell RNA sequencing of individual MSCs has shown that individual MSCs have variable basal expression of both early and late differentiation markers, and that markers of multiple lineages can be co-expressed in the same cell (Cote *et al*., 2016; Freeman *et al*., 2015). Furthermore, DNA methylation patterns are mosaic within individual clonal populations (Noer *et al*., 2006; Noer *et al*., 2007). Thus, cell-to-cell variation exists at every level where inter-clonal heterogeneity has been noted, and this must be considered in any basic science study or clinical application that utilises this cell type.

Potential origins & mechanisms of MSC heterogeneity

It is tempting to speculate that MSC heterogeneity mirrors the diversity of environments present in the *in vivo* stem cell niche (Huang, 2009). *In vivo*, MSCs reside in niches characterised by broad cellular communities that present variable chemical and mechanical conditions. Indeed, micro-anatomical heterogeneity within the bone marrow niche has been shown to dictate cell-to-cell variation in osteo-lineage cells (Silberstein *et al*., 2016). Upon isolation, MSCs from these heterogeneous environments mix together, and extant *in vivo* variation may persist into *in vitro* cultures. Indeed, there is mounting evidence that cultured cells retain a "memory" of their previous environments (Li *et al*., 2017; Yang *et al*., 2014). Furthermore, the mechanical properties of the stem cell microenvironment influence self-renewal capacity and regenerative potential (Gilbert *et al*., 2010). Perhaps then, the mechanisms responsible for *in vitro* cellular memory may also facilitate the maintenance of heterogeneity in primary cultures.

In addition to any heterogeneity derived from the *in vivo* niche, there is also apparent plasticity in the MSC phenotype. In the study of intra-clonal spatial variation discussed above, the subculture of either "inner" or "outer" cells yielded new colonies with their own distinct inner and outer populations, suggesting that, in at least some dimensions, cellular variation is dynamic and reversible (Ylöstalo *et al*., 2008). Potential dynamics of shifting phenotypic variability have been carefully studied in the context of haematopoietic stem cell (HSC) lineage commitment towards erythroid and myeloid fates. Clonal HSC populations heterogeneously express the surface protein Sca-1, a marker associated with the erythroid transcriptional signature. Subcultures of cells sorted for either the lowest or highest Sca-1 expression shift with time to reconstitute the original distribution of Sca-1 expression (Chang *et al*., 2008). Similar behaviour is observed in MSCs, which express Sca-1 heterogeneously within and among clonal populations (Hamidouche *et al*., 2016). At early passage, MSC fractions with either low or high Sca-1 expression are able to regenerate the distribution of Sca-1 expression in the parent population (Hamidouche *et al*., 2016). However, after extended passaging, sorted MSCs are less able to reconstitute the full range of Sca-1 expression (Hamidouche *et al*., 2016). Potential explanations of this behaviour include spontaneous transcriptional fluctuations (of either transcriptome-wide programs (Chang *et al*., 2008) or individual regulators (Pina *et al*., 2012)) and epigenetic bistability (Hamidouche *et al*., 2016).

Transcriptional fluctuations in the expression of individual genes might arise from the stochasticity inherent to many biological processes. While transcription at the population level is often considered a process that proceeds at a constant, defined rate, transcription in individual cells is highly stochastic. Fundamentally, transcription requires the chemical interaction of RNA polymerases with an accessible promoter sequence and any requisite transcription factors. Thus, even if two stem cells were identical in every way, the transcriptional processes in each would be dictated by the random collisions of molecules within the nuclear milieu. The importance of such probabilistic interactions was elegantly shown in a now-classic experiment

where two distinguishable, yet near-identical, genes were inserted into a cell (Elowitz *et al*., 2002). Within individual cells, the expression of these two genes deviated, suggesting the existence of "intrinsic" random noise in gene expression. Notably, this intrinsic noise is superimposed upon any cell-to-cell variation controlled by "extrinsic" factors (*e.g.* epigenetic differences among cells, cell size, *etc*. – many of the types of variation described in the previous sections). Even so, intrinsic noise can give rise to substantial variation in copy number, and may drive cellular decision-making and phenotypic divergence (Balázsi *et al*., 2011; Raj and van Oudenaarden, 2008).

Population dynamics also likely contribute to the variation that emerges as the stem cells proliferate in culture. Upon cell division a single, self-renewing, cell splits into two daughter cells of approximately equal size. If the division is symmetrical, both daughter cells will possess the same self-renewal capacity: they will either both divide, or not divide. In contrast, asymmetric cell division will yield one self-renewing cell, and one that senesces in culture. Such dynamics allow an initially small fraction of cells to give rise to the majority of the population several days later. For example, one study reported that after 6 d of culture, 50 % of progeny cells were derived from 9 % of the initial population (Whitfield *et al*., 2013). Thus, much of the cell-to-cell variation observed in polyclonal populations may actually be the heterogeneity that emerges from within a single dominant clone. Longer term tracking of MSC lineages over 12 passages confirms this notion of clonal dominance (Selich *et al*., 2016) (Fig. 7). Interestingly, initially dominant clones are sometimes overtaken by other clonal subpopulations (Selich *et al*., 2016).

This delayed dominance may be related to variation in the onset of cellular senescence. With extended passage, MSCs suffer from decreased multilineage potential (Digirolamo *et al*., 1999; Kretlow *et al*., 2008; Russell *et al*., 2011; Schellenberg *et al*., 2011). In parallel, the clonogenicity of MSCs decreases and proliferation slows (Digirolamo *et al*., 1999; Schellenberg *et al*., 2011). Functional capacity may also grow increasingly restricted with progressive culture. For example, the hierarchical lineage commitment hypothesis posits that through divisions, stem cells progressively lose the ability to commit to certain lineages. Alternatively, MSC functional heterogeneity may also be explained in part by trans-differentiation, or transitions between partially restricted differentiation capacities (Pevsner-Fischer *et al*., 2011). The existence, structure, and governance of any such hierarchy or trans-differentiation processes remain to be elucidated (Pevsner-Fischer *et al*., 2011).

Notably, spontaneous genetic mutations are not thought to be the source of cell-to-cell variation amongst MSCs. Estimates of the mutation rate required to obtain the observed diversity are unfeasibly high: approximately one in three cells would need to experience a phenotype-altering mutation (Rennerfeldt and Van Vliet, 2016). While such rates are possible, they are improbable, and thus genetic mutation is unlikely to be a dominant mechanism in the evolution of *in vitro* MSC heterogeneity (Rennerfeldt and Van Vliet, 2016).

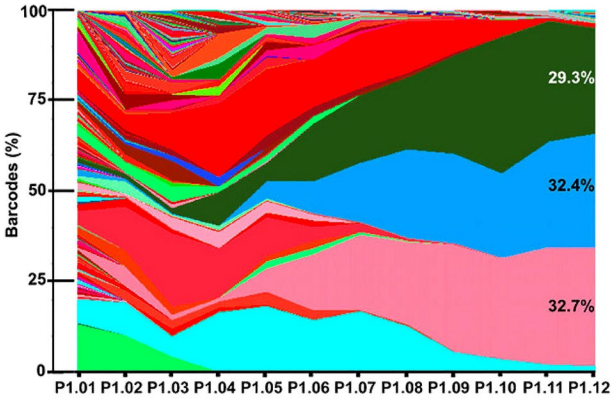


Fig. 7. Clonal dominance amongst MSC during passaging. The relative abundance of barcoded subpopulations shifts with time, as measured in umbilical cord-derived MSCs cultured over 12 passages. Reproduced with permission from Selich *et al*., 2016.

Taken together, the above discussion identifies numerous mechanisms through which heterogeneity may emerge in MSC clonal populations. To identify the timing and mechanism of such changes, however, requires new tools that can operate at the single cell level, the topic of the next section.

Measuring cell-to-cell molecular variation

Studies to discern the underpinnings of stem cell heterogeneity increasingly rely on methods to assay the molecular content of individual cells. To this end, adaptations of traditional methods and new techniques now allow one to assess gene and protein expression at the single cell level. Broadly, these approaches can be classified on the basis of their timing (continuous observation *vs*. fixed endpoint), their modality (imaging based *vs*. lysate based), and their ability to support multiplexed observations (high *vs*. low). In this section, we highlight select methods that may be of particular utility for assessing stem cell heterogeneity; a summary is provided in Table 1.

Assaying gene expression in single cells

Single molecule RNA fluorescent *in situ* hybridisation (FISH) is an imaging-based method that quantifies the absolute amount of mRNA in fixed cells. Sets of fluorescently labelled oligonucleotide probes tile along individual mRNA molecules in a sequence-specific manner, allowing mRNA molecules to be visualised as diffraction-limited spots (Fig. 8**a**) (Femino *et al*., 1998; Raj *et al*., 2008). In standard RNA

Table 1. Single cell methods to assay mRNA and protein abundance.

Method	Timing	Modality	Ability to multiplex
mRNA abundance			
RNA FISH	Endpoint	Image based	Low
Single cell qPCR	Endpoint	Lysate based	Intermediate
Single cell RNA sequencing	Endpoint	Lysate based	High
Molecular beacons	Continuous	Image based	Low
Spherical nucleic acids	Continuous	Image based	Low
Transgenic fluorescent reporters	Continuous	Image based	Low
Protein abundance			
Immunostaining	Endpoint	Image based	Low
Flow cytometry	Endpoint	Image based	Intermediate
Mass cytometry	Endpoint	Lysate based	Intermediate
Proximity assays	Endpoint	Lysate based	Low
Single cell western blot	Endpoint	Lysate based	Low
Single cell mass spec	Endpoint	Lysate based	High
Transgenic fluorescent reporters	Continuous	Image based	Low

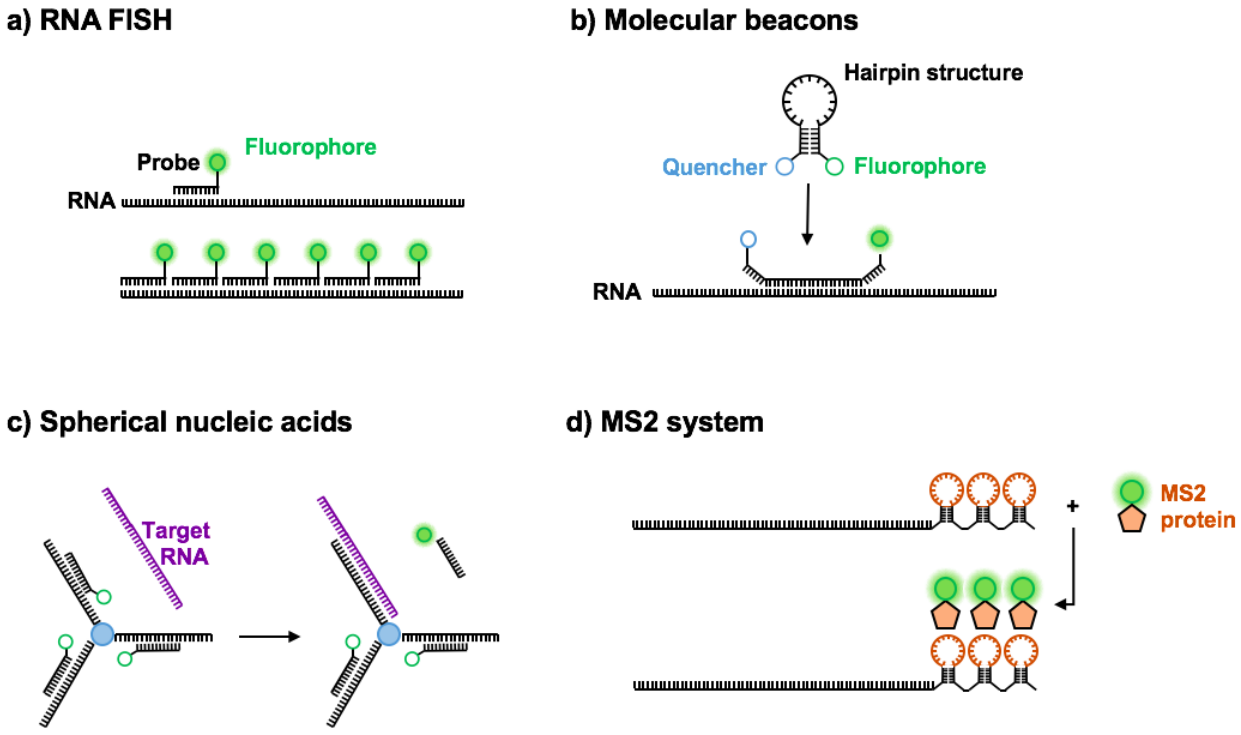


Fig. 8. Schematics of select methods to measure mRNA in single cells. **a**) In RNA FISH, fluorescently labelled oligonucleotide probes tile along the target mRNA. **b**) Molecular beacons emit fluorescence upon binding to target mRNA. **c**) Spherical nucleic acids quench fluorescence until target mRNA binding occurs. **d**) In the MS2 system, fluorescently tagged proteins bind to motifs engineered into the mRNA sequence. Adapted by permission from Macmillan Publishers Ltd: Nature Cell Biology, Hoppe *et al*., copyright 2014.

FISH, the number of genes simultaneously assayed is restricted by the availability of microscope filter sets (approximately 4 genes). However, recent strategies utilising combinatorial and sequential barcoding substantially increase the potential number of genes measured (Lubeck *et al*., 2014; Lubeck and Cai, 2012).

Other endpoint methods for assaying single cell gene expression include single cell RT-qPCR, microarrays, and RNA-seq (Saliba *et al*., 2014; Ståhlberg and Bengtsson, 2010). Each of these approaches measures the abundance of cDNA amplified from mRNA in the lysates of individual cells. The ability of single cell RNA-seq to report transcriptome-wide expression holds particular promise as the field develops an increased understanding of the many dimensions of cell-to-cell variation. However, these

methods also require normalisation (*vs*. absolute quantification), and cannot provide information about the spatial distribution of individual mRNA within an individual cell.

Alternative techniques allow gene expression to be monitored continuously in live cultures through imaging. Strategies such as molecular beacons and spherical nucleic acids (*e.g.* nanoflares) rely on oligonucleotides, fluorophores, and fluorescent quenchers to report gene expression (Fig. 8**b**,**c**) (Seferos *et al*., 2007; Tyagi and Kramer, 1996). When the oligonucleotide probe binds to a target mRNA, the quencher separates from the fluorophore and permits fluorescent signalling. These methods are used to monitor gene expression in live MSCs undergoing osteogenesis, and can be combined with FACS techniques to sort individual cells on the basis of their gene expression (Fig. 9) (Li *et al*., 2016; Marble *et al*., 2014).

Other efforts to monitor gene expression in real-time rely on transgenic methods. Expression of genes modified to include repetitive stem-loop motifs can be monitored using fluorescent bacteriophage proteins that bind to these sequences with high affinity (Fig. 8**d**) (Fusco *et al*., 2003; Shav-Tal *et al*., 2004). Alternatively, short-lived fluorescent reporter proteins are considered as proxies for the expression of genes under the control of the same promoter (Suter *et al*., 2011).

Assaying protein expression in single cells

In addition to quantifying mRNA at the single cell level, it is also essential to map and measure the protein output from this message content on a cell-by-cell basis. Single cell measurements of protein expression are possible using a variety of techniques, many of which rely on antibody-based detection. The simplest of these is standard immunostaining, imaged at high magnification and quantified on a per-cell basis. Flow cytometry offers high throughput measurements of fluorescent antibody signal, and can be coupled with cell sorting and multiplexed to accommodate the measurement of 10-15 proteins (Spitzer *et al*., 2016). Mass cytometry allows further multiplexing by leveraging mass spectroscopy to detect the levels of metallically-conjugated antibodies bound to individual cells (Spitzer *et al*., 2016). Recently, imaging mass spectrometry has further extended this approach to enable the measurement of protein abundance in histological sections while preserving spatial information (Chang *et al*., 2017).

An additional category of assays includes those based on proximity, including proximity ligation (PLA) and proximity extension (PEA). In these approaches, pairs of antibodies conjugated to oligonucleotides are used to probe cell lysates (Greenwood *et al*., 2015). When an antibody pair binds to the protein of interest, the two oligos are brought together and either ligated (PLA) or hybridised (PEA) to create a template for the synthesis of reporter DNA that is ultimately detected using qPCR or sequencing (Greenwood *et al*., 2015). Interestingly, this method is compatible with lysate-based assays of single cell gene expression, and has recently been used to simultaneously examine the proteomic and transcriptomic state of single cells (Darmanis *et al*., 2016).

Techniques for assaying protein expression in bulk lysates have also recently been scaled down to accommodate single cells. Western blots can be performed on individual cells that have settled into microwells in a polyacrylamide gel; the cells are lysed in their wells immediately prior to in-gel electrophoresis, blotting and detection (Hughes *et al*., 2014; Kang *et al*., 2016). There is also active development surrounding single cell mass spectrometry, which was recently used to quantify the abundance of thousands of proteins at the single cell level during embryonic stem cell differentiation (Budnik *et al*., 2017 – non-peer reviewed e-publication).

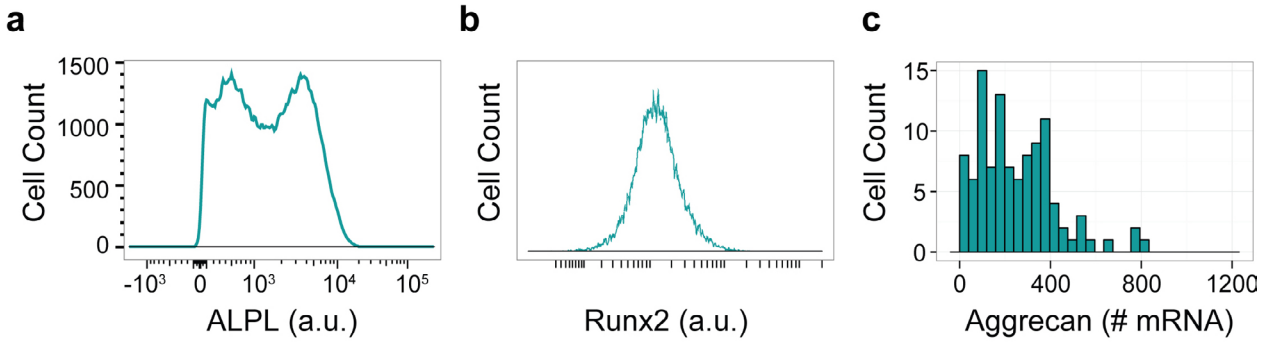


Fig. 9. Multiple techniques identify high cell-to-cell variation in single cell gene expression amongst individual MSCs. **a**) Molecular beacons (Marble *et al*. 2014), **b**) spherical nucleic acids (Li *et al*. 2016) and **c**) single molecule RNA FISH (Cote *et al*. 2016) have been used to query the single cell abundance of various differentiation markers (ALPL, Runx2 and ACAN, respectively). Subfigures reproduced under the terms of the Creative Commons Attribution License.

Conclusions & Outlook

Mesenchymal stem cells demonstrate many dimensions of heterogeneity: they differ among donors, as well as among and within clonal populations. Indeed, cell-to-cell variation seems inherent to the cell type, and we speculate that heterogeneity will be found in every place it is sought. The facets of MSC variability discussed here are non-exhaustive, and even within the categories reviewed, we have highlighted only a subset of relevant studies. Moreover, even papers that do not specifically discuss heterogeneity are impacted by its presence in their cell populations, though this may be underappreciated in the community.

Given the pervasiveness of MSC variability, how can we as a community best acknowledge and respond? As a first step, we recommend that, whenever possible, studies performed at the population level should be validated in terms of the principal findings using clonally expanded populations. This would clarify whether the response is universal to all MSCs, or only to selected subpopulations. A second approach would be to screen cells prior to use in experiments (*e.g.* by FACS or mechanical sorting), to more precisely understand the properties of the cells being used, and potentially select for specific subpopulations. As our understanding of heterogeneity grows, such selection strategies may become more refined and easy to implement, and eventually standard for the field.

With respect to the emergence of genome-wide single cell techniques, these hold the potential to identify new molecular targets that vary among cells and correspond to differences in functional potential. However, as our technological ability to interrogate biology at the single cell level grows, we will also need to distinguish biological noise from variation that represents actionable signal. Our ability to discern such signal may be enhanced by the choice of measurement approach. For example, biological noise often has a temporal component (*e.g.* stochastic gene expression fluctuations). In these situations, endpoint measurements may reveal substantial variation, even if cells' time-averaged behaviour is similar. In contrast, continuous measurements could be integrated or averaged over time to potentially smooth stochastic temporal fluctuations while retaining evidence of major cell-to-cell variation. Regardless of measurement technique, the further study of MSC variation holds the potential to clarify the mechanisms and implications of cell-to-cell heterogeneity.

From a clinical perspective, heightened appreciation of MSC variation may also ultimately improve the efficacy of regenerative medicine applications. If we are able to succeed in identifying determinants or correlates of an individual cell's propensity to differentiate along a specified lineage, it may be feasible to either select for the best cells, or deplete the worst. Such enrichment techniques would ideally be implementable at the time of treatment, and most ideally during the time frame of a normal surgery, so that these technologies could be applied without undo cell manipulation. However, the most efficient sorting may require priming of the population prior to implementation so as to sufficiently distinguish between high- and low-performing cells. Short of this, when we interpret MSC functionality relative to other cell types, we could consider not only total cell number, but also the fraction of MSCs expected to exhibit the desired performance; this may guide dosing guidelines for clinical applications.

Taken together, this review outlines the many dimensions and potential mechanisms in which mesenchymal stem cells exhibit heterogeneity. We also outline emerging tools, working at the single cell level, which may shed new light on the mechanisms that govern the emergence and persistence of such heterogeneity in these populations. Once understood more completely, sorting and selection may be tractable, in a cost-efficient and practical fashion, ultimately improving the clarity and efficacy with which MSC-based therapeutics are understood and applied.

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