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Orthogonal Potency Analysis of Mesenchymal Stromal Cell Function During Ex Vivo Expansion

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Abstract

Adult bone marrow mesenchymal stromal cells (MSCs) have cross-functional, intrinsic potency that is of therapeutic interest. Their ability to regenerate bone, fat, and cartilage, modulate the immune system, and nurture the growth and function of other bone marrow hematopoietic stem/progenitor cells have all been evaluated by transplant applications of MSCs. These applications require the isolation and expansion scaled cell production. To investigate biophysical properties of MSCs that can be feasibly utilized as predictors of bioactivity during biomanufacturing, we used a low-density seeding model to drive MSCs into proliferative stress and exhibit the hallmark characteristics of *in vitro* aging. A low-density seeding method was used to generate MSCs from passages 1 to 7 to simulate serial expansion of these cells to maximize yield from a single donor. MSCs were subjected to three bioactivity assays in parallel to ascertain whether patterns in MSC age, size, and shape were associated with the outcomes of the potency assays. MSC age was found to be a predictor of adipogenesis, while cell and nuclear shape was strongly associated to hematopoietic-supportive potency. Together, these data evaluate morphological changes associated with cell potency and highlight new strategies for purification or alternatives to assessing MSC quality.

Keywords

Mesenchymal stromal cell; Aging; Cell morphology; Adipogenesis; Hematopoiesis; Immunosuppression

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Introduction

There are currently over 200 active mesenchymal stromal cell (MSCs) clinical trials for regenerative medicine and immunomodulatory applications (www.clinicaltrials.gov; keyword: mesenchymal stem cell). Bone marrow-derived MSCs have been a long standing cornerstone in cell therapy and contain a subpopulation of stem cells that possess the innate ability to repopulate connective tissue cells in the bone marrow (BM) cavity [1–4]. This adipogenic, osteogenic, or chondrogenic capacity, when induced *in vitro* [5], has led to orthopedic applications to replace or reconstruct tissue defects as a local injection. These stem cell-containing MSCs also support the bone marrow compartment through the nurture and trafficking of hematopoietic stem cells (HSCs) as well as their differentiated progeny [6–8]. This hematopoietic supportive function has been harnessed clinically through an MSC:bone marrow co-infusion to enhance the transplant engraftment speed for blood reconstitution [9, 10]. It soon became clear that IV administered MSCs were also modulating the recipient immune response in early safety studies in graft-versus-host-disease (GvHD) patients [11, 12]. This immunomodulatory effect was prospectively applied in the treatment of steroid-refractory GvHD and has since evolved to numerous immune-mediated disease applications since the early 2000s [13, 14]. These orthogonal functions of MSCs – differentiation, hematopoietic support, and immunomodulation – involve different MSC-containing subpopulations [15–17], transcriptional machinery [18–20], signaling networks, and secretory outputs [21–23] and would benefit from quantitative benchmarking for a given therapeutic application.

The MSC pool is heterogeneous and contains primitive stem cells and a spectrum of downstream progenitors with different functional attributes [24]. Collectively, both stem cells and progenitors within the MSC pool are expanded (likely at differing growth rates) by the serial passaging of whole BM aspirates. These bulk expansion methods are the foundation of current clinical practices for the isolation and expansion of MSCs. The ability of MSCs to proliferate *ex vivo* further enhances their use as a scalable clinical cell therapeutic [13, 25]. Yet, the large-scale production of cells and processing towards a cell therapeutic may, in-and-of-itself, effect the functional quality of resulting clinical batches. The composition of the MSC pool will greatly influence batch-to-batch variation. Quality control assays, specifically potency assays, are critical in linking the bioactivity of MSCs to an expected clinical outcome [26]. The specific therapeutic use of MSCs requires a defined mechanism of action (MoA) including an in-depth dissection of its heterogeneity for optimizing towards each clinical application [27]. In the case of regenerative medicine, there are several classic potency assays such as *in vitro* MSC differentiation assays [28]; for hematopoietic support, an MSC-marrow co-culture; and for immunomodulation, a T cell modulation assay [29]. A cross-selection from this wide panel of tissue bioassays were chosen for study, herein.

In an organism, these three functional characteristics of MSCs deteriorate with age of the organism [30–32]. The potency of MSCs to differentiate with varying donor ages have been evaluated in the field [32], with a consensus that MSCs derived from younger subjects have faster growth kinetics and differentiation potential. It remains unclear however whether these investigations into the chronological aging (i.e. age of the donor) of MSCs, can directly

translate to what happens when a cell ages biologically; i.e. through expansion *in vitro* [33]. The effects of MSC expansion on individual potency activity [34], correlative potency, and potential altered phenotypes to use in prediction of MSC potency has not been clearly defined and was the scope of this study [35, 36].

We used an *in vitro* model of low-density MSC growth to determine whether the numerous therapeutic potencies are measurably altered with age. We observed age-associated morphological changes within the MSC pool and hypothesize the use of morphological characteristics as metrics to predict MSC function through established MSC potency assays.

Materials and Methods

Isolation of MSCs and Whole BMCs

Primary human mesenchymal stem cells were isolated from whole human BM aspirates (Lonza, Basel, Switzerland). BM was diluted in sterile phosphate buffered saline (PBS) at a 1:1 ratio. Ficoll-Paque (GE, PA) was added to the bottom of the tube without disrupting the BM layer, at a 1:2 ratio and then spun at 1,500 rpm with no brake. The mononuclear cell layer or buffy coat was collected, washed with 5 mL PBS, and spun at 1,500 rpm with high brake. The cell pellet was resuspended in MSC culture medium; α -MEM (Gibco, MA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Atlanta Biologicals, GA), 1% penicillin/streptomycin (Gibco, MA), 1% antibiotic-antimycotic (Gibco, MA), and 2.5 μ g/L human fibroblast growth factor (hFGF) (R&D Systems, MN), and seeded at a density of 10,000 cells/cm² in T175 flasks (Corning) in a 37°C, 5% CO₂ incubator. Adherent cells could grow for 10 days before media change. Next media change occurred at day 17. Subsequent media changes occur on a 3-day frequency. Cells were harvested by quick trypsin digestion and cryopreserved at a concentration of 1x10⁶ cells/mL in freezing medium (culture medium with 10% DMSO) as p1 when they reached a confluency of approximately 80–90%.

Culture and Cryopreservation of MSCs

Passage 1 (P1) cells were thawed and sub-cultured in culture media. Cells were continuously expanded for 56 days, with each passage lasting 8 days for 7 passages. Passages were initiated by seeding at a density of 200 cells/cm². MSCs were harvested at the end of 8 days by trypsin (GIBCO, MA) digestion. Each passage was cryopreserved at a concentration of 1x10⁶ cells/mL in freezing medium for analysis. Normal human dermal fibroblasts (NHDFs) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, MA) supplemented with 10% FBS (Atlanta Biologicals, GA) and 1% penicillin/streptomycin (Gibco, MA).

Adipogenic Differentiation

Human MSCs were plated at a density of 2.1x10⁴ cells/cm² in 400 μ L of MSC culture media in a 24 well plate (Corning). Media was replaced with fresh media every 3 days until they reached a confluency of 100%, at which point the media was replaced with 400 μ L of Adipogenic Induction Media (AIM) (Lonza, Switzerland). After 3 days in Induction Media, media was changed to Adipogenic Maintenance Media (AMM) (Lonza, Switzerland). This constituted one cycle of induction/maintenance. MSCs were subjected to 3 cycles of

induction/maintenance for optimal adipogenic differentiation. Non-induced controls were cultured in only Adipogenic Maintenance Media on the same schedule. At the end of the 3rd cycle, MSCs were cultured in Adipogenic Maintenance Media for 7 days, with media changes occurring every 3 days. Differentiation was analyzed at the end of the 7th day by Oil Red O (Sigma, USA) staining. All cells were incubated at 37°C, in 5% CO₂ incubators for the duration of the differentiation assay.

Staining & Analysis of Adipogenic Differentiation

Oil Red O working solution should be prepared up to 3 hours before use. For stock solution: 350mg Oil Red O (Sigma, USA) was dissolved in 100mL isopropanol and incubated at room temperature for 1 hour. Solution was then filtered using a 0.2µm filter. Working solution is made at a ratio of 6mL stock solution to 4mL distilled water, incubated at room temperature for 1 hour and filtered using a 0.2µm filter. AIM was aspirated off of cells and cells were gently washed twice with PBS. Cells were fixed using 4% neutral buffered formaldehyde (Polysciences Inc., PA) and incubated at 4°C for 1 hour on a shaker. Fixative was removed and cells were gently washed twice with PBS, leaving the last wash in the wells, about 300µL. Cells were imaged on a Zeiss 200M (Zeiss Microscopy, Germany). PBS was aspirated and 300µL Oil Red O working solution was added to the cells. Cells were incubated for 15 minutes at room temperature and then gently washed with distilled water, 500µL. Washing step was repeated 6–8 times until aspirated water is clear. Cells were covered with 300µL of distilled water and imaged using a Nikon TMD inverted microscope (Micro Video Instruments, MA). Distilled water was aspirated and Oil Red O stain was extracted from cells by adding 300µL 100% isopropanol and incubating for 5 minutes at room temperature. Absorbance was read at 492nm using a Benchmark Plus microplate spectrophotometer (Bio-Rad, CA).

MSC Support of Whole BM Culture

Human whole BM aspirate was cultured in IMDM (Gibco, USA) supplemented with 12.5% FBS at 4x10⁶ cells/well in a 24 well plate (Corning). Whole BMCs were either cultured with or without human MSC support at a ratio of 1:10 (MSC: whole BMCs). Human MSCs and NHDFs were recovered from cryopreservation 24 hours prior to co-culture in 24 well plates. On the day of co-culture, human MSCs and NHDFs were pre-treated with 10µg/mL Mitomycin C (Sigma, USA) for 2 hours. Mitomycin C-containing media was removed and cells washed in culture media and allowed to grow for 48 hours prior to being seeded with 2x10⁶ whole BMCs. Supporting stromal cells were seeded at 75x10³ cells per a well. Cells were co-cultured for 7 days and then analyzed by fluorescence activated cell sorting (FACS). All cells were incubated at 37°C, in 5% CO₂ incubators for the duration of the assay.

Immuno-modulatory Assay

Human MSCs and NHDFs were recovered from cryopreservation 24 hours prior to co-culture in 24 well transwells. 75x10³ cells were plated per a well in Roswell Park Memorial Institute Medium 1640 (RPMI; Gibco, USA) supplemented with 10% FBS, 1% penicillin/streptomycin, 10mM HEPES (Gibco, USA), and 1mM Sodium Pyruvate (Gibco, USA). Human peripheral blood mononuclear cells (PBMCs) were thawed from liquid nitrogen storage on the day of assay initiation. PBMCs were first stained with carboxyfluorescein

succinimidyl ester (CFSE; Sigma, USA) then added under the transwells with seeded stromal cells and activated with 10 μ g/mL Concanavalin A (Sigma, USA) and 100ng/mL recombinant IL2 (Sigma, USA). The non-contact co-culture was allowed to incubate for 4 days at 37°C, in 5% CO₂ incubators. At the end of the assay, transwells were discarded and PBMCs mechanically dissociated and analyzed on the FACS Calibur (BD, USA).

Immuno-phenotypic Analysis by Flow Cytometry

Cells were stained for 20–30 minutes at 4°C in the dark with directly labelled human monoclonal antibodies directed against CD45, CD34, and CD38 (BD Biosciences, USA). Cells were washed and then resuspended with PBS supplemented with 2% FBS and 2mM Ethylenediaminetetraacetic acid (EDTA; Gibco, USA). Flow cytometric analysis was performed using FACS Calibur (BD Biosciences, USA) and FlowJo Software (USA).

Population Doubling Times and Level

35x10³ MSCs were seeded into each T 175 flasks (Corning, USA) and were harvested by Trypsin (Gibco, USA) digestion at the end of 8 days. MSCs were cultured in culture medium. Cells were counted using a hemacytometer. Population doubling time and level were calculated using these formulae:

$$\text{Population Doubling Time} = [8 * \text{Log}(2)] / [\text{Log}(\text{Harvest Density}) - (\text{Log}(\text{Seeding Density}))]$$

$$\text{Population Doubling Level} = 3.32 * [\text{Log}(\text{Harvest Density}) - \text{Log}(\text{Seeding Density})]$$

Analysis of Cell Morphological Characteristics

Cells were stained with Calcein AM (Life Technologies, USA) and DAPI (Life Technologies, USA) and assessed using the EVOS microscope. Cell size was measured using Image J. Aspect ratio was calculated as the factor of the major axis to the minor axis.

$$\text{Aspect Ratio} = \text{Major Axis} / \text{Minor Axis}$$

Statistical Analyses

All statistical tests were performed using GraphPad Prism. Differences between groups were tested using Student's t-test whereby a p-value of < 0.05 were considered to be statistically significant. All comparisons were performed relative to p1 MSCs. A linear regression model was used to determine relationships between variables. Both correlation coefficient (r) and correlation of determination (r²) were used to describe strength, direction, and variation pertaining to the relationships between variables.

Results

Low-density seeding expansion induce *in vitro* aging of human MSCs

A defining characteristic of cells approaching senescence is reduced proliferative ability, attributed to progressive telomere shortening [37]. To induce MSCs into an aging-like phenotype, we induced proliferative pressure through a low-density (200 MSCs/cm²)

seeding model. This method allows for a greater number of doublings to reach confluency for a fixed flask size (T175). We assessed the effect of serial subculture on the expansion ability of MSCs to determine whether our low-density seeding model drives senescence. MSCs were harvested and counted after each passaging cycle. The expansion potential gradually declined with increasing sub-cultivation frequency, indicating passaging-induced *in vitro* aging. Reduction in absolute cell numbers recovered from each passaging cycle was clearly evident (Fig.1A). The proliferative capacity of passages 2–7 were statistically lower than young MSCs ($p < 0.0001$) (Fig.1A). We will refer to p1 as young MSCs in the subsequent sections of this article. In addition, population doubling times steadily increased with passage number with an approximate 3-fold increase between p1 and p7 (Fig.1B). Population doublings (PDs) are a metric for determining the *in vitro* age of a cell. It is defined as the number of doublings a cell undergoes from its primary isolation [38, 39]. Our low-density seeding model was associated as expected with a progressive decline in PDs with increasing passaging (Fig. 1B). This indicates a steep drop in expansion potential beyond the passage 1. Cumulative PDs were seen to increase with progressive sub-culturing and approaching the Hayflick limit for primary fibroblasts (Fig.1C). These findings indicate that the low-density seeding model of MSC expansion induces MSC aging towards a senescent phenotype.

Adipogenic differentiation capacity of MSCs diminishes with serial cryopreservation and passaging

Adipogenesis is an established test of MSC potency. Lipid-containing adipocytes were analyzed at the end of the 21-day assay. NHDF controls showed oil droplet levels comparable to non-adipogenic induced MSCs (Fig.S1 and Fig.2A–B). Adipogenesis was seen to be a MSC-specific function, with statistically higher levels of oil droplets observed in young (p1) up to p5 MSCs when compared to NHDF controls (Fig. 2B). While p4 MSCs were not found to be statistically significant compared to NHDF controls, it fit within the trend of showing elevated fluorescence levels (Fig. 2B). Consistent with previous data on aged MSCs, there was progressive decline in the ability of aging-induced MSCs to differentiate into adipocytes with increasing passages (Fig.2A–B). p1 and p2 have the highest propensities for adipogenic development (Fig.2B). Significant declines in adipogenic differentiation ability was observed with p3 and all subsequent passages when compared to young (p1) MSCs. Overall, this data suggests that the low seeding density proliferation of MSCs can mimic the reduced potency seen with MSCs in differentiating into lipid-producing adipocytes with age.

***In vitro* aging does not impact upon MSC immune-modulatory capacity**

To assess whether serial passaging-induced aging alters the ability to suppress immune cell proliferation, MSCs were co-cultured with activated human PBMCs in a non-contact co-culture. Unstimulated human PBMC cells were used as negative controls while NHDF cells as mock cell control to determine MSC-specific effects (Fig.3A). Proliferation was represented by the cumulative cell proportions within all generations of cells that were a result of immune activation (Fig.3A–B). Interestingly, unlike trends seen with expansion and adipogenic potentials, MSC aging conferred no impact on their modulation of immune activation with all passages showing no statistical differences in activation compared to

young p1 MSCs (Fig.3A–B and Supp. Table 1A). Immune-modulation of activated PBMCs was greatly enhanced by MSCs with significantly reduced PBMC proliferation observed across all passages when compared to NHDF controls which had only a modest effect (Fig. 3B).

To determine if there were differences within the degree of proliferation, we dissected the total CFSE positive population into individual generations. Peaks were termed F0 (parent peak) through to F4 (Fig.3C). Unstimulated controls showed no proliferation beyond the F2 phase (Fig.3D). Stimulated controls without co-culturing with MSCs showed the largest F4 population compared to all groups indicating the highest degree of activation. There were no significant age-associated differences in the sizes of the F0-F4 groups between p1-p7 MSCs (Fig.3D).

Maintenance of hematopoietic stem- and progenitor-containing populations requires young MSCs

BM-MSCs play a pivotal role in extrinsically regulating hematopoietic stem and progenitor cell (HSPC) proliferation *in vivo* [40]. These MSCs lie both near bone endosteal regions as well as in peri-vascular spaces, in close contact to BM HSPCs and consequently engaged in cross-talk with the HSPCs they regulate [40]. Since age severely affects hematopoiesis, we co-cultured aged MSCs and whole human BM. We utilized a contact dependent co-culture system to mimic the physiological cross-talk of the two cell types *in vivo* [8]. HSPCs express the pan leukocyte marker CD45 and can be found within a low side scatter population by flow cytometric analysis, while excluding MSCs which are CD45⁻ (Fig. S2). All statistical tests were performed relative to p1 MSCs or NHDF controls.

Only young (p1) and p3 MSC cultures showed significantly greater CD45⁺ cell counts at the end of the culture period when compared to NHDF (Fig.4A). Young p1 MSCs were the most supportive in maintaining the progenitor enriched CD45⁺ population (SSC-A^{lo} CD45⁺) after a 7-day co-culture (Fig. 4B). This SSC-A^{lo} CD45⁺ population was further dissected to target the CD34⁺CD38⁺ progenitor population (Fig.S3). Only young (p1) MSCs were able to significantly expand the progenitor-enriched CD34⁺CD38⁺ population (Fig.4C). MSCs older than p1, apart from p3, were significantly deficient in their ability to enhance the size of the progenitor pool (Fig. 4C and Supp. Table 1A).

Of greater clinical importance is the hematopoietic stem cell (HSC), which is capable of long-term, multilineage repopulation [41]. We further sub-fractionated the CD45⁺ parent population into the CD34⁺CD38⁻ stem cell-enriched subset (Fig.S3). Consistent with the CD34⁺CD38⁺ subset, only young p1 MSCs could enhance the HSC enriched pool (Fig. 4D). Only these young (p1) and p3 MSCs were able to support the expansion of the CD34⁺CD38⁻ stem cell enriched compartment above the capacity of NHDF and non-supported BM cells (p=0.0067 and p=0.0404 respectively) (Fig.4D). All MSCs older than p1, except for p3, were significantly inferior in their hematopoietic supporting potential when compared to young p1 MSCs (Fig. 4D and Supp. Table 1A).

Variability in MSC cell and nuclei morphology increases with increasing *in vitro* age

Recent evidence suggests that alterations in MSC morphology correlates with cellular function [35, 42]. To characterize morphologic changes that may be associated with biological aging, we analyzed MSC cell size through fluorescent imaging. Morphological analysis showed a progressive, statistically significant increase in cell area with increasing passages (Fig.5A and Supp. Table 1A). With continuous sub-culturing there was also an increase in the variance of cell area (Fig.5A). There was an additional correlation between MSC aspect ratio, reflecting cell shape, to age (Fig.S4 and Supp. Table 1A). A strong inverse relationship exists in which progressive *in vitro* aging results in decreased nuclei area spread as well as nuclei size (Fig.5B). This trend also applies to nuclei shape, whereby a more elongated nuclei shape (larger aspect ratio) correlates with a linear fit coefficient of 0.7881 to younger MSCs (Fig.5C).

In vitro age is a strong predictor of MSC adipogenic and hematopoietic-supporting potencies

We show that both MSC and nuclei size change with increasing *in vitro* age and that age influences MSC function (Fig.6A and Supp. Table 1A). We proceeded to parametrically assess the relationship between the functions experimentally shown to be affected by age (differentiation, immunomodulation, and HSC support). We performed linear regression modeling to determine the Pearson correlation coefficient associated to MSC function and age (Supp. Table 1). Age was a strong indicator of diminishing adipogenic potentials with a coefficient of 0.8356 (Fig.6A). While the correlations for hematopoietic-supporting potency and age was not above the conventional standard threshold of 0.8 which indicates a strong relationship, the *r* value for HPC-containing (0.6511) and HSC-containing (0.6939) populations were higher than that for immunoproliferative potency (*r*=0.2006) (Supp. Table 1A). We interestingly correlated, for the first time, that cell shape (aspect ratio) is a strong predictor of hematopoietic-supporting potency of MSCs (Fig.6B–C). Larger aspect ratios were seen to strongly correlate to enhanced MSC support of both hematopoietic-progenitor and stem cell (*r*=0.9338 and 0.9806 respectively) (Fig.6B–C). Cell aspect ratios also had a *r* value (0.7881) close to 0.8 with age (Supp. Table 1A).

Discussion

MSCs continue to be widely tested in clinical trials as cell infusion therapy candidates, through the innate propensity of their stem cell subpopulation to regenerate damaged tissue, support hematopoiesis, and modulate the immune response. While their clinical promise is encouraging, much is still unknown about their therapeutic MoAs and respective sensitivities. The clinical use of MSCs as therapeutics would benefit from an end-to-end understanding of MSC heterogeneity and associated potency for a given process and clinical indication prior to patient administration. These potency assays should ideally link *in vitro* readouts that reflected a sufficient bioactivity for clinical outcomes. The goal of this study was to verify MSC potency assay specificity and quantify morphological changes of MSCs during expansion that were associated with age-sensitivity of MSCs in several potency bioassays for regression modeling.

The effect of progressive cell expansion, reflective of a biomanufacturing expansion process, is of particular relevance to the testing of MSC potency. Most previous reports within the field demonstrate profound consequences on MSC proliferative and regenerative potentials with progressive passaging *in vitro* towards a senescent state [34, 37, 43]. This provides a direct link for therapeutic potency to their stem cell subpopulation. We utilized a low-seeding density (200cells/cm²) model of *in vitro* passaging, whereby MSCs were cryopreserved at the end of each passage and then thawed out before being subjected to each potency assay. This methodology represents an industrially relevant model for large expansions of MSCs. Similar to previous reports in literature, our model demonstrated progressive loss of proliferative capacity with continuous passaging, indicative of MSC biological aging [30, 44]. Our model of MSC doubling during expansion approached, but did not reach, the Hayflick limit as a historical metric of senescence of a primary fibroblast [38, 39]. Although our expansion model represents current industrial practices, it does not address the issue of MSC heterogeneity pertaining to the specific expansion and harvesting of their self-renewing stem cell subpopulation [27]. In fact, the understanding of heterogeneity within the MSC pool will directly guide the biomanufacturing of MSCs towards lowering batch-to-batch variation. One study investigated the clonogenicity and hence homogeneity of MSC expansion cultures by manipulating the seeding densities to as low as 1.5 to 12 cells per cm² [24]. They found that lower seeding densities resulted in greater yields within singular colonies [24].

Traditionally, the potency of MSCs for orthopedic and cosmetic regenerative applications have been measured by the ability of their stem cells to differentiate into three lineages; osteogenic, chondrogenic, and adipogenic [45]. Consistent with literature, we confirm that adipogenic differentiation potential is a unique property of MSCs and not seen with human skin fibroblasts. We also observe a progressive loss of adipogenic potential with increasing population doublings, or MSC age *in vitro*. While this assay is often chosen to reflect MSC fitness and their expected clinical outcome, it is important to note the discrepancy between this *in vitro* phenomenon of MSC aging to that seen *in vivo*. In stark contrast, with age there is enhanced adipogenesis within the BM at the expense of osteolineage differentiation and hematopoiesis [46, 47]. The *in vitro* nature of the assay presents many differences to events that occur in the BM. The other stromal components of the BM themselves are subjected to intrinsic aging as well as extrinsic factors from interacting immune cells, that are themselves undergoing intrinsic aging [48]. This intricate cross-talk between niche compartments and immune cells may contribute to this discordance and can be highly influenced by the composition of the heterogeneous pool of MSCs. Furthermore, this age-induced deterioration in adipogenic potential highlights the need for applying these cells as therapeutics for orthopedic applications at earlier stages of expansion.

The same batches of MSCs were subjected to an immunomodulation assay to investigate cross-functional potencies with age. MSCs have been shown to suppress an activated immune cell milieu through contact and non-contact dependent communication [11]. Interestingly, unlike adipogenesis, age did not influence the ability of MSCs to suppress activated PBMCs. BM-derived MSCs appear to only decline in their potency to immunomodulate after passage 8, whereas umbilical cord-derived MSCs show age-induced impairments within earlier passages [49]. While this may indicate that MSC age does not

influence their ability to modulate the activated immune system, it also opens up the possibility that this assay is not sensitive enough to reflect age-associated changes within MSC biology, particularly in earlier passages. As such, this potency assay may not be suitable to report quality of MSCs between batches, donors, before and/or after cryopreservation. Another implication this finding makes is on MSC production efficiency. While P1-P7 MSCs appear to have the same immunomodulatory abilities, their growth kinetics are still quite different. Thus, at a production scale, one should still opt for younger MSCs because they will proliferate more within a fixed amount of time.

The nurturing of hematopoiesis through the support of hematopoietic stem and progenitor compartments by MSCs represents a key function of clinical importance [50]. The potential for a combination of cell-contact [51–53] and soluble factor pre-conditioning [54] of the host by a co-infused MSC population with CD34+ bone marrow progenitors may be viable mechanisms at hand with historical learnings in mouse models. We observed an enhanced ability for young MSCs to expand HPC and HSC containing populations in 7 days, without cytokine supplementation. This effect was lost with any MSC older than at the p1 age. This is quite consistent with previous reports in the literature [8, 55]. Interestingly, the ability of younger MSCs to increase the HSC pool *in vitro* through increased cell cycling is not what happens in aged BM [8, 56]. These results, however, do not necessarily inform on the fitness of HSCs which corresponds to expansion of the HSC pool while supplying HSC differentiated functions [57]. Furthermore, a larger panel of phenotypic markers will enable more specific targeting of primitive HSCs than that seen within the scope of this study.

Our findings indicate that individual potencies do not comprehensively report fitness of a singular batch of MSCs. Whole genomic and transcriptomic snapshots, though effective to show differences with cell aging, may help to identify a feasible molecular marker test for the large scale manufacturing of MSCs [31]. The field can benefit from molecular biomarkers that are causative of MSC therapeutic function but have been challenging to substantiate before introduction as standard practices [58, 59]. A convenient approach to predicting MSC-potency could lie in morphological changes. The use of cell morphology to inform cellular function is not new and in fact dates back to the 1950's [60]. Numerous studies have further corroborated that morphological changes correlate with MSC age and differentiation potential [32, 35, 36, 42, 61]. We herein performed similar analyses on cell and nuclei size and shape with during *ex vivo* expansion. We found that cell area increased progressively with age while nuclei size and shape became smaller and less elongated. Alterations in nuclear biology during MSC aging continue to be dissected as a way to characterize cell state [62, 63]. In keeping with the trends mentioned above, we found strong correlations between age and adipogenic potential, consistent with both our previous findings as well as that of the field. Utilizing linear regression modeling, we expanded our analyses to evaluate associations between cell morphology and other MSC potencies. Our data suggests, for the first time, that cell shape is a strong predictor for hematopoietic supporting potency of BM MSCs. More spindle-shaped MSCs with larger aspect ratios were better able to expand both hematopoietic progenitor- and stem- containing cell populations from whole BM. This is in accordance with recent a report within the MSC field in which investigators demonstrate a strong predictive capacity for cell size within the early days of osteogenic induction on endpoint mineralization levels [61], however specific *in vivo*

assessments of HSPC differentiation and self-renewal are required to better define our HSPC-containing populations. Another study proposes the collective usage of three biophysical parameters; cell diameter, stiffness, and nuclear membrane fluctuation, to inform of BM MSC multipotency [64, 65]. Further investigations looking at shifts in MSC cellular composition and alterations in their biophysical properties will guide biomanufacturing processes and the establishment of quality control assays. These findings, combined with our data, provide strong evidence that purification methods based on the size and shape of MSCs could lead to cell products that are enriched for stromal supportive functions. Future investigations are required to validate results across several tissue sources, ages, sexes and other donor selection criteria and ultimately ascertain the best practices for the scalable sorting of MSCs based on size or other biophysical properties as an indicator of potency.

Potency assessment of MSCs continues to be a major issue in developing MSC therapeutics. A combination of sensitivity, specificity, and time must be balanced. It is unlikely that a single test will suffice to truly encompass the efficacious potential of MSCs even for a single indication. We herein show differential effects of cell expansion with respect to cell differentiation, immunomodulation, and hematopoietic support. Based on these findings, we believe that it is of great importance to perform diverse, parametric analyses of culture-sensitivities to MSC function in order to accurately characterize their potency during stages of considered therapeutic use.

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Highlights

- MSC potencies for their different functions are not identically affected by age.
- *In vitro* age can strongly predict MSC adipogenic and hematopoietic-supporting potencies.
- MSC shape can also strongly predict for hematopoietic-supporting potency.

Figure 1. Serial passaging with low initial seeding density shows progressive deterioration of MSC expansion potential

Analysis was performed on cells seeded at a density of 200cells/cm² and cultured for 8 days, with 7 repeats of this sub-culturing process. All values are means + standard deviation. (A) Yield of cells at the end of the 8-day culture passage progressively decline with continuous sub-culturing. * indicates p-values between all passages compared to young MSCs (p1). *p<0.05, **p<0.01, and ***p< 0.0001. (B) Data points are matched to axis color. Longer population doubling times and lower cumulative population doubling levels are shown to be associated with increasing passages. (C) MSCs undergo more doublings throughout progressive sub-culturing.

Figure 2. *In vitro* aging of MSCs is associated to a decline in adipogenic differentiation capacity Adipogenic assessment was performed on cells at the end of the 8-day culturing for each passage. Cells were subjected to adipogenic differentiation media and maintenance media for 21 days and then analyzed by staining with Oil Red O. (A) Oil Red O staining of MSCs and NHDF subjected to adipogenic induction conditions. (B) Adipogenic differentiation potential was specific to MSCs, specifically from earlier than passage 5. Red lines indicate p-value differences compared to NHDF controls while black lines represent p-values between all passages compared to young MSCs (p1). All values are means + standard deviation. * indicates p-values between all passages compared to young MSCs (p1). *p<0.05, **p<0.01, and ***p< 0.0001, with red lines comparing to NHDF controls and black lines to young (p1) MSCs.

Figure 3. MSCs modulation of human primary T cells is unaffected by *in vitro* aging
 Analysis was performed on CFSE-pulsed human PBMCs activated with 10 μ g/mL Concanavalin A and 100ng/mL recombinant IL-2, and co-cultured with stromal cells for 4 days. (A) Flow cytometric analysis of CFSE from stimulated human PBMCs co-cultured with serially passaged MSCs or NHDF controls compared to CFSE from unstimulated PBMCs. (B) MSC-specific function to suppress immune activation was unaffected *in vitro* age. Red lines indicate p-value differences compared to NHDF controls. (C) Flow cytometric analysis of CFSE incorporation of peripheral lymphocytes were further dissected to individual cell generations referred to as F0 (parent generation) through to F4. (D) All MSC age groups demonstrated similar percentage of cells within each generation. All values are means + standard deviation. * indicates p-values between all passages compared to NHDF controls. *p<0.05, **p<0.01, and ***p< 0.0001.

Figure 4. Human bone marrow hematopoietic-support function of MSCs declines upon sub-culturing

Analysis was performed on whole human BM co-cultured with stromal cells for 7 days. All values are means + standard deviation. * indicates p-values between all passages compared to young MSCs (p1). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$, with red lines comparing to NHDF controls and black lines to young (p1) MSCs. (A) Young (p1) MSCs showed the ability to expand hematopoietic cells. (B) Young (p1) MSCs supported the maintenance of hematopoietic stem- and progenitor-containing population. (C) Young (p1) MSCs demonstrated the ability to expand the hematopoietic progenitor population. (D) Young (p1) and p3 MSCs were able to increase the numbers of stem cell containing CD34⁺ population.

Figure 5. Age-associated alteration in MSC and nuclei size *in vitro*

Analysis was performed on MSCs at the end of each 8-day sub-culturing cycle. Cells were stained with Calcein and nuclear stain DAPI to quantify size and shape by microscopy. All values are presented as means + standard deviation. * indicates p-values between all passages compared to young MSC controls. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$. (A) Cell size increases with increasing *in vitro* age. (B) Nuclei become smaller with progressive sub-culturing. (C) Shape of MSC nuclei become less elongated with increasing *in vitro* age.

Figure 6. Age and cell size can, respectively, predict for MSC adipogenic and hematopoietic-supporting potency

Analysis was performed for potency assays and morphological analyses as previously described. Linear regression analysis was performed to determine strength of relationship between parameters. (A) Adipogenic potential of MSCs shows a strong linear inverse relationship to *in vitro* age. MSCs with high aspect ratios show enhanced ability to support (B) progenitor and (C) stem cell containing BM population.