TITLE:

Hypoxia Depletes Contaminating CD45⁺ Hematopoietic Cells from Bone Marrow Stromal Cell (BMSC) Cultures: Methods for BMSC Culture Purification

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SUMMARY

Culture expanded bone marrow stromal cells (BMSCs) are easily isolated, can be grown rapidly *en masse*, and contain both skeletal stem cells (SSCs) and multipotent mesenchymal progenitors (MMPs). Despite this functional heterogeneity, BMSC cultures continue to be utilized for many applications due to the lack of definitive and universally accepted markers to prospectively identify and purify SSCs. Isolation of BMSCs is widely based on their adherence to tissue culture plastic; however, this method poses unique challenges when working with murine models. In particular, hematopoietic cell contamination is a significant impediment during the isolation of murine BMSCs. Several strategies to reduce hematopoietic contamination have been developed with varying levels of success. In this study, we found that up to 60% of cells isolated in standard BMSC cultures expressed the hematopoietic marker CD45. Remarkably, when cultured at a physiological oxygen tension of 1% O₂, there was a 10-fold reduction in hematopoietic cells associated with a concomitant increase in PDGFR α ⁺ expressing stromal cells. This finding was due, in part, to a differential response of the two populations to low oxygen tension, or hypoxia. Specifically, in standard tissue culture conditions of 21% O₂, CD45⁺ hematopoietic cells showed increased proliferation coupled with no observable changes in cell death when compared to their counterparts grown at 1% O₂. In contrast, PDGFR α ⁺ stromal cells responded to hypoxia by increasing proliferation and exhibiting a 10-fold decrease in cell death. In summary, we describe a simple and reliable method exploiting the divergent biological response of hematopoietic and stromal cells to hypoxia to significantly increase the PDGFR α ⁺ stromal cell population in murine BMSC cultures.

INTRODUCTION

The skeleton houses SSCs which arise in post-natal bone and are defined by their *in vivo* ability to self-renew and to differentiate into hematopoietic supporting stroma, osteogenic, chondrogenic and adipogenic lineages[1-3]. Currently, there remains a lack of definitive cell surface markers to identify and/or prospectively isolate a homogenous population of SSCs [4-6]. In lieu of this, BMSCs have been shown to support hematopoiesis and contribute to all functions of the skeleton. BMSCs were first isolated based on their rapid adherence to tissue culture (TC) plastic [7]. At non-clonal density, they can be quickly expanded and are capable of differentiating into distinct mesodermal lineages *in vitro* demonstrating the presence of MMPs [8]. When plated at clonogenic density, some BMSCs exhibit density independent growth, forming discrete colonies derived from single cells which are referred to as colony forming units-fibroblastic (CFU-F) [9-11]. Upon transplantation, some CFU-Fs have the capacity for *in vivo* multipotent differentiation and self-renewal, the defining features of SSCs [12, 13]. For these reasons, BMSCs serve as a powerful tool for cell-based therapies to treat musculoskeletal disorders by repairing damaged mesenchymal tissues. Moreover, with the breadth of transgenic and genetically engineered mouse models, BMSCs also serve as an invaluable means to harness information regarding the cellular and molecular mechanisms regulating MMP and SSC function. Importantly, the successful use of BMSCs for cell-based therapies and meaningful interpretation of genetic studies is dependent upon the ability to isolate pure populations of mesenchymal cells.

In humans, preferential attachment to TC plastic can be used with success for the isolation of mesenchymal populations; however, for murine models, this technique presents several challenges [14, 15]. Most notably, this includes high hematopoietic contamination because murine hematopoietic cells can directly adhere to TC plastic and both hematopoietic stem cells (HSCs) and their differentiated progeny can attach directly to stromal cells [16, 17]. Moreover, stromal cells have the capacity to support *in vitro* granulopoiesis in the absence of exogenous cytokines [18]. Collectively, these factors contribute to the persistent contamination of hematopoietic cells in murine BMSC cultures even after prolonged culturing [16, 19].

Several methodologies such as subculturing, immunodepletion, and low-density seeding have been tested to eliminate hematopoietic cells from BMSC cultures; however, these techniques have only

achieved limited success. While frequent and prolonged subculturing improves purity, it also results in a replicative senescent phenotype with the possibility of altered differentiation potential [20, 21]. Furthermore, this method increases the potential of selecting for clones carrying inactivating mutations in p53, thereby increasing the likelihood of cellular transformation [22, 23]. Immunodepletion can result in a limited proliferative capacity of BMSCs [24]. Because only a small number of cells are recovered, initiation of cultures at low densities is prohibitively time consuming where rapid expansion of multipotent mesenchymal stromal cells is required [25]. Taken together, these limitations demonstrate that hematopoietic contamination continues to serve as a significant impediment to the isolation of BMSCs based on plastic adherence [26].

The bone microenvironment is characterized by vascular heterogeneity associated with regional areas of hypoxia [27-29]. Under hypoxic conditions, cells upregulate the hypoxia inducible factor (HIF) signaling pathway to drive expression of genes which facilitate oxygen delivery and cellular adaptation to low oxygen [30]. Importantly, activation of HIF target genes is highly context dependent with cellular responses exquisitely sensitive to oxygen concentration [31]. Imaging analysis of HSC and SSC cell surface markers demonstrate that these cells are localized to perivascular regions where direct and indirect measurements reveal oxygen tensions ranging from 4 - 1% [1, 32]. While both human and murine BMSCs demonstrate improved proliferation capacity at 5% O₂, the differential response of mesenchymal stromal cells and hematopoietic progenitors to physiologic oxygen tensions in the bone marrow is less well defined [22]. For these reasons, we aimed to determine the impact of culturing BMSCs under physiologic hypoxic conditions of 1% O₂. Importantly, we discovered that 1% O₂ strikingly reduced the percentage of CD45 expressing hematopoietic cells and this reduction remained consistent over time. Here, we report an easy and reliable method by which hematopoietic cells can be selectively eliminated while simultaneously enriching for stromal cells during BMSC preparations.

RESULTS

The Majority of Bone Marrow Cells Isolated Based on Adherence to Tissue Culture Plastic Express the Hematopoietic Marker CD45

We isolated BMSCs based on the rapid adherence of cells to TC plastic when collected from bone marrow flushes. We noted that high density BMSC ($1.3 \times 10^5 \text{ cm}^2$) cultures were phenotypically heterogeneous when incubated under 21% O_2 , with many cells exhibiting small rounded morphology indicative of monocytes (**Figure 1A, C**). Given this morphological heterogeneity, we sought to determine the proportion of presumptive MMP and SSCs in our cultures. For this purpose, we first evaluated the expression of a known bone marrow stromal cell marker which enriches for SSCs, the leptin receptor (LepR). LepR marks a population of bone marrow stromal cells which arise perinatally and contribute to osteoblast, chondrocyte and adipocyte lineages *in vivo* [33]. Flow cytometry and imaging analysis of BMSCs isolated from *Lep^rCre; Rosa26^{tdTomato/+}* conditional reporter mice revealed only a small proportion of cells expressed tdTomato (Td/Tm) (**Figure 1A**). To confirm our analysis we evaluated the expression of PDGFR α , an early mesenchymal marker which is broadly expressed in the bone marrow stroma, highly expressed in CFU-Fs, and multipotent when subjected to *in vitro* differentiation assays [34]. Consistent with other reports, LepR⁺ bone marrow stromal cells uniformly express PDGFR α , supporting the concept that bone marrow stromal cells express both PDGFR α and LepR (**Figure 1A**). Notably, the proportion of tdTomato⁺, PDGFR α ⁺ or tdTomato⁺;PDGFR α ⁺, double positive cells constituted only a minority of the total cell population when using standard BMSC isolation methods (**Figure 1A**).

Given the low percentage of MMP and SSCs, we next sought to determine the remaining cellular constituents within our cultures. Remarkably, using a gating strategy in which isotype controls acted as a negative reference, flow cytometry analysis revealed that the majority of cells in BMSCS cultures express the hematopoietic cell surface marker CD45 $63.98 \pm 2.03\%$ (**Figure S1A, 1A, B**). Given the high hematopoietic contamination present in these cultures, we sought to determine if vigorously rinsing dishes 3 hours after plating would reduce the number of CD45⁺ cells (**Figure 1B**) [10]. While the majority removed cells were CD45⁺ (**Figure S1B**); despite vigorous rinsing, a high percentage of contaminating CD45⁺ cells remained in our BMSC cultures (**Figure 1C**). Strikingly, in unrinsed samples, only $48.43 \pm 10.0\%$ of cells expressed PDGFR α (**Figure 1D**). Vigorous rinsing after plating led to an increasing trend of PDGFR α ⁺

cells; however, these findings were not statistically significant suggesting that rinsing may remove only CD45 expressing cells that take longer to adhere to TC plastic or to stromal cells and not enrich for PDGFR α ⁺ cells (**Figure 1D, S1B**). Notably, PDGFR α expression was primarily restricted to CD45⁻ cells (**Figure 1D**). Taken together, these data suggest that isolation of BMSCs with conventional techniques leads to a high contamination of CD45⁺ hematopoietic cells coupled with moderately low numbers of mesenchymal stromal cells. These findings highlight the need for methods to increase the purity of stromal cells during BMSC isolation.

Hypoxia Reduces CD45⁺ Cells While Increasing PDGFR α ⁺ Cells Present in BMSC Cultures

Given the physiological relevance of hypoxia in regulating cell function within the bone microenvironment, we sought to determine if this environmental factor could influence the cellular composition of BMSC preparations. To test this, BMSCs were isolated, rinsed and grown either at atmospheric (21%; normoxia) or physiologic (1%; hypoxia) oxygen tensions. In contrast to normoxic cultures which remained heterogeneous, phase microscopy revealed that hypoxic cultures consisted of a homogeneous population of large bipolar spindle shaped cells (**Figure 2A**). Interestingly, flow analysis demonstrated that larger cells constituted the majority of CD45⁻, PDGFR α ⁺ stromal cells (**Figure S2**). Consistent with the observed changes in cellular morphology, cultures grown in hypoxic conditions showed a significant reduction in CD45⁺ cells (45.12 ± 4.44 % vs 5.78 ± 0.95 %) coupled with a substantial increase in PDGFR α ⁺ cells ($48.90\% \pm 5.73$ % vs 81.48 ± 2.86 %) when compared to cells grown in normoxic conditions (**Figure 2B**). Supporting these findings, BMSCs isolated from *LepRCre;Rosa26^{tdTomato/+}* mice and grown at 1% O₂ also showed a significant decrease in CD45⁺ cells which was associated with an increase in both tdTomato⁺, PDGFR α ⁺ and tdTomato⁺;PDGFR α ⁺ double positive cells (**Figure 2C, D**). Taken together, these findings demonstrate that modifying culture conditions by decreasing oxygen levels to 1% profoundly diminishes the percentage of hematopoietic contaminants while enriching for PDGFR α ⁺/LepRCre⁺ derived stromal cells in BMSC cultures.

Hypoxic Reduction of CD45⁺ Cells Remains Constant Over Time, Passage Number, and Cell Density

To further explore the relationship between hypoxia and the reduction in hematopoietic cells, we first sought to determine if CD45 expression remained consistent over time in low oxygen. We noted that over a period of seven days, CD45⁺ cells remained consistently high in normoxic cultures but remained

below 6% in hypoxic cultures at all time points (**Figure 3A**). Moreover, after two rounds of trypsinization, CD45⁺ cells continued to persist in normoxic cultures but were reduced to $1.36 \pm 0.19\%$ of cells in hypoxic cultures (**Figure 3B**). While there was an increase in PDGFR α cells cultured under normoxic conditions in passage 2 (P2) when compared to passage 1 (P1), this was likely due to an increase in CD45⁺;PDGFR α ⁺ double positive cells, suggesting that over time in normoxia, CD45⁺ cells increase their expression of the PDGFR α receptor (**Figure 3B**).

Culturing cells at low density has been shown to reduce hematopoietic contaminants [25]. As such, we next sought to determine if hypoxia would also enrich the cellular composition of stromal cells in BMSC cultures grown at clonogenic density. Consistent with previous reports, when cells were plated at 6×10^5 cells/cm² with rinsing, crystal violet staining highlighted the presence of discrete colonies, derived from single cells (**Figure 3C**). Importantly, hypoxia increased both the total number of colonies and their overall size (**Figure 3C**). Accordingly, flow cytometry analysis revealed a significant reduction in the proportion of hematopoietic cells when compared to high density BMSC cultures, however, $21.82\% \pm 1.73\%$ of cells were still of hematopoietic origin. In contrast, when grown in hypoxia, $5.04\% \pm 0.53\%$ of cells expressed CD45 (**Figure 3C**). Additionally, the percentage of PDGFR α ⁺ cells in hypoxia was also increased relative to normoxia (**Figure 3C**). While at clonogenic density, the number of CD45⁺ cells are fewer than when plated at high density cultures; importantly, hypoxia is still able to reduce hematopoietic cells in these conditions. Taken together, our data demonstrates that BMSCs grown under physiological hypoxia results in a dramatic decrease of contaminating CD45⁺ hematopoietic cells while increasing PDGFR α ⁺ cells which is consistent over time, passage number and cell density.

Hypoxia Reduces Proliferation of CD45⁺ Cells While Inhibiting Apoptosis of PDGFR α ⁺ Cells.

The differential effect of hypoxia on hematopoietic and stromal components isolated during BMSC preparations has not been previously reported. To determine the cellular mechanisms regulating hypoxia dependent reduction of CD45⁺ cells in our cultures, we first assessed proliferation by incubating cells with EdU. Flow cytometry analysis revealed CD45⁺ cells exhibit increased EdU incorporation in normoxia when compared to hypoxia (**Figure 4A,C**). Conversely, in normoxic conditions we noted a decrease in PDGFR α ⁺;EdU⁺ double positive cells when compared to hypoxic conditions (**Figure 4B,C**). Importantly, in

normoxia, CD45⁺ cells had higher levels of EdU incorporation when compared to PDGFR α ⁺ cells; while in hypoxia, CD45⁺ cells were less proliferative than PDGFR α ⁺ cells (**Figure 4C**). We next evaluated apoptosis by performing TUNEL staining assays to assess DNA fragmentation. We did not detect statistically significant differences between CD45⁺ cells subjected to varying oxygen tensions (**Figure 4D, F**). However, there was a 10-fold decrease of PDGFR α ⁺ cells undergoing programmed cell death in hypoxia when compared to normoxia (**Figure 4E, F**). Moreover, in normoxia, we detected a 6-fold increase in PDGFR α ⁺ cells undergoing apoptosis when compared to CD45⁺ cells (**Figure 4F**) but no differences between the two cell populations were detected under hypoxia. Taken together, these data indicate that CD45⁺ hematopoietic cells are more proliferative while PDGFR α ⁺ cells undergo much greater cell death at 21% oxygen. However, in hypoxia, PDGFR α ⁺ cells are more proliferative while both populations undergo cell death at the same low rate. Hence, this differential response in proliferation and apoptosis likely contributes to diminished CD45⁺ cells and the associated expansion of PDGFR α ⁺ cells at low oxygen tensions.

DISCUSSION

Although physiologic oxygen tensions in the bone microenvironment range from 4% – 1%, tissue culture incubators are standardly set to mimic atmospheric oxygen conditions of 21% [28]. Consistent with other reports, we found at this oxygen tension, the majority of cells in murine BMSC cultures expressed the hematopoietic marker, CD45 [22, 24]. Standard protocols that incorporate a vigorous rinsing step three hours after plating reduced CD45⁺ hematopoietic contaminating cells by ~50%. Here, we demonstrate incubation at a physiological oxygen tension of 1% reduced hematopoietic contaminants by over 90% during murine bone marrow preparations (Figure 2B). Importantly, culturing at 1% oxygen also had the added benefit of selecting for PDGFR α ⁺ stromal cells. It is important to note that a previous study culturing BMSCs at 5% O₂ demonstrated improved murine BMSCs lifespan and differentiation potential; however, CD45 expressing cells remained prevalent, constituting 49% of the total cell population. To reduce hematopoietic contamination, the authors performed serial immunodepletion utilizing antibodies against lineage markers [22, 35]. In contrast, our data indicates culturing BMSCs directly in 1% O₂ is sufficient to dramatically reduce contaminating CD45⁺ cells to less than 6% of the total cellular population, thus potentially eliminating the need for immunodepletion. Moreover, this method reduces the need for additional passaging of cells required for immunodepletion; thereby decreasing the possibility of cellular transformation or immortalization which is associated with increased passage number [36, 37].

The bone microenvironment provides the supportive niche for HSCs and SSCs while also housing their differentiated progeny. During HSC differentiation and maturation, hematopoietic cells switch from glycolysis to oxidative phosphorylation to allow for cells to respond to increasing energy demands associated with differentiation [38-40]. Hence, despite its high vascular density, the bone marrow microenvironment harbors regions which are strongly hypoxic due in part to the high oxygen consumption by resident hematopoietic cells [27-29]. Indeed, Kroghian models of the bone microenvironment estimate that a three-cell layer of granulocytic progenitors is sufficient to deplete the oxygen of a nearby sinusoid [41]. Accordingly, this differentiation process is associated with an increase in mitochondrial reactive oxygen species (ROS) created during oxidative phosphorylation [42, 43]. We noted in normoxic conditions, CD45 expressing cells exhibited an increase in EdU incorporation when compared to cells grown in hypoxic conditions with no observed differences in TUNEL⁺ staining. It is possible that differentiated hematopoietic

cells present in BMSC cultures are inherently resistant to ROS generated at 21% O₂ due to the metabolic reprogramming that occurs during differentiation. Murine HSCs, are localized to areas of low oxygen tension, are defined by strong retention of the hypoxia marker, pimonidazole, and require HIF-1 α for survival and quiescence [44, 45]. As such, we cannot rule out the possibility that these biological characteristics leads to the retainment of HSC in hypoxia thereby contributing to low level of CD45⁺ cells persisting at 1% O₂. Collectively, the differential response seen between CD45⁺ and PDGFR α ⁺ cells suggests that inherent differences in biological response to oxygen can be exploited to enhance purification of BMSC cultures.

Supraphysiologic levels of oxygen can result in a several fold increase in ROS causing extensive damage to DNA leading to genomic instability, cell cycle arrest and cell death [46]. Indeed, high oxygen tension has been shown to inhibit proliferation and survival of both human and murine BMSCs, attributed in part, to p53 dependent mitochondrial ROS production [22]. Consistent with these studies, our data indicates PDGFR α ⁺ BMSC are exquisitely sensitive to high oxygen tension, undergoing rapid cell death as indicated by the high percentage of TUNEL positive cells when compared to CD45⁺ hematopoietic cells. BMSC cultured in 1% oxygen were characterized by a 10-fold reduction in programmed cell death when compared to their normoxic counterparts. These data are consistent with studies demonstrating reduced BMSC apoptosis when cultured at 3-5% oxygen [22, 47].

Previous reports in murine models demonstrated that hematopoietic cells are required to provide paracrine factors necessary for stromal cell growth. Specifically, after 8 days of culture, immunodepleted BMSCs isolated from FVB/N and Balb/C mice exhibited limited growth potential relative to non-immunodepleted cells [11, 13, 24]. However, this growth inhibition was not observed in immunodepleted cells in hypoxic conditions [22]. In our study, hypoxic cultures passaged after 7 days showed incorporation of EdU suggesting the retainment of proliferative capacity. Moreover, our studies were performed using BMSCs isolated from C57BL/6 mice which have growth rates much lower than both FVB and Balb/C mice suggesting that this technique could be used to purify and overcome the inherent growth limitations in BMSCs isolated from C57BL/6 strain of mice [48]. However, we cannot rule out the possibility that the low level of CD45⁺ cells present in hypoxic cultures was sufficient to provide paracrine factors for stromal cells.

In summary, several methodologies have been developed for the isolation of more homogeneous populations of BMSCs including prospective isolation using cell surface markers [32], plating at low

249 densities [13], and immunodepletion; however, these protocols have not been widely adopted. Difficulties
 250 in purifying BMSCs arise from limited lifespan, the capacity to undergo transformation and high
 251 hematopoietic content. In this report, we identified that isolating and culturing BMSCs at 1% O₂ affords an
 252 easy and reproducible method to improve the purity of BMSC cultures that can be universally adopted.
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EXPERIMENTAL PROCEDURES

Culture of bone marrow-derived stromal cells

Bone marrow stromal cells were isolated from 8-16-week-old male and female C57BL/6J mice (Jackson Laboratory, 000664). All experimental procedures were approved by the Institutional Animal Care and Use Committee at Duke University. Mice were euthanized by CO₂ asphyxiation and long bones were subsequently extracted. BMSCs were isolated by flushing the bone marrow of the extracted femurs and tibias. Prior to plating, red blood cells were removed using an ammonium chloride-based red blood cell lysis buffer (Sigma-Aldrich, R7757). Cells were cultured in αMEM containing nucleosides (Gibco, 12571-063) supplemented with 20% fetal bovine serum (FBS) (GE Healthcare Life Sciences, SH30071.03), and 1% Penicillin-Streptomycin (P/S) (Gibco, 15140-122) at 37°C in 5% CO₂ atmosphere. Three hours post-plating, the BMSCs cultures were subjected to a 3x PBS rinse to remove non-adherent cells.

For unrinsed cells, cells were cultured as described above, however, the 3 hours post-plating rinse was not performed. Media was changed 2 days post-plating and cells were grown for 7 days in their respective conditions.

To assess clonogenic densities, cells were cultured and grown for 7 days as described above and seeded at 6×10^4 per cm².

For the passage 0 time point, cells were seeded at a density of 40 million cells per 150 mm dish for normoxic (21% O₂) cultures and 13 million cells per 100 mm dish for hypoxic cultures prior to rinsing. Hypoxic cell cultures were grown in an InvivoO₂ 400 chamber (Baker Ruskinn) at 1% oxygen tension. Following a 3-hour post-plating rinse, cells were grown for 7 days in their respective conditions.

For passage 1 time points, cells were initially seeded at the densities described in the passage 0 time point and grown for 7 days in their respective conditions. After 7 days, cells were fully trypsinized using 0.25% Trypsin-EDTA (Gibco, 25200-056) and gently scraped to collect all adherent cells. Afterwards, cells were plated in 100mm dishes at a density of 3.0×10^5 , 2.0×10^5 , and 1.5×10^5 for days 3, 5, and 7 respectively under normoxic conditions. For hypoxic conditions, cells were plated in 100mm dishes at a density of 1.1×10^5 , 0.7×10^5 , and 0.35×10^5 for days 3, 5, and 7 respectively. Stated densities were chosen so that cells were 80% confluent at the time of harvest.

Flow cytometry

For flow cytometry, BMSCs were cultured as describe previously. At the indicated end points, BMSCs were harvested and stained with the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen, L10119) for 30 minutes at room temperature. For antigen staining, antibodies were used at the following dilutions: CD45-PE (1:250, Invitrogen, 12-0451-81), CD45-PerCP-Cyanine5.5 (1:250, Invitrogen, 45-0451-82), PDGFR α -APC (1:250, Invitrogen, 17-1401-81). A complete list of antibodies can be found in Table S1. Cells were stained with antibodies at room temperature for 30 minutes. Flow cytometry was performed on a BD FACSCanto II (BD Biosciences, 338962) and analyzed using FlowJo software (version 10.6.1). Compensation was conducted using OneComp eBeads Compensation Beads (Invitrogen, 01-1111-41) and ArC Amine Reactive Compensation Beads (Invitrogen, A10346) according to manufacturer's instruction using matching fluorophore-conjugated antibodies to the antibodies used for each instance. Live cells were gated for lack of anime reactive dye fluorescence. Subsequent gating was based on fluorescence-minus-one and unstained controls.

EdU staining to assess proliferation

Proliferation was assessed through incorporation of 5-ethynyl-2'-deoxyuridine (EdU) using the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Invitrogen, C10425). One day prior to harvest, cells were incubated with 50mM EdU for 24 hours to assess for changes in proliferation. At the time of harvest, cells were trypsinized and dishes were gently scraped to collect all adherent cells. After harvest, cells were fixed with 4% paraformaldehyde, permeabilized, and incorporated EdU was detected by a click reaction using a fluorescent Alexa Fluor 488 dye according to the manufacturer's protocol. Following EdU detection, cells were stained with additional antibodies and subsequently analyzed with flow cytometry using the parameters described above.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining to assess apoptosis

Apoptosis was assessed through the incorporation of BrdUTP at the sites of DNA damage and detected with an Alexa Fluor 488 dye-labeled anti-BrdU antibody using the APO-BrdU TUNEL Assay Kit (Invitrogen, A23210). At the desired endpoints, cells were trypsinized and dishes were gently scraped to collect all adherent cells. Following harvest, cells were treated according to the manufacturer's instructions for the detection of TUNEL-positive cells. Briefly, cells were fixed with 1% paraformaldehyde, permeabilized in ice

cold 70% ethanol at -20°C for 60 minutes, and incubated in a mixture of TdT and BrdUTP for DNA labeling for 60 mins in a 37°C water bath. Afterward, cells were incubated for 30 minutes at room temperature in an antibody staining solution containing Alexa 488 labeled anti-BrdU. During this incubation step, antibodies against markers of interest were also added at the concentrations indicated previously. Samples were analyzed within 3 hours of completion.

Statistical Analysis

Statistical differences were determined by one-way ANOVA followed by Tukey's post-hoc testing or unpaired Student's t-test, as appropriate (GraphPad, Prism 7, version 7.04). Results are expressed as mean $\pm$ SEM. $p < 0.05$ was considered significant.

REFERENCES

1. Sacchetti, B., et al., *Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment*. Cell, 2007. **131**(2): p. 324-36.
2. Chan, C.K., et al., *Identification and specification of the mouse skeletal stem cell*. Cell, 2015. **160**(1-2): p. 285-98.
3. Chan, C.K.F., et al., *Identification of the Human Skeletal Stem Cell*. Cell, 2018. **175**(1): p. 43-56 e21.
4. Chen, K.G., K.R. Johnson, and P.G. Robey, *Mouse Genetic Analysis of Bone Marrow Stem Cell Niches: Technological Pitfalls, Challenges, and Translational Considerations*. Stem Cell Reports, 2017. **9**(5): p. 1343-1358.
5. Baryawno, N., et al., *A Cellular Taxonomy of the Bone Marrow Stroma in Homeostasis and Leukemia*. Cell, 2019. **177**(7): p. 1915-1932 e16.
6. Tikhonova, A.N., et al., *The bone marrow microenvironment at single-cell resolution*. Nature, 2019. **569**(7755): p. 222-228.
7. Friedenstein, A.J., R.K. Chailakhjan, and K.S. Lalykina, *The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells*. Cell Tissue Kinet, 1970. **3**(4): p. 393-403.
8. Bianco, P., et al., *Multipotential cells in the bone marrow stroma: regulation in the context of organ physiology*. Crit Rev Eukaryot Gene Expr, 1999. **9**(2): p. 159-73.
9. Kuznetsov, S.A., et al., *Enumeration of the colony-forming units-fibroblast from mouse and human bone marrow in normal and pathological conditions*. Stem Cell Res, 2009. **2**(1): p. 83-94.
10. Robey, P.G., et al., *Bone marrow stromal cell assays: in vitro and in vivo*. Methods Mol Biol, 2014. **1130**: p. 279-293.
11. Friedenstein, A.J., J.F. Gorskaja, and N.N. Kulagina, *Fibroblast precursors in normal and irradiated mouse hematopoietic organs*. Exp Hematol, 1976. **4**(5): p. 267-74.
12. Bianco, P., et al., *Postnatal skeletal stem cells*. Methods Enzymol, 2006. **419**: p. 117-48.
13. Kuznetsov, S.A., et al., *Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo*. J Bone Miner Res, 1997. **12**(9): p. 1335-47.

14. Sotiropoulou, P.A., et al., *Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells*. Stem Cells, 2006. **24**(2): p. 462-71.
15. Schallmoser, K., et al., *Rapid large-scale expansion of functional mesenchymal stem cells from unmanipulated bone marrow without animal serum*. Tissue Eng Part C Methods, 2008. **14**(3): p. 185-96.
16. Bearpark, A.D. and M.Y. Gordon, *Adhesive properties distinguish sub-populations of haemopoietic stem cells with different spleen colony-forming and marrow repopulating capacities*. Bone Marrow Transplant, 1989. **4**(6): p. 625-8.
17. Kerk, D.K., et al., *Two classes of primitive pluripotent hemopoietic progenitor cells: separation by adherence*. J Cell Physiol, 1985. **125**(1): p. 127-34.
18. Iguchi, A., et al., *Selective stimulation of granulopoiesis in vitro by established bone marrow stromal cells*. Cell Struct Funct, 1997. **22**(3): p. 357-64.
19. Gordon, M.Y., et al., *Haemopoietic stem cell subpopulations in mouse and man: discrimination by differential adherence and marrow repopulating ability*. Bone Marrow Transplant, 1990. **5 Suppl 1**: p. 6-8.
20. Krebsbach, P.H., et al., *Bone formation in vivo: comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts*. Transplantation, 1997. **63**(8): p. 1059-69.
21. Krebsbach, P.H., et al., *Bone marrow stromal cells: characterization and clinical application*. Crit Rev Oral Biol Med, 1999. **10**(2): p. 165-81.
22. Boregowda, S.V., et al., *Atmospheric oxygen inhibits growth and differentiation of marrow-derived mouse mesenchymal stem cells via a p53-dependent mechanism: implications for long-term culture expansion*. Stem Cells, 2012. **30**(5): p. 975-87.
23. Harvey, D.M. and A.J. Levine, *p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts*. Genes Dev, 1991. **5**(12B): p. 2375-85.
24. Baddoo, M., et al., *Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection*. J Cell Biochem, 2003. **89**(6): p. 1235-49.

25. Wang, Q.R. and N.S. Wolf, *Dissecting the hematopoietic microenvironment. VIII. Clonal isolation and identification of cell types in murine CFU-F colonies by limiting dilution*. Exp Hematol, 1990. **18**(4): p. 355-9.
26. Suire, C., et al., *Isolation of the stromal-vascular fraction of mouse bone marrow markedly enhances the yield of clonogenic stromal progenitors*. Blood, 2012. **119**(11): p. e86-95.
27. Kusumbe, A.P., S.K. Ramasamy, and R.H. Adams, *Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone*. Nature, 2014. **507**(7492): p. 323-328.
28. Spencer, J.A., et al., *Direct measurement of local oxygen concentration in the bone marrow of live animals*. Nature, 2014. **508**(7495): p. 269-73.
29. Parmar, K., et al., *Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia*. Proc Natl Acad Sci U S A, 2007. **104**(13): p. 5431-6.
30. Bayer, C. and P. Vaupel, *Acute versus chronic hypoxia in tumors: Controversial data concerning time frames and biological consequences*. Strahlenther Onkol, 2012. **188**(7): p. 616-27.
31. Hu, C.J., et al., *Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation*. Mol Cell Biol, 2003. **23**(24): p. 9361-74.
32. Morikawa, S., et al., *Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow*. J Exp Med, 2009. **206**(11): p. 2483-96.
33. Zhou, B.O., et al., *Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow*. Cell Stem Cell, 2014. **15**(2): p. 154-68.
34. Takakura, N., et al., *PDGFR alpha expression during mouse embryogenesis: immunolocalization analyzed by whole-mount immunohistostaining using the monoclonal anti-mouse PDGFR alpha antibody APA5*. J Histochem Cytochem, 1997. **45**(6): p. 883-93.
35. Boregowda, S.V., V. Krishnappa, and D.G. Phinney, *Isolation of Mouse Bone Marrow Mesenchymal Stem Cells*. Methods Mol Biol, 2016. **1416**: p. 205-23.
36. Prowse, K.R. and C.W. Greider, *Developmental and tissue-specific regulation of mouse telomerase and telomere length*. Proc Natl Acad Sci U S A, 1995. **92**(11): p. 4818-22.

37. Wadhwa, R., et al., *The ARF-p53 senescence pathway in mouse and human cells*. *Histol Histopathol*, 2004. **19**(1): p. 311-6.
38. Takubo, K., et al., *Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells*. *Cell Stem Cell*, 2013. **12**(1): p. 49-61.
39. Yu, W.M., et al., *Metabolic regulation by the mitochondrial phosphatase PTPMT1 is required for hematopoietic stem cell differentiation*. *Cell Stem Cell*, 2013. **12**(1): p. 62-74.
40. Kohli, L. and E. Passegue, *Surviving change: the metabolic journey of hematopoietic stem cells*. *Trends Cell Biol*, 2014. **24**(8): p. 479-87.
41. Chow, D.C., et al., *Modeling pO₂ distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models*. *Biophys J*, 2001. **81**(2): p. 685-96.
42. Klimmeck, D., et al., *Proteomic cornerstones of hematopoietic stem cell differentiation: distinct signatures of multipotent progenitors and myeloid committed cells*. *Mol Cell Proteomics*, 2012. **11**(8): p. 286-302.
43. Simsek, T., et al., *The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche*. *Cell Stem Cell*, 2010. **7**(3): p. 380-90.
44. Takubo, K., et al., *Regulation of the HIF-1alpha level is essential for hematopoietic stem cells*. *Cell Stem Cell*, 2010. **7**(3): p. 391-402.
45. Nombela-Arrieta, C., et al., *Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment*. *Nat Cell Biol*, 2013. **15**(5): p. 533-43.
46. Parrinello, S., et al., *Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts*. *Nat Cell Biol*, 2003. **5**(8): p. 741-7.
47. Fehrer, C., et al., *Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan*. *Aging Cell*, 2007. **6**(6): p. 745-57.
48. Phinney, D.G., et al., *Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation*. *J Cell Biochem*, 1999. **72**(4): p. 570-85.

FIGURE LEGENDS

Figure 1 CD45⁺ cells constitute the majority of cells in BMSC cultures. **A)** Phase contrast and overlay of immunofluorescent and phase contrast images of BMSCs isolated from *LepRCre;Rosa26^{tdTomato/+}* mice. Red depicts cells in which CRE recombinase was active. Representative flow analysis plots and quantification of the percentage of CD45⁺, tdTomato⁺ (Td/Tm), and PDGFR α ⁺;tdTomato⁺ double positive cells isolated from *LepRCre;Rosa26^{tdTomato/+}* mice. **B)** Schematic representation of BMSC isolation methods. **C)** Phase contrast images of passage 0 BMSCs after 7 days in culture. Representative flow analysis plots and quantification of the percentage **C)** CD45⁺, **D)** PDGFR α ⁺, and PDGFR α ⁺;CD45⁺ double positive cells in WT BMSCs. Gates for positive cells denoted by outlined areas. Each graph represents an individual trial using BMSCs isolated from 2-3 month male and female mice. n $\geq$ 3, unpaired two tailed t-tests, significance p < 0.05.

Figure 2 Hypoxia depletes CD45⁺ cells in BMSC cultures. **A)** Phase contrast images of passage 0 BMSCs after 7 days in either 21% O₂ (normoxia, Nx) or 1% O₂ (hypoxia, Hx). White arrowheads denote small rounded cells. Representative flow analysis plots and quantification of the percentage of CD45⁺, PDGFR α ⁺ and PDGFR α ⁺;tdTomato⁺ double positive cells in **B)** WT or **C)** *LepRCre;Rosa26^{tdTomato/+}* BMSCs cultured in either normoxia or hypoxia. Gates for positive cells denoted by outlined areas. Each graph represents an individual trial using BMSCs isolated from 2-3 month male mice were used for analysis, n $\geq$ 3, unpaired two tailed t-tests, significance p < 0.05

Figure 3 Hypoxic depletion of CD45⁺ cells is maintained over time and passage number. **A)** Quantification of flow analysis showing the percentage of CD45⁺ and PDGFR α cells over 7 days in either normoxia (grey) or hypoxia (red). **B)** Quantification of the percentage of CD45⁺, PDGFR α ⁺, and CD45⁺;PDGFR α ⁺ double positive cells after two passages in either normoxia or hypoxia. **C)** Crystal violet staining of BMSCs plated at clonogenic density (left) and representative flow analysis plots and quantification of the percentage of CD45⁺ and PDGFR α ⁺ (right). Each graph represents an individual trial

using BMSCs isolated from 2-3 month male mice were used for analysis, $n \geq 3$, unpaired two tailed t-tests, significance $p < 0.05$

Figure 4 Differential effect of hypoxia on proliferation and apoptosis within CD45 and PDGFR α subsets. **A)** Representative flow analysis plots of CD45⁺ cells or **B)** PDGFR α ⁺ and percentage of cells which have incorporated EdU under normoxic or hypoxic conditions **C)** Quantification of flow analysis for CD45⁺;EdU⁺ and PDGFR α ⁺;EdU⁺ cells in either normoxic or hypoxic conditions. **D)** Representative flow analysis plots of CD45⁺ cells or **E)** PDGFR α ⁺ and percentage of TUNEL⁺ cells under normoxic or hypoxic conditions **F)** Quantification of flow analysis for CD45⁺;TUNEL⁺ and PDGFR α ⁺;TUNEL⁺ cells in either normoxic or hypoxic conditions. BMSCs isolated from 2-3 month old male and female mice, $n \geq 3$ for each condition. One-way ANOVA, post-hoc Tukey's multiple comparisons test, significance $p < 0.05$.

SUPPLEMENTAL MATERIALS

Supplemental Figure 1 A) Gating strategy used for flow cytometry analysis to demark live BMSCs, CD45⁺ and PDGFR α ⁺ cells staining in WT BMSC. **B)** Flow analysis for CD45⁺ and PDGFR α ⁺ in non-adherent cells isolated 3 hours after plating.

Supplemental Figure 2 A) Flow analysis of BMSCs stained for CD45 and PDGFR α gated based on size.

Supplemental Table 1 Key Resources

Resource	Source	Identifier
<i>Antibodies</i>		
CD45 Rat anti-Mouse, PE, Clone: 30-F11, eBioscience™	Invitrogen	12-0451-81
CD140a (PDGFRA) Monoclonal Antibody (APA5), APC, eBioscience™	Invitrogen	17-1401-81
CD45 Monoclonal Antibody (30-F11), PerCP-Cyanine5.5, eBioscience™	Invitrogen	45-0451-82
Mouse IgG1 Isotype Control (MOPC-21), Alexa Fluor 488	Invitrogen	MA5-18167
<i>Compensation Beads</i>		
OneComp eBeads™ Compensation Beads	Invitrogen	01-1111-41
ArC™ Amine Reactive Compensation Bead Kit	Invitrogen	A10346
<i>Commercial Assays</i>		
Click-iT™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit	Invitrogen	C10425
APO-BrdU™ TUNEL Assay Kit, with Alexa Fluor™ 488 Anti-BrdU	Invitrogen	A23210
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit	Invitrogen	L10119
<i>Cell Culture</i>		
MEM α , nucleosides	Gibco	12571-063
Penicillin-Streptomycin (10,000 U/mL)	Gibco	15140-122
Trypsin-EDTA (0.25%), phenol red	Gibco	25200-056
Red Blood Cell Lysing Buffer Hybri-Max™	Sigma-Aldrich	R7757
HyClone™ Fetal Bovine Serum (U.S.), Characterized	GE Healthcare Life Sciences	SH30071.03
<i>Equipment</i>		
InvivoO ₂ 400	Baker Ruskinn	https://bakerco.com
BD FACSCanto II (8 color)	BD Biosciences	338962
<i>Animals</i>		
C57BL/6J	The Jackson Laboratory	000664
<i>Software</i>		
FlowJo v10.6.1	FlowJo, LLC	https://www.flowjo.com/
Graphpad Prism 7 (version 7.04)	GraphPad	https://www.graphpad.com

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

SUPPLEMENTAL FIGURE 1

SUPPLEMENTAL FIGURE 2