Identification and Isolation of Small CD44-Negative Mesenchymal Stem/Progenitor Cells From Human Bone Marrow Using Elutriation and Polychromatic Flow Cytometry

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INTRODUCTION

Mesenchymal stem/stromal cells (MSCs) are rare, nonhematopoietic adult stem cells originally found to reside in bone marrow (BM) [1, 2]. These plastic-adherent cells were originally characterized by their ability to (a) give rise to the colony forming unit-fibroblast (CFU-F), (b) support hematopoiesis, and (c) possess osteogenic potential [3, 4]. MSCs have been isolated successfully from a number of non-BM tissue sources, including dental pulp [5], lung [6, 7], adipose tissue [8], placenta [9], and umbilical cord [10]; however, BM is still the most extensively studied source to date [11]. Because of the ease of isolating them from BM, expansion capability in vitro, and differentiation potential and immunomodulatory properties, MSCs represent a promising cell-based therapy option for enhancing endogenous tissue repair [12–14] and suppressing autoimmunity [15–17].

BM-MSCs are rare cells, with an estimated frequency of 1 in every 10,000–100,000 nucleated BM cells [18]. Although MSCs have been suggested to occupy a perivascular niche in the BM [19], there is a paucity of data pertaining to the physical characteristics and antigenic profile of the candidate progenitor population in vivo that gives rise to cultured MSCs. Moreover, cultured MSCs represent a heterogeneous population of cells [20, 21], as they are expanded from plastic-adherent cells obtained from unfractionated BM, which contains a number of other cell types that have the ability to adhere to a plastic surface, including endothelial cells, fibroblasts, and monocyes.

There is great interest in the in vivo identification of MSCs. Despite an emerging consensus regarding the topography of MSCs within the BM [19], there is a lack of agreement regarding their antigenic profile [22]. This is related to the fact that the antigenic profile of MSCs has been elucidated postculture in an artificial environment. However, using a limited set of markers, a number of studies have attempted to identify the immunophenotype of prospectively isolated cells that subsequently behave as MSCs in culture [22, 23]. One
of the markers, CD271, has received considerable attention, as it has been used to isolate cells from BM with MSC-like properties [24– 26]. However, even within this population there is a lack of accord on the antigenic profile [22]. Indeed, there is a growing need within the MSC field, similar to the evolving hematopoietic stem cell (HSC) [27] and endothelial progenitor cell fields [28], to develop expanded antibody panels (involving four or more surface markers) for the prospective identification and purification of unique BM mesenchymal stem and progenitor cell (MSPC) populations. This approach would apply to therapeutic applications as well as for basic research, since the identity of the isolated MSPC population may be linked to its efficacy for specific clinical applications, such as regenerative or immunomodulatory therapies [22].

In addition to immunomagnetic- or fluorescence-activated cell sorting (FACS), counterflow centrifugal elutriation (CCE) represents an alternative approach for the separation and enrichment of cells on the basis of their size and cell mass [29]. The concept of using elutriation for cell separation was first introduced by Lindahl [30, 31]. For clinical applications, CCE has been used to enrich monocytes from a large volume of peripheral blood mononuclear cells [32]. Recently, there has been interest in CCE as a method to separate lymphocyte subpopulations from monocytes and granulocytes [33], as well as to enrich tumor cell [34] and progenitor cell populations [35]. Primitive progenitors within mouse BM have been isolated using CCE from the earliest elutriated fractions on the basis of size and shown to contribute to lymphohematopoietic reconstitution [36], as well as multilineage engraftment to epithelial tissues in recipient mice [37]. However, to our knowledge, whether CCE can be used to isolate and identify early primitive cells within human BM with mesenchymal potential is not known.

In the present study, a five-antibody marker panel, which included CD45, CD73, CD90, CD105, and CD44, representing several of the most commonly used markers for immunophenotypic analysis of culture-expanded MSCs, was used to prospectively isolate BM MSPCs that display MSC activity in culture. Surprisingly, MSC activity was found to reside in a population of small, CD45−CD73−CD90+CD105− cells that lacked expression of CD44s, the standard isoform of the CD44 cell adhesion molecule that is commonly used to identify MSCs. These rare CD45−CD73−CD90+CD105− cells, which were between 5 and 12 μm in diameter, expanded rapidly in culture and demonstrated trilineage mesenchymal differentiation potential into osteoblasts, chondrocytes, and adipocytes in vitro. By CCE, these rare, small CD44− cells cofractionated with lymphocytes and were easily separated from plastic-adherent monocytes. Following expansion, the cells displayed phenotypic markers commonly associated with MSCs, including acquisition of CD44. These observations demonstrate that human BM contains a previously undescribed population of small MSPCs that lack expression of CD44. Isolation of these MSPCs to a high level of purity combined with ease of ex vivo expansion will enable their further characterization and comparison with conventional plastic-adherent MSCs for clinical applications of tissue regeneration and immune modulation.

**Materials and Methods**

**Sample Processing**

Fresh, unprocessed BM was obtained from healthy donors (Lonza, Walkersville, MD, http://www.lonza.com). Samples were processed under aseptic conditions. After red blood cell lysis (Pharm Lyse 1 × lysis buffer; BD Pharmingen, San Diego, CA, http://www.bdbiosciences.com), cells were washed with 0.5% human serum albumin (HSA) in Dulbecco’s phosphate-buffered saline (DPBS) and centrifuged at 680g for 15 minutes at 4°C. Next, cells were counted for viability and resuspended in 0.5% HSA/DPBS and processed for cell isolation. Fresh, mobilized leukapheresis products were purchased from AllCells (Emeryville, CA, http://www.allcells.com) or collected from healthy volunteers at NeoStem Laboratory (Cambridge, MA, http://www.neostem.com) under an institutional review board-approved protocol. Three days prior to apheresis, healthy donors received daily subcutaneous injections of granulocyte-colony stimulating factor (G-CSF) (480 μg/day; Neupogen; Amgen, Thousand Oaks, CA, http://www.amgen.com). A certified staff technician conducted the collection of the apheresis product over the course of 2–3 hours. After the collection of the mobilized apheresis product, cells were diluted to a final concentration of 2.5 × 10^6 cells per milliliter in 300 ml of 0.5% HSA/phosphate-buffered saline (PBS) prior to elutriation as described below.

**Fluorescence-Activated Cell Sorting**

After cell viability of the lysed BM was determined, CD34- and CD133-expressing cells were depleted using MACS CD34 and CD133 microbead kits (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotech.com) performed with the MACS LS column and QuadroMACS separator (Miltenyi Biotech) according to the manufacturer’s instructions. Both the enriched and the depleted fractions were examined for cell viability, cell number, and cell size distribution using a Cellometer analyzer (Nexcelom Biosciences, Lawrence, MA, http://www.nexcelom.com). CD34/CD133-depleted fractions were resuspended in FACS staining buffer (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com) and incubated with the following antibodies: CD45-Pacific blue (Beckman Coulter, Fullerton, CA, http://www.beckmancouler.com), CD73-allophycocyanin (APC; BD Biosciences, San Diego, CA, http://www.bdbiosciences.com), CD90-fluorescein isothiocyanate (BD Biosciences), CD105-phycocerythrin (PE; BD Biosciences), and CD44-APC-H7 (BD Biosciences) on ice for 30 minutes. Following staining, cells were washed with DPBS, centrifuged at 680g for 10 minutes, resuspended in buffer, and passed through a 40-μm filter (BD Biosciences). The viability dye 7-aminoactinomycin D (7-AAD; Beckman Coulter) was added prior to sorting.

Cell sorting was carried out with a high-speed MoFlo XDP cell sorter (Beckman Coulter). The MoFlo XDP was equipped with four lasers (488, 642, 405, and 355 nm). The forward scatter threshold was carefully set low to ensure inclusion of small cells. Cells were analyzed and sorted using a sequential gating strategy. An initial gate was set on CD45 versus 7-AAD, where CD45−live (7-AAD−) cells were then displayed on a CD73 versus CD90 plot, and then a second gate was drawn to include the cluster of CD73−CD90− cells. Following this, CD45−CD73−CD90− viable cells were further applied on a third plot of CD105 versus CD44 with quadrant gates delineated for CD105− or CD44− cells. Populations of the following four (if any) CD45−/CD73−/CD90−/CD105−/CD44−, CD45−/CD73−/CD90+/CD105−/CD44−, CD45−/CD73−/CD90−/CD105+/CD44−, and CD45−/CD73−/CD90−/CD105−/CD44− were sorted directly to tubes containing ice-cold (4°C) chemically defined, serum-free culture medium (MSCGM-CD; Lonza). Cells from the population of CD45−/CD73−/CD90−/CD105−/CD44−
CD105+/CD44− were also back-gated and displayed again on a side scatter/forward scatter (SSC/FSC) color density plot to reveal their location, and standardized flow cytometric beads were used to confirm their size (supplemental online data).

The sorted cells were centrifuged at 680g for 15 minutes at 4°C, resuspended in MSCGM-CD and seeded into either six-well or 10-cm dishes. Cultures were maintained in a humidified incubator with 5% CO2 and low oxygen (5% O2) at 37°C. The cells were left untouched for 5 days. On day 6 nonadherent cells were aspirated off, and then fresh MSCGM-CD medium was added. Following this, adherent cultures were maintained by changing the medium twice weekly. The cultures were continuously fed for 10–14 days until they reached 70%–80% confluence. Cells were expanded following subculturing and used for differentiation assays and flow cytometric analysis as described below. Unstained cells and isotype negative control samples were used to set photomultiplier voltage for baseline fluorescence and to set quadrant statistics for analyzing positive fluorescence above baseline. Compensation was manually adjusted using known positive single color-stained samples together with an unstained control. Data acquisition and analysis were performed using Summit software (Beckman Coulter). A minimum of 500,000 events were recorded as a list mode file for further analysis.

Enrichment of CD44+ Bone Marrow Stem/Progenitor Cells by Elutriation

The Elutra cell separation system (CaridianBCT, Lakewood, CO, http://www.caridianbct.com) uses CCE and was programmed to enrich the different cell types based primarily on size and secondarily on density to separate populations of cells into more specific cell fractions. Briefly, approximately 2–3 × 109 nucleated cells from lysed BM were resuspended in 100 ml of 0.5% HSA/DPBS and loaded onto the Elutra system. Lysed BM cells or apheresis products (see above for details) were fractionated under a constant centrifugation rate of 2,400 rpm, and five successive elutriated fractions of 450–900 ml were collected in 0.5% HSA/DPBS using progressive increases in pump speed. Each collected fraction was centrifuged at 680g for 20 minutes at 4°C to pellet the cells. Elutriated cells were used for CD34/CD133 depletion and FACS as described above.

Density Gradient/Plastic Adherence Conventional Method to Isolate Bone Marrow MSCPs

Mononuclear cells (MNCs) from donor-matched BM were isolated by Ficoll density gradient fractionation (Ficoll-Paque Premium, 1.077; GE Healthcare, Little Chalfont, U.K., http://www.gehealthcare.com). MNCs were carefully removed, washed, and resuspended MSCGM-CD (Lonza) or culture medium composed of α-minimal essential medium (αMEM)/GlutaMAX (Gibco, Grand Island, NY, http://www.invitrogen.com) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Following enumeration of MNCs, cells were plated onto plastic dishes at a ratio of 1.0 × 106 cells per cm2 growth area in MSCGM-CD or αMEM/GlutaMAX with 10% FBS. Cultures were maintained in a humidified incubator with 5% CO2 and low oxygen (5% O2) at 37°C. After 72 hours, nonadherent cells were aspirated off, the adherent cells were washed with 5 ml of prewarmed PBS, and then fresh MSCGM-CD medium or αMEM/GlutaMAX with 10% FBS culture medium was added. Following this, adherent cultures were maintained by changing the medium twice weekly. The cultures were continuously fed for 10–14 days until they reached 70%–80% confluence. Cells were expanded following subculturing and used for differentiation assays and flow cytometric analysis as described below.

CFU-F

To examine the CFU-F potential of the elutriated fractions prior to FACs, 1.0 cell per cm² was plated in a six-well dish in MSCGM-CD. Cells were grown for 14 days; thereafter, the medium was removed and cells were washed with DPBS, fixed with methanol (BDH) for 5 minutes at room temperature. Next, the methanol was removed, and cells were air dried for 5 minutes at room temperature. To stain cultures, 2 ml of Giemsa (EMD Chemicals, Billerica, MA, http://emdmillipore.com) staining solution was added to each well and incubated for 10 minutes at room temperature. Afterward, the staining solution was removed and cells were washed with distilled water to remove unbound stain and further washed until wells were clear.

Adipogenic, Chondrogenic, and Osteogenic Differentiation

Culture-expanded (passage 3) cells were grown in defined in vitro culture conditions to examine their multipotentiality toward the mesenchymal lineage, as previously described. Briefly, following 3 weeks of standard culture conditions for adipogenesis, cells were fixed and stained with Oil Red O (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Following the extraction of Oil Red O using 70% isopropanol, the supernatant was measured spectrophotometrically at 510 nm to quantify adipogenesis. To induce chondrogenesis, cell pellets were formed and grown in chondrogenic induction medium (Lonza) supplemented with 10 ng/ml of transforming growth factor-β3 (Lonza). After 21 days, the cell pellets were fixed in 10% formalin and stained with safranin-O (Sigma-Aldrich). To induce osteogenesis, cells were grown in osteogenic induction medium. After 21 days, cells were fixed with 10% formalin and stained with 40 mM alizarin red S (Sigma-Aldrich) (pH 4.2).

Single-Color Flow Cytometric Analysis of Culture-Expanded Cells

Immunophenotypic analysis of culture-expanded (passage 3) FACs-sorted or elutriation/FACS-sorted cells and donor-matched MSCs was performed using a Gallios flow cytometer (Beckman Coulter). For analysis, 30,000 events were collected and analyzed using Kaluza software (Beckman Coulter). A list of the antibodies used is given in supplemental online Table 1.

G-CSF Treatment

To examine the effects of G-CSF treatment on the cell surface phenotype of elutriated/FACS-sorted cells versus BM-MSCs isolated using conventional methods, cells were treated with 10 ng/ml recombinant human G-CSF (R&D Systems) for 4 consecutive days in serum-free αMEM/GlutaMAX medium. Following treatment, cells were harvested using HyQTase, counted and resuspended in 100 µl of staining buffer with CD105-PE and CD44-APC-H7 for 15 minutes on ice. Flow cytometric analysis was performed using a Gallios flow cytometer (Beckman Coulter), and 50,000 events were collected and analyzed using Kaluza software (Beckman Coulter).

Statistical Analysis

All results were expressed as mean ± SEM. One-way analysis of variance and post hoc Student-Neuman-Keuls was used on data
sets when there were more than two groups for multiple comparisons. \( p < .05 \) was used as the significance level.

**RESULTS**

**Human Bone Marrow Contains a Population of Small CD45−CD73−CD90−CD105−CD44− Cells That Lack CD44 but Exhibit Mesenchymal Stem/Progenitor Cell-Like Properties**

The phenotype of human BM MSPCs in vivo is not known; therefore, a panel of antibodies representing the most common markers for plastic-adherent BM-MSCs was used for their prospective identification. This six-color panel, which includes antibodies to CD45, CD73, CD90, CD105, and CD44 and the cell viability marker 7-AAD, was used for fluorescence-activated cell sorting (FACS) to analyze CD34/CD133-depleted BM for populations that contain MSC activity. First, CD45−7-AAD− (live, gate R1) cells were identified and subgated to display a cluster of CD73−CD90−CD105−CD44− cells (gate R2) (Fig. 1C), which were then displayed as a quadrant gate (D) to identify the CD105−CD44− subset of cells. The sort window of CD105−CD44− cells is indicated as R3. (E, F): Back-gating of the original CD45−CD73−CD90−CD105−CD44− population revealed their location near the lymphocyte population within the SSC-height/FSC-height color density plot (E), and standard-sized flow cytometric beads confirmed their small size (right) (F). (G): Representative image of typical colony forming unit-fibroblast from CD45−CD73−CD90−CD105−CD44− sorted cells and higher power image of a single colony (×4, right). (H): Fluorescence-activated cell sorting-sorted CD44− cells were expanded in culture and demonstrated trilineage differentiation potential in vitro toward adipocytes detected using Oil Red O stain for lipids (×10, left), osteoblasts detected using alizarin red S stain (middle), and chondroblasts detected using safranin-O stain (right). Similar results were seen in four other BM samples from different donors. Abbreviations: 7-AAD, 7-aminoactinomycin D; FSC, forward scatter; SSC, side scatter.
of standard size demonstrate that the CD44<sup>−</sup> cells were between 5 and 12 μm (Fig. 1F). Proper placement of our gates was verified using “fluorescence minus one” controls (supplemental online Fig. 1). Next, the sorted CD45<sup>−</sup>CD73<sup>−</sup>CD90<sup>−</sup>CD105<sup>−</sup>CD44<sup>−</sup> cells were plated onto plastic in chemically defined, serum-free medium (MSCGM-CD). Surprisingly, MSC activity, in the form of characteristic CFU-F, was found in the fraction lacking expression of CD44, widely considered an important MSC marker. The CD44<sup>−</sup>cells rapidly proliferated and formed characteristic CFU-F by day 12 (Fig. 1G). Following expansion, these cells were able to differentiate into osteoblasts, chondrocytes, and adipocytes under defined conditions in vitro (Fig. 1H), indicating that they function as MSPCs. Differential cell counts from the BM, final yield of the FACS-sorted CD44<sup>−</sup>fraction, and their growth potential are depicted in Figure 2A–2E.

Elutriation Enriches for CD44<sup>−</sup> MSPCs and Confirms Their Small Size

The unexpectedly small size of the FACS-sorted CD44<sup>−</sup> MSPCs led to studies to determine whether elutriation could be used to enrich these cells on the basis of their small size prior to FACS. CCE was used to separate lysed BM from healthy donor subjects into five distinct fractions on the basis of size. Platelets were removed almost entirely in the first elutriated fraction (35 ml/minute; fraction 35), which contained very few nucleated cells and was discarded. The cell yield and composition from all the subsequent collected fractions is shown in Figure 3A–3D. CCE was able to separate the lymphocyte population (fractions 70 and 90) from the granulocytes, which were collected primarily in the largest cell fractions (fractions 110 and >110), and to a lesser extent in fraction 110. The unexpected small size of the FACS-sorted CD44<sup>−</sup> MSPCs led to studies to determine whether elutriation could be used to enrich these cells on the basis of their small size prior to FACS.
The majority of nucleated cells elutriated in the later fractions, with 45% of cells found in fraction >110 alone (Fig. 3A). The cell separation in each fraction based on size can also be seen by the change in the SSC/FSC pseudo-color density plots (Fig. 4A–4D). CD<sup>+</sup>CD73<sup>-</sup>CD90<sup>-</sup>CD105<sup>-</sup>CD44<sup>-</sup> cells were found in fraction 70 and peaked in fraction 90, confirming their small size (Figs. 3E, 4A–4D). The CD44<sup>-</sup>MSPC population was extremely rare, with approximately 1 in 25,000 events in fraction 90 being live CD45<sup>-</sup>CD73<sup>-</sup>CD90<sup>-</sup>CD105<sup>-</sup>CD44<sup>-</sup> cells (Fig. 3F). Despite their rarity, sorted cells from fraction 90 underwent a 10,000-fold expansion in culture 21 days after the initial sort (Fig. 3G).

**CD44<sup>-</sup> MSPCs Expanded in Culture Acquire CD44 and Express Other Phenotypic Markers Characteristic of MSCs**

To determine whether FACS-sorted CD44<sup>-</sup> MSPCs expanded in culture were phenotypically similar to conventional MSCs, elutriated/
FACS-sorted CD44^- cells from fraction 90 that had been cultured for three passages were compared with donor-matched MSCs isolated from BM by the conventional method of Ficoll purification followed by plastic adherence (Fig. 5). Single-color FACS analysis showed that the small, CD45^-CD73^-CD90^-CD105^-CD44^- MSPCs elute as small cells (70 and 90 ml/minute). (A–D): Representative display of SSC/FSC color density plots of the elutriated fractions 50/70 (A), 90 (B), 100–110 (C), and >110 (D). Display of CD45/7-AAD density plot was used to identify CD45^-7-AAD^- (live) cells (gate R3), which was subgated onto a CD73/CD90 antigen plot and identified a cluster of live CD45^-CD73^-CD90^- cells (gate R4), which was further subgated onto a CD105/CD44 antigen plot. Shown is the display of CD105^-CD44^- on live CD45^-CD73^-CD90^- gated cells. The sort window shows a cluster of CD105^-CD44^- cells (R5). The sorted live CD45^-CD73^-CD90^-CD105^-CD44^- cells (gate R5) are distributed close to the location of lymphocytes when back-gated onto an SSC-height/FSC-height dot plot (far right). Representative images from one experiment are shown. Similar results were seen in four other BM samples from different donors. Abbreviations: 7-AAD, 7-aminoactinomycin D; Frac, fraction; FSC, forward scatter; SSC, side scatter.

G-CSF Diminishes CD44 Expression in FACS-Sorted MSPCs but Not Conventional MSCs

There has been speculation about the concept that MSCs can mobilize from the BM and circulate in peripheral blood, yet there is little evidence in support of this controversial view [40]. To address this question, mobilized peripheral blood products obtained from healthy donors following a 3-day course of G-CSF were evaluated for the presence of rare CD44^- cells using our method of either FACS or elutriation/FACS. However, only very rare CD45^-CD73^-CD90^-CD105^-CD44^- cells were detected in a G-CSF-mobilized peripheral blood collected as an apheresis product (Fig. 6A). These cells were not expandable, either as a typical CFU-F or growth in a nonclonal fashion (data not shown).

The effects of G-CSF on the MSC population were investigated further. In vitro culture of elutriated/FACS-sorted BM-derived CD44^- cells resulted in acquisition of CD44 expression, which is sensitive to G-CSF (Fig. 6B, 6C). Treatment of culture-expanded MSPCs, derived from the CD44^-fraction via elutriation/FACS, with recombinant human (rh) G-CSF (10 ng/ml) for 3...
consecutive days resulted in diminished expression of CD44, which was demonstrated by the appearance of a CD44lo population (Fig. 6B) and quantified by a decrease in mean fluorescence intensity (MFI) compared with vehicle-treated cells (3.2 $/10^5$ vs. 8.4 $/10^5$; p = .037) (Fig. 6C). In comparison, CD44 expression on culture-expanded donor-matched MSCs isolated using the conventional method was not altered following rhG-CSF treatment (MFI 2.4 $/10^5$ vs. 2.7 $/10^5$; not significant) (Fig. 6B, 6C).

**CD44− MSPCs Exhibit a More Robust Expansion and Differentiation Capacity Compared With Conventionally Isolated MSCs**

To further investigate the relative capacity of elutriated CD44− cells to expand and differentiate into tissues of mesenchymal lineage compared with conventionally isolated MSCs, differentiation assays were performed comparing elutriated/FACS-sorted CD44− cells from fraction 90 to donor-matched BM-MSCs prepared by Ficoll purification and plastic adherence. Both preparations were expanded in culture until passage 3. Representative colony appearance from elutriated/FACS-sorted cells on day 12 is shown in Figure 6A (left), as well as cell morphology after passage 1 (Fig. 7A, middle). Furthermore, elutriated/FACS-sorted CD44− cells demonstrated a greater expansion capacity over three passages compared with BM-MSCs isolated using the conventional method (Fig. 7A, right, p < .041). Following this, cells were used for the various differentiation assays in defined culture conditions in vitro.

After 21 days in osteogenic medium, sorted CD44− cells stained more densely with alizarin red S compared with conventional MSCs, indicating an enhanced mineralization in culture (Fig. 7B). In addition, pellets of CD44− cells expanded in chondrogenic induction medium for 21 days demonstrated more staining for safranin-O, a marker of cartilage, compared with donor-matched conventionally isolated MSCs (Fig. 7C). Finally, when plated in adipogenic medium, elutriated/FACS-sorted CD44− cells quickly acquired an adipogenic phenotype demonstrated by significant uptake of Oil Red O staining in lipid (Fig. 7D). Quantification of adipogenesis following isopropanol extraction of Oil Red O confirmed that CD44− MSCPs formed more lipid compared with conventionally isolated MSCs (Fig. 7D, far right) (p < .034).

**DISCUSSION**

The data presented in this study demonstrate that a rare BM population of small, CD45−CD73−CD90−CD105+CD44− cells function as MSPCs. These MSPCs can readily be isolated from CD34/CD133-depleted BM by a combination of CCE and FACS. Once expanded in culture, these MSPCs acquire CD44 and exhibit a similar immunophenotype but a more robust expansion and
differentiation potential compared with MSCs obtained by standard plastic adherence methods. Contrary to the widely held views that MSCs in vivo are large cells [38] and that CD44 represents a reliable and highly expressed marker on postcultivated BM-MSCs [39], our observations indicate that BM contains a primitive population of small MSPCs that simultaneously express CD73, CD90, and CD105 and lack CD44 and that the acquisition of CD44 expression is a postculture phenomenon.

There is a lack of consensus on the antigenic profile and morphology of bone marrow-derived MSCs in vivo. Immunophenotypic analysis of conventionally isolated MSCs is generally performed following cultivation of cells that were initially selected on their ability to adhere to plastic. Although antibodies to several markers have been used for the prospective isolation of MSC subsets from BM, these studies have typically used a more limited number of antibodies in their marker panels [22, 23]. Introduction of a multicolor panel of antibodies (more than four colors) against more surface markers as presented here does appear to allow for better resolution of novel cell subsets and rare event analysis. Such multiparameter FACS procedures are widely used in the fields of hematopoietic [27] and endothelial stem and progenitor cell research [28].

Remarkably, the BM MSPCs identified in this study, as well as in a recent study by Qian et al. [40], lack expression of CD44, a multifunctional class I integral transmembrane glycoprotein that is widely expressed on a number of cells in the bone marrow with varying functions [41, 42]. In concordance with Qian et al., the CD44- cells in the present study acquired CD44 expression during culture, indicating that adherence and growth on plastic alters the in vivo antigenic profile of BM MSPCs. However, several differences between our observations are notable. First, Qian et al. [40] were able to identify a prominent CD44-positive fraction, which they were able to prospectively sort, and compare its CFU-F potential to that of the CD44-negative fraction. However, the CD44-negative fraction formed CFU-F only when plated at nonclonal densities of 2,000 cells per cm². Using a different antibody matrix and gating strategy, we did not detect a CD44-negative fraction, and therefore, we were not able to compare CFU-F potentials. Second, CD105 served as a key positive marker for the prospective isolation of CD44-negative MSPCs in our study. This is contrast to the report from Qian et al. [40], where CD105 expression was diminished or absent on primary CD44-negative cells. However, one study
reported that the administration of noncultured BM-derived cells immunoselected based entirely on CD105 expression resulted in robust bone formation through the endochondral pathway [43]. In addition to this difference in CD105 expression, the CD44/H11002 fraction identified by Qian et al. [40] coexpressed the low-affinity nerve growth factor receptor CD271 and STRO-1, markers that have previously been used to prospectively isolate cells with MSC-like potential [24 –26, 44]. However, neither marker was shown to present on in vitro culture-expanded elutriated/FACS CD44/H11002 cells nor the donor-matched MSCs isolated using the conventional method. The stability of these markers in culture has been questioned and shown to be downregulated following expansion [45, 46]. However, there are conflicting reports describing this phenomenon [47]. At present, it is not

Figure 7. Trilineage differentiation potential of small CD45−CD73−CD90−CD105−CD44− cells obtained from fraction 90 following expansion. (A): In vitro growth of CCE/FACS CD44− cells obtained from fraction 90. Shown are a representative Giemsa-stained colony forming unit-fibroblast colony at day 12 (left) and the typical colony appearance by phase contrast (middle). After three passages, CCE/FACS-sorted cells demonstrated a greater expansion capacity compared with conventional mesenchymal stem/stromal cells (MSCs) (right). (B–D): After passage 3, cells were placed in differentiation conditions in vitro to induce osteogenesis, chondrogenesis, and adipogenesis. (B): To induce chondrogenesis, cell pellets were incubated with 10 ng/ml recombinant human transforming growth factor-β3. Chondrogenesis was detected following staining with safranin-O. A representative image of a CCE/FACS CD44− pellet (left) shows more intense staining compared with donor-matched conventional MSCs (right). (C): Osteogenesis was detected by staining cultures with 2% alizarin red S solution (pH 4.2). Shown is a representative image comparing osteogenesis cultures for CD44− cells (left) with cultures of donor-matched conventional MSCs (middle). The monolayer of CCE/FACS CD44− cells stained more densely with alizarin red S. (D): Adipogenesis was detected following staining of cultures with Oil Red O, which detects lipids. Representative images of CCE/FACS CD44− culture (left) compared with donor-matched conventional MSCs (middle) are shown (×20). Right panel shows spectrophotometric quantification of adipogenesis following isopropanol extraction of Oil Red O. Data are presented as mean ± SEM, n = 4–5, *p < .05 versus conventional isolated MSCs, using an unpaired t test.

Abbreviations: CCE, counterflow centrifugal elutriation; FACS, fluorescence-activated cell sorting.
known whether the CD44− fraction discovered in our study using elutriation/FACS overlaps with the CD44− BM-MSPCs cells described by Qian et al. [40], and this will be the subject of future investigations.

Since there is a lack of data concerning the physical size of MSPCs within the BM, it was not known where these cells would fractionate using CCE. We anticipated that MSPCs may elutriate in the later fractions, which contain contaminating monocytes and granulocytes, as MSCs are suggested to be large cells in vivo [38]. However, the CD44− MSPCs were enriched in early fractions (Fig. 3) that also contain other stem and progenitor cell populations such as HSCs and very small embryonic-like stem cells, a population of developmentally early stem cells residing in adult tissues [48]. A previous study in mice similarly demonstrated that BM cells from the earliest elutriated fractions using CCE were endowed with a primitive potential and contribute to multiple tissues in recipient mice [36, 37]. Colter et al. also reported the presence of small, rapidly self-renewing cells isolated from human BM that demonstrated robust differentiation for both osteocytes or adipocytes [21]. In a follow-up study, MSCs were isolated on the basis of the FSClo/SSclo property, which enabled the enrichment of these small rapidly proliferating cells [49]. However, it is not known whether these cells, which appear to be early progenitors based on size, represent the same population as identified here, as the antigenic profile of these cells was not determined prior to plating. In further support of our finding, Rasini et al. recently demonstrated the existence of small, round/polygonal CD73−CD105−CD44− cells in the BM stroma that are positive for a number of pluripotency markers [50]. These findings reinforce the need for a continued assessment of the human BM for putative MSC subpopulations and importantly, to link these cells with in vivo function.

Recent evidence indicates that MSCs play a supportive role in maintaining HSCs in a specialized niche within the BM [19]. This raises the question of whether MSCs, along with HSCs, may be mobilized by G-CSF from the BM to the peripheral circulation. The concept that MSCs mobilize and circulate remains controversial [51] with a lack of supportive evidence [40]. BM-MSCs respond to G-CSF by promoting the transmigration of CD34+ cells in culture [52]. Using our method of FACS or elutriation/FACS, very rare CD45−CD73−CD90−CD105−CD44− cells could be detected in G-CSF mobilized peripheral blood products obtained from healthy donors; however, in our hands, these cells did not expand. In contrast, FACS or elutriation/FACS culture-expanded CD44− cells do respond to G-CSF, with loss of their acquired CD44 expression, an observation that was not seen with conventionally plastic purified MSCs (Fig. 6). The physiological significance and biological consequences of this difference are not known. Whether CD44+ MSPCs might mobilize in response to other combinations of cytokines and growth factors remains to be determined [53].

**CONCLUSION**

In summary, we report here a distinct subset of small, highly proliferative MSPCs within human BM that lack CD44 but that exhibit mesenchymal-like multilineage differentiation capabilities. CCE represents a method that can enrich MSPCs from the BM; it also has the potential to obviate the need to FACS sort cells, since major contaminating populations of plastic-adherent cells such as monocytes are largely depleted by elutriation and could easily be completely removed by CD14 immunodepletion. In vivo studies are now needed to determine the fate and function of these MSPCs in normal tissue homeostasis and in various disease conditions, as well as to assess their therapeutic potential.

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**AUTHOR CONTRIBUTIONS**

S.R.R.H.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; Y.J.: conception and design, collection and/or assembly of data, data analysis and interpretation; E.L. and G.Y.: collection and/or assembly of data; S.E.: data analysis and interpretation; D.W.O.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; W.A.M.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

W.A.M. owns stock in NeoStem and was a paid consultant during this study, and G.Y. and D.W.O. have compensated employment from NeoStem.

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