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Density Gradient Centrifugation for the Isolation of Cells of Multiple Lineages

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ABSTRACT

We recently developed a simple strategy for the enrichment of mesenchymal stem cells (MSCs) with the capacity for osteoblast, chondrocyte, and adipocyte differentiation. On transplantation, the progenitor-enriched fraction can regenerate bone with multiple lineages of donor origin. Although comprising multiple precursor cell types, the population is enriched >100-fold in osteoprogenitors, hence the name "highly purified osteoprogenitors" (HipOPs). To establish a new modified method of purifying pure MSCs, it is useful to know the expression patterns of surface markers on heterogeneous MSCs and committed cells such as osteoblasts, adipocytes, and chondrocytes. However, calcium deposition by osteoblasts is a critical obstacle in visualizing the expression patterns of surface markers. We now report a new method of separating differentiated osteoblastic HipOPs (OB-HipOPs) from calcium deposits using the Percoll density gradient centrifugation technique. After centrifuge separation, calcium deposits were observed at the bottom of the centrifuge tube, and living OB-HipOPs were harvested from the 10–70% fractions. However, there were no living cells in the 70–80% fraction. We concluded that living OB-HipOPs are separated by one 10–70% Percoll gradient. Furthermore, we analyzed the expression patterns of putative MSC markers on differentiated HipOPs. FACS analysis revealed that Sca-1, CD44, CD73, CD105, and CD106 were decreased in OB-HipOPs. In adipogenic- and chondrogenic-HipOPs, Sca-1, CD73, CD105, and CD106 were decreased. This new technique is a helpful tool to identify MSC surface markers and to clarify in more detail the differentiation stages of osteoblasts. J. Cell. Biochem. 116: 2709–2714, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: METHODOLOGY; OSTEOBLAST; PERCOLL GRADIENT; SURFACE MARKER; DIFFERENTIATION

Adult bone marrow generally comprises two stem cell populations with distinct progenies: hematopoietic stem cells and mesenchymal stem cells (MSCs). Friedenstein and coworkers reported that fibroblast-like cells elaborated from bone marrow via attachment to tissue culture plastic were inherently osteogenic [Friedenstein et al., 1976; Prockop, 1997; Phinney et al., 1999; Bianco et al., 2006]. Thereafter, many studies demonstrated that these osteogenic cells are capable of differentiating into multiple connective tissue cell types at a clonal level, validating the concept of MSCs [Caplan, 1994; Pittenger et al., 1999]. MSCs are typically defined as adherent, fibroblastoid-like cells that differentiate into osteoblasts, adipocytes, and chondrocytes in vitro, and are thought to escape immune recognition by alloreactive cells or at least exhibit low immunogenicity [Dominici et al., 2006].

Phenotypically, MSCs express several markers, but none are specific to MSCs. It is established that murine MSCs do not express the hematopoietic markers CD45, CD34, CD14, CD11, and CD79, but can express CD105, CD73, CD44, and CD90 [Horwitz et al., 2005; Dominici et al., 2006]. The characteristics of MSCs differ between laboratories and species, and there is no marker or combination of markers specific to MSCs in vivo or in vitro. Two approaches for purifying murine MSCs have been described: the first involves serial passaging to enrich stromal precursors over hematopoietic "contaminants;" the second employs selection based on surface markers [Van Vlasselaer et al., 1994; Baddoo et al., 2003; Javazon et al., 2004; Chamberlain et al., 2007; Hachisuka et al., 2007]. However, positive selection of mesenchymal cells based on surface markers [Van Vlasselaer et al., 1994; Hachisuka et al., 2007], including the putative "mesenchymal stem cell" markers CD90, Sca-1, CD44, CD105, and CD73 [Javazon et al., 2004; Chamberlain et al., 2007] is also used, as are combinations of negative and positive selection.

We developed a simple strategy for the robust enrichment and co-purification of MSCs with the capacity for osteoblast, chondrocyte, and adipocyte differentiation in vitro [Itoh and Aubin,

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2009]. On transplantation, the progenitor-enriched fraction regenerates bone with multiple lineages of donor origin: osteoblasts, osteocytes, osteoclasts, sinusoidal, and bone marrow cells. Although comprising multiple precursor cell types, the population is enriched >100-fold in osteoprogenitors, hence their name "highly purified osteoprogenitors" (HipOPs). HipOPs are a purified progenitor fraction of MSCs, but remain a heterogeneous fraction, because the frequency of MSCs is around 1/1,000 as determined by a limiting dilution assay. Thus, further purification of this fraction is necessary to yield pure MSCs. To establish a new modified method of purifying pure MSCs by positive sorting using MSC-specific surface markers, knowledge of the expression patterns of surface markers on both heterogeneous MSCs and committed cells, such as osteoblasts, adipocytes, and chondrocytes, is necessary. We speculated that the expression patterns of surface markers would differ between MSCs and committed cell lineages. Although the expression patterns of surface markers maybe different between MSCs, osteoblasts, adipocytes, and chondrocytes, the precise expression patterns for these cells remain unclear. One obstacle in observing the expression patterns of surface markers is calcium deposition by osteoblasts during culture, which hinders FACS analysis. We now report a new method, which separates differentiated cells from mineral deposits using Percoll density gradient centrifugation. We also analyzed and compared the expression patterns of putative MSC markers on MSCs, osteoblasts, adipocytes, and chondrocytes.

MATERIALS AND METHODS

ISOLATION OF HIGHLY PURIFIED OSTEOPROGENITORS

The tibiae and femurs of C57BL/6J mice (4–6 weeks old) were harvested and immersed in α-minimum essential medium (α-MEM; Life Technologies, Carlsbad, CA) with penicillin and streptomycin (PC/SM). After removing the femoral heads, the marrow was obtained by repeatedly flushing out the shafts with a syringe containing α-MEM supplemented with antibiotics and 10% heat-inactivated fetal calf serum (FCS; Nichirei Corporation, Tokyo, Japan). To remove cell aggregates, we strained the cell suspension. Collected cells were plated in α-MEM containing antibiotics and 10% FCS. After 3 days, non-adherent cells were cleared by washing three times with phosphate- buffered saline (PBS; Sigma–Aldrich Corporation, St. Louis, MO). Approximately 2 weeks after seeding, when adherent cells had increased to approximately 80% subconfluence, they were detached with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Life Technologies). The cells were purified by negative selection using anti-CD5, CD45, CD11b, Gr-1, 7-4, Ter-119, and CD45R in combination with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and the resultant extracted cells were HipOPs [Itoh and Aubin, 2009]. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Osaka University, Osaka, Japan (AD-21-002-0).

DIFFERENTIATION ASSAY: OSTEOGENESIS

Cells (1×10^5 cells/ml) were cultured in the osteogenic induction medium containing α-MEM, 10% FCS, PC/SM, 50 μg/ml ascorbic acid (Sigma–Aldrich Corporation), 10 mM β-glycerophosphate (Sigma–Aldrich Corporation), and 10^{-8} M dexamethasone (Dex; Sigma–Aldrich Corporation). The osteogenic induction medium was replaced every 2 or 3 days. After 7 days of culture, cells were fixed in 10% neutral-buffered formalin for 30 min. All buffer was removed by suction, the dishes were rinsed once with distilled water, then they were left in distilled water for 15 min. Simultaneously, the substrate solution was prepared and filtered (5 mg of Naphthol AS MX-PO4 was dissolved in 200 μl of DMF, and 25 ml of 0.2 M Tris-HCl buffer pH 8.3 and 25 ml of distilled water were added. Prior to use, 30 mg of Red Violet LB Salt was added, and the solution was filtered through 3-mm Whatmann filter paper). Next, the distilled water was removed by suction, the substrate solution was added, and the dishes were left at room temperature for 45 min for alkaline phosphatase (ALP) staining. Subsequently, the ALP substrate was removed by suction, and the cell were washed four times with distilled water. After the last wash, the distilled water was removed by suction, and then 2.5% silver nitrate solution (2.5 g silver nitrate in 100 ml distilled water) was added, and the dishes were incubated at room temperature for 30 min (von Kossa staining). The dishes were then washed with distilled water three times. Colony-forming unit- osteoblasts (CFU-Os) were defined as colonies with ALP-positive cells associated with mineralized matrix (von Kossa-positive).

DIFFERENTIATION ASSAY: ADIPOGENESIS

Cells (1×10^5 cells/ml) were cultured in the adipogenic induction medium comprising α-MEM, 10% FCS, PC/SM, 50 μg/ml ascorbic acid, and 10^{-6} M BRL-49653 (Rosiglitazone; Cayman Chemical Company, Ann Arbor, MI). The adipogenic induction medium was replaced every 2 or 3 days. After 7 days of culture, adipocytic cells (with Oil red O-positive lipid droplets) were identified by fixing in 10% neutral-buffered formalin and staining with Oil Red O (stock solution [0.5% Oil Red O in 100% isopropanol] was diluted 3:2 with distilled water, allowed to stand for 30 min, and filtered to remove undissolved Oil Red O [working solution]), for 30 min followed by rinsing with distilled water.

DIFFERENTIATION ASSAY: CHONDROGENESIS

Cells (1×10^5 cells/ml) were cultured in the chondrogenic induction medium comprising α-MEM, 10% FCS, PC/SM, 50 μg/ml ascorbic acid, 10^{-8} M Dex, and 50 ng/ml hrBMP2 (R&D Systems, Minneapolis, MN). The chondrogenic induction medium was replaced every 2 or 3 days. After 7 days of culture, cells were fixed in 10% neutral-buffered formalin, and colony-forming unit-chondrocytes were identified by staining with goat anti-Type II Collagen antibody (Santa Cruz Biotechnology, Inc., Dallas, TX), followed by biotinylated anti-goat Immunoglobulin G (IgG) and a Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA).

FLOW CYTOMETRY

Harvested cells were suspended in ice- cold PBS containing 2% FCS (1×10^6 cells/ml). We placed 100 μl of cell suspension into 96-well round-bottomed plates and blocked with rat anti-mouse CD16/CD32 antibody (BD Biosciences, Franklin Lakes, NJ) for 10 min. Cells were washed three times by centrifugation at 1200 rpm for 5 min, and resuspended in ice-cold PBS containing 2% FCS. Cells were reacted

with the respective mouse antibodies directly labeled with Biotin (Sca-1 and CD90, BD Biosciences; CD34, CD44, CD73, CD105, and CD106, Biolegend, Inc., San Diego, CA) for 15 min on ice, washed three times by centrifugation at 1,200 rpm for 5 min, and resuspended in ice-cold PBS containing 2% FCS. Cells were labeled with APC-conjugated Streptavidin (BD Biosciences) to reveal Biotin-coupled antibodies for 15 min on ice. After labeling, cells were washed three times by centrifugation at 1,200 rpm for 5 min, and resuspended in ice-cold PBS containing 2% FCS. After washing, cells were reacted with propidium iodide (PI; Sigma–Aldrich Corporation) for 10 min on ice, washed three times by centrifugation at 1,200 rpm for 5 min, resuspended in ice-cold PBS containing 2% FCS, and analyzed using guava easyCyte (EMD Millipore Corporation, Billerica, MA). The settings for FASC were adjusted to detect dead cells in the corner area of forward scatter (FSC) and side scatter (SSC) [Itoh et al., 2002].

PERCOLL DENSITY GRADIENT CENTRIFUGATION

HipOPs were cultured in the osteogenic induction medium containing α-MEM, 10% FCS, PC/SM, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 10^{-8} M Dex. The osteogenic induction medium was replaced every 2 or 3 days. After 7 days, HipOPs differentiated into osteoblasts (OB-HipOPs) were detached with trypsin-EDTA solution. Eight concentrations (10%, 20%, 30%, 40%, 50%, 60%, 70%, and 80%) of Percoll (GE Healthcare Life Sciences, Tokyo, Japan) were prepared by Hank's balanced salt solution (HBSS; Life Technologies). A Percoll gradient was made by layering 2 ml of each Percoll solution in a centrifuge tube. Finally, 1 ml of cell suspension (OB-HipOPs) was placed on top of the gradient and centrifuged at 3,000 rpm for 10 min at 4°C. Fractions from the center of one layer to the center of the next were collected.

STATISTICAL ANALYSIS

All results were representative of these independent experiments. All data were expressed as mean ± standard error of the mean. Statistical comparisons were made using the Student's *t*-test (StatView; SAS Institute Inc., Cary, NC). $P < 0.05$ was considered significant.

RESULTS

CALCIUM DEPOSITION BY OSTEOBLASTS HINDER FLOW CYTOMETRY

HipOPs cultured in the osteogenic induction medium for 7 days differentiated into osteoblasts (Fig. 1A). HipOPs that differentiated into osteoblasts (OB-HipOPs) were analyzed using flow cytometry. Flow cytometry did not detect living cells and detected only calcium deposits (Fig. 1B). Furthermore, autofluorescence was observed (Fig. 1C).

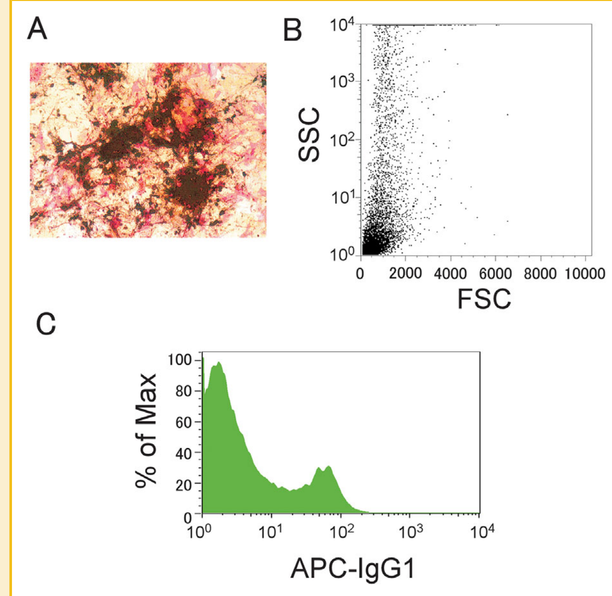

Fig. 1. Calcium deposits produced by osteoblasts perturb flow cytometry. (A) HipOPs cultured in the osteogenic induction medium for 7 days were stained with alkaline phosphatase and von Kossa staining, or analyzed by flow cytometry (B). (C) HipOPs cultured in the osteogenic induction medium were treated with biotinylated anti-IgG1 antibody. Biotinylated antibody was detected with APC-labeled Streptavidin.

A NOVEL METHOD FOR THE PURIFICATION OF DIFFERENTIATED OSTEOBLASTS

Figure 2 presents a schematic illustration of the purification of differentiated osteoblasts from 10% to 20% Percoll gradients. First, 10% and 20% concentrations of Percoll were layered in the centrifuge tube. Then, OB-HipOPs were placed on the top of the gradient and centrifuged. A fraction from the center of the 10% and 20% Percoll layers was collected and used for flow cytometry. Living cells were detected in the 10–20%, 20–30%, 30–40%, 40–50%, 50–60%, and 60–70% fractions. However, living cells were not observed in the 70–80% fraction (Fig. 3). Living cells were therefore

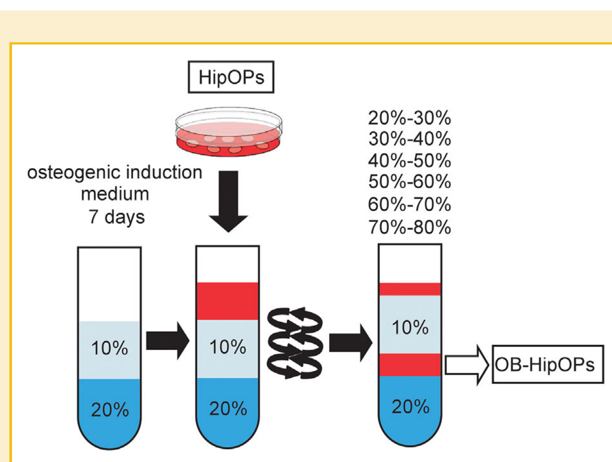

Fig. 2. The method of purifying OB-HipOPs. HipOPs cultured in the osteogenic induction medium for 7 days were detached with trypsin-EDTA solution. Eight concentrations of Percoll were prepared using HBSS as diluent. A layered Percoll gradient was made by layering each Percoll solution in the centrifuge tube (10–20%, 20–30%, 30–40%, 40–50%, 50–60%, 60–70%, and 70–80%). Finally, the cell suspension was placed on the top of the gradient and centrifuged at 3,000 rpm for 10 min at 4°C. Fractions from the center of one layer to the center of the next were collected and used for experiments.

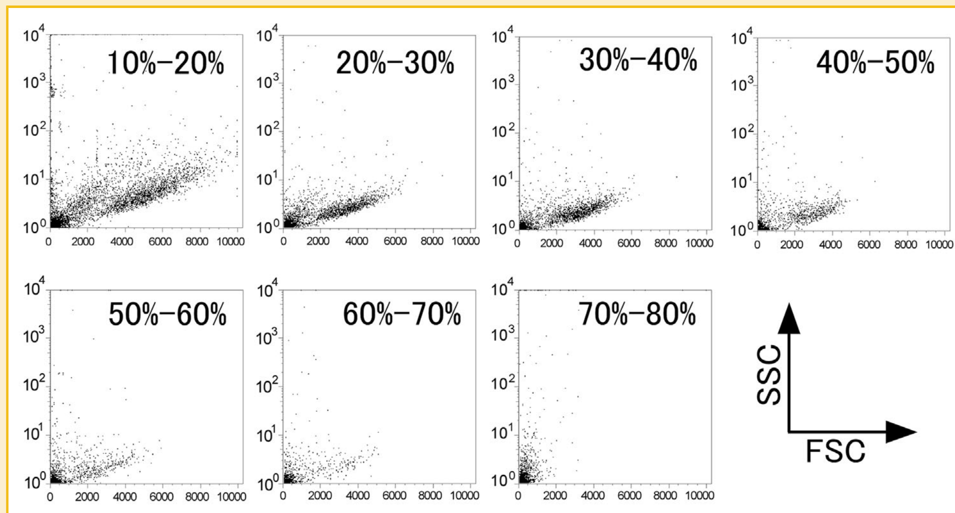

Fig. 3. FACS analysis of fractions from the center layer of the Percoll gradients. OB-HipOPs were placed on the top of the gradient and centrifuged at 3,000 rpm for 10 min at 4°C. Fractions from the center of one layer to the center of the next were collected and used for FACS analysis.

present in the 10–70% fractions. Finally, we tried to separate living cells from calcium deposits using a 10–70% Percoll gradient (Fig. 4A). The number of OB-HipOPs before Percoll density gradient centrifugation was 1.5×10^5 cells/ml (dead cells: 1.0×10^4 cells/ml), and the number of OB-HipOPs after Percoll density gradient centrifugation was 1.0×10^5 cells/ml, based on Trypan blue staining. Living cells (the percentage of PI-negative cells was $87.4 \pm 9.2\%$) were separated using a 10–70% Percoll gradient. Furthermore, FACS analysis showed that autofluorescence observed for unseparated OB-HipOPs disappeared in cells sorted by the Percoll density gradient centrifugation technique (Fig. 4B).

PUTATIVE MESENCHYMAL STEM CELL MARKERS ON CELLS SORTED USING THE PERCOLL DENSITY GRADIENT CENTRIFUGATION TECHNIQUE

We analyzed the expression levels of Sca-1, CD34, CD44, CD73, CD90, CD105, and CD106 on HipOPs and OB-HipOPs sorted by the Percoll density gradient centrifugation technique. The expression levels of Sca-1, CD44, CD73, CD105, and CD106 drastically decreased on OB-HipOPs (Table I). We also compared the expression levels of these markers on HipOPs cultured in the adipogenic induction medium (AD-HipOPs). AD-HipOPs differentiated into Oil red O-positive cells (data not shown). The expression levels of Sca-1, CD73, CD105, and CD106 drastically decreased on AD-HipOPs (Table I). Finally, we compared the expression levels of these markers on HipOPs cultured in the chondrogenic induction medium (Ch-HipOPs). Ch-HipOPs differentiated into Type II Collagen-positive cells (data not shown). The expression levels of Sca-1, CD73, CD105, and CD106 also drastically decreased on Ch-HipOPs (Table I).

DISCUSSION

Osteoblasts, the primary bone-making cells, secrete a unique combination of extracellular proteins, including osteocalcin, ALP,

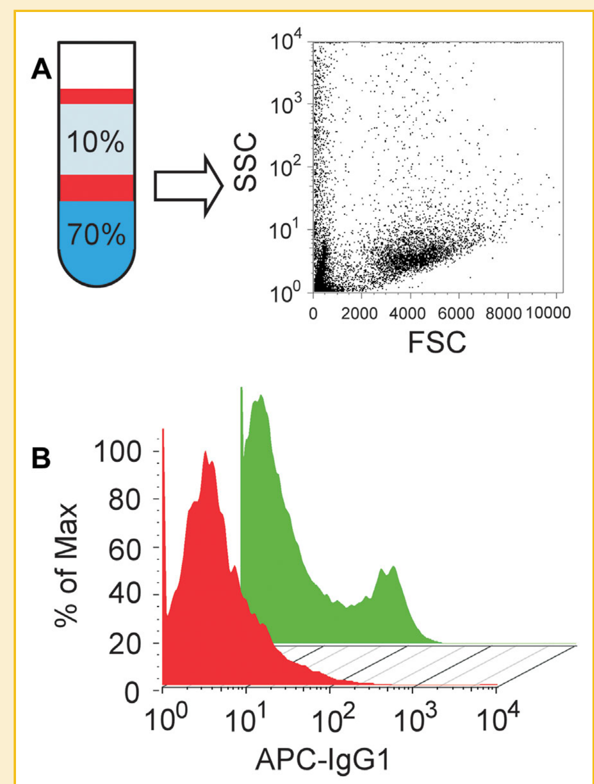

Fig. 4. One Percoll gradient of 10–70% is enough to separate OB-HipOPs from calcium deposits. (A) Cells in the center of 10–70% Percoll gradients were collected and subjected to FACS analysis. (B) Green histogram indicates OB-HipOPs. Red histogram indicates OB-HipOPs sorted by the Percoll density gradient centrifugation technique (cells in the center of 10–70% Percoll gradients).

TABLE I. Flow Cytometry Analysis of Differentiated HipOPs

	Positive cells (%)			
	HipOPs	OB-HipOPs	AD-HipOPs	Ch-HipOPs
Sca-1	96.1 ± 2.6	6.6 ± 3.3***	49.7 ± 5.3***	30.3 ± 11.1**
CD34	6.7 ± 2.1	2.0 ± 0.8*	7.8 ± 1.3	3.3 ± 1.3
CD44	89.4 ± 3.3	18.7 ± 8.3***	94.8 ± 3.2	77.9 ± 10.0
CD73	81.7 ± 9.5	44.3 ± 13.4*	22.7 ± 2.5***	20.7 ± 1.6**
CD90	7.0 ± 1.9	4.9 ± 3.6	5.5 ± 0.7	3.6 ± 0.9
CD105	47.0 ± 5.9	2.1 ± 0.5**	26.8 ± 1.6**	10.1 ± 0.9**
CD106	72.8 ± 4.3	3.4 ± 0.6***	26.8 ± 7.1***	16.1 ± 7.0***

Values are the means ± S.D. of three independent experiments. Asterisks indicate statistically significant differences: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.005$ (***).

and large amounts of Type I Collagen [Shokrgozar et al., 2010]. The extracellular matrix, which is rich in Type I Collagen, is known as the osteoid when first deposited but not yet mineralized. It is subsequently mineralized through the accumulation of calcium phosphate in the form of hydroxyapatite. This process results in the hard but lightweight composite material that is the major constituent of the bone. The process of osteoblast differentiation progresses from MSCs to preosteoblasts to osteoblasts. Preosteoblasts encompass all cells transitioning from progenitors to mature osteoblasts and are by definition, heterogeneous. Although this division is useful for the discussion of osteoblast biology, the identities of these multiple-stage cells are poorly understood. Much remains to be elucidated about the process of osteoblast differentiation. One approach to understanding osteoblast differentiation is to examine the expression pattern of surface markers on cells at each stage, including MSCs, preosteoblasts, and osteoblasts. Although we speculated that the expression pattern of surface markers changes during the differentiation of MSCs into osteoblasts, calcium deposits produced by osteoblasts during culture obstruct the observation of the expression patterns of surface markers. Flow cytometry is unable to detect any differentiated living cells because it detects each calcium deposit as one cell (Fig. 1B). Furthermore, as autofluorescence occurs (Fig. 1C), there is a critical risk of false-positive results if calcium deposits contaminate samples during FACS analysis. To address this critical problem, we developed a novel method of separating living cells from calcium deposits. We recently succeeded in purifying novel multipotential progenitors or stem cells from bone marrow stromal cells (BMSCs) [Itoh and Aubin, 2009]. This population exhibited a very high frequency of CFU-Os (100 times higher than those in BMSCs) and high expression levels of osteoblast differentiation markers. Furthermore, large masses of mineralized tissue were observed in in vivo transplants of this new population, which we designated HipOPs. CFU-Os only developed after 7 days of culture in the osteogenic induction medium. In this report, we used HipOPs as the MSC population. CFU-Os already exist among HipOPs cultured in the osteogenic induction medium for 7 days. These differentiated HipOPs were harvested and placed on top of a two-layered Percoll gradient. Although living differentiated HipOPs were harvested from the 10–70% fractions, there were no living cells in the 70–80% fraction (Fig. 3). Thus, we tried to remove calcium deposits using one Percoll gradient of 10–70%, and succeeded in separating differentiated living cells (Fig. 4).

Phenotypically, MSCs express several non-specific markers, none of which either individually or in combination, exhibit high levels of expression [Haynesworth et al., 1992; Digirolamo et al., 1999; Colter et al., 2000; Javazon et al., 2001]. It is accepted that MSCs are devoid of hematopoietic and endothelial markers such as CD11b, CD31, and CD45 [Chia et al., 2015]. Although human and rat MSCs have been shown to be CD34-negative, murine MSCs demonstrate variations in CD34 expression [Colter et al., 2000; Javazon et al., 2001; Peister et al., 2004]. MSCs express numerous surface adhesion molecules, including CD44, CD49e, and CD62, and are typically positive for MHC I and Sca-1 [Pittenger et al., 1999; Colter et al., 2000; Javazon et al., 2001; Martin et al., 2002; Sun et al., 2003]. Because MSC populations are heterogeneous between species and within cultures, variable expressions of CD90, CD117 (c-kit), CD105, CD73, and STRO-1 (a specific marker for human MSC populations) are often observed [Haynesworth et al., 1992; Pittenger et al., 1999; Kobune et al., 2003; Rombouts and Ploemacher, 2003]. These discrepancies arise due to differences in isolation methods, tissue and species of origin, and culture conditions; therefore, we tried to analyze the putative murine MSC markers Sca-1, CD44, CD73, CD90, CD105, CD106, and CD34. FACS analysis revealed that the expression levels of Sca-1, CD44, CD73, CD105, and CD106 were drastically decreased in OB-HipOPs (including preosteoblasts and osteoblasts) compared with HipOPs. Differentiated adipocytes and chondrocytes also showed decreased expression of Sca-1, CD73, CD105, and CD106. If the expression pattern of surface markers changes during the differentiation of MSCs into committed cell lineages, Sca-1, CD73, CD105, and CD106 may represent candidate MSC markers. Notably, the expression of CD44 was unchanged in AD-HipOPs and Ch-HipOPs, but changed in OB-HipOPs. This implies that the expression of CD44 relates to osteoblast differentiation. Also an experiment using human MSCs showed that CD44 expression was a post-culture phenomenon [Hall et al., 2013]. However, the expression levels of transcription factors related to osteoblast differentiation should also be considered. Preosteoblasts, transitioning from progenitors to mature osteoblasts, are believed to express the runt-related transcription factor 2 (RUNX2) and at more advanced stages of differentiation, both RUNX2 and osterix [Long, 2012]. Thus, in future, we aim to identify the surface markers expressed at each stage of differentiation using our novel technique and consider the expression patterns of transcription factors at each stage in conjunction with these findings.

In summary, we demonstrated that one Percoll gradient of 10–70% is sufficient to separate OB-HipOPs from calcium deposits. In addition, we showed that the expression levels of Sca-1, CD73, CD105, and CD106 drastically decreased in differentiated HipOPs.

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