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A Novel Purification Method for Multipotential Skeletal Stem Cells

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ABSTRACT

At least some cells within bone marrow stromal populations are multipotential (i.e., differentiate in vitro into osteoblasts, chondrocytes, and adipocytes) and thus designated skeletal stem cells (SSCs) or mesenchymal stem cells (MSCs) amongst other names. Recently, a subpopulation of stromal cells, notably osteoblasts or their progenitors, has been identified as a definitive regulatory component of the hematopoietic stem cell (HSC) niche. Thus, the development of methods for purifying not only SSCs but cells comprising the HSC niche is of interest. Here, we report a method for purifying a novel bone marrow-derived population with a high frequency of osteoprogenitors and high expression levels of osteoblast differentiation markers (highly purified osteoprogenitors (HipOPs)) as well as markers of the bone niche for HSCs. In vivo transplantation experiments demonstrated that donor HipOPs differentiated into not only osteoblasts, osteocytes and cells around sinusoids but also hematopoietic cells. Thus, HipOPs represent a novel population for simultaneous reconstruction of bone and bone marrow microenvironments. J. Cell. Biochem. 108: 368–377, 2009.

KEY WORDS: MULTIPOTENTIAL SKELETAL STEM CELLS; TRANSPLANTATION; BONE MARROW MICROENVIRONMENT; METHODOLOGY; MOUSE

As established originally by Friedenstein, at least some cells within bone marrow stromal populations are multipotential and can differentiate in vitro into osteoblasts, chondrocytes, adipocytes and myoblasts, which has led to the population frequently being designated skeletal stem cells (SSCs), mesenchymal stem cells or multipotential marrow stromal cells (MSCs) amongst other names [Friedenstein et al., 1976; Piersma et al., 1985; Owen, 1988; Owen and Friedenstein, 1988; Prockop, 1997; Bianco et al., 2006]. SSCs/MSCs from human and rat bone marrow have been the most extensively characterized, because they are relatively easy to isolate by their phenotype of adherence to plastic and extensive expansion capacity in culture. In contrast, murine SSCs are far more difficult than that of at least certain other species both to isolate from bone marrow and to expand in culture [Phinney et al., 1999], at least in part due to significant contamination by hematopoietic cells that are also directly adherent to plastic and bind to bone marrow stromal cells via adhesion molecules, cytokine receptors, and extracellular matrix proteins [Bearpark and Gordon, 1989; Simmons et al., 1992; Deryugina and Muller-Sieburg, 1993]. Further, unambiguous characterization of the intrinsic phenotypic and functional properties of murine SSCs requires processes for depletion of contaminating cell types including lineage committed hematopoietic cells from bone marrow cultures.

Bone marrow harbors both an endosteal/osteoblast niche and a vascular/sinusoidal endothelial niche for hematopoietic stem cells (HSCs) [Arai and Suda, 2007; Frisch et al., 2008; Kiel and Morrison, 2008]. Tie2/Angiopoietin1 (Ang1) signaling between HSCs and osteoblasts respectively has been described to enhance N-cadherin-mediated adhesion, providing a niche for quiescent HSCs [Zhang et al., 2003; Arai et al., 2004]. Parathyroid hormone 1 receptor (PTH1R) signaling induces not only osteoblast differentiation but also activates osteoblastic cells to increase their surface expression of the Notch ligand jagged1 (Jag1) which supports an increase in the number of HSCs in the bone marrow niche [Calvi et al., 2003]. CXCL12-CXCR4 signaling (on osteoblasts and HSCs respectively and reticular cells around sinusoids and HSCs) has also been shown to play an important role for the maintenance of the HSC pool [Sugiyama et al., 2006]. A CD146-positive SSC and reticular stromal cell (also called mural or pericyte) niche has also been described in human bone [Sacchetti et al., 2007]. However, understanding of the relationship(s) between the cells comprising these various bone niches and the nature or maturity of

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the osteoblastic subpopulation comprising the niche remain incomplete [Kiel and Morrison, 2008]. Thus, the development of methods for purifying not only SSCs but cells comprising the HSC niche is of interest.

Several purification strategies for SSCs have been reported, and can be roughly divided into two categories: one involves serial passaging to enrich for a stromal precursor versus hematopoietic "contaminants," while the other employs selection on the basis of surface markers [Van Vlasselaer et al., 1994; Baddoo et al., 2003; Javazon et al., 2004; Chamberlain et al., 2007; Hachisuka et al., 2007]. Negative selection methods utilize several antibodies to exclude the significant contamination of hematopoietic cells in murine bone marrow stromal cell populations [Van Vlasselaer et al., 1994; Baddoo et al., 2003; Hachisuka et al., 2007]. On the other hand, positive selection with surface markers to select the mesenchymal population [Van Vlasselaer et al., 1994; Hachisuka et al., 2007], including the putative "mesenchymal stem cell" markers CD90 (Thy1.1), Sca-1, CD44, SH2 (CD105 or endoglin), and SH3 or SH4 (CD73) [Javazon et al., 2004; Chamberlain et al., 2007] are often used, as well as combinations of negative and positive selection. Here, we report a method for significant enrichment of a novel SSC population from murine bone marrow by using a magnetic micro-beads technique. The enriched population is multipotential, with a very high frequency of CFU-O and high expression levels of osteoblast differentiation markers in the fractionated compared to the unfractionated population, indicating significant enrichment of osteoprogenitor cells (highly purified osteoprogenitors or HipOPs), amongst other mesenchymal progenitors. To address the in vivo differentiation potential, HipOPs were transplanted on collagen sponges into immunodeficient mice and found to be significantly enriched in cells with a high potential for reconstitution of the skeletal system in vivo. Interestingly, donor cells differentiated into not only osteoblasts, osteocytes and cells around sinusoids but also bone marrow cells. Thus, the HipOP fraction has potential for reconstruction of bone and the bone marrow microenvironments, and may be a useful population for robust regenerative therapies.

MATERIALS AND METHODS

ISOLATION OF HipOPs

Femurs of C57BL/6 mice (4–6-week-old) were harvested under sterile conditions and immersed in α-minimum essential medium (α-MEM) with antibiotics. After removal of the femoral heads, the marrow was collected by flushing repeatedly through the shafts with a syringe containing α-MEM supplemented with antibiotics and 10% heat-inactivated fetal calf serum (FCS), and sieving the cell suspension to remove cell aggregates. Recovered cells were plated in α-MEM supplemented with antibiotics as above and 10%FCS. After 3 days, nonadherent calls were removed by washing 3 times with PBS. Approximately 2 weeks after seeding, when the adherent cells had expanded to ~80% subconfluence, they were detached with trypsin-EDTA solution (0.2% trypsin, 1mM EDTA). HipOPs were purified by negative sorting using anti-CD5, CD45, CD11b, Gr-1, 7-4, Ter-119 and CD45R conjugated magnetic beads (Miltenyi Biotec).

FLOW CYTOMETRY

Cells were blocked with rat anti-mouse CD16/CD32 (BD Biosciences) for 10 min, and then stained with respective mouse antibodies (mAbs) directly labeled with phycoerythrin (PE) or biotin for 15 min on ice (Supplementary Table I). APC-conjugated streptavidin (BD Biosciences) was used to reveal biotin-coupled Abs. After labeling, cells were analyzed using FACSCalibur and CellQuest software (BD Biosciences) [Itoh et al., 2002].

DIFFERENTIATION ASSAYS OSTEOGENESIS

Cells were cultured in osteogenic induction medium comprising α-MEM, 10% FCS, antibiotics, 50 μg/ml ascorbic acid (Fisher Scientific Co.), 10 mM β-glycerophosphate (β-GP) (Sigma Chemical Co.) and 10^{-8} M dexamethasone (Dex). After 4 weeks, cells were fixed in 10% neutral-buffered formalin for 30 min and double-stained for alkaline phosphatase (ALP) activity and mineral deposition (von Kossa). After recording the presence or not of ALP- and ALP-von-Kossa-positive colonies in each well, all wells were re-stained with 0.15% methylene blue for 10 min and rinsed with distilled water. Colony-forming units-osteoblast (CFU-O) were defined as colonies with ALP-positive cells associated with mineralized matrix (von-Kossa-positive) (Supplementary Fig. 2). CFU-ALP colonies were defined as colonies with ALP-positive cells but no detectable mineralization (Supplementary Fig. 2). Those colonies staining with methylene blue alone were defined as CFU-F; thus, the CFU-F category may contain not only cells capable of forming connective tissue, but also other progenitor types not characterized in the current experiments. Multiple discrete colonies present in the same wells were categorized and recorded separately. ALP + Mineral + clusters were defined as small clusters of cells (1–4 cells) with mineralized matrix (Supplementary Fig. 2).

ADIPOGENESIS

Cells were cultured in adipogenic induction medium comprising α-MEM, 10% FCS, antibiotics as above, 50 μg/ml ascorbic acid and 10^{-6} M BRL-49653 (a generous gift from the Sankyo Company), a PPAR-γ selective ligand. After culture for 1 week, adipocytic cells (cells with Oil red O-positive lipid droplets) were identified by fixing in 10% neutral-buffered formalin, and staining with Oil Red O (a 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 (Supplementary Fig. 2).

CHONDROGENESIS

Cells were spun down and resuspended in 1:1 DMEM/Ham F-12 (Gibco Co.), 10% FBS, antibiotics as above, 50 μg/ml ascorbic acid, 10^{-8} M Dex and 50 ng/ml hrBMP2 (R&D Systems). After culture for 2 weeks, cells were fixed in 10% neutral-buffered formalin and CFU-chondrocytes (CFU-Ch) were identified by staining with goat anti-type 2 collagen antibody (Santa Cruz Biotechnology) followed by biotinylated anti-goat IgG and a Vectastain Elite ABC kit (Vector Laboratories).

LIMITING DILUTION

The frequency of progenitor cells was determined by quantifying the fraction of wells not containing CFU-O, CFU-ALP, ALP + Mineral+ cells, Oil Red O+ cells, or CFU-Ch at each cell density tested. From a plot of the fraction of empty wells against cell number plated per well, the progenitor cell number was determined from $Fo = e^{-x}$, where Fo is the fraction of empty wells, and x is the mean number of osteoprogenitors per well; assuming a Poisson distribution, $Fo = 0.37$ is the dilution at which one progenitor is present per well. In limiting dilution experiments, a minimum of 48 wells were plated for each cell density tested and the actual fraction of wells without the colony types of interest was counted and plotted ±95% confidence limits [Aubin, 1999]. Analyses were repeated a minimum of 3 times with independent cell isolates.

QUANTITATIVE ANALYSIS OF GENE EXPRESSION

RNA was extracted with TRIzol® reagent (Invitrogen Inc.) and reverse transcribed using Superscript™II (Invitrogen Inc.). Expression levels of each lineage marker and a housekeeping gene (ribosomal protein L32) were assessed by real-time PCR (ABI Prism® 7000, Applied Biosystems) with primer sequences as listed in Supplementary Table II. The relative amounts of transcripts were normalized to the L32 transcript.

IN VIVO TRANSPLANTATION

In vivo transplantation was performed as reported [Krebsbach et al., 1997; Bianco et al., 2006]. BMSCs or HipOPs (1.5×10^6 cells, H-2K^b: C57BL6 major histocompatability complex) [Zinkernagel and Althage, 1999] were suspended in α-MEM containing 20% FCS. To load sponges (Gelfoam; Pfizer) with cells, the sponges were placed into the cell suspension and incubated for 90 min at 37%. The sponges were transplanted subcutaneously into 8–15-week-old female Crlj:CD1-*Foxn1*^{nu} mice (H-2L^q) [Zinkernagel and Althage, 1999]. The transplants were recovered at 8 weeks after transplantation, fixed in PLP fixative (containing 4% paraformaldehyde) for 6 h at 4°C, and decalcified with 15% EDTA for 1 week at 4°C. Decalcified transplants were frozen in ornithine carbamoyltransferase compound with liquid nitrogen. After sectioning (6 μm), H-2K^b positive cells were detected by staining sections with biotinylated anti-H-2K^b antibody and a Vectastain Elite ABC kit. HipOPs prepared from YFP transgenic mice {129-Tg(ACTB-EYFP)7AC5Nagy/J} were transplanted into nude mice and processed similarly. After sectioning (6 μm), sections were stained with Hoechst 33342 (Calbiochem).

MICROCT

A detailed qualitative and quantitative 3D evaluation of the whole transplants was performed using a Scanco μCT40 scanner with 12 μm resolution (SCANCO Medical AG). A fixed threshold was applied to assess mineralized bone on the gray scale images. The total mineralized tissue volume was used for statistical analysis.

STATISTICAL ANALYSIS

Values are given as means ± SD of a minimum of three independent experiments, except for limiting dilution where the mean and 95% confidence limits of a minimum of 96 wells are plotted. Comparisons between means were made by using a Student's *t*-test, and differences between means were considered significant when *P*-values were less than 0.05.

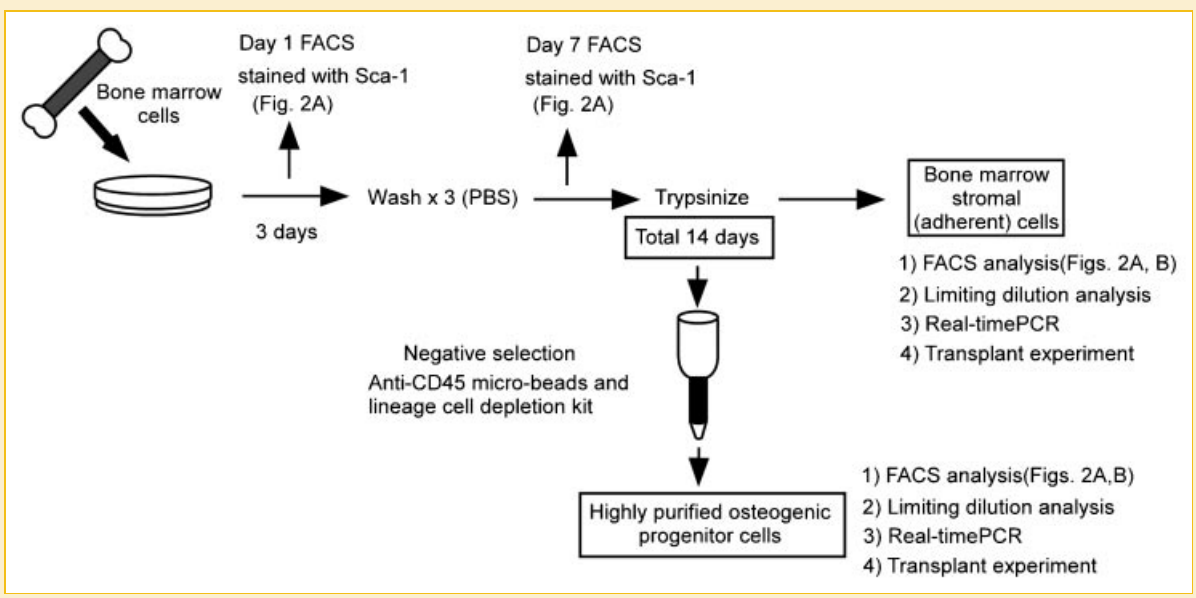

Fig. 1. The method to purify HipOPs. Freshly isolated bone marrow cells were plated into 10 cm dishes, and on day 1 after plating, the total population (floating and adherent cells) were used for FACS analysis (day 1). Cultures were washed 3 times with PBS to remove floating cells on day 3, medium was changed thereafter every 3–4 days, and at day 7, adherent cells were used for FACS analysis (day 7). At day 14, adherent cells were harvested (BMSCs) and sorted by magnetic micro-beads (HipOPs). Both BMSCs and HipOPs were used for FACS analysis, and limiting dilution, real-time PCR and transplant experiments.

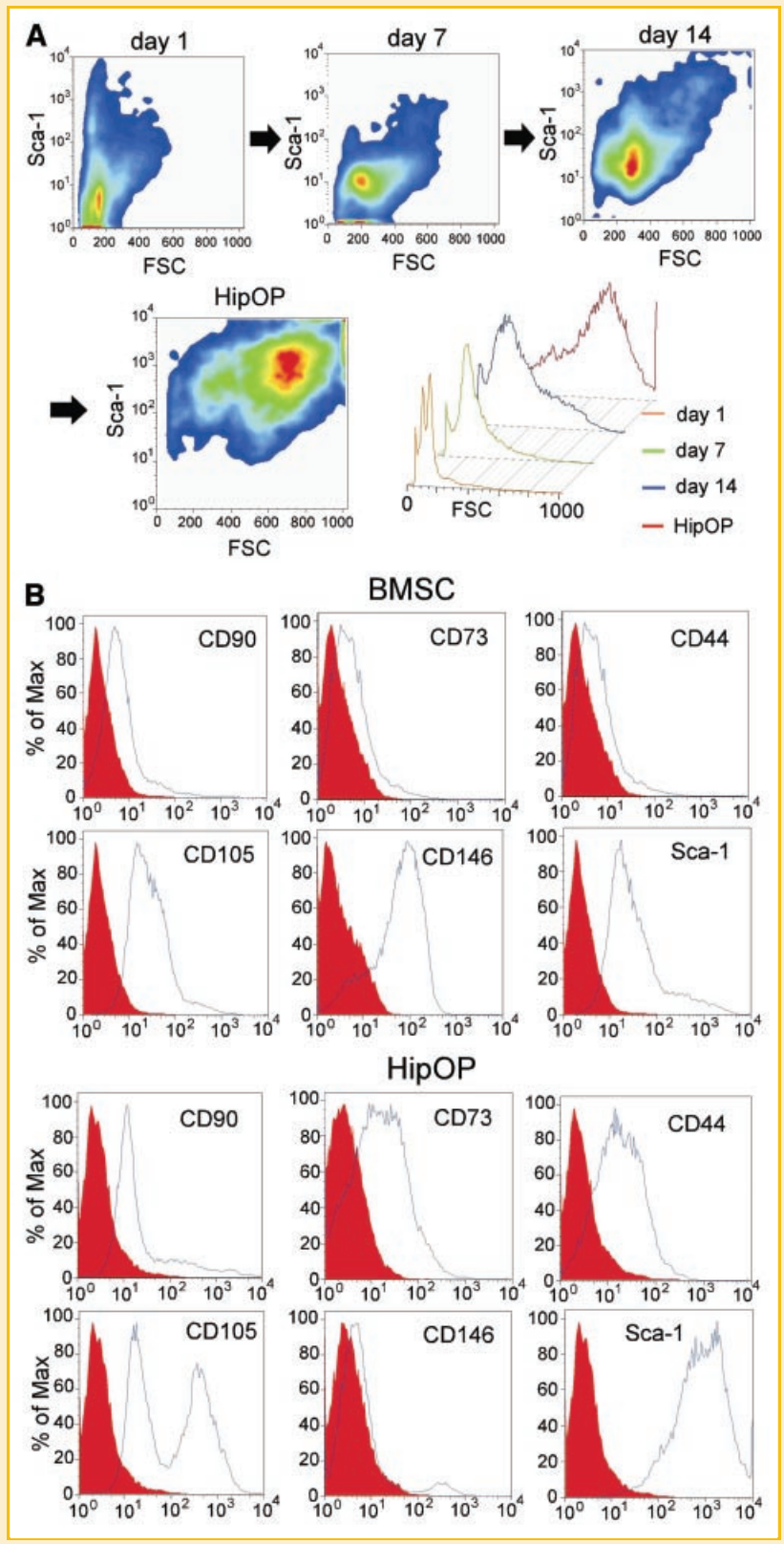

Fig. 2. FACS analysis reveals that HipOPs are large Sca-1-positive cells. A: At the days indicated, BMSCs and sorted HipOPs were labeled with APC-anti-Sca-1 Abs. Graphs are displayed with forward scatter (FSC; *x*-axis) and Sca-1 (*y*-axis). B: BMSCs and HipOPs were labeled with a panel of antibodies against typical "mesenchymal stem cell" markers.

TABLE I. Flow Cytometry Analysis of BMSCs and HipOPs

	Positive cells (%)	
	BMSCs	HipOPs
CD90	20.3 ± 2.4	$26.4 \pm 2.3^{*}$
CD73	10.9 ± 0.7	$43.0 \pm 1.3^{***}$
CD44	92.8 ± 2.3	$36.9 \pm 3.1^{***}$
CD105	84.2 ± 2.2	$62.7 \pm 5.9^{***}$
CD146	81.1 ± 1.2	$10.2 \pm 1.8^{**}$
Sca-1	79.2 ± 2.7	$98.3 \pm 0.5^{***}$
CD34	17.3 ± 1.2	17.6 ± 2.5

Values are the means ± SD of three independent experiments. Asterisks indicate statistically significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

RESULTS

CHARACTERIZATION OF A NOVEL BONE MARROW CELL POPULATION

To purify an SSC-enriched population, we expanded bone marrow stromal (adherent) cells (BMSCs) in vitro for 14 days, then harvested and fractionated the cells by magnetic micro-beads to exclude lineage-committed hematopoietic cells (BMSCs $1.6 \pm 0.34 \times 10^{6}$/mouse; HipOPs $1.1 \pm 0.37 \times 10^{5}$/mouse) (Fig. 1). The cell size of HipOPs was quite large compared to both freshly isolated and culture-expanded BMSCs (Fig. 2A). Surface phenotypic characteristics of cells designated SSCs differ amongst laboratories and species, and there is no one specific marker or combination of markers that unambiguously identifies SSCs either in vivo or in vitro. Phenotypically, it is generally accepted that SSCs variably express the putative "mesenchymal stem cell" markers CD90 (Thy1.1), CD44, SH2 (CD105 or endoglin), and SH3 or SH4 (CD73) [Javazon et al., 2004; Chamberlain et al., 2007]. Both the unsorted BMSCs and HipOPs express CD90, CD73, CD44, CD105, CD146 and Sca-1, but CD90-, CD73-, and Sca-1-positive cells are significantly enriched, CD44-, CD105- and CD146-positive cells are significantly depleted in the HipOP versus BMSC fractions and CD34 is unchanged (Fig. 2B and Table I). Consistent with the presence of some CD34-positive cells in the HipOP fraction, assays for hematopoietic colony forming units revealed that the HipOP fraction has a low hematopoietic multilineage potential and likely contains HSCs (Supplementary Fig. 1).

TABLE II. Frequency of Various Progenitor Cell Types Determined by Limiting Dilution Analysis

	BMSCs	HipOPs
CFU-ALP		
#1	1/6,700	1/280
#2	1/7,800	1/580
#3	1/7,500	1/780
ALP + mineral+		
#1	1/42,000	1/260
#2	1/42,000	1/730
#3	1/43,000	1/880
CFU-O		
#1	1/130,000	1/740
#2	1/140,000	1/820
#3	1/150,000	1/1,000
CFU-F		
#1	1/1,800	1/340
#2	1/1,900	1/340
#3	1/2,900	1/460
Oil Red O+		
#1	1/700	1/300
#2	1/700	1/300
#3	1/1,000	1/400
CFU-Ch		
#1	1/5,000	1/1,000
#2	1/5,400	1/1,000
#3	1/5,700	1/1,200

See Materials and Methods Section for definitions and identification protocols for all colony types.

EVALUATION OF MULTI-LINEAGE DIFFERENTIATION ABILITY OF HipOPs

Since SSCs are expected to have capacity for differentiation along multiple mesenchymal lineages, we next cultured HipOPs under conditions supportive of osteoblastic, adipocytic or chondrocytic development and confirmed differentiation along all three lineages (Supplementary Fig. 2 and Materials and Methods Section). To confirm that the frequency of SSCs was increased in the HipOP versus BMSC populations, we performed limiting dilution analyses. The frequency of CFU-O was 100 times higher in HipOPs than in BMSCs (Supplementary Fig. 2 and Table II). Also increased was the frequency of CFU-ALP and the frequency of individual or small clusters of cells (1–3 cells; not counted as a colony or CFU) that are ALP-positive and have associated mineral deposits (almost 10 times and 60 times, respectively) (Supplementary Fig. 2 and Table II). The frequency of adipocyte (Oil Red O-positive cells) and chondrocyte (CFU-Ch) progenitors was also higher in HipOPs versus BMSCs (almost 2 times and 5 times, respectively) (Supplementary Fig. 2 and Table II). Consistent with colony counts, the expression levels of differentiation markers for all three lineages tested (*OPN*, *OCN*, *BSP*, *ALP*, *Col1a1*, *Runx2*, and *OSX* for osteoblasts; *PPARγ* for adipocytes; *aggrecan* for chondrocytes) were higher in HipOPs than BMSCs (Figs. 3 and 4A). Thus, both limiting dilution and real-time PCR analyses demonstrated that the HipOP fraction is highly enriched in multipotential SSCs, with an especially high capacity for osteogenic differentiation.

ABILITY OF HipOPs TO RECONSTITUTE MULTIPLE LINEAGES AND THE BONE NICHE FOR HSCs IN VIVO

Given the robust osteoblast differentiation capacity of HipOPs in vitro, we next asked whether HipOPs showed enhanced differentiation capacity for skeletal tissue(s) in vivo. When an equal number (1.5×10^{6} cells) of BMSCs or HipOPs in collagen sponges were transplanted subcutaneously into immunodeficient mice (Crlj:CD1-*Foxn1*^{nu}), and analyzed at 8 weeks, both macroscopic observation (not shown) and microCT revealed only a few small areas of mineralization in transplants with BMSCs (Fig. 5A). In contrast, large masses (over 100 times higher than those seen in BMSCs by microCT analysis) of mineralized tissue were seen in transplants with HipOPs (Fig. 5B,C). Histological analyses confirmed the bone-like architecture of HipOP transplants with an exterior of cortical bone and an interior cavity of trabecular bone structures with osteoblasts, osteocytes and osteoclasts, bone marrow cells, adipose tissue and vascular channels (Fig. 5D–G). HLA typing with H-2K^{b} antibodies showed that donor cells were present throughout the

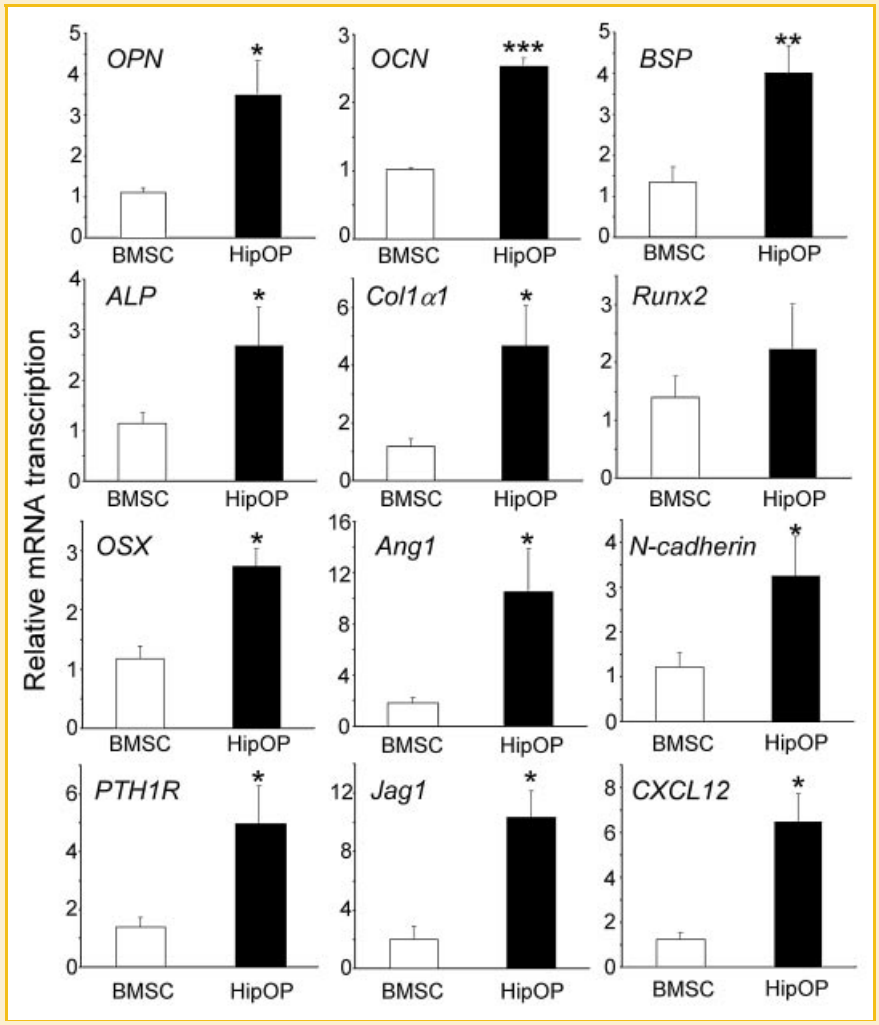

Fig. 3. HipOPs show higher expression of "mesenchymal" cell differentiation and HSC niche marker genes. mRNA extracted from freshly isolated BMSCs and HipOPs reverse transcribed in three independent experiments. Samples were subjected to quantitative real-time PCRs using specific promers for *OPN, OCN, BSP, ALP, Type I collagen, Runx2, OSX, Ang1, N-cadherin, PTH1R, Jag1, and CXCL12*. mRNA expression were normalized to *L32* expression. Values are expressed as means ± SD. Asterisks indicate statistically significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

structures [Zinkernagel and Althage, 1999], with H-2K^{b}-positive osteoblasts, osteocytes and cells around sinusoids (Fig. 5H–K); H-2K^{b}-positive bone marrow cells were also present (Fig. 5H–K). To confirm the presence of donor cells, HipOPs prepared from YFP transgenic mice {129-Tg(ACTB-EYFP)7AC5Nagy/J} were transplanted into nude mice with similar results: donor cells were seen throughout the bone structures and YFP-positive bone marrow cells were also present (Supplementary Fig. 3). Thus, to address whether the HipOP fraction contains cells comprising the HSC niche, we analyzed expression of *Ang1*, *N-cadherin*, *PTH1R*, *Jag1*, and *CXCL12*. All were significantly more highly expressed in HipOPs compared to BMSCs at day 0 (*Ang1*; 5 times, *N-cadherin*; 3 times, *PTH1R*; 3 times, *Jag1*; 5 times, *CXCL12*; 5 times, respectively) (Fig. 3). The data indicate that the HipOP fraction is highly enriched in cells comprising the HSC niche, and indicate that bone niche markers change as the cells undergo osteogenic differentiation (Fig. 4B).

DISCUSSION

Purification of murine SSCs is far more difficult than that of at least certain other species, due to significant contamination by hematopoietic cells that are also directly adherent to plastic and bind to bone marrow stromal cells via adhesion molecules, cytokine receptors, and extracellular matrix proteins [Bearpark and Gordon, 1989; Simmons et al., 1992; Deryugina and Muller-Sieburg, 1993]. To purify an SSC-enriched population, we cultured BMSCs for 14 days and then fractionated by exclusion of lineage-committed hematopoietic cells by using magnetic micro-beads, resulting in a novel significantly enriched SSC population that comprises almost 100% Sca-1-positive cells, even though Sca-1 is not amongst the lineage markers used for fractionation. Earlier studies on murine stromal cells have also suggested that SSCs have high surface expression of Sca-1 [Van Vlasselaer et al., 1994; Javazon et al., 2004; Peister et al., 2004]. Consistent with this, an interesting

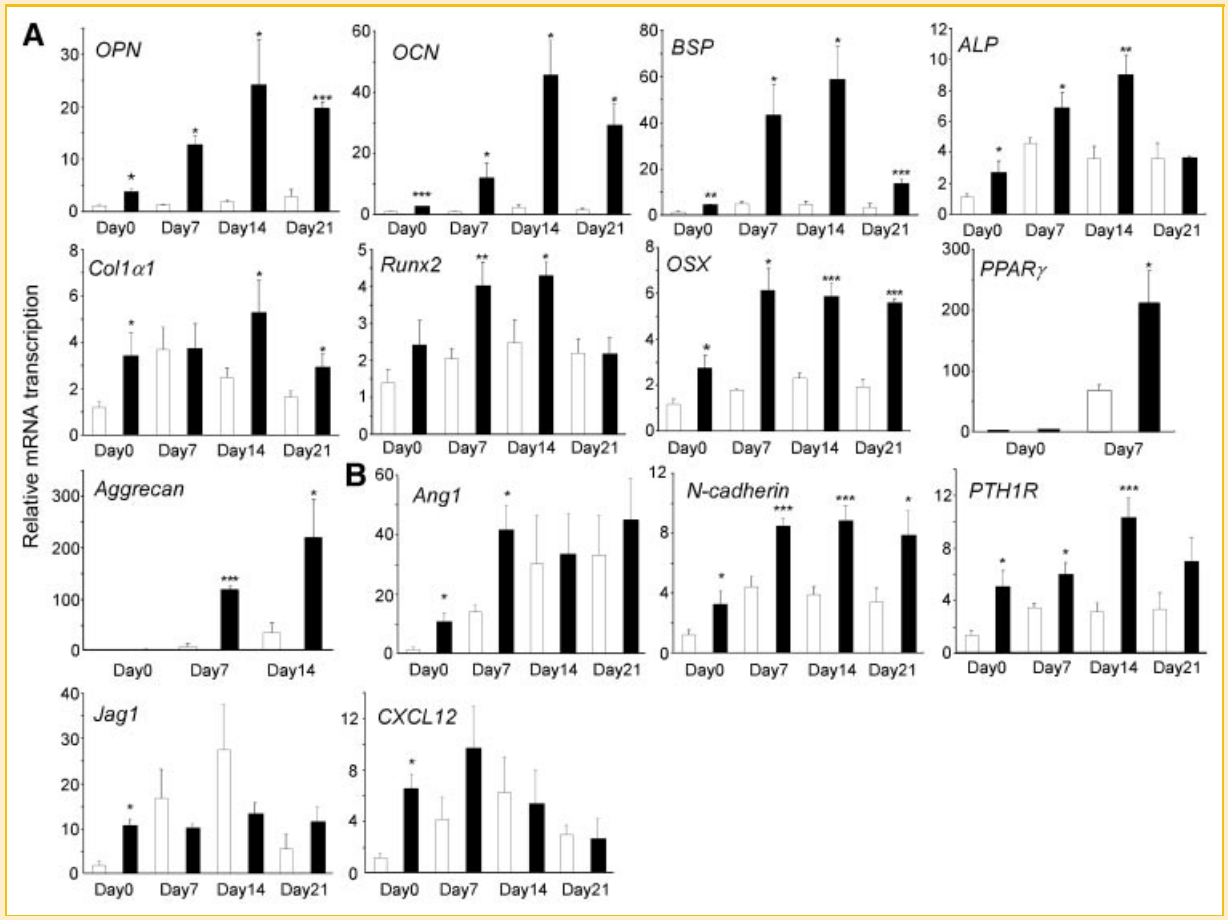

Fig. 4. HipOPs show higher expression of "mesenchymal" cell differentiation marker genes. BMSCs and HipOPs were cultured with osteogenic induction medium, adipogenic induction medium, or chondrogenic induction medium. mRNA was extracted and reverse transcribed in three independent experiments. Samples were subjected to quantitative real-time PCRs using specific promers for (A) *OPN, OCN, BSP, ALP, Type I collagen, Runx2, OSX, PPARγ* and *Aggrecan* (B) *Ang1, N-cadherin, PTH1R, Jag1, and CXCL12*. mRNA expression were normalized to *L32* expression. Values are expressed as means ± SD. Asterisks indicate statistically significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

recent study in Sca-1/Ly-6A null mouse documented normal skeletal development with age-dependent onset of osteoporosis associated with a defect in mesenchymal progenitor self-renewal [Bonyadi et al., 2003]. However, previously, van Vlasselaser et al. reported use of mAbs against Sca-1 and Wheat Germ Agglutinin (WGA) to positively select osteoprogenitor cells. Unexpectedly, although the sorted FSC^{high} WGA^{bright} Sca-1^{+} population contained a high frequency of CFU-O, their osteogenic activity in vitro was lower than that of unsorted cells [Van Vlasselaer et al., 1994]. Because they attributed the loss of osteoblastic activity to the positive selection for Sca-1-positive cells, we employed here negative selection for purifying HipOPs and somewhat surprisingly achieved an almost 100% Sca-1-positive population (Fig. 2B and Table I). In this regard, it is interesting that although the majority of BMSCs are small cells on day 1 of culture, cell size gradually increases with time such that a large and Sca-1 positive (FSC^{high} Sca-$1^{positive}$) fraction is present in BMSCs by day 14 (cf. days of culture in Fig. 2A). Notably, the HipOP fraction isolated at day 14 from BMSCs by exclusion of lineage-committed hematopioetic cells may correspond to the FSC^{high} Sca-$1^{positive}$ cells in the cultured BMSCs. In any case, HipOPs have a higher osteogenic and other mesenchymal cell differentiation capacity than the unsorted BMSC population, suggesting that Sca-1 is associated with the presence of SSCs and may be a significant stem cell marker not only on the surface of hematopoietic stem cells (HSCs) but also murine SSCs.

In our experiments, the same percent BMSCs and HipOPs express CD34, which is widely used as an HSC marker (BMSCs 17.3 ± 1.2%; HipOPs 17.6 ± 2.5%). Previous reports that SSCs may express CD34 have been discrepant. For example, although human and rat SSCs have been reported to be negative for expression of CD34, murine SSCs have variously been described to express or not CD34, with some attributing expression to acquisition of the marker in vitro [Javazon et al., 2004; Peister et al., 2004; Chamberlain et al., 2007]. Our data suggest that CD34 is not uniquely expressed by murine HSCs, but additional work is required to delineate changes in CD34 expression with differentiation, including of osteoblastic cells. Our data are also consistent with the HipOP fraction of cells being enriched not only in SSCs, in particular cells with high osteogenic

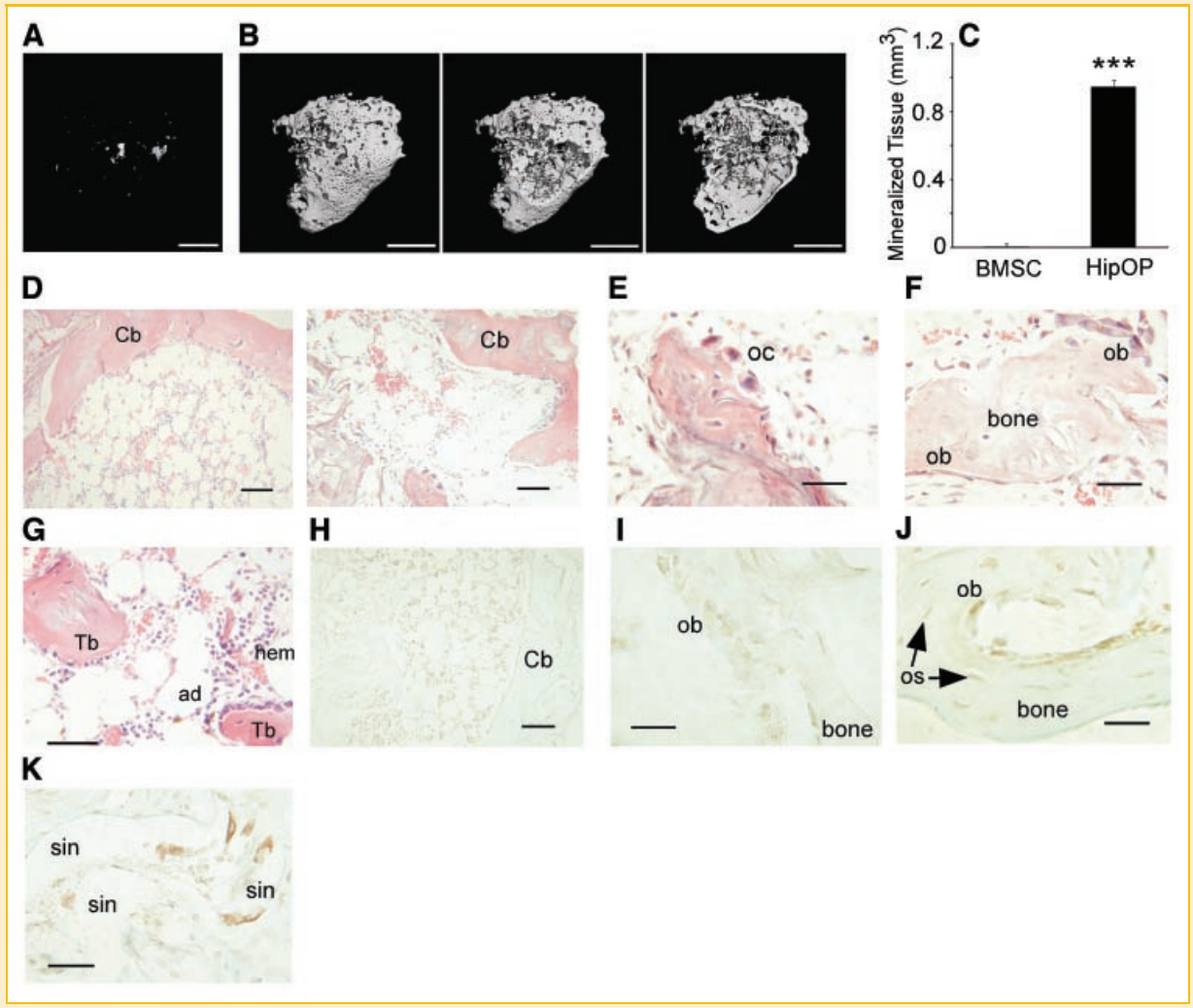

Fig. 5. HipOPs form a complete skeletal organ after transplantation. A: MicroCT 3D reconstructions of a typical transplant of BMSCs at 8 weeks after transplantation. Bar = 1 mm. B: MicroCT 3D reconstructions of a typical transplant of HipOPs at 8 weeks after transplantation. Bars = 1 mm. C: The comparison of volume of total mineralized tissue between BMSCs and HipOPs. Data are expressed as means of three independent experiments ± SD. Asterisks indicate statistically significant differences: $^{***}P < 0.005$. D–G: Histology of transplants of HipOPs harvested at 8 weeks. Cb, cortical bone; Tb, trabecular bone; ob, osteoblast; oc, osteoclast; ad, adipose tissue; hem, hematopoietic cells, H&E. D: 100×; E–G: 400×. D: Bars = 50 μm; E–G: Bars = 30 μm. H–K: Frozen sections of transplants of HipOPs were stained with anti-H-2K^{b} Abs and re-stained with fast green. sin, sinusoid; os, osteocyte. H: 100×; I–K: 400×. H: Bars = 50 μm; I–K: Bars = 30 μm.

differentiation capacity, but also cells comprising the bone niche for HSCs. As already raised, Sacchetti et al. [2007] recently demonstrated the utility of CD146 as a marker for those self-renewing human bone marrow stromal cells/CFU-Fs with a critical role for reconstitution of both osteoblasts making bone and those creating the hematopoietic microenvironment. In contrast, we found that a high percent of cells in the unfractionated murine BMSC expressed CD146, but far fewer did so in the HipOP fraction which was, on the other hand, enriched for all "mesenchymal" progenitor types including osteoprogenitors and the cells expressing markers of the HSC niche (Fig. 3). That expression level of surface markers has been sometimes quite different between human and murine cells is already well-established [Javazon et al., 2004], and our data suggest that alternative marker(s) to CD146 may better characterize the murine SSC and HSC niche population.

Recently, several groups have proposed that Ang1, N-cadherin, PTH1R, Jag1, and CXCL12 on a subpopulation of osteoblasts involved in the maintenance of HSC in bone marrow microenvironments, but the degree of maturity of these cells, that is, whether osteoprogenitors or mature osteoblasts remains unclear [Calvi et al., 2003; Zhang et al., 2003; Arai et al., 2004; Sugiyama et al., 2006]. All these HSC niche markers were significantly more highly expressed in HipOPs compared to BMSCs (Fig. 3). Consistent with earlier reports that expression levels of N-cadherin and Ang1 are correlated [Arai et al., 2004; Arai and Suda, 2007], not only N-cadherin and PTH1R, which are known to be highly expressed by mature osteoblasts [Marie, 2002; Yang et al., 2006], but also Ang1 expression increased during osteogenic differentiation (Fig. 4B). On the other hand, the expression levels of Jag1 and CXCL12 did not change in parallel with differentiation and maturation of the

osteoblastic cells (Fig. 4B). Our data suggest that expression levels of the latter two markers, while obviously highly enriched in the freshly isolated HipOP fraction, may not change with osteoblast differentiation stage, but rather reflect regulation in osteoblastic cells treated under specific conditions, such as with PTH [Calvi et al., 2003; Frisch et al., 2008]. However, we cannot exclude the possibility that other non-osteoblastic (e.g., reticular cells) remain and express these markers in the differentiating HipOP fraction. Nevertheless, our data demonstrate that the HipOP fraction has potential for reconstitution of bone marrow microenvironments, such as the HSC niche, but also support the view that all the niche markers other than Jag1 and CXCL12 are upregulated as osteogenic cells differentiate, supporting the view of a dynamic niche for HSC regulation.

In summary, we have purified a novel bone marrow-derived population that manifests robust enrichment for cells with high potential for reconstitution of a multilineage bone organ structure in vivo, and for cells associated with the HSC niche. The data suggest that this novel population will be useful in regenerative medicine, including those necessary in certain orthopedic or dental applications.

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