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Journal of Cellular Biochemistry 114:1066–1073 (2013)

Journal of Cellular Biochemistry

Osteogenesis and Expression of the Bone Marrow Niche in Endothelial Cell-Depleted HipOPs

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ABSTRACT

The identification and purification of murine multipotent mesenchymal stem cells (MSCs) have been difficult due to their low frequency, the presence of contaminating cell types and lack of unambiguous markers. Using a magnetic micro-beads negative selection technique to remove hematopoietic cells from mouse bone marrow stromal cells (BMSCs), our lab recently isolated a highly purified osteoprogenitor (HipOP) population that was also enriched for other mesenchymal precursors, including MSCs [Itoh and Aubin, 2009]. We now report that HipOPs are also highly enriched in vascular endothelial cells (VECs), which we hypothesized were an accessory cell type regulating osteogenesis. However, when VECs were immunodepleted from HipOPs with anti-CD31 antibodies, the resulting CD31(−) HipOP population had equal osteogenic capacity to the HipOPs in vitro and in vivo. Analysis of gene expression of *Ncad*, *Pth1r*, *Ang1*, *Cxcl12*, *Jag1*, *Pdgfr-β*, *α-sma*, *Desmin*, and *Ng2* suggested that both HipOPs and CD31(−) HipOPs are hemopoietic stem cell (HSC) niche populations. However, the data support the view that osteoblast differentiation and depletion of VECs modulate the HSC niche. J. Cell. Biochem. 114: 1066–1073, 2013.
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KEY WORDS: VASCULAR ENDOTHELIAL CELLS; OSTEOPROGENITORS; MESENCHYMAL STEM CELLS; HEMATOPOIETIC STEM CELL NICHE; IMMUNODEPLETION

Mesenchymal stem cells (MSCs) are thought to give rise to and to play an important role in the repair and regeneration of mesenchymal tissue types, such as bone, cartilage and fat, amongst others [Phinney and Prockop, 2007; Aubin, 2008]. Although the identification and purification of MSCs and their more differentiated progeny, osteoprogenitors, remain challenging due to the lack of specific and unambiguous cellular markers, the ability to isolate populations enriched in cells with a high capacity to form bone remains desirable for the treatment of bone and joint diseases, such as osteoporosis, osteogenesis imperfecta and arthritis.

Bone is a highly vascularized tissue that requires spatial and temporal coordination of both osteogenesis and angiogenesis for its development and repair. Within allogeneic scaffolds, the survival of osteogenic cells is dependent on the vascularisation of the scaffold [Kneser et al., 2006]. Conversely, when the vascular supply to the bone is altered, a variety of pathologies can result including osteoporosis, osteomyelitis or osteonecrosis [Burkhardt et al., 1987; Alagiakrishnan et al., 2003; Lazzarini et al., 2004; Childs, 2005]. This dependence on vasculature—whether supplied by donor or host—must be considered in the quest to find an ideal cell population for bone repair and regeneration.

Data on the regulation of osteogenesis by vascular endothelial cells (VECs) in vitro is diverse, with different results reported with the use of different cells/site of biopsy or cell lines, culture

Abbreviations used: ALP, alkaline phosphatase; BMSCs, bone marrow stromal cells; CD31(−) HipOPs, highly purified osteoprogenitor population depleted in CD31-expressing cells; HipOPs, highly purified osteoprogenitor population; Lin(+), lineage-positive; MACS, magnetic-activated cell sorting; PBS, phosphate buffered saline; RT-qPCR, real-time quantitative polymerase chain reaction; VEC, vascular endothelial cell.

The authors declared they have no conflict of interest.

Grant sponsor: Canadian Institutes of Health Research (CIHR); Grant number: FRN 83704; Grant sponsor: The Edward Dunlop Ontario Graduate Scholarship in Science and Technology; Grant sponsor: Canadian Arthritis Network Trainee Award; Grant sponsor: Skeletal Regenerative Medicine Team (CIHR Team) Studentship; Grant sponsor: Life Science Undergraduate Research Award.

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Manuscript Received: 25 October 2012; Manuscript Accepted: 30 October 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 13 November 2012

DOI 10.1002/jcb.24446 • © 2012 Wiley Periodicals, Inc.

conditions, species studied and donor age. While most studies support the view that VECs positively regulate osteogenesis, whether they do so by direct cell–cell contact or in a paracrine fashion remains uncertain [Deckers et al., 2000; Villars et al., 2002; Kaigler et al., 2005; Xue et al., 2009; Saleh et al., 2010]. In vivo data agree that direct cell–cell contact enhances bone formation, but disagree on whether paracrine factors increase osteogenesis to a lesser extent than direct contact [Sun et al., 2007] or have no effect at all [Kaigler et al., 2005]. Teasing out the interactions between osteogenic and vascular cells becomes even more complicated when one considers recent evidence of transdifferentiation between the cellular lineages [Medici et al., 2010; Hamdan et al., 2011]. In fact, osteogenic cell populations have been reported to express some angiogenic factors [Phinney, 2007; Joggerst and Hatzopoulos, 2009] and vice versa [Shin et al., 2004].

Despite the lack of a unique marker of MSCs, there is a consensus that MSCs do not express hematopoietic markers [Kolf et al., 2007]. Consistent with this, using negative selection with anti-CD5, CD45, CD11b, GR-1, 7-4, TER-119, and CD45R-conjugated magnetic micro-beads to remove hematopoietic cells, our lab recently significantly enriched for various mesenchymal progenitors, including osteoprogenitor cells, the latter evidenced by a high frequency of colony forming units-osteoprogenitor (CFU-O) and high expression levels of osteoblast differentiation markers in vitro [Itoh and Aubin, 2009]. Notably, when this highly purified osteoprogenitor population (HipOPs) was transplanted within collagen sponges into immunodeficient mice, HipOPs had far greater capacity than unfractionated stromal cells to produce a bone-like organ with differentiated osteoblasts, osteocytes, sinusoidal cells, and bone marrow cells of donor origin. This indicated that the HipOPs were able to reconstitute a bone niche for HSCs, which was supported by high expression of HSC niche markers in vitro. The in vivo data also suggested the possibility of the presence of an angiogenic cell population since YFP-labeled donor HipOPs were observed around sinusoids. In this report, we show that VECs are significantly enriched within the HipOP population. Using CD31 in negative selection, we also confirmed that CD31 is not a useful mouse MSC marker [Kolf et al., 2007] and demonstrated that osteogenic capacity in vitro and in vivo is not affected by the removal of VECs from the HipOP fraction. However, the HSC niche is modulated by the removal of VECs and by osteoblast differentiation.

MATERIALS AND METHODS

ISOLATION OF HipOPs

All experimental procedures received approval from the Faculty of Medicine and Pharmacy Animal Care Committee of the University of Toronto and were conducted in accordance with the guidelines of the Canadian Council on Animal Care. HipOPs were isolated as previously described [Itoh and Aubin, 2009]. Typically, the bone marrow from the femurs and tibiae of 10 mice was plated into 6 10 cm plates, which translated into a density of 4–8 $\times$ 10^7 nucleated cells/plate.

Bone marrow stromal cells (BMSCs) and HipOPs were also fractionated according to their expression of CD31, using biotin-conjugated anti-CD31 antibodies (eBioscience, San Diego) and anti-biotin magnetic beads (Miltenyi Biotec, Cologne, Germany) (Fig. 1).

OSTEOGENIC DIFFERENTIATION ASSAYS AND LIMITING DILUTION ANALYSIS

For osteogenic differentiation assays, fractionated or unfractionated cells were plated at a density of 2 $\times$ 10^4 cells/well into 96-well plates.

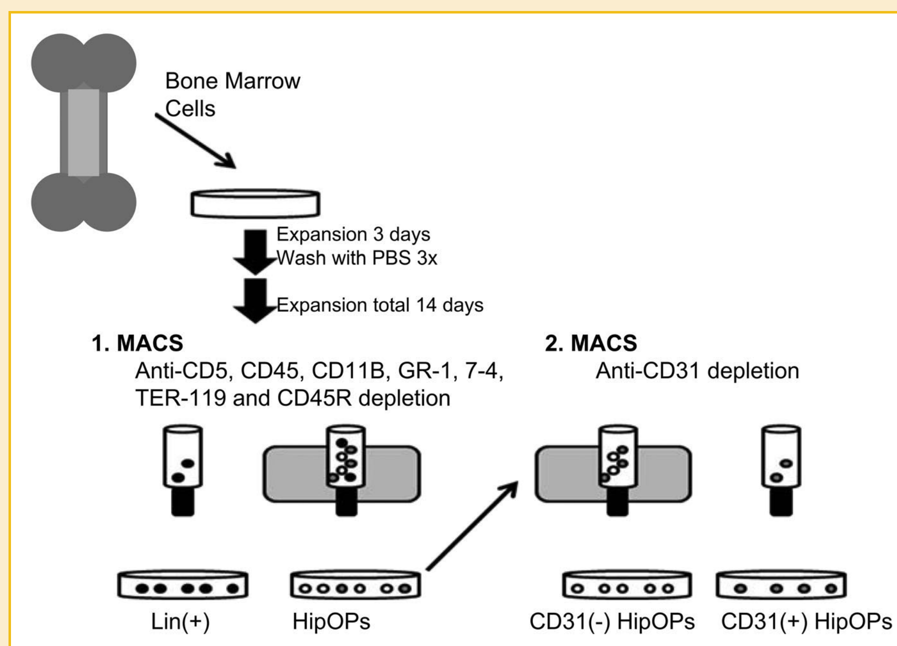

Fig. 1. Schematic of HipOP and CD31(−) HipOP cell isolation. Bone marrow stromal cells were harvested from mice, expanded for 3 days then washed with PBS three times, and then expanded for a total of 2 weeks prior to fractionation. The first MACS sort depleted the BMSCs of lineage-positive (lin(+)) cells expressing CD5, CD45, CD11b, GR-1, 7-4, TER-119, and CD45R, resulting in a highly purified osteoprogenitor population (HipOPs). A second/consecutive MACS sort depleted the HipOP population of CD31(+) cells.

For limiting dilution experiments, cells were plated at several densities, as specified in figures, into 48-wells of 96-well plates for each density. Cells were then cultured for up to 28 days in osteogenic differentiation medium (αMEM, 10% FBS, 1 IU penicillin, 1 μg/ml streptomycin, 50 μg/ml gentamycin, 250 ng/ml fungizone, 50 μg/ml ascorbic acid (Fisher Scientific Co., Ottawa, Canada), 10 mM β-glycerophosphate (β-GP) (Sigma Chemical Co., Oakville, Canada) and 10^{-8} M dexamethasone (Dex) (Sigma Chemical Co.). The medium was changed twice a week. At the end of the culturing period, cells were fixed in 10% neutral-buffered formalin for 15 min and stained sequentially for alkaline phosphatase (ALP) activity, for phosphate deposition with von Kossa stain (VK) and for all cells with methylene blue (MB). Image J software was used to quantify the areas stained with ALP, VK, and MB.

For limiting dilution analysis, the presence or absence of colonies in each well was recorded. Colony forming units-alkaline phosphatase (CFU-ALP) were defined as colonies with ALP-positive cells. CFU-O (osteoblast) was defined as colonies with ALP-positive cells associated with mineralized matrix (VK-positive). CFU-Fs (fibroblasts) were defined as all MB-positive colonies, regardless of other staining; thus, the CFU-F category may contain progenitor types other than those capable of forming connective tissue and not investigated in these experiments.

The frequency of progenitors was determined from the limiting dilution experiments as previously described [Itoh and Aubin, 2009]. A line of best fit was applied to the linear portion of the curve and extrapolated if necessary to the density at which the fraction of empty wells is 0.37.

QUANTITATIVE ANALYSIS OF GENE EXPRESSION

RNA was extracted with Trizol (Invitrogen Inc., Burlington, Canada) at day of isolation (d0) and after 14 and 21 days (d14 and d21, respectively) in osteogenic culture and reverse-transcribed with Superscript II (Invitrogen Inc., Burlington, Canada). Real-time quantitative polymerase chain reaction (RT-qPCR) (BioRad MyiQ5, Mississauga, Canada) was used to determine expression levels of osteoblast lineage markers: osteopontin (*Opn*), osteocalcin (*Ocn*), bone sialoprotein (*Bsp*), alkaline phosphatase (*Alp*), Type I collagen (*Col1a1*), runt-related transcription factor 2 (*Runx2*) and osterix (*Osx*); VEC markers: vascular endothelial cadherin (*Ve-cad*), and *CD31* variants *1* and *2* (*CD31v1* and *CD31v2*); and HSC niche markers: angiopoietin 1 (*Ang1*), neural cadherin (*Ncad*), parathyroid hormone 1 receptor (*Pth1r*), jagged ligand 1 (*Jag1*), and chemokine ligand 12 (*Cxcl12*; also known as stromal cell-derived factor-1 (*Sdf-1*)), platelet-derived growth factor receptor β (*Pdgfr-β*), α-smooth muscle actin (*α-sma*), desmin (*Desmin*), and neuron-glial proteoglycan (*Ng2*). Gene expression levels were normalized to the housekeeping ribosomal protein *L32* transcript. Specific primer sequences used are in Table I.

IN VIVO TRANSPLANTATION

In vivo transplantation was performed as previously described [Krebsbach et al., 1997; Bianco et al., 2006; Itoh and Aubin, 2009]. Cells were suspended in 10% FBS αMEM and antibiotics as above. Sponges (Gelfoam, Pfizer, Kirkland, Canada) were cut into the desired size, squeezed to remove any air and incubated in the cell suspension for 90 min at 37°C. Equal numbers of BMSCs, HipOPs or CD31(−) HipOPs were loaded onto sponges: 1.5×10^6 cells in $5 \times 5 \times 5$ mm^3 "large" collagen sponges, 3.8×10^5 cells in "medium" sponges (one quarter size of large sponges), or 1.9×10^5 cells in "small" sponges (half the size of medium sponges). One experiment was conducted with a large sponge containing only 1.0×10^6 HipOPs. The sponges were transplanted subcutaneously into 8–10 weeks-old female Crl: CD1-Foxn1^{nu} mice. The transplants were harvested at 8 weeks after transplantation, fixed in 4% paraformaldehyde or neutral buffered formalin for 6 h at 4°C, washed in PBS, embedded in paraffin, sectioned (6 μm) and stained with hematoxylin and eosin. There were two sections mounted on each slide. For each small sponge, bone was quantified from four slides spaced five slides apart. For each large sponge, bone was quantified from nine slides spaced five slides apart. Bone area from each CD31(−) HipOP sponge was normalized to bone area of the HipOP sponge of corresponding size. The average fold difference and standard deviation were calculated.

TABLE I. Specific Primer Sequences Used for RT-qPCR

Primer name	Primer sequence
L32-forward	CAC AAT GTC AAG GAG CTG GAA GT
L32-reverse	TCT ACA ATG GCT TTT CGG TTC T
Opn-forward	AGC AAG AAA CTC TTC CAA GCA A
Opn-reverse	GTG AGA TTC GTC AGA TTC ATC CG
Ocn-forward	CTG ACC TCA CAG ATG CCA AGC
Ocn-reverse	TGG TCT GAT AGC TCG TCA CAA G
Bsp-forward	CAG GGA GGC AGT GAC TCT TC
Bsp-reverse	AGT GTG GAA AGT GTG GCG TT
Alp-forward	CCA ACT CTT TTG TGC CAG AGA
Alp-reverse	GGC TAC ATT GGT GTT GAG CTT TT
CollαI-forward	GCT CCT CTT AGG GGC CAC T
CollαI-reverse	CCA CGT CTC ACC ATT GGG G
Runx2-forward	TGT TCT CTG ATC GCC TCA GTG
Runx2-reverse	CCT GGG ATC TGT AAT CTG ACT CT
Osx-forward	ATG GCG TCC TCT CTG CTT G
Osx-reverse	TGA AAG GTC AGC GTA TGG CTT
Cxcl12-forward	CGC TCT GCA TCA GTG ACG GTA
Cxcl12-reverse	GTT CTT CAG CCG TGC AAC AAT C
Ang1-forward	CAT TCT TCG CTG CCA TTC TG
Ang1-reverse	GCA CAT TGC CCA TGT TGA ATC
Ncad-forward	TTG CTG CAG AAA ACC AAG TG
Ncad-reverse	GAC TGA GGT GGG TGC TGA AT
Jag1-forward	CAC TTA TTG CTG CGG TTG CA
Jag1-reverse	TTT TCA GAG GAC GCC TCT GAA C
Pth1r-forward	TTC CAG GGA TTT TTT GTT GC
Pth1r-reverse	AGT CCA ATG CCA GTG TCC AG
Ve-cad-forward	AGC CCC CTG TCT TCC AGC GA
Ve-cad-reverse	GGA GTA CCC GAT GCT GCG CT
CD31v1-forward	AGC CTC ACC AAG CTC TGG GAA CG
CD31v1-reverse	ACG TGC ACA GGA CTC TCG CA
CD31v2-forward	CCT CAC CAA GAG AAC GGA AGG CT
CD31v2-reverse	ACG TGC ACA GGA CTC TCG CA
Pdgfr-β-forward	AGG TCA ATG TCC CCG TCC GT
Pdgfr-β-reverse	GGT CTC TGC AGG TAG ACC AGG TG
α-sma-forward	GCC AGT CGC TGT CAG GAA CC
α-sma-reverse	AGC GAA GCC GGC CTT ACA GA
Desmin-forward	TTC CGA GAA ACC AGC CCC GA
Desmin-reverse	ACG GGG CCA GGA CAC TGA AT
Ng2-forward	TGT CCA GAG TCT GTG CAG CCG
Ng2-reverse	CTG GGG GCC TCT TAC CAC CC

STATISTICAL ANALYSIS

Unless otherwise indicated, values are given as means ± SD of at least three independent experiments, except for limiting dilution analysis where the mean and 95% confidence limits of 48-wells/cell densities are plotted; limiting dilution experiments were repeated at

least three times. Comparisons between the means were made using the Student's t-test, and differences were considered significant when P-values were less than 0.05.

RESULTS

VECs AND OSTEOGENIC CAPACITY OF HipOPs IN VITRO AND IN VIVO

Our previous limiting dilution results indicated that more than one cell type is limiting for formation of CFU-ALP and CFU-O in vitro, that is, that (an) accessory regulatory cell type(s) is/are present in the HipOP population and that HipOPs form a bone organ complete with vasculature when transplanted into immunodeficient mice [Itoh and Aubin, 2009]. Based on these results and the importance of vasculature in terms of bone repair and the MSC niche, we hypothesized that VECs may be present in the HipOP population and may contribute to the osteogenic capacity of HipOPs in vitro and in vivo. Quantification of expression levels of two VEC markers, *Ve-cad*, and both transcript variants of *CD31* (*v1* and *v2*), confirmed that HipOPs were enriched in VECs compared to BMSCs at day of isolation (d0) (Fig. 2A).

To determine whether VECs that were enriched within the HipOP population were an accessory cell type contributing to the high osteogenic capacity of the HipOPs, we fractionated HipOPs into CD31(−) and CD31(+) fractions. The HipOP sort recovery was 63.4 ± 11.5%, with CD31(+) HipOPs representing 6.7 ± 2.9% of the recovered HipOP population. Quantification of VEC marker expression levels confirmed that CD31(−) HipOPs were depleted in VECs compared to HipOPs at d0 and after 14 days in osteogenic conditions (Fig. 2B). When osteogenic capacity was assessed by ALP staining, HipOPs depleted in VECs (i.e., CD31(−) HipOPs) and HipOPs further enriched in VECs (i.e., CD31(+) HipOPs) were not significantly different compared to the baseline VEC-containing HipOPs. However, HipOPs and CD31(−) HipOPs consistently mineralized (Fig. 3A), whereas mineralization of CD31(+) HipOPs was infrequent (data not shown), and this population was not studied further. For comparison, mineralization was also much lower in unfractionated BMSCs versus HipOPs, as expected (Fig. 3A; see also [Itoh and Aubin, 2009]). RT-qPCR analysis was conducted with cDNA from BMSCs, HipOPs and CD31(−) HipOPs. The osteogenic markers, *Osx*, *Opn*, *Bsp*, and *Ocn*, increased with culture time in osteogenic conditions in all three cell populations (Fig. 3B–D). Consistent with the histochemical results, while expression levels of osteogenic transcripts were significantly lower in BMSCs than either HipOPs or CD31(−) HipOPs, there were no significant differences in expression levels between HipOPs and CD31(−) HipOPs at d0, 14 or 21 (Fig. 3E).

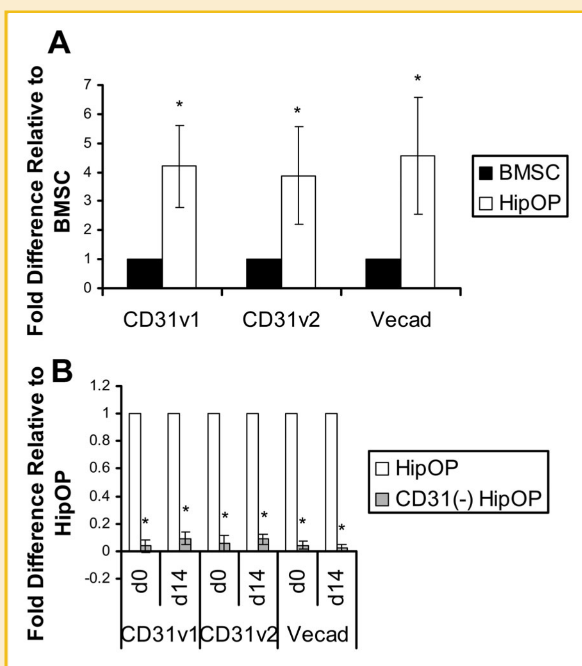

Fig. 2. HipOPs are enriched in VECs, which are depleted by fractionation with antibodies against CD31 (CD31(−)). RT-qPCR results are plotted as means ± SD of at least three independent experiments. The expression of each gene was normalized to that of *L32*. A: At day of isolation (d0), VEC markers were more highly expressed in the HipOPs than in the BMSCs. Graph indicates fold expression in HipOPs normalized to BMSCs. $^*P < 0.001$. B: CD31(−) HipOPs were reduced in expression of *Vecad* and both variants of *CD31* at d0 and d14. Graph indicates fold expression in CD31(−) HipOPs normalized to HipOPs. $^*P < 0.05$.

Limiting dilution assays also confirmed no significant differences in frequencies of CFU-F, -ALP, or -O between CD31(−) HipOPs and HipOPs (Table II). Furthermore, extrapolation of the linear portions of the limiting dilution graphs indicated that there remained one or more accessory population(s) in the CD31(−) HipOPs (Fig. 3F), suggesting that VECs are neither a required accessory population nor limiting for osteogenesis in the HipOP population in vitro.

To test the osteogenic capacity of CD31(−) HipOPs versus HipOPs in vivo, we conducted multiple transplantation experiments. Consistent with the in vitro results, bone was observed in sections of sponges transplanted with either CD31(−) HipOPs or HipOPs (Fig. 4A), and the area of bone formed was not significantly different between the two populations (Fig. 4B).

MARKERS OF THE HSC NICHE IN HipOPs VERSUS CD31(−) HipOPs

Our previous data showed that the HipOP population in vitro expressed high levels of markers associated with the HSC niche(s) [Itoh and Aubin, 2009]. In bone marrow, multiple HSC niches have been described: the endosteal or osteoblastic niche, the vascular or endothelial niche and the reticular stromal niche [reviewed in Bianco et al., 2011; Nagasawa et al., 2011]. Given that the CD31(−) HipOPs are highly enriched in osteoprogenitors and other mesenchymal precursors but depleted of endothelial cells, we next asked how CD31(−) HipOPs compare to HipOPs and unfractionated BMSCs in expression of a variety of markers typically associated with the HSC niches, at day of isolation (d0) and after 21 days in osteogenic culture. Equivalent levels of expression were seen of all markers at both time points, except *Ng2*, which was slightly but significantly higher in the CD31(−) HipOPs versus HipOPs at day of cell isolation (d0) (Fig. 5A). Expression either did not change (*Pth1r*, *Jag1*) or increased (*Ncad*, *Ang1*) over the osteogenic time course in both HipOPs and CD31(−) HipOPs. The exception was *Cxcl12*, which did not change in HipOPs but decreased by d21 in CD31(−) HipOPs (Fig. 5B).

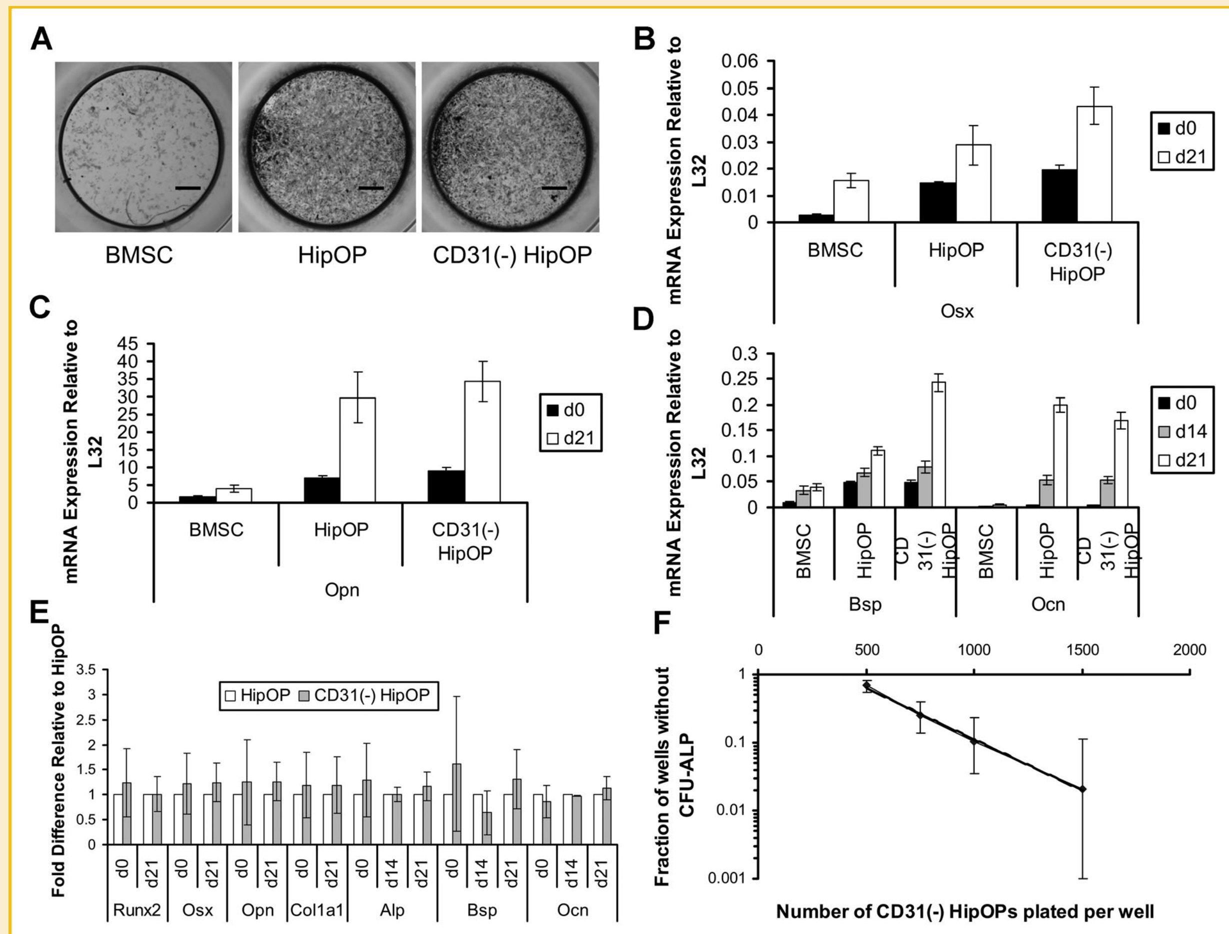

Fig. 3. HipOPs and CD31(−) HipOPs have equal osteogenic capacity in vitro. A: BMSCs, HipOPs and CD31(−) HipOPs were cultured for 28 days in osteogenic medium. The area of the dish covered in ALP and mineral was equal between the HipOPs and the CD31(−) HipOPs. BMSCs did not mineralize, and a much smaller area of the dish was covered with ALP. B–D: Osteogenic markers *Osx*, *Opn*, *Bsp*, and *Ocn* increased across the osteogenic time course in all cell populations. RT-qPCR results of mRNA from cells isolated at d0 and after 14 or 21 days in osteogenic culture. The expression of each gene was normalized to that of *L32*, and a representative experiment is shown. E: Osteogenic markers were equally expressed by HipOPs and CD31(−) HipOPs at all time points tested. RT-qPCR results of mRNA from cells isolated at d0 and after 14 or 21 days in osteogenic culture. The expression of each gene was normalized to that of L32. Fold expression in CD31(−) HipOPs was normalized to HipOPs. Results are plotted as means ± SD of three independent experiments. F: CD31(−) HipOPs contain at least one accessory population for osteogenesis. A representative limiting dilution analysis of three independent experiments is shown.

DISCUSSION

Although the search for unambiguous MSC markers is important for MSC identification and characterization, determining markers that are not expressed by MSCs is also useful and may be more feasible. Previously, we demonstrated that the depletion of cells expressing hematopoietic markers CD5, CD45, CD11b, GR-1, 7-4, TER-119, and CD45R from murine BMSCs yields a highly enriched osteoprogenitor population (HipOPs), also enriched with MSCs [Itoh and Aubin, 2009]. We now report that the HipOP population is also enriched with CD31(+) VECAD(+) VECs. Notably, when we removed the VECs by CD31 magnetic micro-bead antibody cell depletion, we saw no difference in osteogenic capacity or frequency of osteoprogenitors between the HipOP and CD31(−) HipOP populations, consistent with their equivalent capacities for bone formation when transplanted in vivo. These data suggest that endogenous CD31(+) VECAD(+) VECs are not required for the osteogenic capacity of the murine HipOP population.

Our previous studies indicated the presence of (an) accessory cell population(s) regulating osteogenesis in the BMSC and HipOP populations [Itoh and Aubin, 2009] and, for reasons summarized earlier, VECs appeared good candidates [Villars et al., 2002; Kaigler et al., 2005; Meury et al., 2006; Kanczler and Oreffo, 2008; Xue et al.,

TABLE II. There were no Significant Differences in the Frequency of Mesenchymal Progenitors Between HipOPs and CD31(−) HipOPs

Frequency	HipOP	CD31(−) HipOP	*P*-value
CFU-F	1 in 824 ± 140	1 in 551 ± 255	0.18
CFU-ALP	1 in 910 ± 266	1 in 752 ± 155	0.42
CFU-O	1 in 966 ± 261	1 in 1,003 ± 380	0.90

Summary table of data from at least three independent experiments, indicating the average progenitor frequencies ± SD and the *P*-value, derived from the Student's *t*-test, for the comparison of HipOPs to CD31(−) HipOPs within each progenitor type.

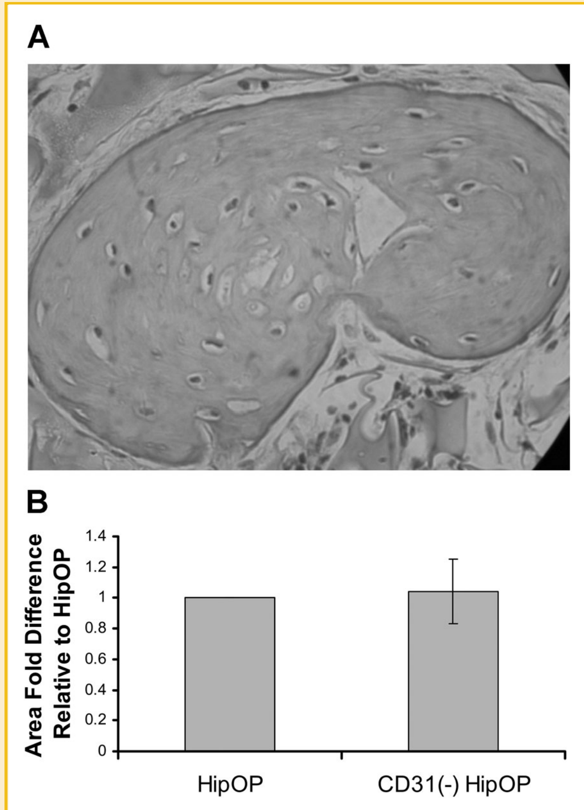

Fig. 4. HipOPs and CD31(−) HipOPs formed equal amounts of bone in vivo. A: A section of bone formed from CD31(−) HipOPs transplanted in sponges for 8 weeks; section shows osteoblast lining cells surrounding a bone matrix with embedded osteocytes. B: Amount of bone formed in each sample; for each sponge size, total amount of bone formed in the CD31(−) HipOP sample was normalized to that of the HipOP sample (set to 1). Normalized CD31(−) HipOP results are plotted as the mean ± SD of three independent experiments.

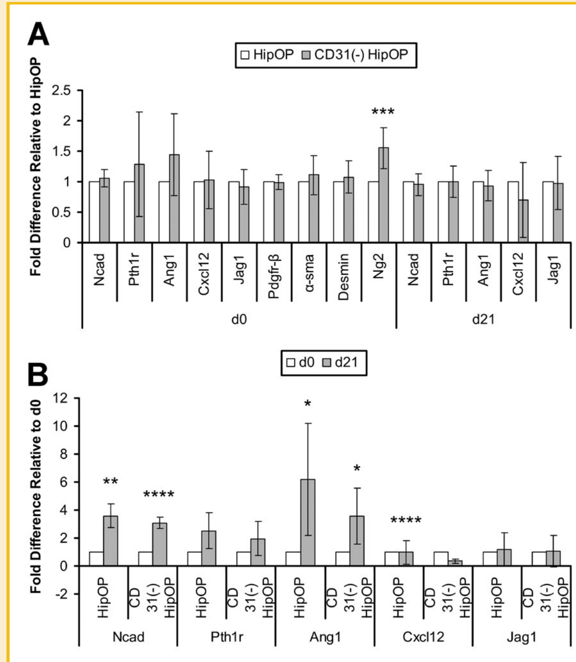

Fig. 5. HSC niche marker expression in CD31(−) HipOPs versus HipOPs across the osteogenic time course. RT-qPCR results of mRNA from freshly isolated cells and cells after 21 days in osteogenic culture. A: Fold expression of d21 normalized to d0, mean ± SD of three independent experiments. The expression of each gene was normalized to that of L32. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$. B: Fold expression of CD31(−) HipOPs normalized to HipOPs, mean ± SD of three independent experiments. The expression of each gene was normalized to that of L32. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$.

2009; Saleh et al., 2010]. We found that cells expressing VEC markers, *Vecad* and *CD31*, were significantly enriched in the HipOPs compared to the BMSCs. This is consistent with earlier studies showing that human bone marrow and blood-derived CD45(−) cells express VECAD and CD31 [Quirici et al., 2001; Medina et al., 2010]. Since both human and murine MSC-containing populations cultured in endothelial growth medium can differentiate into endothelial-like cells [Oswald et al., 2004; Liu et al., 2007; Copland et al., 2008], but VEC marker expression did not increase in either the BMSC or HipOP populations over the time course tested (data not shown), we conclude that expansion or differentiation of the VEC subpopulation did not occur under the osteogenic conditions used here. We also conclude that either such osteogenic conditions do not support VEC differentiation of a common endothelial–osteoblastic precursor [Collett and Canfield, 2005; Towler et al., 2006; Hofbauer et al., 2007; d'Aquino et al., 2007; Crisan et al., 2008; Valarmathi et al., 2008; Medici et al., 2010], or that a common precursor is not present in our populations (i.e., that the populations only contain committed osteoblasts and VEC precursors). In any case, depletion of endogenous VECs from HipOPs using magnetic micro-bead fractionation with anti-CD31 antibodies did not affect osteoprogenitor cell number or osteogenesis in vitro or osteogenesis in vivo. We can therefore conclude that cells expressing CD31 (i.e., VECs) are not an accessory cell population or are not required at high frequency for osteogenesis in our models. Moreover, frequencies of CFU-F, -ALP and -O were the same between HipOPs and CD31(−) HipOPs, which is consistent with the view that MSCs and osteoprogenitors do not express CD31 [Kolf et al., 2007; Modder et al., 2012].

As outlined earlier, multiple hematopoietic stem cell (HSC) niches have been described, including an endosteal/osteoblast niche (expressing *Ncad*, *Pth1r*, *Ang1*, *Cxcl12*, and *Jag1*), a vascular/endothelial niche (expressing *Ncad*, *Ang1*, *Jag1*, *Pdgfr-β*, and *α-sma*) and a reticular stromal niche (expressing *Cxcl12*, *Pdgfr-β*, *α-sma*, *Desmin*, and *Ng2*), although whether these are distinct or the same/overlapping remains uncertain [reviewed in Bianco et al., 2011; Nagasawa et al., 2011]. However, growing evidence supports the view that CXCL12-abundant reticular (CAR) cells, an osteoadipogenic subset of pericytes (CD146-positive subendothelial reticular cells in humans), i.e. non-endothelial cells, are the HSC niche cells [reviewed in Bianco et al., 2011; Nagasawa et al., 2011]. Nevertheless, several studies have suggested that bone marrow endothelial cells play an important role in maintaining a pool of HSCs, but it is not yet clear whether they do so directly or indirectly, i.e. by regulating other HSC niches adjacent to endothelial cells. The

expression analysis of niche markers suggests that both HipOPs and CD31(−) HipOPs are HSC niche populations. The slightly higher expression of *Ng2* in the CD31(−) HipOPs versus the HipOPs at day of isolation (d0) and lower expression of *Cxcl12* at the endpoint of osteogenic differentiation (d21) in CD31(−) HipOPs versus HipOPs suggests, however, that the depletion of VECs does modulate expression of niche markers and presumably niche activity for HSCs. Similarly, within both cell populations, the expression of *Ncad* and *Ang1* increased while *Cxcl12* decreased over the osteogenic culture time, indicating that the bone marrow niche for HSC is modulated as osteoblasts differentiate. Osteoblast ANG1 signaling provides a niche for quiescent HSCs and regulates HSC numbers [Suzuki et al., 2007; Aubin, 2008], whereas osteoblast CXCL12 signaling regulates HSC homing to the endosteal/osteoblast niche [Kopp et al., 2005] and HSC mobilization to the blood [Petit et al., 2002]. The decreasing expression of *Cxcl12* by CD31(−) HipOPs over osteogenic culture time may therefore suggest an in vivo role for VECs residing in or near the endosteal/osteoblast niche in positively regulating osteoblast CXCL12 signaling to maintain HSCs in the endosteal/osteoblast niche. Alternatively or additionally, VECs themselves may increase their expression of CXCL12, ANG1, and JAG1 over the osteogenic time course. Evidence supporting this possibility comes from coculture studies in which endothelial cells express more ANG1 when cocultured with MSCs under osteogenic conditions than when in monoculture [Pedersen et al., 2012].

In summary, depletion of VECs (CD31+ cells) does not significantly affect osteogenesis in HipOPs in vitro or in vivo, but expression of markers of the HSC niche(s) is modulated by osteoblast differentiation in both populations and by depletion of VECs.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Shousaku Itoh and Solmaz Khoshniat for helpful discussions, and Lily Morikawa for paraffin embedding, sectioning, and staining of transplanted cell-containing sponges. This work was supported by the Canadian Institutes of Health Research (CIHR; FRN 83704 grant to J.E.A.), the Edward Dunlop Ontario Graduate Scholarship in Science and Technology, the Canadian Arthritis Network, and the Skeletal Regenerative Medicine Team (CIHR Team, studentships to K.P.M.), and a Life Science Undergraduate Research Award (to D.C.M.).

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