

Osteoblast Lineage Cells Expressing High Levels of Runx2 Enhance Hematopoietic Progenitor Cell Proliferation and Function

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ABSTRACT

Although osteoblasts (OB) play a key role in the hematopoietic stem cell (HSC) niche, little is known as to which specific OB lineage cells are critical for the enhancement of stem and progenitor cell function. Unlike hematopoietic cells, OB cell surface phenotypic definitions are not well developed. Therefore, to determine which OB lineage cells are most important for hematopoietic progenitor cell (HPC) function, we characterized OB differentiation by gene expression and OB function, and determined whether associations existed between OB and HPC properties. OB were harvested from murine calvariae, used immediately (fresh OB) or cultured for 1, 2, or 3 weeks prior to their co-culture with Lin⁻Sca1⁺c-kit⁺ (LSK) cells for 1 week. OB gene expression, alkaline phosphatase activity, calcium deposition, hematopoietic cell number fold increase, CFU fold increase, and fold increase of Lin⁻Sca1⁺ cells were determined. As expected, HPC properties were enhanced when LSK cells were cultured with OB compared to being cultured alone. Initial alkaline phosphatase and calcium deposition levels were significantly and inversely associated with an increase in the number of LSK progeny. Final calcium deposition levels and OB culture duration were inversely associated with all HPC parameters, while Runx2 levels were positively associated with all HPC properties. Since calcium deposition is associated with OB maturation and high levels of Runx2 are associated with less mature OB lineage cells, these results suggest that less mature OB better promote HPC proliferation and function than do more mature OB. *J. Cell. Biochem.* 111: 284–294, 2010.

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Hematopoietic stem cells (HSC) are multipotent cells that give rise to all blood cells. Located within the bone marrow, it is generally believed that HSC reside in more specialized and restricted areas of the bone marrow microenvironment called the hematopoietic niche. The concept of the hematopoietic niche was first introduced by Schofield [1978]. Since then, a more detailed picture of the hematopoietic microenvironment emerged in which two niches, the endosteal, and the vascular niche are now distinguished

[Kopp et al., 2005]. It is generally accepted that quiescent HSC reside in the endosteal region in close proximity to osteoblasts (OB), while more active HSC reside in the vascular niche [Wilson et al., 2007]. Further, many studies provide evidence in support of the physical [Nilsson et al., 2001] and molecular [Calvi et al., 2003; Zhang et al., 2003; Nilsson et al., 2005; Stier et al., 2005; Adams et al., 2006] interactions between HSC and OB and demonstrate the ability of OB to support in vitro hematopoiesis [Taichman and Emerson, 1994]

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and survival of stem cells and hematopoietic progenitor cells (HPC) [Jung et al., 2005].

Although it has been shown that OB are a key player in the HSC niche [Taichman, 2005], very little is known as to which OB lineage cells are critical for the enhancement of HSC properties. Unlike hematopoietic lineage cells where cell surface markers are well established and can allow cells to be separated by specific stages of differentiation, knowledge of the cell surface markers expressed on OB lineage cells is much more limited. As a result, the term OB has been loosely used when describing the hematopoietic niche to refer to any cell of the OB lineage. This issue was recently reviewed by Kiel and Morrison [2008], where they suggested that most investigators term endosteal lining cells as OB while in fact these cells are heterogeneous and include OB lineage cells at many stages of maturation as well as other cells including osteoclasts. In this study we investigated a well-defined population of immature OB and used proven culture methodologies and standard markers of osteoblastic differentiation to examine the effect of OB at various stages of maturation on fundamental HPC functional properties. Like was previously posited by Zhu and Emerson [2004], we believe that clarification of the population of cells responsible for enhancing stem and progenitor cell proliferation and function is of importance as the application of this knowledge would allow for better understanding of how OB contribute to stem cell fate decisions and be used to possibly optimize culture systems to manipulate or expand HSC for clinical applications. We therefore attempted to dissect whether early or late stage OB are better at promoting several hematopoietic stem and progenitor cell properties. Our data illustrate that early or less mature OB have a higher hematopoiesis enhancing potential than their more mature counterparts.

MATERIALS AND METHODS

ANIMALS

C57BL/6 mice (2-day pups) and B6.SJL-PtqcPep3b/BoyJ (BoyJ) mice (6- to 8-week-old) were originally obtained from Jackson Laboratories. Both C57BL/6 and BoyJ mice were subsequently bred, housed, and used to generate F1 progeny at our animal facilities at Indiana University. For transplantation, recipient mice received 1,000 Rad, split dose. Cells were infused via the tail vein. All procedures followed protocols approved by the Laboratory Animal Research Facility of the Indiana University School of Medicine and followed NIH guidelines.

PREPARATION AND CULTURE OF CALVARIAL OB

Neonatal murine calvarial cells were prepared following a modification of published basic methods [Wong and Cohn, 1975; Horowitz et al., 1994; Ciovacco et al., 2009]. Briefly, calvariae from C57BL/6 mice <48 h old were dissected out, pre-treated with EDTA in PBS for 30 min, then subjected to sequential collagenase digestions (200 U/ml). Fractions 3–5 (digestions 20–35, 35–50, and 50–65 min) were collected and used as OB. These cells are >95% OB or OB precursors as previously demonstrated by a variety of criteria [Jilka and Cohn, 1981; Simmons et al., 1982; Horowitz et al., 1994]. Freshly prepared OB (0 weeks culture duration) or OB that were cultured for 1, 2, or 3 weeks were used for all studies.

OB cultured for 1, 2, or 3 weeks were maintained in α MEM supplemented with 10% FBS and were further supplemented with ascorbic acid (50 μ g/ml added on day 0 and at all feedings) and β -glycerophosphate (5 mM added starting on day 7 and all subsequent feedings). Cells were fed 2 \times /week.

LSK PREPARATION AND CELL SORTING

Low-density cells were prepared from BM flushed from the long-bones of BoyJ (CD45.1) mice. These cells were washed once with stain wash (PBS, 1% bovine calf serum, and 1% penicillin-streptomycin) and then stained with antibodies as indicated below for 15 min on ice. Briefly, low-density BM cells from BoyJ (CD45.1) mice were stained with phycoerythrin (PE)-conjugated lineage markers including monoclonal antibodies (Ab) for CD3, CD4, CD45R, Ter119, and Gr1; allophycocyanin (APC)-conjugated cKit (CD117); fluorescein isothiocyanate (FITC)-conjugated Sca1 (BD Biosciences). Lin⁻Sca1⁺cKit⁺ (LSK) cells were identified and sorted on a BD FACS Aria.

OB-LSK CO-CULTURE SET-UP

Freshly prepared OB or cultured OB from C57Bl/6 mice (CD45.2) were plated at a frequency of 40,000 cells per well in a 12-well plate; freshly sorted 1,000 LSK cells from BoyJ mice (CD45.1) were seeded after 24 h. Figure 1 is a representative micrograph showing similar OB morphology and confluence following 4 days of culture in the presence or absence of LSK cells and cytokines. It should be noted that by day 7 cultures containing LSK cells and cytokines have numerous hematopoietic cells making visualization of OB difficult unless hematopoietic cells are removed. Importantly, OB morphology and confluence (~100% confluent) is still virtually identical between cultures (data not shown). All the cultures were supplemented with a cocktail of cytokines containing recombinant murine SCF and IL3 (10 ng/ml), IGF1 and TPO (20 ng/ml), IL6 and Flt3 (25 ng/ml), and OPN (50 ng/ml). Cultures were maintained for 1 week in medium consisting of 1:1 mix of IMDM and α MEM supplemented with 10% FBS, 1% Pen/Strep, and 1% L-glutamine. Cells were harvested on day 7 and counted. Fold increase in the number of cells derived from LSK cells was calculated relative to d0 count of 1,000. It should be noted that in each experiment LSK cells were also seeded in wells containing no OB as a control.

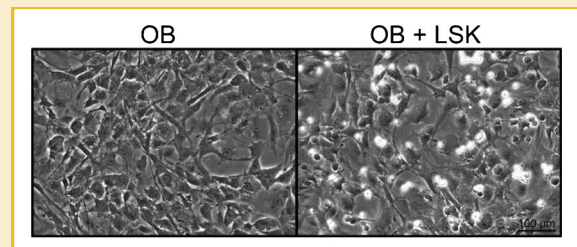


Fig. 1. Micrographs of OB cultured for 4 days in the presence or absence of LSK cells and cytokines. No significant differences were observed in OB morphology or confluence when cultured with LSK cells and cytokines. The hematopoietic cells are the smaller, more spherical cells which refract the light differently than the OB and appear more white in the image.

LSK PHENOTYPING

For the analysis of cultured LSK cells, the entire well of co-cultured cells was harvested and stained with the above Ab combinations except CD117. In addition, cells were also stained with Pacific Blue (PB)-conjugated CD45.1 and phycoerythrin-Cy7 (PE-Cy7)-conjugated CD45.2. CD45.1⁺ cells were gated and phenotypically analyzed for the presence of Lin⁻Sca1⁺ cells. Since c-kit expression (CD117) is quickly downregulated via the internalization of the receptor in cultures supplemented with exogenous SCF [Yee et al., 1994; Danet et al., 2001; Eto et al., 2005], we did not use CD117 to track the phenotypic makeup of cultured cells on day 7. Flow cytometric analysis was performed on a BD LSRII.

CLONOGENIC ASSAYS

Cultured cells or freshly sorted cells were plated in duplicate in 3 cm Petri dishes containing 1 ml methyl cellulose with cytokines (MethoCult GF M3434, Stem Cell Technologies). Cultures were maintained at 37°C in a humidified incubator at 5% CO₂ and colonies were counted on an inverted microscope after 7 days. CFU fold increase among cultured cells was calculated relative to the number of colonies obtained from the plating of 250 fresh LSK cells on d0 under identical conditions.

ALKALINE PHOSPHATASE ACTIVITY

Alkaline phosphatase activity was determined by the colorimetric conversion of *p*-nitrophenol phosphate to *p*-nitrophenol (Sigma) and normalized to total protein (BCA, Pierce) [Hughes and Aubin, 1997]. Briefly, cells were washed 2× with PBS, subsequently lysed with 0.1% (v/v) Triton X-100 supplemented with a cocktail of broad-range protease inhibitors (Pierce), subjected to two freeze-thaw cycles, and cleared via centrifugation. Lysates were incubated with 3 mg/ml *p*-nitrophenol phosphate in an alkaline buffer, pH 8.0, (Sigma) for 30 min at 37°C. The reaction was stopped by the addition of 20 mM NaOH and read at 405 nm (GENios Plus, Tecan). The enzymatic activity of alkaline phosphatase was determined by comparison with known *p*-nitrophenol standards (Sigma). It should be noted that initial alkaline phosphatase refers to levels in OB cultures at the initiation of the experiment (just prior to LSK cells being added) and final alkaline phosphatase refers to levels in OB cultures 7 days later (when HPC cells are removed and hematopoietic properties are tested).

QUANTITATIVE ANALYSIS OF CALCIUM DEPOSITION

Calcium deposition was assessed by eluting Alizarin Red S from cell monolayers as previously described [Stanford et al., 1995]. Briefly, monolayers were washed 2× with PBS, subsequently fixed in ice cold 70% (v/v) ethanol for 1 h, then washed 2× with water. Monolayers were stained with 40 mM Alizarin Red S (pH 4.2) for 10 min (room temperature, shaking), unbound dye was removed by washing with water (5×) and with PBS (1× for 15 min, room temperature, shaking). Bound Alizarin Red was eluted by incubating monolayers with 1% (v/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for 15 min (room temperature, shaking). Absorbance from aliquots was measured at 562 nm (GENios Plus, Tecan), and Alizarin Red concentrations were calculated from measured standards (Ca/mol of dye in solution). Like alkaline phosphatase, initial calcium deposition refers to levels in OB cultures

at the initiation of the experiment (just prior to LSK cells being added) and final calcium deposition refers to levels in OB cultures 7 days later (when HPC cells are removed and hematopoietic properties are tested).

QUANTITATIVE REAL-TIME PCR

Total RNA was isolated using RNeasy kit (Qiagen). DNase (Qiagen)-treated RNA was used to generate cDNAs by reverse transcription according to the manufacturer's instructions (SuperScript II kit; Invitrogen). PCR reactions were performed in an MX3000 detection system using SYBR green PCR reagents following the manufacturer's instructions (Stratagene). For each gene analyzed, a calibration curve was performed and all the oligonucleotides were tested to ensure specificity and sensitivity. For each sample, arbitrary units obtained using the standard curve and the expression of GAPDH was used to normalize the amount of the investigated transcript. The following primer sequences were used:

Runx2 forward primer: 5'-CGACAGTCCCAACTCCTGT

Runx2 reverse primer: 5'-CGGTAACCACAGTCCCATCT

Alkaline phosphatase forward primer: 5'-GCTGATCATTCCCACG
TTT

Alkaline phosphatase reverse primer: 5'-CTGGGCTGGTAGTTG
TTGT

Osteocalcin forward primer: 5'-AAGCAGGAGGGCAATAAGGT

Osteocalcin reverse primer: 5'-TTTGTAGGCGGTCTTCAAGC

Osteopontin forward primer: 5'-ACTCCAATCGTCCCTACAGTCG

Osteopontin reverse primer: 5'-TGAGGTCTCATCTGTGGCAT

Type I collagen forward primer: 5'-CAGGGAAGCCTCTTCTCCT

Type I collagen reverse primer: 5'-ACGTCCTGGTGAAGTTGGTC

GAPDH forward primer: 5'-CGTGGGGCTGCCAGAACAT

GAPDH reverse primer: 5'-TCTCCAGGCGGCACGTCAGA

STATISTICAL ANALYSES

Pearson's correlation coefficients (bivariate correlation) were used to determine R² values. Linear regressions using analysis of variance model were performed to compare groups. All analyses were performed with the Statistical Package for Social Sciences (SPSS 16; Norusis/SPSS, Inc., Chicago, IL) software and were two tailed with a level of significance set at 0.05.

RESULTS

OB ENHANCE HPC PROLIFERATION AND FUNCTION

In preliminary experiments, we pre-determined the numbers of OB and LSK cells required for the initiation and maintenance of co-cultures for 7 days without the need to remove any of the LSK progeny prior to day 7 because of excessive proliferation or the rapid expansion of OB that may result in the sloughing of these cells and loss of integrity of the co-cultures. Cell numbers reported in our studies were determined to be ideal for this mix of cells over 7 days. The 7-day period was chosen based on our historical work with cultured HSC [Ladd et al., 1997; Srour et al., 2005]. Multiple mixes of media at different ratios were also examined and these experiments

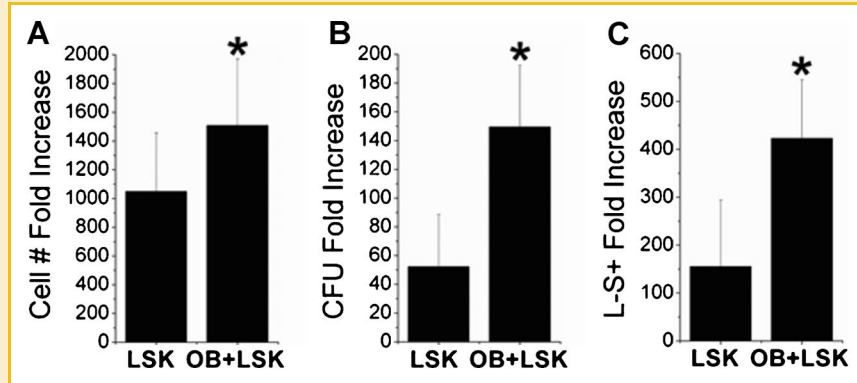


Fig. 2. LSK cells were cultured alone or in the presence of freshly prepared OB for 7 days and the following parameters were measured: (A) hematopoietic cell number fold increase; (B) CFU fold increase; and (C) percentage of $\text{Lin}^{-}\text{Sca1}^{+}$ cell fold increase. Co-culture of LSK cells with OB significantly increased all hematopoietic parameters analyzed as compared to LSK cells cultured alone. Error bars represent the standard deviations associated with the mean. Asterisk (*) indicates, statistically significant differences ($P < 0.05$) compared to LSK cells alone for identical HSC measurements.

indicated that a 1:1 mix of media provided ample support for both cell types.

Although it is well known that OB lineage cells are important in the HSC niche, it remains unclear as to which OB lineage cells are critical for the maintenance of HSC properties. In this study, we worked to dissect whether early or late stage OB are better at promoting several functions of immature hematopoietic cells. To accomplish this, we first had to confirm that in our in vitro experimental model, OB were able to enhance cell function. The HPC properties examined in this study were hematopoietic cell proliferation (defined as the fold increase in cell number from the original 1,000 LSK cells seeded after 1 week of culture), CFU fold increase (defined as the total CFU number after 1 week of culture/total number of colonies derived from freshly sorted LSK cells), and the fold increase of $\text{Lin}^{-}\text{Sca1}^{+}$ cells (defined as the percentage of $\text{Lin}^{-}\text{Sca1}^{+}$ cells among progeny of LSK cells following 1 week of culture multiplied by the total LSK progeny cell number). In eight separate experiments, we have consistently observed enhanced HPC properties when LSK cells were cultured with OB as compared to being cultured alone (on tissue culture grade plastic). Figure 2 illustrates this enhancement in HPC properties for LSKs cultured with OB ($n = 7-9$) as compared to those cultured without OB ($n = 6-$

8). Cell number fold increase was significantly elevated ($P = 0.05$) when LSK cells were cultured with OB ($1,500 \pm 500$) as compared to being cultured alone ($1,000 \pm 400$). Likewise, CFU fold increase was significantly elevated ($P < 0.001$) when LSK cells were cultured with OB (150 ± 43) as compared to being cultured alone (52 ± 36). Similarly, the fold increase of $\text{Lin}^{-}\text{Sca1}^{+}$ cells was significantly elevated ($P = 0.004$) when LSK cells were cultured with OB (420 ± 120) as compared to being cultured alone (160 ± 140). We have previously demonstrated that these changes are associated with the maintenance of repopulating potential of cultured HSC [Chitteti et al., in press] in that LSK cells harvested from OB co-cultures after 10 days repopulated the hematopoietic system of lethally irradiated recipients at levels comparable to those obtained with freshly isolated LSK cells (when expansion equivalent numbers of cells were used) and sustained high levels of chimerism in secondary recipients [Chitteti et al., in press].

ASSOCIATIONS BETWEEN FUNCTIONAL OB MEASURES AND HPC PROPERTIES

The major goal of this study was to determine whether less mature or more mature OB lineage cells were important in the HSC promoting properties observed above (Figure 2). To do this, we examined two

TABLE I. Significant Associations Observed Between OB Culture Properties (Dependent) and HPC Properties (Independent)

	Dependent variable	n	Independent variable	n	R ²	P-value
1	AP initial	6	Cell # fold Increase	15	0.781	0.019
2	Ca initial	6	Cell # fold increase	15	0.650	0.05
3	Ca final	10	Cell # fold increase	15	0.682	0.003
4	Ca final	10	CFU fold increase	15	0.738	0.001
5	Ca final	9	% $\text{Lin}^{-}\text{Sca1}^{+}$ fold increase	13	0.624	0.01
6	Culture duration	14	Cell # fold increase	15	0.588	0.001
7	Culture duration	14	CFU fold increase	15	0.696	<0.001
8	Culture duration	13	% $\text{Lin}^{-}\text{Sca1}^{+}$ fold increase	13	0.679	0.001
9	Culture duration-Trypsin	29	Cell # fold increase	24	0.212	0.02
10	Culture duration-Trypsin	29	CFU fold increase	24	0.542	<0.001
11	Culture duration-Trypsin	29	% $\text{Lin}^{-}\text{Sca1}^{+}$ fold increase	22	0.383	0.002
12	Runx2	8	Cell # fold increase	15	0.845	0.03
13	Runx2	8	CFU fold increase	15	0.790	0.04
14	Runx2	8	% $\text{Lin}^{-}\text{Sca1}^{+}$ fold increase	13	0.874	0.02

AP, alkaline phosphatase activity; Ca, calcium deposition.

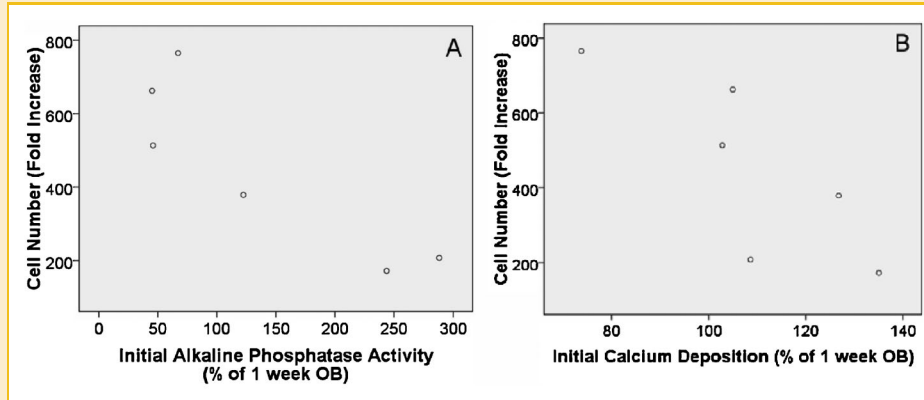


Fig. 3. A: Scatter plot with initial alkaline phosphatase (AP) levels on the x-axis and cell number fold increase on the y-axis. With an R^2 value of 0.781 coupled with ANOVA results ($P=0.02$) it appears that a relationship exists (inverse association) between these variables and that the relationship may be exponential as an R^2 value >0.8 was not achieved (generally thought of as linear). B: Scatter plot with initial calcium (Ca) levels on the x-axis and cell number fold increase on the y-axis. With an R^2 value of 0.650 coupled with ANOVA results ($P=0.05$) it appears that an exponential relationship exists (inverse association). Initial levels of AP and Ca refer to levels measured in OB cultures prior to seeding of LSK cells. Results are reported as a percentage of "1 week OB cultures" (e.g., 1 week OB AP level = 100%). It is important to understand that "1 week OB cultures" have been cultured for 1 week in osteogenic medium prior to co-culture for 1 week with LSK cells and cytokines. Significant relationships were not identified between either initial AP or Ca levels and the other HSC properties tested.

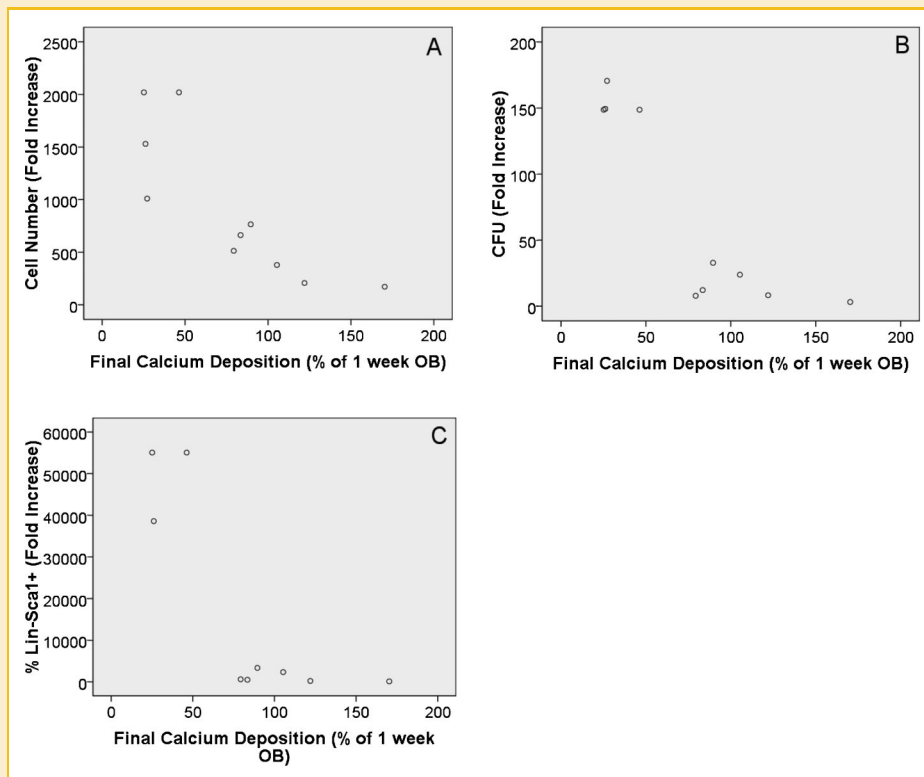


Fig. 4. Scatter plots with final Ca levels on x-axis and cell number fold increase (A), CFU fold increase (B), and percentage of Lin⁻Sca1⁺ cell fold increase (C) on the y-axes, respectively. In all cases the R^2 value coupled with the ANOVA results are suggestive of a significant relationship between the variables tested. Likewise, in all comparisons the relationship was an inverse association, where the HSC parameter tested increased with lower levels of Ca. Further, for all comparisons it appears that the relationship is more exponential in nature as compared to linear. Final Ca levels refer to levels measured in OB cultures 1 week after initial Ca levels were measured (duration of LSK cell co-culture). Results are reported as a percentage of "1 week OB cultures" (e.g., 1 week OB Ca level = 100%). It should be noted that "1 week OB cultures" have been cultured for 1 week alone prior to co-culture for 1 week with LSK cells and cytokines, thus total OB culture duration is 2 weeks.

well-accepted measures of OB function and determined whether associations existed between OB function and HPC properties tested. Specifically, we measured alkaline phosphatase (major enzymatic activity of OB) and calcium deposition (as a marker for mineralization). In parallel cultures, we measured these levels at the initiation of the experiment (when LSK cells were added) and at the termination of the experiment (7 days after initiation, when cells were removed and HSC properties were tested) for fresh OB (assessed only after 7 days culture, final levels) and for OB maintained in culture for 1, 2, or 3 weeks. The other set of cultures was used to measure HPC function after 7 days as described above for Figure 2. Data from these cultures were analyzed by linear regression using analysis of variance model (ANOVA) to determine whether significant associations were found between independent and dependent variables. Pearson's correlation coefficient was used to determine R^2 values. Data from these studies are summarized in Table I. Rows 1 and 2 in Table I demonstrate that initial alkaline phosphatase and initial calcium deposition levels are both significantly associated with cell number fold increase. These data are shown in scatter plot form in Figure 3. It should be noted that the alkaline phosphatase and calcium deposition measurements were normalized to the 1 week OB time point. While it would be more typical to normalize to the fresh OB time point, the calculation could not be determined for fresh OB at the experiment initiation because cells must be adherent and growing in order to complete the functional assays measuring

alkaline phosphatase and calcium deposition. Fresh OB would not be adherent at time 0, so we normalized to the next time point (1 week OB). These data demonstrate that the association is an inverse relationship where the higher LSK progeny cell number is associated with lower levels of alkaline phosphatase and lower calcium deposition levels. Rows 3–5 in Table I show that final calcium deposition levels are associated with all three HPC parameters measured. As depicted in Figure 4, like the initial levels, final calcium deposition levels are inversely associated with the HPC parameters, with higher fold increase in LSK progeny cell number, CFU fold increase, and fold increase of $\text{Lin}^- \text{Sca1}^+$ cells being associated with lower final levels of calcium deposition. Because higher calcium deposition or mineralization generally occurs in more mature OB cultures, these data suggest that less mature or early stage OB lineage cells better promote HPC properties as compared to more differentiated/mature OB lineage cells.

ASSOCIATIONS BETWEEN OB CULTURE DURATION AND HPC PROPERTIES

To further test the idea that less mature OB lineage cells are better at enhancing HPC properties, we examined the ability of freshly prepared OB as well as those cultured for 1, 2, and 3 weeks prior to experiment initiation to enhance HPC properties. In general, OB mature with culture duration [Gerstenfeld et al., 1987]. Therefore, the more mature cells should be found in 3-week cultures. Rows 6–8

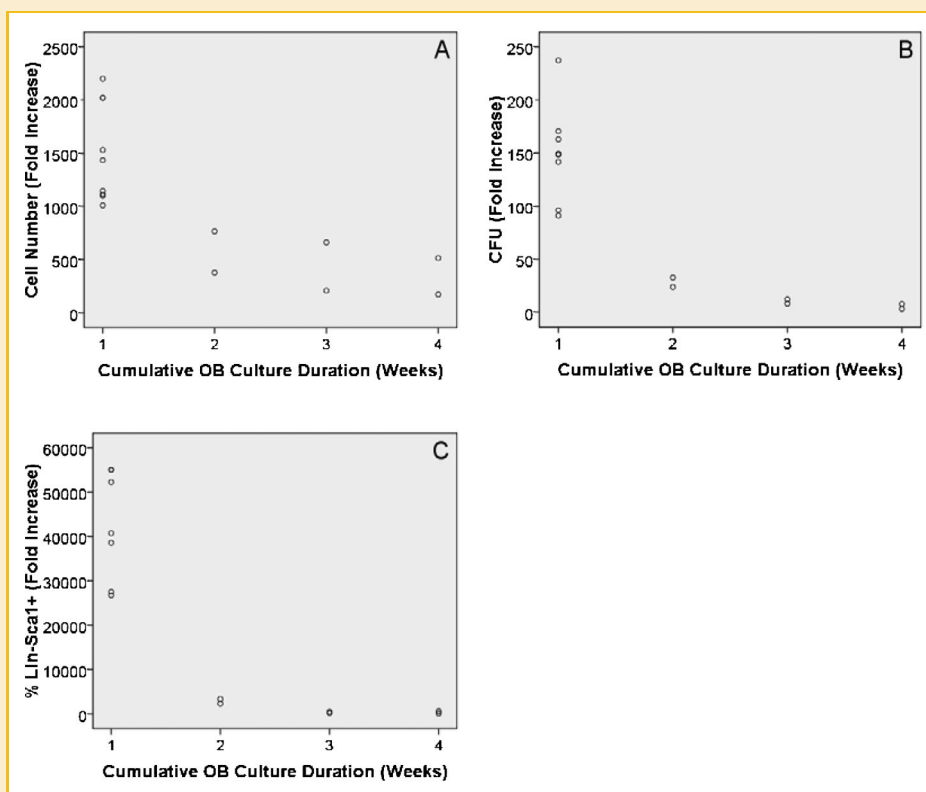


Fig. 5. Scatter plots with final OB culture duration on the x-axis and cell number fold increase (A), CFU fold increase (B), and percentage of $\text{Lin}^- \text{Sca1}^+$ cell fold increase (C) on the y-axes, respectively. In all cases the R^2 value coupled with the ANOVA results are suggestive of a significant relationship between the variables tested. For all comparisons the relationship was an inverse association and the association appears to be exponential.

in Table I show that significant associations were detected between culture duration and all of the HPC properties analyzed. Again, as illustrated in Figure 5 an inverse relationship was observed. As culture duration increased, the ability to promote HPC properties was reduced. Like the calcium deposition data, these data suggest that as OB lineage cells mature, their ability to enhance HPC properties decreases. To further confirm these data, we co-cultured LSK cells with freshly prepared OB or OB that had been cultured for 1, 2, or 3 weeks prior to experimental initiation as before with one difference: In these studies, pre-cultured OB were trypsinized and then seeded at identical cell concentrations as those used for freshly prepared OB. This manipulation assures that the increased cell number in 1, 2, and 3 weeks OB cultures was not responsible for inhibiting LSK expansion and function. Rows 9–11 in Table I show that as was seen with non-trypsinized OB, trypsinized OB culture duration is associated with all three LSK parameters studied. Again, similar to the results from non-trypsinized cultures, as the initial culture duration of trypsinized OB cultures increased, HPC enhancement declined. These data further suggest that OB culture duration and by extension, OB maturation, is inversely related to hematopoietic enhancing properties. This inverse relationship is illustrated in Figure 6.

As detailed in Figure 7, our real-time PCR data is consistent with the notion that as OB culture duration increases so does OB maturation. Specifically we show that (1) Runx2 expression is highest in fresh OB cultures and decreases with increases in OB

culture duration (Runx2 is a transcription factor which is critical for differentiation of early stage OB lineage cells) [Stein and Lian, 1993; Otto et al., 1997; Mundlos, 1999; Lian et al., 2004; Schroeder et al., 2005; Krause et al., 2008]; (2) alkaline phosphatase expression increases with OB culture duration up to 2 weeks (3 weeks cumulative OB culture duration) and then stabilizes; and (3) all of the OB-specific bone matrix proteins tested showed an increase in expression with an increase in culture duration (type I collagen, osteopontin (OPN), and osteocalcin) suggesting that cells in the latter culture durations are mature OB producing osteoid while fresh OB are early stage OB lineage cells.

Finally, we confirmed our in vitro findings by performing in vivo reconstitution studies. LSK cells were co-cultured with either fresh or 3 weeks OB for 7 days prior to transplantation into lethally irradiated recipients and chimerism in the blood was measured monthly for 4 months post-transplantation. While LSK cells from both co-cultures were able to repopulate the hematopoietic system, the levels of chimerism were significantly higher in recipients receiving LSK cells harvested from fresh OB as compared to those harvested from 3 weeks OB (average 7.4-fold increase, $P=0.01$, $n=5/\text{group}$).

ASSOCIATIONS BETWEEN OB GENE EXPRESSION AND HPC PROPERTIES

To further confirm that less mature OB lineage cells are better at enhancing HPC properties, we examined Runx2 expression (high in

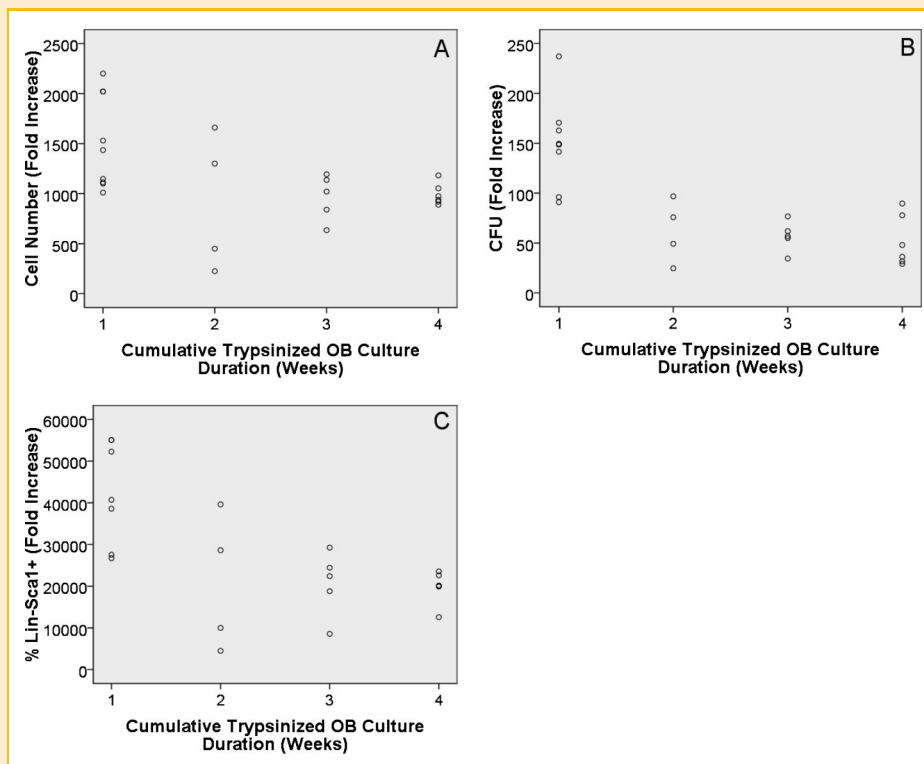


Fig. 6. Scatter plots with final OB culture duration (cumulative duration for trypsinized OB) on the x-axis and cell number fold increase (A), CFU fold increase (B), and percentage of Lin⁻Sca1⁺ cell fold increase (C) on the y-axes, respectively. Although in all cases significance was achieved ($P<0.05$), the R^2 values were lower than those obtained when OB were not trypsinized (0.212, 0.542, and 0.383 for A, B, and C, respectively).

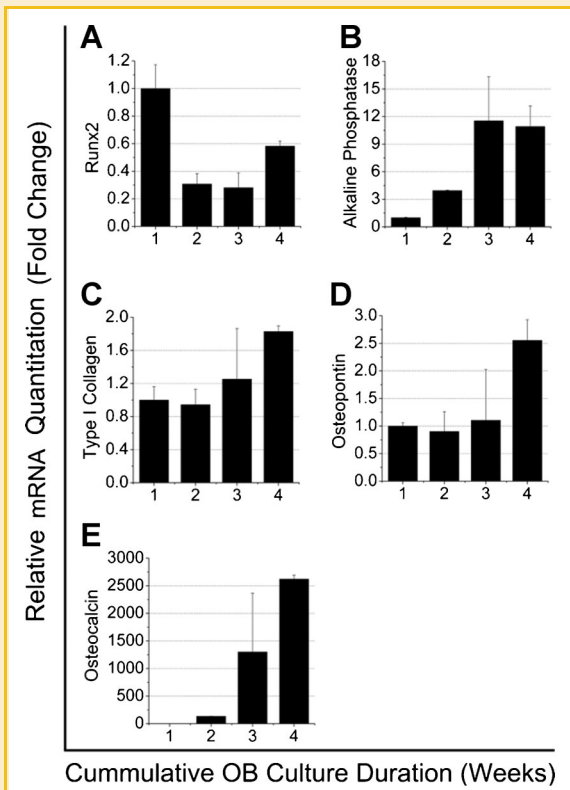


Fig. 7. mRNA expression in freshly prepared OB or OB cultured for 1, 2, or 3 weeks prior to LSK seeding (x-axis shows cumulative OB culture duration, e.g., freshly prepared = 1, 1 week = 2, 2 week = 3, and 3 week = 4). Error bars represent the standard deviations associated with the mean. Runx2 expression was highest in freshly isolated OB and declined with culture duration. Alkaline phosphatase expression increased with culture duration, peaking at week 2 and this level was maintained in week 3 cultures. As expected, expression of type I collagen, OPN, and osteocalcin increased as OB culture duration increased.

early stage OB lineage cells and declines with differentiation) in freshly prepared OB as well as those cultured for 1, 2, or 3 weeks prior to experiment initiation and examined associations with HPC properties. Rows 12–14 in Table I show that significant associations were detected between Runx2 mRNA expression and all of the HPC properties analyzed. As illustrated in Figure 8 as Runx2 expression decreased, the ability of OB to promote HPC properties was also reduced. Like the calcium deposition and the OB culture duration data, results from Runx2 analysis suggest that as OB lineage cells mature, their ability to enhance HPC properties decreases.

DISCUSSION

Although recent work has focused on the role of OB in the HSC niche, little progress has been made in delineating which specific OB lineage cells are important for HSC maintenance and function. In a series of experiments presented here, we provided substantial evidence suggesting that early stage OB expressing high levels of Runx2 better promote HPC proliferation and function than do more

mature OB. Given that unlike hematopoietic cells, cell surface markers for OB are not well defined, we could not proceed with our investigations using prospectively isolated populations of early stage versus later stage OB. Limited consensus is that OB lineage cells are CD45[−]CD31[−]Ter119[−]Sca1[−] cells [Semerad et al., 2005; Arai et al., 2008; Mayack and Wagers, 2008]. Some work has been completed to further separate OB lineage populations using cell surface markers such as ALCAM [Arai et al., 2008] and OPN [Mayack and Wagers, 2008]. Arai et al. [2008] recently defined ALCAM as a marker capable of distinguishing between mature and immature OB from long bones of adult mice when combined with Sca1 and a CD45, CD31, and Ter119 lineage (Lin) cocktail. In their definition, Lin[−]Sca1[−]ALCAM[−] cells were immature OB while Lin[−]Sca1[−]ALCAM⁺ cells were mature OB [Arai et al., 2008]. Of importance, many investigators use ALCAM as a marker of multipotential mesenchymal stem cells as compared to OB lineage cells [Brooke et al., 2008; McCarty et al., 2009]. Kalajzic et al. [2003] indicated that ALCAM is not an OB-specific marker, although it can be used to assess the proportion of undifferentiated mesenchymal stem cells in culture. On the other hand, OPN is commonly used in conjunction with the expression of other bone secreted proteins to identify OB cells and even stages of differentiation [Krause et al., 2008]. However, by itself, OPN would be expressed on many stages of OB cells including pre-OB, immature OB, and mature OB, albeit at different levels of expression [Krause et al., 2008]. Our real-time data in Figure 7 concur that OB cultured for various durations all expressed OPN with 3 weeks culture expressing the highest levels. Therefore, while OPN may serve as a marker for determining whether a cell is in the OB lineage, by itself, it does not provide insight as to the specific stage of differentiation of an OB lineage cell. Our group used a cocktail of these markers to prospectively isolate phenotypically defined populations of OB but were unable to segregate the hematopoiesis enhancing activity of OB into a specific group of cells [Chitteti et al., in press]. These data suggest that, at present, the hematopoiesis enhancing activity of OB cannot be separated even among OB within a single maturational stage.

Because of the limited knowledge of cell surface markers able to characterize specific stages of OB lineage, we approached determining which OB lineage cells are most important for maintenance and expansion of hematopoietic cells from a different standpoint: by analyzing the functional behavior of OB. Here, we measured alkaline phosphatase activity and calcium deposition as a marker for mineralization. As detailed above, the initial alkaline phosphatase and calcium deposition levels were both significantly and inversely associated with hematopoietic cell number fold increase. Likewise, final calcium deposition levels were inversely associated with all of the HPC parameters tested. As calcium deposition generally increases with OB maturation [Gerstenfeld et al., 1987], and since higher calcium deposition was associated with lower hematopoietic proliferation and function, our data seem to suggest that less mature, or early stage OB lineage cells, better promote HPC properties compared to more differentiated/mature OB lineage cells. Our real-time PCR data corroborated our calcium deposition data illustrating the 3 weeks OB cultures (4 weeks cumulative OB culture duration) contain more mature OB in comparison to freshly isolated OB cultures.

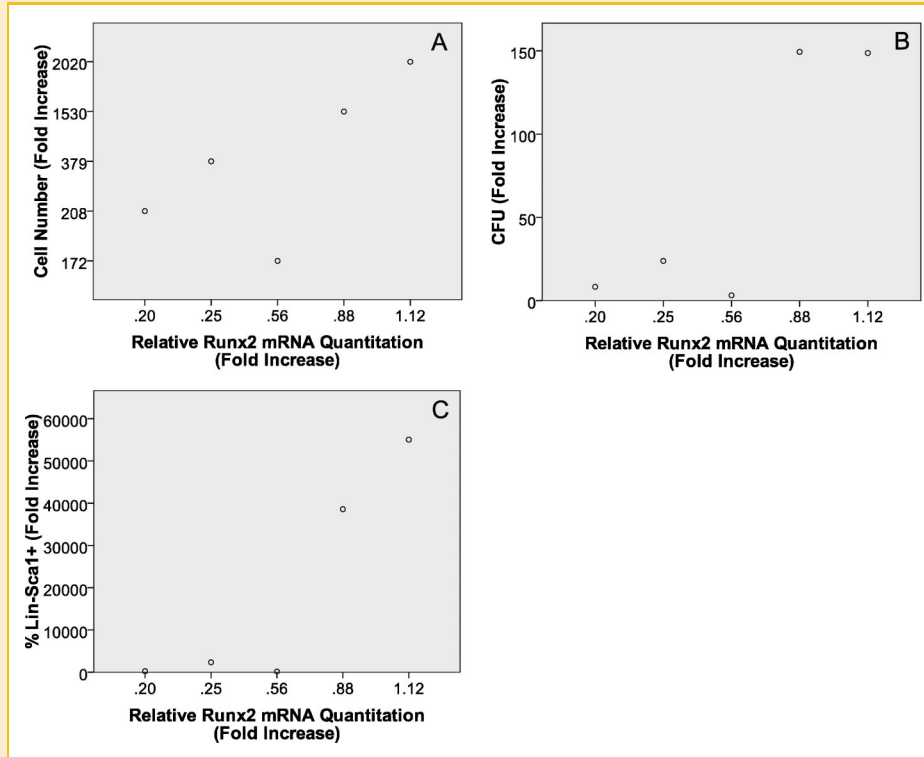


Fig. 8. Scatter plots with Runx2 mRNA expression on the x-axis and cell number fold increase (A), CFU fold increase (B), and percentage of Lin⁻Scal⁺ cell fold increase (C) on the y-axes, respectively. In all cases the R² value coupled with the ANOVA results are suggestive of a significant linear relationship between the variables tested.

It should be noted that our measurement of calcium deposition is a marker for the mineralization occurring in newly formed osteoid bone matrix. Calcium and phosphate form hydroxyapatite crystals which is the mineral content of bone. Our data are not inconsistent with those presented by Adams et al. [2006]. They suggest that preferential localization of stem cells in the endosteal region of the bone marrow microenvironment is likely due to the high concentration of calcium in this niche as HSC express a calcium sensing receptor [Adams et al., 2006]. Indeed, it is well understood that one of the functions of the skeleton is to provide a reservoir of minerals such as calcium and phosphate [Copp and Shim, 1963], and through bone remodeling, osteoclasts release these minerals under the control of various stimuli to maintain homeostasis [Raisz, 1999]. The calcium that is freed by bone resorption through osteoclastic activity is bioavailable; however, the calcium we measured in these studies is not free calcium such as that described by Adams et al. [2006] and is therefore not bioavailable but is rather a component of the mineral matrix of bone. These levels of calcium are more reflective of the OB stage of differentiation.

We also tested whether less mature or early stage OB lineage cells are better at enhancing HPC properties by examining the effects of culture duration. Because OB generally mature with culture duration [Gerstenfeld et al., 1987], more mature cells would typically be present in older cultures. Similar to the calcium deposition data, we saw an inverse relationship where the higher hematopoietic and clonogenic cell number and function were associated with shorter culture durations. In these experiments, OB maintained in long-term

cultures would have undergone multiple cell doublings as well as matured. Therefore, to determine whether the cell number could explain the observed relationships (i.e., whether due to large numbers of OB cells, LSK cells were deprived of nutrients and thus unable to proliferate and function as well), we maintained, in parallel cultures, OB for 1, 2, or 3 weeks, trypsinized these cells and then reseeded them at the same starting concentrations as was used for freshly prepared OB. Results from these experiments demonstrated that as culture duration increased (cumulative culture duration pre- and post-trypsin), HPC proliferation and function decreased. Since in these experiments the number of plated OB was identical, the main difference is in the maturation of the OB cells themselves. Thus, these data are suggestive that early stage OB lineage cells are better at enhancing HPC properties than are later stage OB lineage cells.

To further demonstrate that stage of OB maturation is associated with hematopoietic cell expansion and function, we characterized the expression of several OB-related genes: Runx2, alkaline phosphatase, type I collagen, OPN, and osteocalcin and compared expression levels to hematopoietic parameters. As detailed in Figure 7, our real-time PCR data supports the idea that OB maturation increases with culture duration. With regard to OPN, it is important to note that our data are consistent with previous work demonstrating that OPN inhibits HSC differentiation [Nilsson et al., 2005; Stier et al., 2005] as we see that OB expressing the highest levels of OPN (3 weeks cultures or 4 weeks cumulative OB culture duration) least support hematopoietic properties. Further, our data

also demonstrate that OB cultures expressing high levels of Runx2 (early stage OB lineage cells) better promote hematopoietic proliferation and function than do cultures expressing lower levels of Runx2 (more mature OB lineage cells).

Finally, these *in vitro* data were supported by *in vivo* bone marrow repopulation studies. As differences in the *in vitro* hematopoietic properties were highest between fresh and 3 weeks OB, we chose these culture durations for our *in vivo* studies and found that fresh OB supported an average 7.4-fold higher level of chimerism than did 3 weeks OB. Of importance, these *in vivo* data provide strong support of our assertion that early stage OB better support hematopoietic function than do more mature OB.

Although it is generally taken for granted that hematopoiesis in the adult takes place in long bones, flat bones are also hematopoietically active in the adult. This leaves the question as to whether our results obtained with OB collected from neonatal calvariae can be replicated with OB from long bones. While all of our data presented here are generated using neonatal calvarial OB, we previously demonstrated that OB derived from both long bones and calvariae of neonatal and young adult mice resulted in enhancement of the same hematopoietic properties studied here [Chitteti et al., *in press*]. That being said, the question of whether OB from different sites can support hematopoiesis is relevant especially in view of the recent publication by Chan et al. [2009] in which fetal 14.5 d.p.c. CD105⁺Thy1⁻ cells from long bones but not from mandibular and calvarial bones gave rise to marrow-containing niche when transplanted under the kidney capsula of adult recipient mice. The authors argued that niche progenitors from bones that cannot undergo endochondral ossification (e.g., calvarial bones) fail to develop into a marrow-containing niche. While these results await further confirmation, it is important to point out that calvariae and other flat bones are indeed active sites of hematopoiesis. In fact, harvesting of donor BM-derived HSC for clinical transplantation is performed from the iliac crest demonstrating that the hematopoietic niche in these bones can sustain and produce functionally active long-term repopulating stem cells.

Additional support for the notion that OB from both long bones as well as calvariae are functionally similar and critical for the formation of the hematopoietic niche was also recently demonstrated by two similar *in vivo* studies [Lo Celso et al., 2009; Xie et al., 2009]. In their studies they highlighted the intimate interactions between HSC and OB in the transplantation setting [Xie et al., 2009] as well as in response to physiological challenges [Lo Celso et al., 2009]. Furthermore, a “hierarchical” order of interaction between OB and hematopoietic cells depending on their stage of differentiation as was also demonstrated by Kohler et al. [2009] for aged versus young early HPC.

In summary, our study demonstrated that calvarial OB support hematopoietic progenitor proliferation and function and has begun to narrow which OB lineage cells are best at enhancing HPC proliferation and function; early stage or less mature OB. The knowledge gained from these experiments can be used to enhance *in vitro* expansion of HSC for possible clinical applications. Future studies can focus on identifying cell markers specific to early stage or less mature OB, as compared to all OB lineage cells, for the optimization of HSC culture systems and for a more detailed

understanding of the molecular interaction between OB and HSC in the hematopoietic niche.

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