ORIGINAL ARTICLE

Calcium-Infiltrated Biphasic Hydroxyapatite Scaffolds for Human Hematopoietic Stem Cell Culture

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Long-term *in vitro* expansion of hematopoietic stem cells (HSCs), while maintaining their functionality and multilineage differentiation potential, is still challenging. In this study, three-dimensional (3D) high-porosity hydroxyapatite (HA) foams have been designed to closely mimic the chemistry and physical structure of cancellous bone. Furthermore, calcium oxide was distributed in the HA ceramics to provide surface calcium ion release, hypothesizing that a local surface calcium gradient supports HSC localization and maintenance. Primary human HSCs and osteoblasts were cocultured for 6 weeks. Controls were cultured in two-dimensional dishes, while scaffold cultures were performed with calcium nitrate-infiltrated HA scaffolds and untreated HA scaffolds. Cells were analyzed for surface markers by flow cytometry, metabolic activity, and hematopoietic multilineage differentiation potential. The release of calcium into culture medium was also determined. The implementation of HA scaffolds had a positive effect on erythrocyte colony formation capacity of HSCs, with an increased osteoblast fraction observed when compared to control cultures without scaffolds. The presentation of scaffolds did not affect metabolic turnover when compared to control cultures. In conclusion, 3D open-porous HA scaffolds provide a bone-like structure and enable the long-term maintenance of primary HSCs.

Keywords: hematopoietic stem cells, long-term culture, hydroxyapatite, calcium ions, differentiation

Introduction

H_{EMATOPOIETIC} STEM CELLS (HSCs) initiate hematopoiesis and form hematopoietic tissue in red bone marrow. HSCs have the ability to self-renew and to replenish all cellular components of the blood.¹ To maintain the steady state in the peripheral circulation, HSCs produce 10^{11} – 10^{12} blood cells per day in a healthy human adult.² Those cells include erythrocytes for transporting oxygen, myelocytes such as monocytes and macrophages, and lymphocyte lineages of the adaptive immune system, such as T cells and B cells.³ In consequence of the importance of HSCs in human blood production, there is a high demand for *in vitro* HSC maintenance and expansion for cell-based therapies in clinical applications.^{4,5} Ideally, *in vitro* models should aim to mimic the specific HSCs *in vivo* bone marrow microenvironment, or niche, a term first coined by Schofield.⁶

Two HSC-specific microenvironments have been described: the perivascular niche consisting of a network of thin-walled and fenestrated sinusoidal vessels to stimulate

differentiation of hematopoietic progenitors especially toward megakaryocytes,⁷ and the endosteal niche consisting of osteoblasts and maintaining the long-term HSC population with heterogeneous differentiation potential.⁸ The perivascular niche contains two important cell types, the endothelial cells and the mesenchymal stromal cells (MSCs), which are regarded to have a direct relationship to HSC maintenance.^{9,10} Within the endosteal niche, osteoblasts are an essential cell type.^{11,12} Osteoblasts produce hematopoietic growth factors¹³ and also act as a regulatory component within the HSC niche that influences stem cell function. Importantly, in both, perivascular niche and endosteal niche, osteoblasts have been shown to be present near the HSCs¹⁴ and the lack of osteoblastic differentiation blocks hematopoiesis.¹⁵ *In vivo*, the endosteal niche is close to the surface of the trabecular bone,¹⁶ which consists of hydroxyapatite (HA) as the main inorganic component.

To mimic the endosteal niche microenvironment, a three-dimensional (3D) HA foamed structure was designed with 90% open porosity. HA ceramics have been implemented

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successfully in bone repair in clinical applications (for review, see McMahon *et al.*¹⁷) because of osteoconductive properties. Previously, we have successfully applied HA ceramics to culture human adult bone marrow mononuclear cells in long-term culture.¹⁸ The HA ceramics were processed into macroporous spongy structures that mimic the anatomy of cancellous, or spongy, bone. In those cultures, the various cell types of the bone, including cluster of differentiation 34 (CD34)-positive hematopoietic cells and osteocalcin-positive osteoblasts, attached to the scaffold's surface.

Near the endosteal niche, a high Ca^{2+} is thought to exist¹⁹; a calcium receptor-deficient mice model leads to fewer HSCs residing in the bone marrow and more HSCs released into circulation. In our study, the need to release calcium from the surface of the scaffold was addressed using a calcium oxide minor phase dispersed in the HA scaffold material. On exposure to moisture, the calcium oxide at the surface of the scaffold will transform to calcium hydroxide and calcium carbonate and then quickly dissolve. In consequence, the presence of this minor phase will create a local Ca^{2+} concentration gradient in the medium during culture. The effects of the 3D scaffold and the calcium-rich second phase on the hematopoietic fate of HSCs were studied over a 6-week period.

Materials and Methods

In this study, HSCs and osteoblasts were cultured with HA foams or biphasic HA foams, the latter being processed by an infiltration process.²⁰ These scaffold conditions were then compared to a negative control, in which HSCs and osteoblasts were cultured without any foam.

Material preparation

HA powder [$3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{Ca}(\text{OH})_2$; Sigma-Aldrich, St. Louis, MO] was calcined at 900°C for 1 h to ensure phase purity, and no second phases were detected by X-ray diffraction. After calcination, the powder was milled in water for 24 h, dried, and then granulated by pestle and mortar. The HA foam was prepared by direct foaming. An aqueous suspension was then made with 30 volume percent solids. First, ammonium polymethacrylate polyelectrolyte dispersant (Darvan C; RT Vanderbilt Co., Norwalk, CT) was added to deionized (DI) water and adjusted to pH 5.5 using HCl solution.

After adding the HA powder, the suspension was mixed at 2500 rpm for 20 min and the cationic surfactant (benzethonium chloride; Sigma-Aldrich) was added to the suspension and mixed at 2500 rpm speed for 2 min. Then, the emulsion was created by adding 10% volume of heptane and mixing at high speed for 2 min. The emulsion was poured into a paper mold in an incubator at 60% humidity and kept for 1 h. Subsequently, the humidity was decreased to 40% until the foam had fully dried. The mold containing the foam was then slowly heated in a furnace to 900°C to burn away the mold. The resulting foams were then partially sintered in air at 1000°C in preparation for infiltration. This same partial sintering step was also used on the infiltrated HA samples so that all the scaffold conditions received the same thermal treatments. After partial sintering, the HA foams were full sintered at 1300°C.

To create biphasic HA foams, the partially sintered HA foams were subjected to an infiltration process. The foams were infiltrated with 1 or 2 M calcium nitrate solution for 24 h in a vacuum container, followed by immersion in am-

monia hydroxide solution with pH 12.5–13 for 1 h to transform the calcium nitrate in the open pores to calcium hydroxide. After drying, the foams were fired at 900°C to decompose the calcium hydroxide and any remaining calcium nitrate to form calcium oxide. Finally, the resulting functionally graded biphasic ceramics that contained a gradient of CaO, which decreased from the surface into the interior,²⁰ were sintered to high density at 1300°C. The biphasic HA foams infiltrated with 1 M calcium nitrate solution for 24 h in a vacuum container were named “1 M infiltrated HA”; similarly, the HA foams infiltrated with 2 M calcium nitrate solution for 24 h in a vacuum container were labeled “2 M infiltrated HA.”

HA scaffold characterization

The HA and infiltrated HA scaffolds were coated with palladium using a sputter coater, and examined in an XL30 scanning electron microscopy/energy-dispersive detector (SEM/EDS; Philips/FEI Company, Hillsboro, OR). The EDS was applied to the scaffold surfaces for qualitative analysis of the calcium to phosphate ratio (Ca/P ratio). To determine the scaffold structure and porosity, microcomputed tomography (CT) scanning (Skyscan 1272; Micro Photonics, Allentown, PA) was applied to the HA scaffolds. During the micro-CT scanning, the resolution was kept in the range of 1.2–1.5 μm . The scanning time for each sample was from 6 to 8 h. After the microscanning, the images from the sample slices were reconstructed using CTAN (Micro Photonics) and analyzed for porosity.

Cell culture

Saos-2 osteoblasts were obtained from the American Type Culture Collection (Manassas, VA). The Saos-2 osteoblasts were precultured and passaged three times. After harvest, the osteoblasts were uniformly mixed with fresh human bone marrow-derived CD34⁺ HSCs (Stem Cell Technologies, Vancouver, Canada) at a ratio of 9:1. Experiments were performed with HSCs derived from three different healthy donors (28-year female, 23-year female, and 30-year male). Cell cultures were performed in six-well plate inserts (Stem Cell Technologies).

Cells were plated in blank wells (negative control), on HA foams, or on biphasic HA foams at a density of 5000 cells/ cm^2 in MesenCult Proliferation Kit (human) and MesenCult MSC Basal Medium (human; Stem Cell Technologies), supplemented with 5% human serum (Sigma-Aldrich) and antibiotic/antimycotic mix (Fisher, Brown Deer, WI). In each well, the weight of the foam was 0.100 (± 0.005) g, and 50,000 cells per foam. The average dimensions of the ceramic scaffolds were 2.5 (± 0.5) $\times$ 10 (± 3) $\times$ 10 (± 3) mm. Due to the open porosity of the scaffolds, the total volume for each scaffold per well was expected to be 0.318 (± 0.020) cm^3 .

During the seeding process, the tip of the pipette was placed on the center surface area of the scaffold. By slowly pushing the cell mixture into the open structure scaffolds, the cells flowed through into the scaffold volume. Finally, medium was slowly added drop by drop to the same position area on the scaffold to slowly rinse cells into the scaffold volume. The cells were then cultured in an incubator at 37°C having a humidified atmosphere of 95% air and 5% CO_2 . The culture medium was changed at days 1, 3, 5, 7, 9, 10, 12, 15, 18, 20, 21, 23, 25, 28, 30, 32, 35, 38, 40, and 42 of

culture. At 0, 5, 14, and 42 days, cells were harvested and analyzed using flow cytometry and colony-forming unit (CFU) assay.

Measurement of Ca^{2+} concentration, lactate, and glucose

Lactate and glucose concentrations were measured from medium samples to monitor the metabolic activities of cells. In addition, the net Ca^{2+} concentration in medium samples was determined. Samples were analyzed with a Cobas 221b blood analyzer (Roche Diagnostics, Indianapolis, IN) with a resolution of 0.4–40 mM.

Lactate dehydrogenase activity determination

To determine indirectly cell viability, cell culture medium samples were analyzed for lactate dehydrogenase (LDH) activity. LDH activity can be measured in medium when the enzyme is released from disintegrating cell. The QuantiChrom LDH Kit (BioAssay Systems, Hayward, CA) was used according to the manufacturer's instructions. A negative assay control included water, and a positive assay control included the calibrator of the kit. Medium samples were collected, frozen immediately, and kept at -20°C until assayed. Medium without cells served as blank.

Assays were performed in clear flat-bottomed 96-well plates (Corning Costar, Corning, NY). Into separate wells, 200 μL of water and calibrator were transferred. Ten microliters of each sample was transferred into separate wells, and 190 μL of working reagent was added and mixed. The optical density at 565 nm was read immediately and after 25 min using a Synergy H1 Hybrid Multi-Mode Microplate Reader and Gen5 data analysis software (BioTek, Winooski, VT). LDH activity was calculated as IU/L.

Flow cytometry

Freshly mixed cells (day 0) as well as cells cultured for 5, 14, and 42 days were analyzed for their surface marker expression profiles by fluorescence-activated cell sorting (FACS). At harvest, HSCs were collected first. All liquid in the well was collected, and the scaffold was washed very gently with Dulbecco's phosphate-buffered saline (DPBS; without calcium and magnesium), collecting also the DPBS. Subsequently, osteoblasts were harvested by detachment with trypsin-EDTA. The detached osteoblasts were added to the HSCs. Then, the well and the scaffolds were washed twice, followed by a microscopical check to ascertain no cells remained in the well. After cell harvest with trypsin-EDTA (Fisher), single cells were incubated with blocking buffer containing 20% FcR block (Miltenyi), 0.5% bovine serum albumin, and 2 mM EDTA (Sigma-Aldrich) in DPBS without calcium and magnesium (Fisher), pH 7.2. Controls included nonstained cells, and cells incubated with corresponding isotype controls (Becton Dickinson, Bedford, MA).

For surface marker staining, cells were incubated with fluorochrome-conjugated antibodies FITC-Linage cocktail, CD34-PerCPCy5, CD31-BV421, CD38-PE, ALP-AF647, and CD235a-AF700 (all Becton Dickinson). Saos-2 osteoblasts were also analyzed separately before coculture to confirm their typical surface marker expression profiles. Antibodies included CD10-PE, CD51-AF647, CD44-

APCH7, and ALP-AF647 (all Becton Dickinson). Compensation beads (Becton Dickinson) were used to compensate for potential spectral fluorochrome overlap. Cells were analyzed with an FACS Aria II (Becton Dickinson). For data analysis in FlowJo software version 9.5.2 (Tree Star, Ashland, OR), a forward versus side scatter gate was applied to exclude cell debris and cell doublets.

CFU assay

CFU assays were performed to investigate the potential of hematopoietic progenitors to form colonies of different hematopoietic lineages. Freshly mixed cells (day 0) and cultured cells of days 5, 14, and 42 were assayed in ultralow attachment dishes (Stem Cell Technologies). A complete MethoCult methylcellulose-based assay was used according to the manufacturer's instructions (Stem Cell Technologies).

After 14 days of culture, colonies were classified and counted by phase microscopy (Zeiss Invertoskop C; Carl Zeiss, Jena, Germany). Four different types of colonies were identified, and their frequencies compared to expected numbers. These included the following: CFU-E (colony-forming unit—erythrocyte), BFU-E (burst-forming unit—erythrocyte), CFU-GM (colony-forming unit—granulocyte and macrophage), and CFU-GEMM (colony forming unit—granulocyte, erythrocyte, macrophage, and megakaryocyte).

Statistics

All data are given as mean from three biological repeats $\pm$ standard deviation. Statistical significant differences were calculated by using one-way analysis of variance and Bonferroni posttest, using Prism 7.0c version software (GraphPad, La Jolla, CA).

Results

Characterization of HA scaffolds

In this long-term HSCs and osteoblast coculture, the HA and infiltrated HA scaffolds provided a 3D structure for cell growth and proliferation. As the infiltrated HA scaffolds were based on HA scaffolds processed by the same emulsion method, there was no observable difference between the architecture of the two kinds of scaffolds. SEM images of the open-porous structure of an HA scaffold and an infiltrated scaffold are shown (Fig. 1a, b).

Each piece of scaffold has high porosity, with large, connected pores. The total porosity in the scaffolds was $\sim 90\%$, with open porosities of $\sim 87\%$, as reported previously.²¹ With the micro-CT scanning, the porosity of the scaffolds was confirmed to be in the same range ($87\% \pm 2\%$ for open porosity). The micro-CT scanning reconstructed image was shown in Figure 1c. EDS results collected from the surface of the scaffolds gave the chemical composition (Fig. 1d) and allowed for calculation of the Ca/P ratio. At certain positions on the surface of the infiltrated HA, the Ca/P ratio was 1.72, which is higher than the HA stoichiometric ratio of 1.67. This suggests that the surface contained a mixture of HA and a calcium-rich phase that was identified as CaCO_3 by X-ray diffraction of the ground foam.²⁰ The EDS result for a large area of the uninfiltrated HA surface of the glaze resulted in a value of 1.62, suggesting that the HA was slightly calcium deficient.

FIG. 1. (a) SEM of the as-processed HA scaffold, (b) SEM of the as-processed infiltrated HA scaffold, (c) micro-CT scanning image for the HA scaffold, and (d) EDS result at the surface of the infiltrated HA scaffold. CT, computed tomography; EDS, energy-dispersive detector; HA, hydroxyapatite; SEM, scanning electron microscopy.

Medium Ca^{2+} concentrations

The calcium concentration of the cell culture medium was measured at every medium exchange. There were significant differences in the variations of the Ca^{2+} concentrations surrounding the infiltrated scaffolds and the pure HA scaffolds over time. Figure 2 shows the calcium concentrations relative to the calcium concentration in the media before the culturing experiments [measured Ca^{2+} at the time point minus the original Ca^{2+} concentrations in the medium; the measured blank Ca^{2+} concentration in medium was $0.68 (\pm 0.05)$ mM].

At first, the Ca^{2+} concentration in the medium increased for all the scaffold conditions. However, the infiltrated scaffolds provided a higher Ca^{2+} concentration over the first 3 days. In the HA scaffold condition, the Ca^{2+} concentration was significantly lower than in the medium with infiltrated scaffold but significantly higher than in the medium of the nonscaffold control condition at day 1.

From days 5 to 14, the Ca^{2+} concentrations of the media samples decreased below the calcium concentration of the original medium for all culture conditions, suggesting cells are consuming calcium ions. Interestingly, the calcium concentration in the medium of the infiltrated scaffolds

decreased sharply and was significantly lower than those of HA scaffolds and the no-scaffold control.

From days 16 to 42, the Ca^{2+} concentrations in the four conditions became similar, with no significant differences between each other. The Ca^{2+} concentration in the medium of all culture conditions rose once more to values slightly lower than that of the fresh medium and peaked at around 20 days for all the scaffold conditions. The calcium concentrations in all the scaffold conditions subsequently decreased once again to a minimum between 25 and 30 days, and finally increased again toward the end of culture period.

Glucose and lactate concentrations

The consumption of glucose and the secretion of lactate increased with time in much the same way for all conditions during 42 days as shown in Figure 3. This suggests the cells were healthy over most of the culture period. Over the first 20 days, the rate of increase in glucose consumption and lactate secretion was relatively low but then increased after 20 days and was maintained up until 40 days. During the last 2–3 days in culture, glucose consumption (Fig. 3a) started to

FIG. 2. Ca^{2+} concentration in the medium of HSC and osteoblast cocultures over 42 days of culture. Data are expressed as net Ca^{2+} concentration, with blank medium values subtracted. An overall decrease in the net Ca^{2+} concentration in the medium indicates cells take up calcium. The mean $\pm$ standard deviation from $n=3$ biological repeats are given for each time point. HSC, hematopoietic stem cell.

plateau. There was no significant difference between the scaffold conditions in glucose consumption and lactate secretion at any particular time point in culture (Fig. 3a, b).

Cell viability

The viability of cells in culture was determined indirectly by measuring LDH activity in medium samples (Fig. 3c). Statistically, we could not observe significant differences between culture conditions. In general, measured LDH activity increased slightly overall during culture. During the first days of culture, controls seemed to have lower activity than scaffold cultures, but higher activity than scaffold cultures at the later phase of culture. These differences, however, were not statistically significant.

Flow cytometry

To track the fate and differentiation of HSCs and osteoblasts in coculture, we investigated cell-type-specific surface marker expressions in flow cytometry at day 5, 14, 28, and 42 in culture (Fig. 4). We also confirmed typical cell surface marker expression profiles of Saos-2 osteoblasts before coculture experiments (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea); we could confirm that osteoblasts were strongly positive for CD10, CD51, CD44, and alkaline phosphatase (ALP).

In cocultures, CD235a was implemented to detect erythrocytes. A lineage cocktail (Lin) consisting of CD3, CD14, CD16, CD19, CD20, and CD56 was used to stain for lymphocytes, monocytes, macrophages, eosinophils, and neutrophils. For the detection of osteoblasts, staining for ALP was included. CD34 was used in combination with CD38 to determine the presence of $\text{CD34}^+\text{CD38}^-$ HSCs. Due to the biological variation between different human HSC donors, we observed relatively high standard deviations. To overcome the interindividual differences, we also applied normalization to the FACS results and give data as non-normalized and normalized. For normalized results, the FACS data for the four scaffold conditions were normalized to the day 0 uncultured controls. Figure 4 shows both the non-normalized and the normalized results for each marker at the different

time points. Figure 5 depicts exemplary FACS plots of cocultures at days 0 and 42.

Compared with day 0 fresh cells (Fig. 4a), the absolute percentage of erythrocytes decreased in the control (nonscaffold) condition by day 5, and although the percentage in the control increased somewhat over the culture period, it never regained that of the cells at day 0. A similar decrease was not observed for the scaffold conditions at day 5 or any other time point. After normalization to the nonscaffold control condition, the cell percentages for the HA scaffold condition and the 1 M infiltrated HA scaffold condition were significantly higher than those of the nonscaffold control on day 5 in Figure 4b ($p=0.0025$ and 0.0002 , respectively). The 2 and 1 M HA-infiltrated scaffold conditions were also significantly higher in CD235a^+ cells than the nonscaffold control condition on day 14 ($p=0.0451$ and 0.0014 , respectively). After long-time culture of 42 days, the percentages of erythrocytes on all scaffold conditions were maintained.

Lin-positive cells (Fig. 4c) showed no significant change in their percentages in all conditions until 42 days of culture when there was an increase for the scaffold conditions but not the control. When normalized (Fig. 4d), a significant increase in the percentage of cells expressing Lin at 42 days was detected for most of the scaffold conditions but not in the nonscaffold control condition, in which a low but stable percentage of Lin-positive cells could be observed (with p -values for HA, 1 M infiltrated HA, or 2 M infiltrated HA condition compared with control being 0.0091 , 0.0475 , and 0.0045 , respectively).

An increase of ALP-positive osteoblasts was observed after 5 and 14 days for all conditions, but at longer times the fraction of osteoblasts decreased in the control samples but was maintained for the scaffold conditions. After 42 days, this led to a significant difference between the fraction of osteoblasts for the control and the 2 M infiltrated scaffold ($p=0.0455$). When normalized (Fig. 4f), significantly more osteoblasts were maintained in the two infiltrated scaffold conditions compared with the nonscaffold control condition ($p=0.0282$ and 0.0287 , respectively) already at day 28. After 42 days in culture, the differences became more significant ($p=0.0057$ and 0.0004). Between the cultures on HA and the nonscaffold control cultures, there was no

FIG. 3. Net (a) glucose consumption, (b) lactate secretion, and (c) lactate dehydrogenase activity as indirect viability measurement of cells in cocultures over 42 days. Continuous glucose consumption and lactate secretion indicate metabolic activity throughout culture; low lactate dehydrogenase activity reflects high cell viability that was not significantly different between culture conditions. Mean $\pm$ standard deviation from $n=3$ biological repeats are given for each time point.

significant difference in the 42-day culture. In addition, there was no significant difference between the HA condition and the two infiltrated HA conditions.

The percentages of early HSCs as defined by the marker combination of $\text{Lin}^- \text{CD34}^+ \text{CD38}^-$ are presented in Figure 4g and h. Although 10% of the original cell population comprised HSCs, the fraction of $\text{Lin}^- \text{CD34}^+ \text{CD38}^-$ cells detected

by our FACS analysis at day 0 was $\sim 6\%$. This can be explained by the fact that manufacturer-provided HSCs had been selected for CD34 only, and thus, $\text{Lin}^- \text{CD34}^+ \text{CD38}^-$ cells excluded cells in the original population that were CD34^+ and also coexpressed Lin and/or CD38. During culture, we observed a decrease in the absolute fraction of HSCs to $<1\%$ within the first 5 days. This percentage was then maintained throughout the remainder of the culture period. This percentage is higher than the $\text{Lin}^- \text{CD34}^+ \text{CD38}^-$ HSC percentage in bone marrow *in vivo*.⁵ After normalization (Fig. 4h), the two infiltrated HA conditions demonstrated significantly higher ($p=0.00227$ and 0.0260 , respectively) HSC percentages than the nonscaffold control condition at day 42. The results at day 42 indicate that the HSCs were maintained in long-term culture, especially in scaffold conditions, with cell percentages being higher than 0.5% .

CFU assay

During long-term culture, there is a high possibility that HSCs will lose their functionality and multilineage differentiation potential. To investigate the differentiation potential of cultured HSCs, CFU assays were performed (Tables 1, 2). HSCs, including day 0 noncultured controls, did not form BFU-E or CFU-GEMM colonies under any culture condition. Table 1 shows that the ability of HSCs to form CFU-E (erythrocyte colonies) dropped at day 5 for all culture conditions compared with day 0 but then recovered at the longer culture times for infiltrated scaffold conditions (Table 1). The variation between the repeats led to large standard deviations, but HSCs that had been cultured for 28 days formed significantly more CFU-E colonies under the 2M infiltrated HA condition than those under the nonscaffold control condition. At day 42, both 1 and 2M infiltrated HA scaffold conditions gave significantly higher numbers of CFU-E colonies than the nonscaffold control condition.

Table 2 shows that the ability of HSCs to form CFU-GM colonies decreased after 5 days of culture for all culture conditions. At longer culturing times, the ability of HSCs to form these colonies seemed to recover for all culture conditions. Again, the variation between the biological repeats was high. Although not statistically significant, cells cultured on infiltrated scaffolds gave higher CFU-GM colony numbers than those in the control condition harvested after 42 days in culture.

Discussion

A long-term culture of HSCs and osteoblasts was performed in an effort to mimic important aspects of the endosteal niche microenvironment in bone marrow by using 3D HA-based scaffolds. It was hypothesized that simulating the basic aspects of the endosteal niche, including the 3D architecture and the ability to release calcium ions, could promote HSC maintenance. In long-term culture, cell fractions with characteristics of HSCs were maintained (as seen in Fig. 4g, h), and more cells expressing mature hematopoietic markers were present at the end of the culture (as shown in Fig. 4c, d) compared to nonscaffold conditions. These results suggest that HA scaffolds support long-term maintenance of HSCs.

The maintenance of HSC populations using *in vitro* models has been described in the past (for review, see Wilson and Trumpp⁵). Mohamed *et al.*²² cultured HSCs with cytokines

FIG. 4. FACS results for percentages of cells being positive for (a) CD235a expressed by erythrocytes, (c) lineage cocktail expressed by various mature hematopoietic cell types but not erythrocytes, (e) ALP expressed by osteoblasts, and (g) Lin⁻/CD34⁺/CD38⁻ defining HSCs for HSC/Saos-2 osteoblasts over 42 days of coculture. FACS results, normalized to the control, are also shown for (b) CD235a, (d) lineage cocktail, (f) ALP, and (h) Lin⁻/CD34⁺/CD38⁻ for HSC/Saos-2 over 42 days of coculture. Total numbers of HSCs are shown in (i). FACS results for cell-type-specific surface markers indicate significant differences between cultures for different cell types. Data are given as mean $\pm$ standard deviation from $n=3$ biological repeats; asterisks indicate statistically significant differences (*** $p<0.001$, ** $p<0.01$, * $p<0.05$). ALP, alkaline phosphatase; CD, cluster of differentiation; FACS, fluorescence-activated cell sorting; Lin, lineage.

FIG. 5. Exemplary FACS plots of HSCs and Saos-2 in coculture at (a) day 0, (b) control condition at day 42, and (c) 2 M infiltrated HA condition at day 42 for (I) initial FSC versus SSC cell gate to exclude cell debris and cell doublets, (II) Lin⁺ and Lin⁻ gates, (III) Lin⁺CD34⁺CD38⁻ gate, (IV) ALP⁺ gate, and (V) CD235a⁺ positive gate. FSC, forward scatter; SSC, side scatter.

TABLE 1. COLONY-FORMING UNIT ERYTHROCYTE ASSAY OF HEMATOPOIETIC STEM CELLS

CFU-E colonies per 500 cells				
Culture time (days)	Control	HA scaffold	HA with 1 M $\text{Ca}(\text{NO}_3)_2$ infiltrant	HA with 2 M $\text{Ca}(\text{NO}_3)_2$ infiltrant
0	28.0±9.0	28.0±9.0	28.0±9.0	28.0±9.0
5	0.0±1.0	4.0±4.0	2.2±1.5	5.3±5.7
14	1.3±1.3	4.7±4.7	8.0±7.0	10.7±9.3
28	5.0±3.0	12.7±5.7	18.3±3.7	25.0±10.0
42	3.3±2.3	6.0±5.0	28.3±35.3	26.7±24.7

Fresh cells at day 0 and cells that had been cultured for 5, 14, or 42 days in control conditions (no scaffold), on plain HA scaffold, or infiltrated HA scaffolds were cultured for 15 days in methyl cellulose-based hematopoietic assay medium. Colony types were counted microscopically, and data are given as mean±standard deviation from $n=3$ biological repeats.

CFU-E, colony-forming unit—erythrocyte; HA, hydroxyapatite.

and successfully expanded HSCs in a short-term experiment. Winkler *et al.*²³ demonstrated that E-selectin in the vascular niche regulated HSC dormancy, self-renewal, and chemoresistance. Also, Zhang *et al.*²⁴ cultured HSCs with MSCs resulting in a significant increase in the amount of HSC, which was attributed to the mimicry of the endosteal niche. Compared to their experimental setup, we chose to coculture HSCs with osteoblasts as based on their proposed role in immobilizing the HSCs in the endosteal niche cells. In addition, we chose to exclude growth factors from the medium to examine the effect of the scaffold material and this resulted in lower HSC expansion rates in our experiment.

Other attempts have been made to simulate the relationship between HSCs and MSCs in the endosteal niche. In short-term culture, Jing *et al.*²⁵ demonstrated the positive effect of an MSC layer on proliferation of HSCs. In their report, CD34⁺/CD38[−] were used for selection of HSCs. The numbers of HSCs increased rapidly to 96- or 248-fold in two-dimensional or 3D conditions, respectively, after 7 days of culture. However, the absence of an exclusion for Lin-positive cells might have led to a larger gate for HSC identification. These researchers attributed the strong increase in HSC expansion to the important role of MSCs in the endosteal niche. The MSCs could maintain the immature properties of the HSCs, which is a key point to maintain proliferation. Compared to our experiment, MSCs might affect HSC proliferation more dramatically than osteoblasts. In addition, the effect of the different cell culture media and the use of growth factors should be considered in any comparison. In longer term culture, Salati *et al.*²⁶ also reported that osteoblasts have the ability to increase the

number of immature progenitors of the HSC pool over 14 days, but none of these studies examined HSC proliferation and maintenance in 42-day long-term coculture.

In addition to cellular interactions, there is evidence that the 3D architecture of the endosteal niche is important to the *in vivo* physiological conditions that affect cell fate.^{25,27} Indeed, HA scaffolds have been applied in tissue engineering^{28,29} and stem cell culture,^{30–33} commonly for bone tissue engineering rather than HSC culturing. For example, osteogenic differentiation of mesenchymal stem cells has been reported, using HA alginate scaffolds³³ or HA nanoparticles.³⁴

In our previous work,¹⁸ successful implementation of HA scaffolds in perfusion bioreactors has been accomplished for long-term culture of primary human bone marrow mononuclear cells. This study further advances the application of HA scaffolds to bone marrow stem cell culturing by calcium infiltration to provide local calcium enrichment toward the surface of the scaffold. The effect of this functionally graded scaffold material on hematopoietic progenitors was then investigated. The scaffolds maintained an HSC population over 42 days (Fig. 4h) and the potential for the formation of erythrocyte colonies increased on the infiltrated HA scaffolds after long-term culture over 42 days (Table 1). Hence, the infiltrated HA scaffolds played an important role in maintaining the HSC differentiation potential.

As a primary component of the endosteal niche, osteoblasts have the ability to regulate HSC fate.^{35,36} Calvi *et al.*¹³ and Zhang *et al.*³⁶ have both demonstrated that osteoblasts are an important regulatory component of the HSC niche and can control the number of HSCs in bone marrow

TABLE 2. COLONY-FORMING UNIT GRANULOCYTE AND MACROPHAGE ASSAY OF HEMATOPOIETIC STEM CELLS

CFU-GM colonies per 500 cells				
Culture time (days)	Control	HA scaffold	HA with 1 M $\text{Ca}(\text{NO}_3)_2$ infiltrant	HA with 2 M $\text{Ca}(\text{NO}_3)_2$ infiltrant
0	28.0±6.0	28.0±6.0	28.0±6.0	28.0±6.0
5	1.8±2.2	5.2±3.8	2.7±3.3	7.3±5.3
14	4.3±6.7	7.3±8.7	9.3±6.7	11.0±7.0
28	20.0±27.0	28.7±46.3	37.3±42.7	31.0±39.0
42	20.0±32.0	23.0±37.0	57.0±53.0	43.0±49.0

Fresh cells at day 0 and cells that had been cultured for 5, 14, or 42 days in control conditions (no scaffold), on plain HA scaffold, or infiltrated HA scaffolds were cultured for 15 days in methyl cellulose-based hematopoietic assay medium. Colony types were counted microscopically, and data are given as mean±standard deviation from $n=3$ biological repeats.

CFU-GM, colony-forming unit—granulocyte, macrophage.

through notch and BMP signaling. Osteoblasts have been shown to prefer high Ca^{2+} concentrations and they consume Ca^{2+} ions from the medium during culture.³⁷ Therefore, a high local Ca^{2+} concentration at the scaffold surface as provided by the infiltrated scaffolds is expected to benefit osteoblast maintenance and activity in culture, which has also been suggested by Dvorak *et al.*³⁸ and Maeno *et al.*³⁹ Indeed, increased osteoblast cell numbers were observed and maintained over 42 days of culture (as determined by percentages of ALP-positivity in flow cytometry) with infiltrated scaffolds (Fig. 4f).

Calcium is also known to be important for stem cell differentiation and proliferation.⁴⁰ For HSCs, different calcium receptors have been described (CaR¹⁹ and GPCRs⁴¹). These receptors are required to maintain HSCs near the endosteal surface of the bone,⁴² indicating that HSC maintenance is sensitive to the local Ca^{2+} concentration. This is consistent with Lin[−]CD34⁺CD38[−] FACS results in this study (Fig. 4h). When normalized to the results for the control populations, infiltrated scaffolds, providing a higher Ca^{2+} concentration in the early stages of culture, resulted in significantly increased percentages of HSCs over the controls at 42 days.

In vivo, human erythrocytes have a life span of about 90–120 days.⁴³ However, during *in vitro* culture, the life span of erythrocytes is shortened due to the aging of the erythrocytes (for review, see Bosman⁴⁴). With their inability to undergo cell division, erythrocytes are generated solely from HSCs (for review, see Hamidi and Tajerzadeh⁴⁵). In our experiments, during the 42 days of culture, erythrocytes were present at all time points, indicating that HSCs differentiated into erythrocytes after seeding. Indeed, the fractions of the erythrocytes in the scaffold conditions were higher than those in the non-scaffold condition after short-term culture (Fig. 4b). Furthermore, HSCs differentiated to mature, Lin⁺ hematopoietic cell types, with significantly increased cell percentages in the infiltrated HA condition compared with the nonscaffold control condition after 42 days (Fig. 4d).

Conclusion

In conclusion, the effects of highly porous, functionally graded HA scaffolds during long-term coculture of HSCs and osteoblasts were investigated. HA scaffolds *per se* increased the percentages of cells positive for erythrocyte markers, mature hematopoietic lineage markers, and HSC makers, and significantly increased CFU activity. In addition, the infiltrated HA scaffolds resulted in higher fractions of hematopoietic cells after long-term culture, indicating that the functionally graded biphasic HA scaffolds provide an improved *in vivo*-like scaffold for hematopoietic bone marrow tissue engineering.

Acknowledgment

The authors acknowledge the financial support of the National Institute of Health (NIH) under award No. 1R01HL108631-01.

Disclosure Statement

No competing financial interests exist.

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Received: January 22, 2018

Accepted: April 30, 2018

Online Publication Date: June 1, 2018