Effect of Pulse Frequency on the Osteogenic Differentiation of Mesenchymal Stem Cells in a Pulsatile Perfusion Bioreactor

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Perfusion bioreactors are a promising in vitro strategy to engineer bone tissue because they supply needed oxygen and nutrients and apply an osteoinductive mechanical stimulus to osteoblasts within large porous three-dimensional scaffolds. Model two-dimensional studies have shown that dynamic flow conditions (e.g., pulsatile oscillatory waveforms) elicit an enhanced mechanotransductive response and elevated expression of osteoblastic proteins relative to steady flow. However, dynamic perfusion of three-dimensional scaffolds has been primarily examined in short term cultures to probe for early markers of mechanotransduction. Therefore, the objective of this study was to investigate the effect of extended dynamic perfusion culture on osteoblastic differentiation of primary mesenchymal stem cells (MSCs). To accomplish this, rat bone marrow-derived MSCs were seeded into porous foam scaffolds and cultured for 15 days in osteogenic medium under pulsatile regimens of 0.083, 0.050, and 0.017 Hz. Concurrently, MSCs seeded in scaffolds were also maintained under static conditions or cultured under steady perfusion. Analysis of the cells after 15 days of culture indicated that alkaline phosphatase (ALP) activity, mRNA expression of osteopontin (OPN), and accumulation of OPN and prostaglandin E₂ were enhanced for all four perfusion conditions relative to static culture. ALP activity, OPN and OC mRNA, and OPN protein accumulation were slightly higher for the intermediate frequency (0.05 Hz) as compared with the other flow conditions, but the differences were not statistically significant. Nevertheless, these results demonstrate that dynamic perfusion of MSCs may be a useful strategy for stimulating osteoblastic differentiation in vitro. [DOI: 10.1115/1.4004919]

Keywords: mesenchymal stem cells, PLGA, pulsatile perfusion, alkaline phosphatase, osteopontin, PGE₂

1 Introduction

Engineered bone tissues are promising materials for the repair of large tissue deficits, but to be clinically effective they must be biologically active and capable of stimulating the normal bone remodeling processes upon implantation (e.g., integration, vascular infiltration, and new bone formation). One strategy for creating such materials is to culture mesenchymal stem cells (MSCs) [1–3] within porous three-dimensional scaffolds, as these cells are capable of synthesizing a bone-like extracellular matrix (ECM) containing bioactive growth and differentiation factors (e.g., bone morphogenetic proteins (BMPs) and vascular endothelial growth factor (VEGF) [4]) that can enhance osteoblastic differentiation of MSCs in vitro [5]. However, a recent in vivo study showed that bone-like ECM alone was not able to demonstrate a significant osteogenic response [6], underscoring the need for developing new strategies to enhance formation of a bone-like ECM.

Medium perfusion may be an important component for forming a bone-like ECM in vitro as it serves for two complementary purposes. First, perfusion delivers oxygen and nutrients to the cells deep within large (> 1 cm) three-dimensional scaffolds [7–9]. This overcomes diffusional mass transport limitations, which otherwise restricts cell viability and ECM deposition to the outer surfaces of biomaterial scaffolds [7]. Second, perfusion enhances several phenotypic markers of osteoblastic differentiation, including alkaline

phosphatase (ALP) activity [7,10], synthesis of type I collagen [11], osteocalcin (OC) [11], and osteopontin (OPN) [11], and mineral deposition [10,12]. Further, this biological response is sensitive to the flow conditions. For example, increasing the perfusion rate [12] or the fluid viscosity [13] has been shown to increase mineral deposition. However, perfusion has been shown to decrease cell density [14], which suggests that higher flow rates or medium viscosities might reduce the quality of bone-like ECM formed. Alternatively, dynamic perfusion regimens (e.g., oscillatory and pulsatile flow) may improve engineered bone tissue quality. To date only a handful of studies have examined the effect dynamic perfusion in porous three-dimensional scaffolds. A few short term studies (≤ 49 h) involving MC3T3-E1 model osteoprogenitor cells in three-dimensional porous scaffolds have demonstrated an increase in synthesis of prostaglandin E₂ (PGE₂) [14,15] and cyclooxygenase-2 (COX-2) [16] relative to steady flow. In addition, one long term study (14 days) in perfused scaffolds showed an increase in OPN mRNA expression with dynamic flow for MC3T3-E1 cells [17].

In contrast, dynamic flow regimens have been studied extensively in two-dimensional culture, and have demonstrated that osteoblastic cells are more responsive to dynamic flow conditions. In particular, dynamic flow has been shown to enhance mRNA expression of osteopontin, BMP-2, BMP-7 [18], and VEGF-A [18,19], synthesis of PGE₂, and activation of the mitogen-activated protein kinases ERK and p38 [15,19–21]. In addition, evidence suggests that cell response is sensitive to the frequency of pulsatile flow [20,22,23], although varying trends have been reported. Jacobs et al. [20]—who measured intracellular calcium release in response to both oscillatory and pulsatile flow at 0.5 to 2 Hz—reported a decrease in cell response with increasing

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frequency. In contrast, Nauman et al. [23] reported that PGE₂ production increased with increasing frequency, while Mullander et al. [22] reported no change in PGE₂ production but an increase in nitric oxide release with increasing frequency (over the range from 1 to 9 Hz).

To date the effect of dynamic perfusion on osteoblastic differentiation of MSC in three-dimensional porous scaffolds has not been described. Therefore, the goal of this study was to determine the effect of pulsatile perfusion on osteogenic differentiation of MSCs in three-dimensional culture. To accomplish this MSCs—expanded from bone marrow explants—were seeded into poly(D,L-lactic-co-glycolic acid) (PLGA) foam scaffolds and subjected to pulsatile flow at frequencies of 0.083, 0.050, and 0.018 Hz for 15 days. These very low frequencies were chosen to ensure that the shape of the pulsatile waveforms would not depend on frequency, and previous evidence indicates that these low frequencies stimulate late markers of osteoblastic differentiation in two-dimensional cultures [18]. Cell density, ALP activity, mRNA expression of OPN and OC mRNA expression, and accumulation of PGE₂ and OPN in conditioned medium were measured, and compared with cells cultured statically or under steady perfusion.

2 Materials and Methods

2.1 Materials. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and all cell culture materials were obtained from Fisher Scientific (Pittsburgh, PA) unless otherwise specified.

2.2 Scaffold Fabrication. Scaffolds for these studies were prepared from 75:25 poly(D,L-lactic-co-glycolic acid) (PLGA, Lactel Biodegradable Polymers, Birmingham, AL) by solvent casting/particulate leaching [7,24]. Briefly, approximately 10% (w/v) PLGA solutions in dichloromethane were cast over NaCl crystals (sieved 300–500 μ m) at a ratio of 15 wt. % PLGA/85 wt. % NaCl, dried, and then cut into 2–3 mm pieces. The PLGA/NaCl composite pieces were loaded into a cylindrical Teflon-lined mold (12.7 mm internal diameter) and heated to 100 °C for 30 min under 1.2 MPa of pressure. The composite material was cut into 2.5 mm disks with a low speed diamond wheel saw (South Bay Technology, San Clemente, CA), leached for 48 h in deionized water to remove the NaCl particulates, and dried. This approach has previously been reported to achieve a porosity of 79% and a mean pore diameter of 70 μ m [7,24]. The resulting scaffolds were press-fit into acrylic cassettes, and sterilized by gamma irradiation.

Prior to cell seeding, scaffolds (in cassettes) were immersed in 70% ethanol and a vacuum was drawn to release air bubbles trapped within the pores of the scaffolds. Mechanical agitation was applied to facilitate the escape of air bubbles and the ethanol was removed. The scaffolds were rinsed three times with phosphate buffered saline (PBS) and then soaked overnight in PBS to remove any additional ethanol. Scaffolds were incubated in a 2 μ g/ml fibronectin (Invitrogen) in PBS for 1 h prior to cell seeding.

2.3 Bone Marrow-Derived Mesenchymal Stem Cell Culture. MSCs were developed from bone marrow explants harvested from the tibias and femurs of 125–150 g male Sprague-Dawley rats (Harlan, Dublin, VA) in accordance with the Institutional Animal Care and Use Committee at Virginia Tech [25,26]. Briefly, explants were dispersed in growth medium (α -MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini, Calabasas, CA) and 1% antibiotic/antimycotic (Invitrogen) and expanded for approximately 10 days with medium changes every 3 or 4 days. After 10 days the cells were rinsed twice with PBS, lifted with trypsin/ethylenediaminetetraacetic acid (EDTA) (Invitrogen), and split 1 to 2. After the cells

reached approximately 90% of confluence the passing process was repeated until the cells were approximately 90% confluent at passage 3 or 4. Cells were then lifted and suspended in growth medium at a density of 2×10^6 cells/ml, and seeded by dropwise addition of 750 μ l of cell suspension to each scaffold. (Here, the estimated void volume of the scaffolds is 250 μ l so that the theoretical seeding density was 5×10^5 cells per scaffold. An excess volume of cell suspension was used to ensure a uniform distribution of cells and a concentration close to the theoretical density [7].) The seeded scaffolds were placed into 12 well plates for 1 h to permit initial cell attachment. Then, 2 ml of growth medium were added to each well and the plates were transferred to a CO₂ incubator and allowed to attach overnight.

2.4 Perfusion Bioreactor. The perfusion bioreactor system consisted of bioreactor chambers (Fig. 1(a)) that could each accommodate one 12.7 mm diameter cylindrical scaffold. Three bioreactor chambers were connected in parallel into a single flow loop that included a medium reservoir, a peristaltic pump (Cole Parmer, Vernon Hills, IL), a manometer, and 60 ml of medium (Fig. 1(b)) [7,27]. A microprocessor-controlled actuator and pinch valves were used to step the flow rate between 3.1 and 6.1 ml/min and flow was elevated for 25% of the cycle period. Sub-physiologic frequencies of 0.083, 0.050, and 0.017 Hz were selected to ensure that the minimum, maximum, and time-average shear stresses for all frequencies were similar. Flow rates for these three pulsatile conditions were measured with a FLR-1603A flow meter (Omega, Stamford, CT) using water at 31 °C (to match the viscosity of culture medium at 37 °C) and acquired to a computer using LabView software. Analysis of the flow rates indicated that the pulse amplitude was not affected by frequency (Fig. 2). In addition, the measured time-average flow rates of 3.9, 3.8, and 3.9 ml/min for 0.017, 0.050, and 0.083 Hz, respectively, compared well with theoretical time-average flow rate of 3.9 ml/min.

On the day after cell seeding, the scaffolds (in acrylic inserts) were loaded individually into bioreactor chambers, the perfusion bioreactor systems assembled, and each system filled with 60 ml of osteogenic medium (growth medium supplemented with 2 mM β -glycerophosphate, 0.13 mM ascorbate-2-phosphate, 10 mM

Fig. 1 Diagram of (a) a perfusion flow chamber containing a seeded scaffold and (b) schematic of perfusion flow system

Fig. 2 Flow profiles of (a) 0.017 Hz, (b) 0.050 Hz, and (c) 0.083 Hz pulsatile perfusion conditions

dexamethasone, and 25 mM HEPES). Three bioreactor systems were used to delivered pulsatile perfusion, while a fourth delivered steady flow rate at 3.9 ml/min. A separate set of seeded scaffolds (in acrylic inserts) were maintained statically in 12 well culture plates with 2 ml of osteogenic differentiation medium per well and served as the no flow (control) group. The bioreactors were placed within 37°C, 5% CO₂ incubators for 15 days, and half of the medium (30 ml) was replaced every 3 days. Samples of conditioned medium were collected at each media change and stored at -70°C for subsequent analysis for PGE₂ and OPN. After 15 days of perfusion, the scaffolds were removed from the flow systems, rinsed twice with PBS, cut into quarters, and the pieces weighed. To facilitate the extraction of cellular materials, the scaffolds were frozen in liquid nitrogen before being crushed in the appropriate collection buffer for analysis of cell number, ALP activity, and gene expression.

2.5 Cell Number. Cell number was determined at day 15 using PicoGreen reagent (Invitrogen). One quarter of each sample was crushed and collected in 300 μ l digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5 wt. % sodium dodecylsulfate, and 0.1 mg/ml proteinase K, pH 8.0). The DNA was isolated and recovered using a phenol chloroform extraction process and the DNA was resuspended 0.5 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). DNA standards of 0.016 to 1 μ g/ml were prepared in TE buffer, according to manufacturer's instructions. A volume of 100 μ l of samples and standards were added to a 96-well plate in duplicate. PicoGreen reagent was diluted 1:200 in TE buffer and then 100 μ l of the diluted reagent was added to each well. Fluorescence was measured with a SpectraMax fluorescent plate reader (Molecular Devices, Sunnyvale, CA) using excitation and emission frequencies of 488 and 525 nm, respectively, and a linear standard curve was plotted correlating fluorescence to DNA concentration. Cell number was calculated per gram of scaffold from the standard curve using a conversion factor of 8.1 pg DNA/cell (determined experimentally), and then reported relative to the cell number for the no flow group.

2.6 ALP Activity. After 15 days of flow, ALP activity was determined using a commercially available kit (Biotron Diagnostics, Hemet, CA). Scaffold quarters were homogenized in 500 μ l

TGT solution (50 mM Tris-HCl, 100 mM glycine, 0.1% Triton X100, pH 10.5) containing 5 μ l of protease inhibitors (2.0 μ g/ml aprotinin, 2.0 μ g/ml leupeptin, and 1.0 μ g/ml pepstatin) (Calbiochem, La Jolla, CA) [28]. A volume of 100 μ l of homogenized sample was combined with 500 μ l ALP reagent and incubated at 37°C for 15 min as previously described [28]. After 15 min the reaction was stopped with 500 μ l of 0.3 M NaOH and the absorbance of the reaction mixture was measured at 405 nm using a Spectronic Genesys 5 spectrophotometer (Spectronic Analytical Instruments, Leeds, United Kingdom). Enzyme activity—defined as the rate of conversion of *p*-nitrophenol phosphate to *p*-nitrophenol—was calculated per cell, and then normalized relative to the no flow (control) group.

2.7 mRNA Expression. Expression of mRNA for OPN and OC was determined after 15 days of flow by quantitative RT-PCR as described previously [29]. Briefly, RNA was isolated from the samples using the RNeasy mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Next, 200 ng of isolated RNA was reverse transcribed to cDNA using the Superscript kit (Invitrogen) and random hexamers as primers. Quantitative RT-PCR was performed using an ABI 7300 (Applied Biosciences, Foster, CA), SYBR green master mix (Applied Biosciences), and specific primers for β -actin (β A), OPN, and OC (Integrated Technologies, Coralville, IA) [25]. The $2^{-\Delta\Delta C_t}$ method was used to quantify the gene expression of OPN and OC relative to the no flow group using β A as the internal reference [30].

2.8 OPN and PGE₂ Content. Accumulation of OPN and PGE₂ in conditioned medium was determined at each media change using enzyme immunoassay (EIA) kits (Assay Designs, Ann Arbor, MI) according to the manufacturer's instructions. Absorption was measured with a Labsystems Multiskan RC ELISA plate reader (Labsystems, Frankfurt, Germany) at 450 nm and 405 nm for the OPN and PGE₂ ELISA kits, respectively. OPN and PGE₂ protein contents were calculated from curves generated using the standards provided with the kits.

2.9 Statistics. Data were analyzed with JMP 8.0 (SAS, Cary, NC) using a Tukey-Kramer post hoc test and a 95% confidence criterion to test for differences between experimental groups. Each experiment was replicated and $n = 3$ samples were prepared per experiment. Data from the two replicates were combined and data are presented as mean $\pm$ standard deviation for $n = 5$ or 6 samples. An asterisk denotes a statistically significant difference between a flow condition and the no flow control.

3 Results

The goal of this study was to determine the effect of pulsatile perfusion culture on the osteoblastic differentiation of MSCs in three-dimensional porous scaffolds. To accomplish this, a traditional gravity-driven perfusion apparatus—designed to deliver steady flow—was modified to introduce controlled pulses. The flow rate was stepped between 3.1 and 6.1 ml/min at frequencies of 0.017, 0.050, and 0.083 Hz at a time-average flow rate of 3.9 ml/min (Fig. 2), and compared with steady flow at 3.9 ml/min and static culture (i.e., the no flow group). To determine the effect of pulsatile flow on MSC density, cell number was calculated per gram of PLGA scaffold. However, because cell densities varied considerably between the two replicate studies, data for each replicate were normalized by the mean of the no flow group, and then the two replicates were combined. The resultant data indicated that cell density within the PLGA foam scaffolds was reduced by pulsatile flow, but that the differences were not statistically significant (Fig. 3).

The effect of pulsatile flow on the osteoblastic differentiation of MSCs was determined by analyzing ALP activity, gene expression for OPN and OC, and the concentration of OPN in conditioned medium. ALP activity at day 15 was calculated on a per

Fig. 3 Cell density in PLGA porous scaffolds at 15 days normalized to the no flow control. Data were combined from two replicates of $n=3$ samples, and data for each replicate were normalized by the mean for the no flow control. Bars correspond to the mean $\pm$ SEM for $n=6$ (or $n=5$ for continuous flow) samples, and no differences between groups were statistically significant ($p>0.05$).

Fig. 4 ALP activity per cell for MSCs seeded in PLGA scaffolds at day 15. Data were combined from two replicates of $n=3$ samples, and data for each replicate were normalized by the mean for the no flow control. Bars correspond to the mean $\pm$ SEM for $n=6$ (or $n=5$ for continuous flow) samples. No differences between groups were statistically significant.

cell basis and then normalized to the no flow control of the respective replicate (Fig. 4). Analysis revealed that all flow conditions enhanced ALP activity over the no flow group, with the higher frequencies (0.050 and 0.083 Hz) yielding the highest ALP activity. However, differences between conditions were not statistically significant. Analysis for OPN and OC mRNA indicated that all flow conditions significantly induced OPN gene expression compared with the no flow group (Fig. 5(a)). In contrast, OC gene expression was down-regulated for all flow conditions relative to the no flow group (Fig. 5(b)), but these differences were not statistically significant. To confirm the elevated expression of OPN with flow, conditioned medium—collected at each media change—was assayed for OPN (Fig. 6(a)). All flow regimens enhanced the synthesis of OPN protein over the no flow control at every time point after day 0. The two highest concentrations of OPN protein corresponded to the two highest pulse frequencies (0.050 and 0.083 Hz).

In addition, the conditioned media were also assayed for PGE₂—an eicosonoid marker of osteoblastic cells response to shearing flow [14,15,22,23]. Measured concentrations revealed that all flow conditions elevated PGE₂ relative to the no flow control group (Fig. 6(b)). Among the flow groups, the highest concentration of PGE₂ corresponded to the lowest pulse frequency (0.017 Hz) while the lowest concentration corresponded to the highest frequency (0.083 Hz).

Fig. 5 Gene expression of (a) OPN and (b) OC by MSCs cultured in PLGA scaffolds for 15 days. β -actin was used as the housekeeping gene and data are normalized to the no flow condition. Data are mean $\pm$ standard error for $n=6$ (or $n=5$ for continuous flow) samples. An asterisk denotes a significant difference with respect to the no flow control.

Fig. 6 Accumulation of (a) OPN and (b) PGE₂ per scaffold at 0, 3, 6, 9, 12, and 15 days. Data are mean of $n=2$ replicates (with three scaffolds per replicate).

4 Discussion

Perfusion bioreactors are widely acknowledged to facilitate osteoblast viability in large three-dimensional biomaterials scaffolds [7,10,12,13]. In addition, several recent model studies—involving the measurement of early markers of mechanotransduction in short-term planar cultures—have suggested that osteoblastic cells are more sensitive to dynamic flow regimens [18,20,22,23]. However, to date only a few studies have examined

the effect of dynamic perfusion on osteoblastic differentiation and the development of a bone-like ECM in three-dimensional scaffolds. In this study MSCs derived from rat bone marrow were seeded into porous PLGA foam scaffolds cultured under pulsatile flow with frequencies of 0.017, 0.050, or 0.083 Hz, under steady flow, or statically. These sub-physiologic frequencies were selected to ensure that the minimum, maximum, and time-average shear stresses for all frequencies were similar among groups. Fifteen days of perfusion culture was found to enhance ALP activity, gene expression of OPN, and accumulation of OPN protein in conditioned medium, but to suppress OC gene expression. In general, pulsatile flow did not elicit significantly different effects than steady flow and no frequency-dependent trends were observed. However, cells cultured at 0.050 Hz exhibited slightly higher levels of ALP activity, OC and OPN gene expression, and OPN protein accumulation than cells cultured under the other three perfusion conditions.

The increases in OPN and ALP activity with perfusion flow relative to static culture demonstrated in this study are consistent with previous reports. In particular, the increase in OPN mRNA expression with 15 days of both steady and pulsatile flow regimens (Fig. 5(a)) is consistent with MC3T3-E1 preosteoblast studies involving 2 days and 14 days of rest-inserted steady and dynamic flow [14, 17]. Similarly, the increase in OPN accumulation (Fig. 6(a)) is consistent with previous studies involving 16 days of steady flow for bone marrow-derived MSCs [12,31,32] and Saos-2 osteosarcoma cells [11]. Further, a continuous increase in OPN concentration in circulating medium over the culture period (Fig. 6(a)) has also been demonstrated by others [11,31,32]. With regard to ALP activity, the increase in its measured activity under steady and dynamic perfusion (Fig. 4) is consistent with previous studies involving 16 days of steady perfusion culture for bone marrow-derived MSCs [13,31–33]. However, it contrasts with two studies that involved MC3T3-E1 cells: one that showed no effect on mRNA expression of ALP when pulsatile perfusion (0.033–0.067 Hz) was applied for 1 h out of every 8 h [17], and one that showed a decrease in mRNA expression at low steady perfusion rates (i.e., 0.1 mL/min) [34]. Thus, the differences between those two studies and the one presented here could reflect differences in either the cell type or the perfusion regimen.

Despite increases in ALP activity and accumulation of OPN and PGE₂ with pulsatile flow relative to no flow, systematic frequency-dependent trends were not observed in this study. Similarly, Plunkett et al. [17] found no trend for different durations of rest-inserted flow (corresponding to frequencies of 0.033 to 0.067 Hz). In contrast, two-dimensional studies have shown that the rates of NO [22] and PGE₂ synthesis [23] and the occurrence of calcium transients [20] are sensitive to pulse frequency. However, the studies presented here and by Plunkett et al. [17] were performed at significantly lower frequencies (0.017–0.083 Hz as compared with 0.3–9 Hz [20,22,23]).

In a previous study, the effect of 24 h of pulsatile perfusion on rat bone marrow-derived MSC was examined using a model two-dimensional system and the same range of frequencies (0.017–0.083 Hz) that were employed in this study [18]. In that study no trends were observed with respect to pulse frequency. However, a maximum in mRNA expression of BMP-2, OC, bone sialoprotein BSP, and type I collagen α_1 was observed at 0.05 Hz in that study, which is the same frequency that yielded slightly—but not significantly—elevated levels of ALP activity (Fig. 4) and OPN expression (Fig. 5(a)) in this study. Together, these two studies suggest that MSCs may exhibit heightened mechanosensitivity to particular frequencies of pulsatile flow.

In this study hydrodynamic shear markedly increased PGE₂ release and OPN induction. However, it has been noted that neither of these two markers are unique to osteoblastic cells. PGE₂ is expressed by numerous tissues—including gastrointestinal where it is thought to aid in ulcer healing [35]—and under ischemia conditions where it may contribute to reperfusion injury, tissue necrosis, and cell apoptosis [36]. Likewise, OPN expression is induced

by cell stress and is expressed in response to mechanical trauma (e.g., reperfusion injury [37], cardiac failure [38]) as part of an inflammatory response. Further, siRNA knock-down studies suggest that OPN expression protects cells from apoptosis [39]. Thus, the increased levels of PGE₂ and OPN observed in this study—while consistent with the development of the osteoblastic phenotype—are also consistent with inflammatory and mechanical stresses. Therefore, an analysis of late markers of osteoblastic maturation (e.g., expression of OC and bone sialoprotein, calcium accumulation [40]) is needed to more definitively assess osteoblastic differentiation. These markers are detectable within 15 days in vitro—the endpoint of this study—however they increase by several fold during the mineralization stage, which typically begins after 3 weeks of MSC culture in osteogenic medium [40].

A limitation of pulsatile flow relative to steady flow was the diminished cell density (Fig. 3), which may be a consequence of cell detachment. Cell detachment has been observed in two dimensional cultures when flow is applied at high shear stresses (e.g., 88–264 dyn/cm² [41]), and has been proposed previously to explain decreases in MSC density with time for steady flow at moderate shear stresses (e.g., 1.6 dyn/cm² [25]). For the studies presented here the shear stresses are estimated to be in the range of 0.21–0.42 dyn/cm² for perfusion rates of 3.1–6.1 mL/min (based on the assumptions of fully-developed laminar flow, 70 μ m diameter pores, and a scaffold porosity of 79% [7]). However, instability in the flow field may have been generated by pulsatile flow—especially where recirculation or separation of flow occurs—and this could have contributed to cell detachment.

Finally, perfusion has been shown to induce expression of growth factors and morphogens associated with bone formation, including transforming growth factor- β 1 (TGF- β 1), fibroblast growth factor-2 (FGF-2), VEGF, BMP-2 and BMP-7 [4,18]. In particular, elevated expression of BMP-2 and BMP-7 by 24 h of pulsatile perfusion (relative to steady flow) has been demonstrated previously [18], and correlated with enhanced expression of mRNA for OC and BSP 12 days later. Thus, the enhanced expression of ALP (Fig. 4) and OPN (Fig. 5(a)) under perfusion in this study may be a consequence of elevated expression of various osteogenic growth factors and morphogens. However, further studies are necessary to characterize the expression and accumulation of these proteins in biomaterial scaffolds under perfusion culture [5].

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