

Construction of Mesenchymal Stem Cell–Containing Collagen Gel with a Macrochanneled Polycaprolactone Scaffold and the Flow Perfusion Culturing for Bone Tissue Engineering

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Abstract

A novel bone tissue-engineering construct was developed by using poly(ϵ -caprolactone) (PCL)-macrochanneled scaffolds combined with stem cell-seeded collagen hydrogels and then applying flow perfusion culture. Rat mesenchymal stem cells (MSCs) were loaded into collagen hydrogels, which were then combined with macrochanneled PCL scaffolds. Collagen hydrogels were demonstrated to provide favorable growth environments for MSCs and to foster proliferation. Cell number determination identified retention of substantially fewer (50–60%) cells when they were seeded directly onto macrochanneled PCL than of cells engineered within collagen hydrogels. Additionally, the cells actively proliferated within the combined scaffold for up to 7 days. MSC-loaded collagen–PCL scaffolds were subsequently cultured under flow perfusion to promote proliferation and osteogenic differentiation. Cells proliferated to levels significantly higher in flow perfusion culture than that under static conditions during 21 days. A quantitative polymerase chain reaction (QPCR) assay revealed significant alterations in the transcription of bone-related genes such as osteopontin (*OPN*), osteocalcin (*OCN*), and bone sialoprotein (*BSP*), such as 8-, 2.5-, and 3-fold induction, respectively, after 10 days of flow perfusion relative to those in static culture. *OPN* and *OCN* protein levels, as determined by Western blot, increased under flow perfusion. Cellular mineralization was significantly enhanced by the flow perfusion during 21 and 28 days. Analyses of mechanosensitive gene expression induced by flow perfusion shear stress revealed significant upregulation of *c-fos* and cyclooxygenase-2 (*COX-2*) during the initial culture period (3–5 days), suggesting that osteogenic stimulation was possible as a result of mechanical force-driven transduction. These results provide valuable information for the design of a new bone tissue-engineering system by combining stem cell-loaded collagen hydrogels with macrochanneled scaffolds in flow perfusion culture.

Key words: 3D scaffolds; bone tissue engineering; collagen gel; flow perfusion; osteogenic differentiation

Introduction

SUCCESSFUL TREATMENTS OF DEFECTIVE BONE TISSUE have been effected through tissue engineering, in which osteoprogenitor or stem cells are combined with scaffolding materials and cultured to develop bone-analogous constructs.^{1,2} A key component in tissue engineering is the material or scaffold used, which must be appropriate for host cells and provide them with space for spreading and multiplication while facilitating their differentiation into bone-specific cell types.^{3,4} Degradable polymeric scaffolds have been frequently adap-

ted for bone tissue engineering, mainly due to their lack of toxicity while maintaining cellular processes, including initial adhesion, spreading, mitosis, and differentiation.^{5–8} Moreover, polymeric scaffolds are easy to fabricate into various shapes, which has enhanced their application potential to many different scaffolding technologies. Among these techniques, robocasting or robotic dispensing is a potential tool to generate scaffolds with a defined pore configuration in terms of pore shape, pore size, and porosity.^{8–12} This methodology is highly useful to produce scaffolds with complex shapes tunable to patient defects. The pores produced are

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generally macrochanneled to facilitate connectivity, which is helpful for nutrient supply and waste removal.^{8,12}

Cell seeding onto scaffolds and their manipulation for proliferation or osteogenic induction requires stringent control. For example, initial cell loading onto porous scaffolds, particularly macrochanneled scaffolds, requires special care to achieve a homogeneous distribution of a large quantity of cells throughout the scaffold. Cells generally descend to the bottom of the channels without adhering to the scaffold.¹² This is due not only to the geometry of the macrochanneled pores but also to the hydrophobic nature of degradable polymers. Despite the beneficial features of macrochannels, this cell seeding issue may limit potential applications of such scaffolds for bone tissue engineering.

In this study, we improved initial cell loading onto a macrochanneled polymer scaffold by pooling cells in a collagen solution that was loaded onto macrochanneled scaffolds. Collagen gelation permits effective manipulation of the cell-containing matrix into the scaffold framework. We further cultured these cell-collagen-loaded scaffolds under flow perfusion conditions based on the concept that flow perfusion might enhance cellular behaviors such as proliferation and differentiation into specific tissue lineages.^{8,13–16} A flow perfusion system significantly enriches the population of cells, including endothelial cells, osteoblasts, and smooth muscle cells, in the culture with polymeric scaffolds.^{13–15} Mesenchymal stem cells (MSCs) are stimulated to develop into osteogenic lineages under appropriate flow perfusion culture.¹⁶ Our group has also recently observed that adipose-derived stem cells cultured on composite scaffolds were highly stimulated to proliferate and develop osteogenically in flow perfusion culture.⁸ Perfusion culture enables a continuous supply of fresh medium while removing metabolites consumed during growth, and additionally, the shear stress may act as a mechanical stimulus to promote differentiation.^{8,17,18}

Herein, we designed a new bone tissue-engineering paradigm in which cells were first embedded in a collagen gel that was combined with macrochanneled biopolymer scaffolds. Then, we applied flow perfusion culture conditions to the cell-scaffold constructs. Rat bone marrow-derived MSCs were used as a cell source. We addressed the efficacy of the combined cell-scaffold system and probed the effects of flow perfusion on the osteogenic development of MSCs.

Materials and Methods

Macrochanneled biopolymer scaffolds

Poly(ϵ -caprolactone) (PCL, MW = 80,000; Sigma-Aldrich) was used as the biopolymer source. Macrochanneled PCL scaffolds were fabricated using the robotic dispensing technique, as described in our previous report with slight modifications.^{8,12} A viscous solution of PCL dissolved in acetone was dispensed into an ice-cooled ethanol bath through a nozzle (diameter, 520 μm) assisted by a force-controlled plunger to regulate the mass flow rate using robocasting equipment (Ez-ROBO3; Iwashita). Dispensed fibers were constructed layer by layer using a positional control unit linked to a personal computer. The intended pore size was 500 μm \times 500 μm in the x - and y -plane, and the pore dimension along the z -axis was determined by the diameter of the fibers dispensed. Scaffolds were constructed to have a dimension of 10 mm \times

10 mm \times ~3 mm. In particular, dispensing was conducted in a cooled ethanol bath for effective solidification; moreover, the sample was dried and then heated to 50°C to aid contact fusion of the scaffold framework after direct deposition. The samples were treated with 70% ethanol, dried under laminar flow, and UV-irradiated for 1 h before cell assays.

Culture conditions for rat bone marrow-derived MSCs

Bone marrow-derived MSCs were isolated from adult rats (180–200 g) as described previously.¹⁹ All protocols involving animals were approved by the Animal Care and Use Committee of Dankook University. The proximal and distal epiphyses of the rat femora and tibiae were cut off, and bone marrow tissue was flushed out with dispase II and type II collagenase solution in α -minimal essential medium (α -MEM; WelGene). The tissue was centrifuged and resuspended in normal culture medium (α -MEM supplemented with 10% fetal bovine serum containing 1% penicillin/streptomycin) and then placed in a culture dish in an incubator under a humidified atmosphere of 5% CO₂ in air at 37°C. Nonadherent hematopoietic cells were removed from the medium during medium changes (every 3 days), and the cells that had undergone three passages were used for further experiments.

Embedding MSCs in collagen gel and combining them with PCL scaffolds

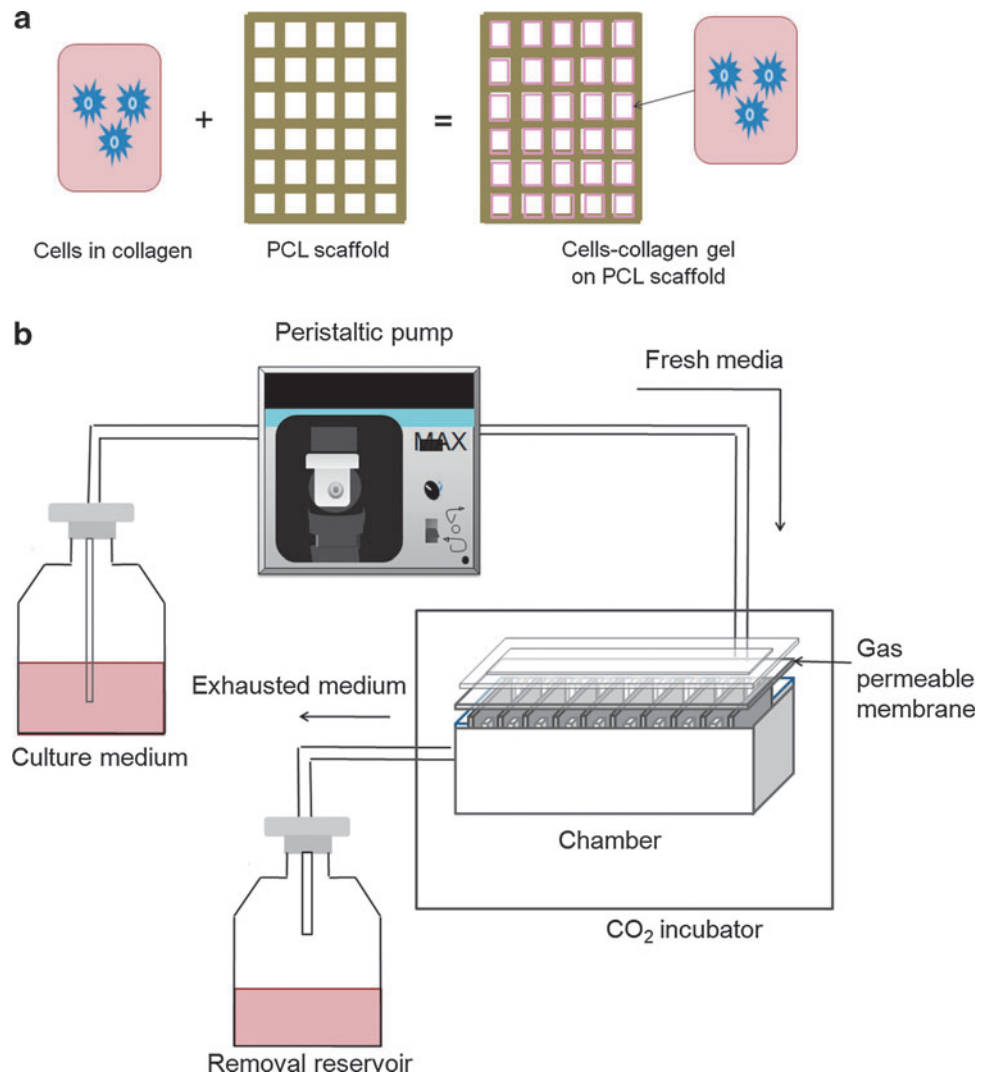
Rat-tail type I collagen was purchased from BD Biosciences (cat. 354236). The collagen solution was diluted with an equivalent amount of distilled water, and 10 \times Dulbecco's modified Eagle's medium (Gibco) containing 1% penicillin/streptomycin was added. The pH of the collagen solution was adjusted to 7.4 with 1 N NaOH. The collagen solution becomes a gel during a 30-min incubation at 37°C. Cellular mixtures were made by adding MSCs prepared in normal culture medium to the collagen solution; cell concentrations were adjusted to 1 \times 10⁶ cells/mL.

An 80- μL aliquot of the MSC-collagen mixture was gently dropped onto each PCL scaffold to allow infusion into the macrochannels. Care was taken to load the mixture only into the scaffold and not the underlying culture-well surface. Combined scaffolds were incubated for 30 min, and then 1 mL normal culture medium was added to each well to allow cell growth. A schematic of the MSC-collagen gel and its combination with the PCL-macrochanneled scaffold is depicted in Figure 1a.

Flow perfusion culture and osteogenesis

MSC-collagen-combined PCL-macrochanneled scaffolds were cultured for 24 h in normal culture medium before applying flow perfusion to allow enough time for the cells to settle and enter into the growth stage within the collagen gel matrix. Cell-scaffold constructs were then transferred to the perfusion culture system (Fig. 1b). The system was comprised of a peristaltic pump (Masterflex[®] L/S[™]; Cole-Parmer Instruments), a culture chamber, and a medium reservoir. Cell-scaffold constructs were positioned in a holder contained in the chamber, which was maintained in an incubator at 5% CO₂ and 37°C. The top surface of the chamber was made of a gas-permeable polymer, and the chamber holder was designed to fit the scaffold with no medium leakage around

FIG. 1. Schematic illustration of the experimental design of the study: **(a)** cell-scaffold design and **(b)** flow perfusion system. MSCs were initially pooled in collagen solution, which was subsequently applied to the macrochannels of PCL scaffolds to form cell-collagen-embedded scaffold constructs **(a)**. The constructs were cultured using a flow perfusion system supplied with osteogenic culture medium **(b)**. The constructs were placed into a chamber contained in an incubator with a humidified atmosphere of 5% CO₂ at 37°C. Fresh medium was supplied continuously at a constant rate (0.6 mL/min) using a peristaltic pump, and the exhausted medium was collected and discarded. MSCs, mesenchymal stem cells; PCL, poly(ϵ -caprolactone).



the chamber surface. Osteogenic medium (normal culture medium plus 50 $\mu\text{g}/\text{mL}$ sodium ascorbate, 10^{-2} M sodium β -glycerol phosphate, and 10^{-8} M dexamethasone)¹⁹ was supplied continuously at a constant rate of 0.6 mL/min by the peristaltic pump; consumed medium was not reused. The flow perfusion culture was continued for up to 21 days for cell proliferation and osteogenic differentiation assays. For the purpose of comparison, a conventional static culture in a culture dish (not subjected to flow perfusion) was also maintained alongside the cell-scaffold construct in osteogenic medium.

Cell viability and proliferation assay

First, cell viability was compared between groups of cells that were directly seeded onto a PCL scaffold versus when they were seeded within the collagen gel and then combined into a PCL scaffold cultured under a static condition. For both groups, cells were cultured under a static condition for 3 and 7 days, and the viable cells were quantified with an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay kit (CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay; Promega).²⁰

Cells directly seeded onto PCL scaffolds were detached by trypsinization for the MTS assay. In the case of cells contained within the collagen gel, the cell-scaffold constructs were washed with phosphate-buffered saline (PBS) and then transferred to a fresh plate. The sample was treated with 10 mg of collagenase (type I, 1250 U/mg; Worthington) to digest collagen and liberate the cells. The collagenase concentration used was determined from a pilot study, and showed good cell viability (no cellular toxicity) and sufficient collagen digestion for the cell assays. Samples were then centrifuged at room temperature for 5 min at 1500 rpm to collect cells, which were then suspended in the normal culture medium.

The MTS assay was performed by mixing 400 μL of sample to 40 μL of MTS solution, followed by a 3-h incubation at 37°C. Absorbance was read at 490 nm using an ELISA Plate Reader (BioRad). Five replicates were used for each experimental condition.

Next, the proliferative potential of cells cultured in the collagen gel-combined PCL scaffold was assessed between the static and flow perfusion conditions for up to 21 days. After each culture period, the cells were gathered by digestion with collagenase, and cell viability was assessed by the MTS method, as described above.

Cell observations by confocal laser-scanning microscopy

Viable cells in the scaffolds during culture were observed by confocal laser-scanning microscopy (CLSM, LSM700; Carl Zeiss). Cells were fixed with 4% paraformaldehyde and treated with 0.1% Triton X-100, after which Alexa Fluor 488 phalloidin (A12379; Invitrogen) diluted in 1% bovine serum albumin (BSA; Sigma) was added to stain F-actin. Nuclei were additionally stained using the ProLong[®] Gold antifade reagent with 4',6-diamidino-2-phenylindole (P36935; Invitrogen).

Alkaline phosphatase determination

Osteogenic differentiation of MSCs cultured within scaffold constructs was assessed by alkaline phosphatase (ALP), an early osteogenic marker.¹⁹ After culturing for up to 14 days, cells were harvested by digesting with collagenase, as described above. Lysis buffer was added to the cell pellets for disruption by the freeze/thaw process. Samples were added to the ALP reaction media for enzymatic reaction, according to the manufacturer's instructions (Sigma). The sample quantity added was determined through total protein content, which was measured with a commercial DC protein assay kit (BioRad). *p*-Nitrophenol generation was measured spectrophotometrically at an absorbance of 405 nm. Five replicate samples were used for each experimental condition.

Gene expression by real-time polymerase chain reaction

Expression levels of the bone-associated genes, including osteopontin (*OPN*), bone sialoprotein (*BSP*), and osteocalcin (*OCN*), were determined quantitatively by real-time polymerase chain reaction (QPCR).¹⁹ After culturing for 5 or 10 days, cells were gathered from each sample, and total RNA was isolated using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Next, RNA was reverse transcribed to cDNA using the Superscript kit (Invitrogen) with random hexamers as primers. PCR amplification was performed using the Sensimix Plus SYBR Master Mix (Quantace). The comparative CT method was used for analysis by normalizing the PCR product accumulated for each gene to the β -actin housekeeping gene.²¹

The expression of genes involved in the initial intracellular signal transduction after mechanical stimulation, including *c-fos* and cyclooxygenase-2 (*COX-2*), was also investigated by QPCR. Cell-scaffold constructs were cultured for short time periods (1, 3, and 5 days) under static or flow perfusion conditions. A quantitative *c-fos* and *COX-2* mRNA analysis was conducted by following the procedure described above. Triplicate samples were tested in the QPCR assay. Primer sequences used for the assay are summarized in Table 1.

Immunofluorescent staining

Expression of *OPN*, *BSP*, and *OCN* was visualized by immunofluorescence. MSC-scaffold constructs were cultured for 10 days, fixed with 4% paraformaldehyde for 5 min, washed with PBS, and dried. Samples were treated with 0.3% Triton X-100 and blocked with 2% BSA, then incubated with primary antibody against *OPN* (rabbit anti-*OPN*, with cross reactivity to rat, 1:100; Abcam), *OCN* (rabbit anti-*OCN*, with cross reactivity to rat, 1:100; Abcam), or *BSP* (mouse anti-*BSP*, with cross reactivity to rat, 1:50; Developmental Studies Hybridoma Bank) at 4°C overnight. The fluo-

TABLE 1. PRIMER SEQUENCES OF GENES RELATED WITH OSTEOGENESIS OF MSCs (*OPN*, *OCN*, AND *BSP*), AS WELL AS THOSE INVOLVED IN THE INITIAL INTRACELLULAR SIGNAL TRANSDUCTION AFTER MECHANICAL STIMULATION (*C-FOS* AND *COX-2*) USED FOR THE QPCR ANALYSIS

Gene	Primer sequence
<i>OPN</i>	F: ATCTGATGAGTCCTTCACTG B: GGGATACTGTTTCATCAGAAA
<i>OCN</i>	F: TGAGGACCCTCTCTCTGCTC B: GGGCTCCAAGTCCATTGTT
<i>BSP</i>	F: ATAGGCAACGAGTACAACAC B: GTATCCAGATGCAAAGACAG
<i>c-fos</i>	F: CGCAGATCTCGTCTCTA B: CCTGACATGGTCTTACCAC
<i>COX-2</i>	F: CTGAGGGGTTACCACTTCCA B: TGAGCAAGTCCGTGTTCAAG

MSCs, mesenchymal stem cells; *OPN*, osteopontin; *OCN*, osteocalcin; *BSP*, bone sialoprotein; *COX-2*, cyclooxygenase; QPCR, quantitative polymerase chain reaction.

rescence images were labeled by fluorescein isothiocyanate-conjugation (goat anti-rabbit for *OPN* and *OCN*, and goat anti-mouse for *BSP*; Santa Cruz Biotechnology), and the stains were visualized by CLSM (M700; Zeiss).

Protein detection by Western blotting

Protein expression of *OPN* and *OCN* was analyzed by Western blotting based on the results of QPCR and immunofluorescence. Extracts of cells harvested after 20 days of culture were prepared in radio immuno precipitation assay buffer, and proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes blocked with 2.5% BSA in Tris-buffered saline with 0.1% Tween-20, and probed with a primary antibody (rabbit anti-*OPN*, 1:1000 or rabbit anti-*OCN*, 1:1000, with cross reactivity to rat; Abcam). Blots were then incubated with a secondary antibody (goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase; Santa Cruz Biotechnology), and immunoreactive bands were detected using an enhanced chemiluminescence detection reagent (Pierce). Two different sets of experiments were carried out to confirm the Western blot analysis.

Mineralization assay by measuring calcium content

After 21 and 28 days of culture, the cell-cultured scaffolds were added with a 1-N HCl solution to decalcify the mineralized nodules. The calcium produced during cellular mineralization was determined quantitatively using a Calcium E-Test kit (Wako Pure Chemical Industries, Ltd.). The absorbance of the reaction products was measured at 560 nm using a microplate reader (Molecular Devices). The concentration of calcium ions was calculated from the absorbance value relative to a calcium standard curve. Each measurement was performed in triplicate.

Results

Combined scaffold capacity for cell loading and proliferation

Figure 2 shows scanning electron micrographs of a macrochanneled PCL scaffold produced by the robotic dispensing

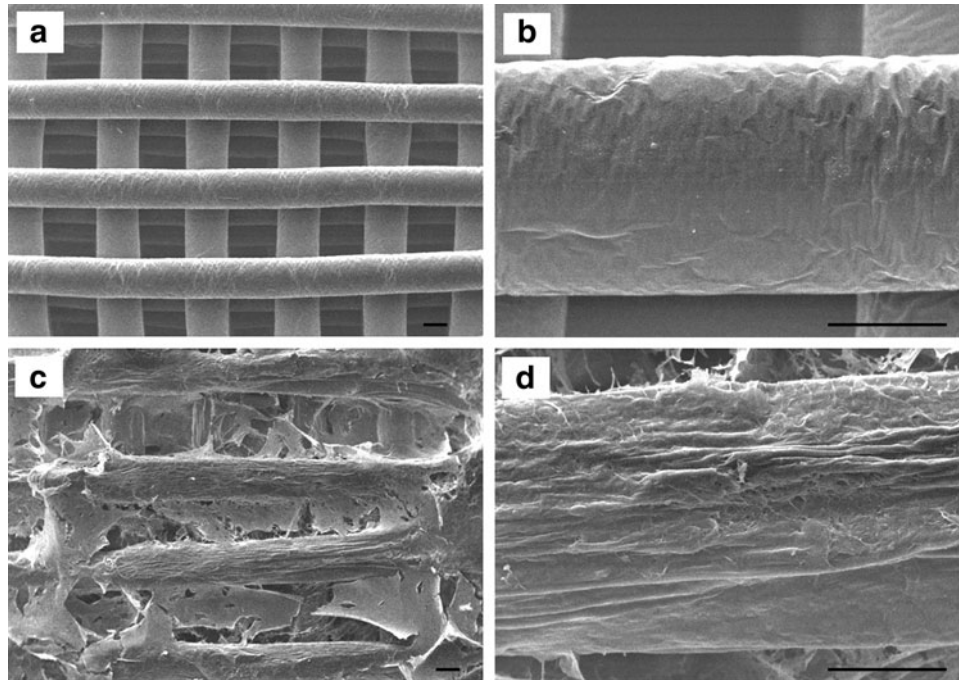


FIG. 2. Scanning electron microscopy morphology of (a, b) macrochanneled PCL scaffold produced by robotic dispensing and (c, d) collagen-treated PCL scaffold at different magnifications. The PCL scaffold showed a well-defined pore configuration with pore size of $225 \mu\text{m}$ ($\pm 21 \mu\text{m}$) and framework diameter of $213 \mu\text{m}$ ($\pm 16 \mu\text{m}$). Square-shaped pores were developed to form channels penetrating through the scaffold (a). The surface of the scaffold was smooth, as is characteristic of a solidified PCL polymer (b). A layer-by-layer deposition process resulted in 10 consecutive layers. The scaffold was constructed with a dimension of $10 \text{ mm} \times 10 \text{ mm} \times \sim 3 \text{ mm}$ for cell culture. PCL scaffolds were soaked in collagen solution and then freeze-dried to show the appearance of dried collagen mostly filling the pore channels (c) and the fibrous morphology of collagen on the surface (d). Scale bar = $100 \mu\text{m}$.

technique. Scaffold pore configuration was well defined, with a pore size of $225 \mu\text{m}$ ($\pm 21 \mu\text{m}$) and a framework diameter of $213 \mu\text{m}$ ($\pm 16 \mu\text{m}$); square-type pores developed to form channels through the scaffold (Fig. 2a). The scaffold surface appeared smooth, and was characteristic of the solidified PCL polymer (Fig. 2b). The layer-by-layer construction process resulted in the deposition of 10 consecutive layers; the scaffold size was designed to be $10 \text{ mm} \times 10 \text{ mm} \times \sim 3 \text{ mm}$ for cell experiments.

We performed a pilot study before applying the cell-seeded collagen to the macrochanneled PCL scaffolds. PCL scaffolds were soaked with collagen solution, which was then freeze-dried, to observe the status of collagen throughout the scaffold macrochannels. The presence of freeze-dried collagen could clearly be observed throughout the PCL scaffold (Fig. 2c). The pores were mainly filled with dried collagen (Fig. 2c), and the scaffold surface showed the fibrous morphology of collagen (Fig. 2d). The hydrogel characteristics were predicted to be capable of effectively retaining living cells.

Therefore, we proceeded by pooling MSCs in a collagen solution and then combining the solution with macrochanneled PCL scaffolds. Initial cell loading in the collagen gel was compared with direct seeding onto the PCL scaffolds (Fig. 3). As expected, a large quantity of cells seeded directly immediately penetrated through the scaffold macrochannels and sedimented in the culture dish, such that adherent cells comprised fewer than 50% of those initially seeded. In contrast,

cells contained in the collagen gel were predominantly retained on the scaffold. As a result, cell viability was significantly different between the groups when cells were loaded into the collagen gel and directly seeded, almost doubling in the former case (data at 24 h in Fig. 3).

Cells were further cultured under static conditions with normal culture medium for up to 3 days to investigate cell proliferation within the collagen gel supported on the PCL scaffold. The cell population demonstrated a large increase at 3 days and a further increase at 7 days for both cases (data at 3 and 7 days in Fig. 3). However, a significant difference in the cell population was maintained throughout the culture period tested, demonstrating that initial high cell loading is important for a substantial cell population during prolonged culture.

Flow perfusion culture and osteogenic differentiation of MSCs

MSCs in collagen-combined PCL scaffolds were cultured in an osteogenic medium containing ascorbic acid, dexamethasone, and β -glycerophosphate to induce osteogenesis. Flow perfusion culture was introduced at this osteogenic culture stage. MSC-scaffold constructs were cultured either under normal static conditions with medium refreshed every 2 days or under flow perfusion at a constant rate of 0.6 mL/min controlled by a peristaltic pump. At 24 h after the

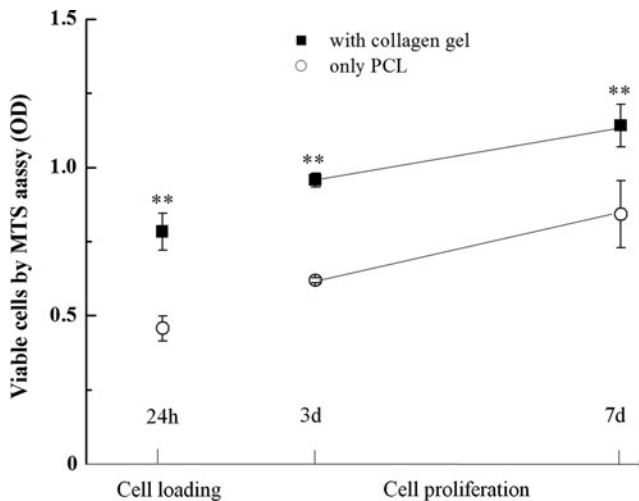


FIG. 3. Comparison of initial cell loading (at 24 h) and extended proliferation (for up to 7 days) between cells directly seeded onto PCL scaffolds and those pooled in collagen gel combined with PCL scaffolds. More than 50% of cells easily penetrated through scaffold macrochannels to sediment in the bottom of the culture dish, resulting in a reduction in the number of adherent cells when seeded directly. In contrast, cells contained within collagen gels were predominantly retained on the scaffolds. The MTS assay showed that cell viability almost doubled with the use of collagen gels (data at 24 h; $**p < 0.01$, $n = 5$). Cells continued to proliferate for up to 7 days in both cases; however, significant differences in initial cell loading were maintained during the proliferative stage (data at 3 and 7 days; $**p < 0.01$, $n = 5$), demonstrating that high initial cell loading is important for securing a large cell population during prolonged culture. MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium).

cell–collagen gel treatment (set as $t=0$), constructs were placed under static or perfusion conditions, and cell proliferation was measured by the MTS assay for culture periods up to 21 days. As shown in Figure 4, 14 and 21 days of static culture increased cell proliferation approximately three and five times, respectively, with respect to that at the initial time point. Cell proliferation was stimulated to a greater degree under flow perfusion, an approximate five- or six-fold increase at 14 or 21 days, respectively. A comparison between static and perfusion culture showed significantly higher cell proliferation under the perfusion condition during the entire culture period ($**p < 0.01$ at day 14 and $*p < 0.05$ at day 21).

Figure 5 shows cell morphology under CLSM during static and flow perfusion culture at different time points. MSCs proliferated actively based on nuclear and cytoskeletal staining. Compared to cells cultured under the static condition (Fig. 5a, c, e), those in perfusion culture (Fig. 5b, d, f) generally exhibited more pronounced staining of nuclei and F-actin, particularly after prolonged culture periods. While the cells initially loaded with collagen gel were distributed within the PCL scaffold macrochannels, they appeared to move toward the scaffold surface, and this was more pronounced in the perfusion culture.

We assessed levels of the early osteogenic marker ALP during static and perfusion culture of the MSC–scaffold constructs (Fig. 6). ALP activity increased with increasing

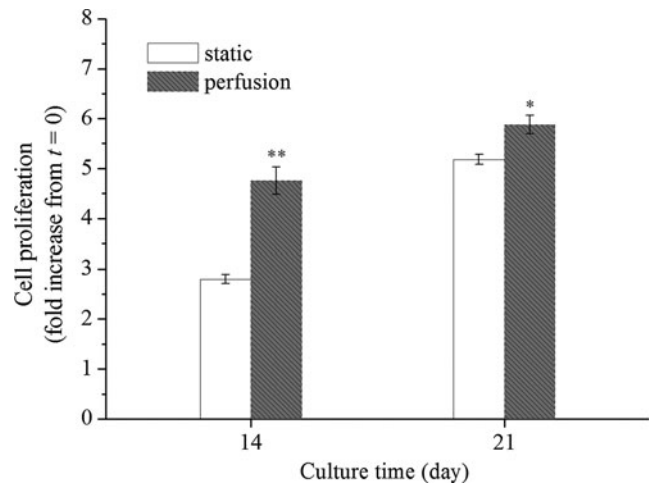


FIG. 4. Effects of flow perfusion culture on the proliferation of MSCs contained in collagen/PCL scaffolds over culture periods of up to 21 days. Twenty-four hours after applying the MSC–collagen gel (set as $t=0$), cell–scaffold constructs were cultured either under a normal static condition with the medium refreshed every 2 days or under flow perfusion at a constant rate of ~ 0.6 mL/min using a peristaltic pump. Osteogenic medium containing ascorbic acid, dexamethasone, and β -glycerophosphate was used to induce osteogenesis of MSCs. After culture for 14 and 21 days, the MTS assay was conducted to measure cell proliferation; data are represented with respect to the onset of osteogenic culture (24 h after MSC–collagen gel treatment, which was set as $t=0$). The flow perfusion condition resulted in significantly higher cell proliferation at all culture periods compared to that of static culture ($**p < 0.01$ at day 14 and $*p < 0.05$ at day 21, $n = 5$).

culture time for both culture methods, but more pronounced increases were detected during perfusion. Indeed, ALP levels in flow-perfused cells were significantly higher than those in cells under static culture at 14 days ($**p < 0.01$), suggesting an improvement in MSC osteoblastic differentiation under the former condition.

MSC gene expression associated with osteoblastic differentiation was analyzed by QPCR, and the expression levels were presented relative to the values of static culture at each culture time (Fig. 7). OPN, OCN, and BSP mRNA levels were significantly upregulated under flow perfusion culture at 10 days ($**p < 0.01$ for all genes), although no significant difference was observed at 5 days. Stimulation was as high as 8-, 2.5-, and 3.0-fold for OPN, OCN, and BSP, respectively, relative to that in static culture.

Expression of these proteins was also assessed by immunofluorescence staining of cells during a 10-day culture (Fig. 8). No autofluorescence or nonspecific binding signals were observed from the materials. OPN, OCN, and BSP protein expression (Fig. 8a–f) was detected by CLSM. Fluorescent staining was qualitatively more pronounced in cells cultured under flow perfusion than those cultured under a static condition, particularly for OPN and BSP expression.

We additionally examined expression of OPN and OCN by Western blot analysis (Fig. 9). Levels of both proteins were higher in the flow perfusion–cultured cells than those in cells under static culture (Fig. 9a). Protein expression was

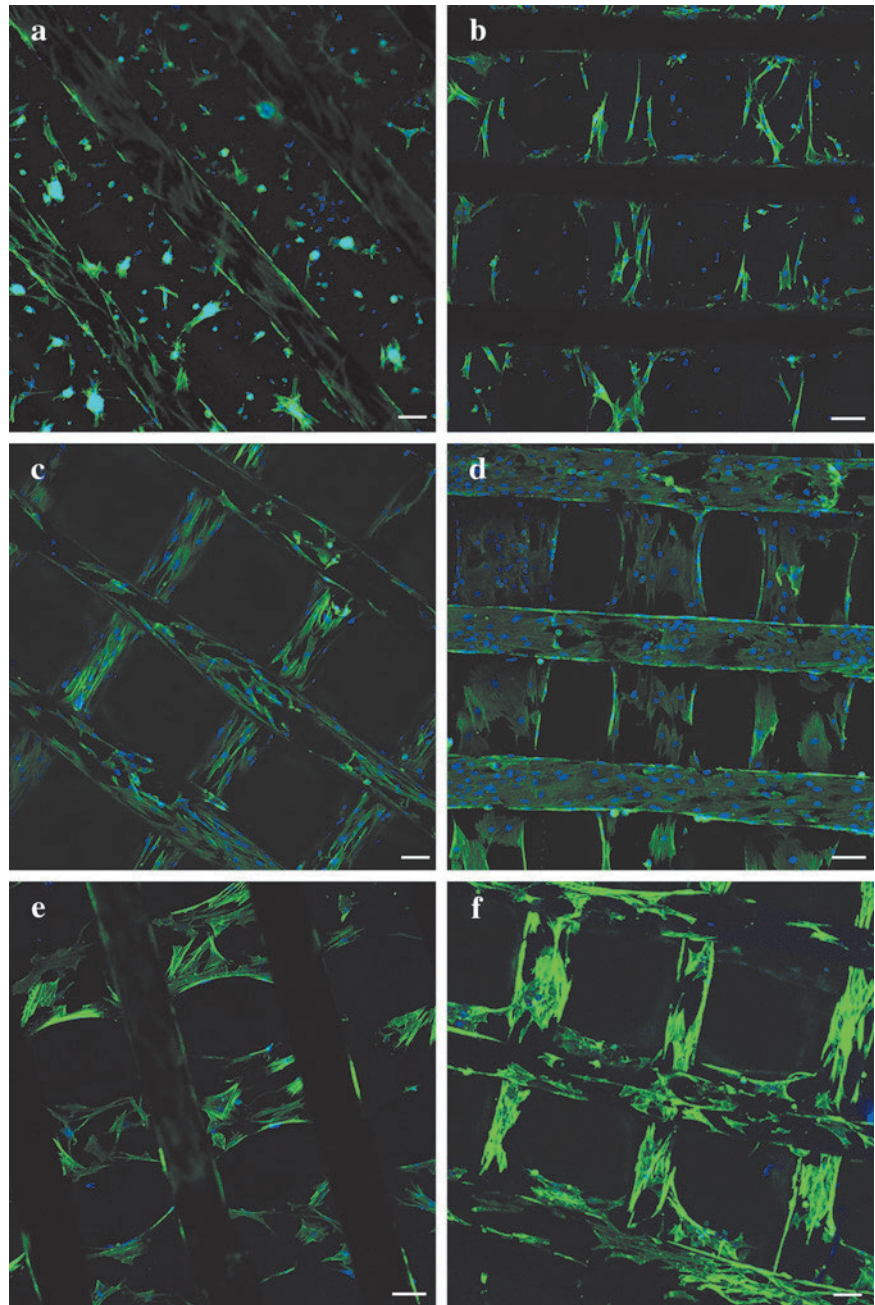


FIG. 5. CLSM cell morphology during static (a, c, e) and flow perfusion (b, d, f) culture at different time points: (a, b) 3, (c, d) 14, and (e, f) 21 days. Perfusion culture significantly enhanced cell proliferation, particularly for prolonged periods. While the cells loaded in concert with the collagen gel were distributed within the macrochannels of the PCL scaffold at the initial culture period, prolonged culture allowed cells to move toward the surface of the PCL scaffold, which was more pronounced by perfusion culture. Scale bar = 100 μm . CLSM, confocal laser-scanning microscopy.

quantified by densitometry and normalized to that of GAPDH (Fig. 9b). As a result, perfused cells showed significantly higher expression than that of statically cultured cells.

Cellular mineralization behavior was assessed by measuring calcium content after culturing cells for 21 and 28 days either in static or in perfusion conditions (Fig. 10). Cellular mineralization was pronounced after 28 days, particularly in the perfusion culture, with a significant difference compared with that of static culture (** $p < 0.01$).

Further analyses of *c-fos* and *COX-2* genes were performed using QPCR to determine whether the mechanical stress induced by the flow perfusion elicited osteogenic downstream signaling (Fig. 11). MSC-scaffold constructs were cultured for relatively short periods (1, 3, or 5 days) under either static or

flow perfusion conditions; QPCR analyses showed significantly enhanced stimulation of these genes in cells under flow perfusion, particularly after 3 and 5 days, (** $p < 0.01$).

Discussion

Bone tissue engineering requires appropriate use of three-dimensional (3D) matrices that can support initial cellular adhesion and subsequent migration and proliferation.²² The loading of cells uniformly and with high efficiency within 3D pore channels is the first key step to realize successful tissue engineering. A wealth of approaches have been reported to this end, including vibration or agitation, mechanical stirring, and gravity-driven centrifugation.^{23–25} Cell loading is

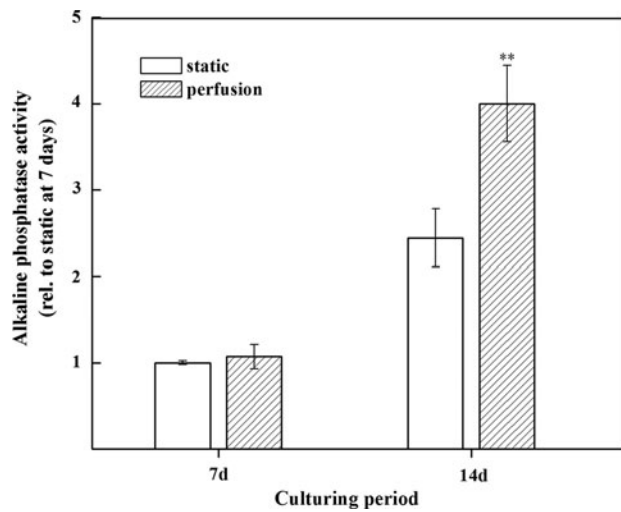


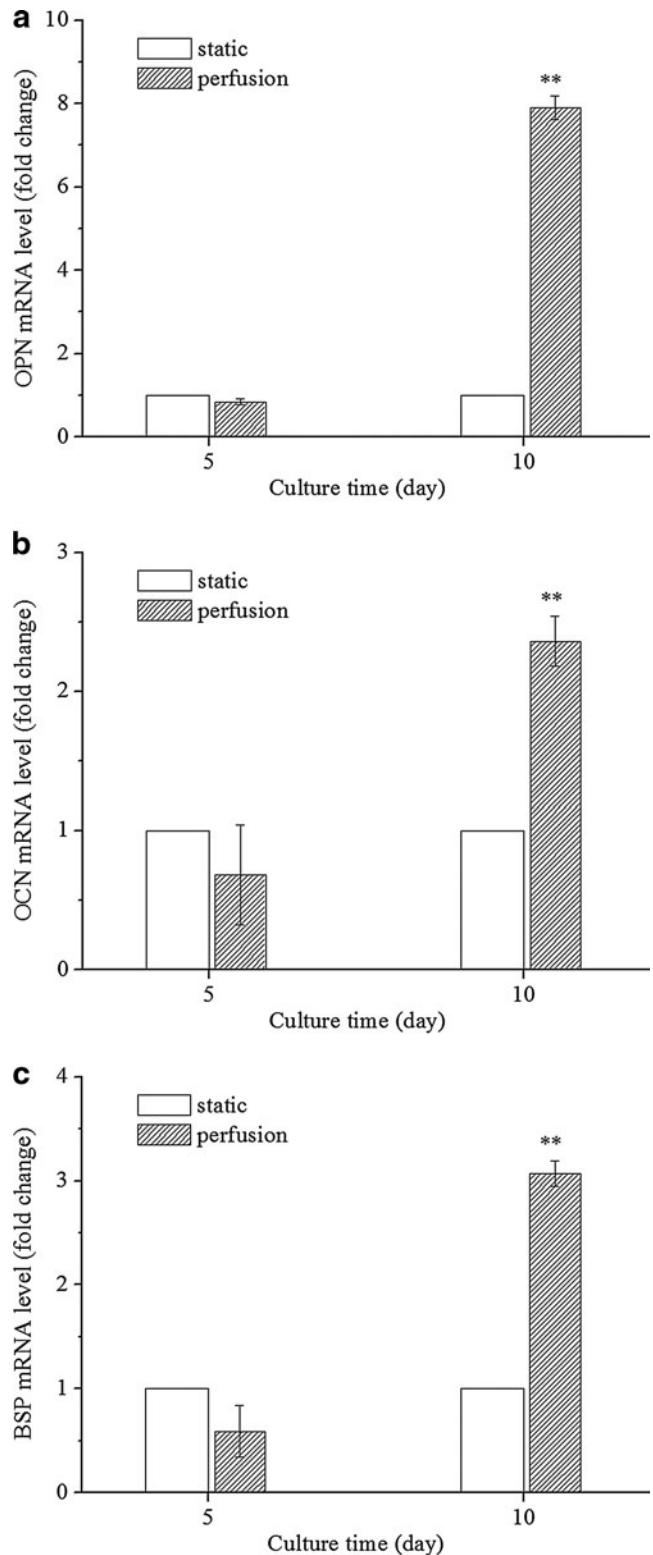
FIG. 6. ALP activity, an early osteogenic marker, in MSCs during the culture of combined scaffold–MSC constructs under a static or perfusion condition. ALP enzymatic activity was normalized to total protein content, and results are represented with respect to that of static culture at 7 days. ALP levels increased with culture time under both culture methods; the increase was greater under flow perfusion system, resulting in significantly higher enzymatic activity at 14 days in flow perfusion culture than that under the static condition (** $p < 0.01$, $n = 5$). ALP, alkaline phosphatase.

highly dependent on scaffold pore configuration. In the case of the PCL scaffolds used in this study, which typically retain a macrochanneled pore structure, it is difficult to maintain seeded cells within the scaffolds primarily because of the ease of cellular penetration through the macrochannels to the bottom of the culture dish.¹² This problem is exacerbated by the low hydrophilicity of PCL, which leads to poor cell affinity and substantial cell loss.

In fact, this type of scaffold, generally manufactured by rapid prototyping methods, has unique benefits, particularly that the pore configuration is highly effective for transporting body fluids and blood, removing waste products, and cellular ingrowth and vascularization, which are highly significant phenomena under *in vivo* conditions. However, homogeneous and efficient initial cell loading into scaffolds is essential to apply PCL-macrochanneled scaffolds to bone tissue engineering, where cell loading into scaffolds and *ex vivo* culture over extended periods are both required, and adequate proliferation and specific differentiation into bone cells must also occur.

FIG. 7. MSC expression of genes associated with osteoblastic differentiation as analyzed by QPCR. Gene expression levels are presented with respect to those in static culture at each culture time. The mRNA levels of essential osteogenic genes, including (a) *OPN*, (b) *OCN*, and (c) *BSP*, were significantly upregulated by flow perfusion culture at 10 days (** $p < 0.01$ for all genes, $n = 3$), although no significant difference was noted at 5 days. Induction was as high as 8-, 2.5-, and 3.0-fold for *OPN*, *OCN*, and *BSP*, respectively. QPCR, quantitative polymerase chain reaction; *OPN*, osteopontin; *OCN*, osteocalcin; *BSP*, bone sialoprotein.

Thus, we employed collagen hydrogels to load and deliver cells within macrochanneled PCL scaffolds. We used MSCs derived from rat bone marrow. MSCs were pooled in collagen solution to fill the PCL macrochannels, and subsequent gelation produced cell-containing hydrogel-impregnated PCL scaffolds. While a PCL network preserves a rigid



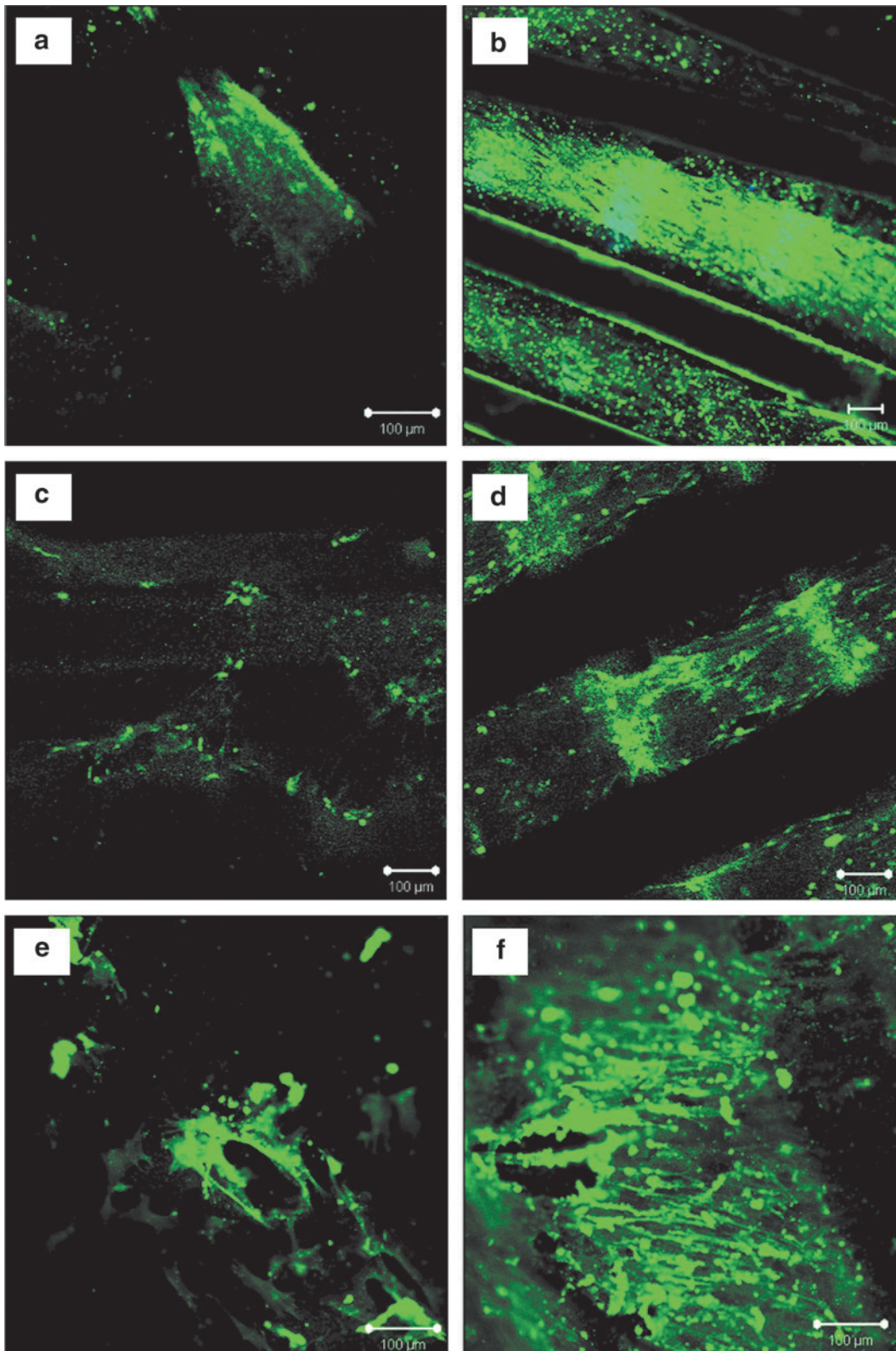


FIG. 8. CLSM immunofluorescent images of cells cultured under (a, c, e) static and (b, d, f) perfusion conditions showing green fluorescent staining of OPN (a, b), OCN (c, d), and BSP (e, f) expression after 10 days of culture. Fluorescence was qualitatively more pronounced in cells cultured under flow perfusion than those under a static culture condition.

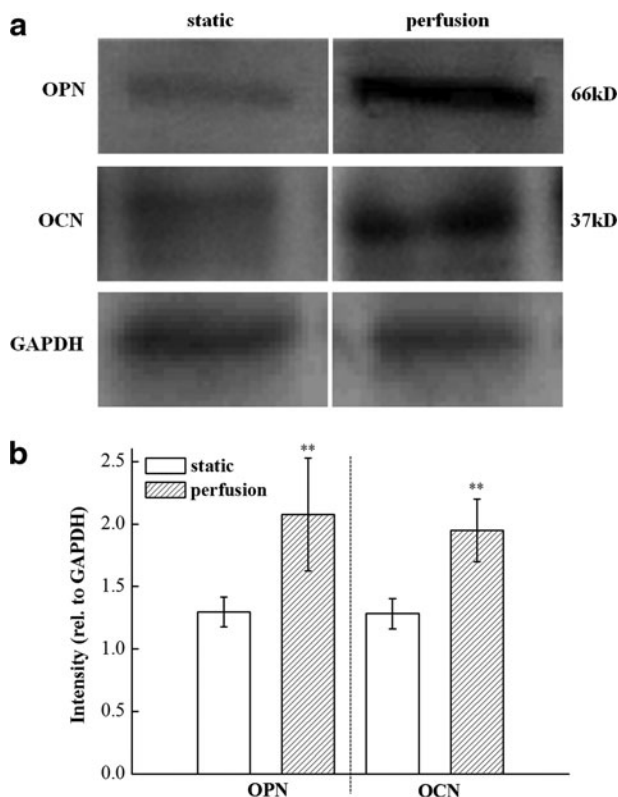


FIG. 9. Western blot analysis of OPN and OCN proteins during culture for 20 days: **(a)** protein expression bands (OPN at 66 kDa and OCN at 37 kDa, and glyceraldehyde 3-phosphate dehydrogenase used as a reference) and **(b)** quantification of band intensity by image densitometry. Of special note is the more distinct expression of both protein bands (especially OPN) under flow perfusion than that under static culture. Image densitometry analysis from two sets of experiments showed significantly higher expression levels under flow perfusion than those under static culture (** $p < 0.01$, $n = 2$).

framework for tissue-engineered constructs adaptable to the size and shape of a defect, collagen gels not only provide a means of cell loading but also provide microenvironments for cell proliferation and osteogenic differentiation.

Indeed, cell loading into collagen gels was as much as 50–60% more efficient than that of direct cell seeding (Fig. 3 at 24 h). After the gelation stage, we observed that the collagen gels were initially able to fill the PCL macrochannels, and the central part of the channel appeared to shrink toward the surface. This was presumably due to the exudation of water from the gel, as well as the surface tension exerted on the collagen, which resulted in a small gap in the central region of the infiltrated collagen. This was likely due the use of a relatively dilute collagen solution. When a much more viscous collagen solution was used, the gelled collagen preserved the initial solution volume quite well, filling the entire space without shrinkage during gelation. However, despite this slight shrinkage, cells appeared safely contained within the collagen gel and could undergo cellular processes such as spreading, proliferation, and differentiation during perfusion culture.

We next sought to culture the cell-loaded scaffolds under a dynamic flow perfusion condition. A 1-day culture after combining the MSC–collagen with the scaffolds was thought to be

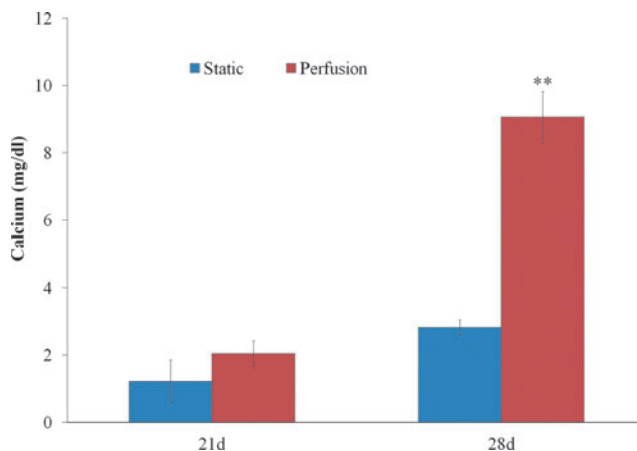
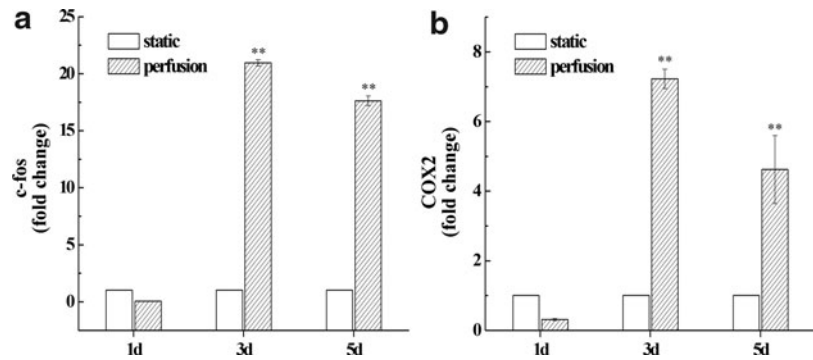


FIG. 10. Mineralization assay of the cells cultured under either static or perfusion conditions for 21 and 28 days. Ca content was determined quantitatively using a Calcium E-Test kit. The mineralization level was significantly higher in perfusion culture than in static at day 28 (** $p < 0.01$, $n = 3$).

enough time for cells to settle in the collagen hydrogel matrix. Constructs were then cultured under perfusion flow at a constant rate (0.6 mL/min), and the proliferation and differentiation behaviors were monitored relative to static culture control. Flow perfusion culture of cell-loaded 3D scaffolds has been utilized in creating a variety of tissues, including bone, cartilage, muscle, and blood vessels.^{14–16,26–30} Cells undergoing flow perfusion on 3D scaffolds have been shown to possess enhanced proliferative potential and exhibit better differentiation into specific tissues when biochemical factors are properly supplied.^{27–30} Scherberich et al. showed that flow perfusion of endothelial and osteoblastic progenitors plays a significant role in generating osteogenic constructs.²⁹ We have also observed a higher rate of proliferation of adipose-derived MSCs under constant perfusion, which further stimulates development into the osteogenic lineage over prolonged culture.⁸ The continuous, higher-volume input of culture medium, which can facilitate refreshing of exhausted cellular metabolites during culture, is considered the principal reason for improved cellular behavior.^{8,30} Moreover, mechanical factors such as stress, possibly exerted by perfusion flow on cellular compartments, including cell membranes, adhesion complexes, and the cytoskeleton, are believed to be the primary cause of altered cell behaviors, in contrast to those in static culture.³¹ Flow perfusion cultures require consideration of many design parameters, including scaffold pore geometry, perfusion rate, consequent microflow dynamics, and stress distribution in the cells (or even individual cells), to optimally develop engineered tissues.³²

Our results demonstrate the significant impacts of flow perfusion on the biological capacity of MSCs engineered within a combined scaffold system. Indeed, the proliferative potential of cells developing within the collagen gel supported on the channel surface was substantially enhanced by flow perfusion; cell proliferation in the collagen gel/PCL scaffold almost doubled during 14 days of culture with respect to that under static conditions (Fig. 4), suggesting that dynamic flow enhanced the metabolism and division of cells present in the collagen gels. Further culture up to 21

FIG. 11. Quantitative analysis of *c-fos* and COX-2 gene expression by QPCR; these genes are related to osteogenic differentiation downstream of mechanotransduction. Combined scaffold-MSC constructs were cultured for relatively short periods (1, 3, or 5 days) under static or flow perfusion conditions. QPCR analyses showed significantly enhanced induction of both genes after 3 and 5 days in flow perfusion culture compared to that under a static condition (** $p < 0.01$, $n = 3$). COX-2, cyclooxygenase-2.



days diminished differences between flow perfusion and static conditions, possibly indicative of a switch in the proliferative status of MSCs toward osteogenic differentiation.

Prolonged culture appears to alter collagen gel shape, as the collagen gel and cells moved toward the framework surface. Flow dynamics and the consequent distribution of stress should cause the relatively flexible collagen gel component to rearrange along the rigid PCL framework during culture, which could additionally alter cell positioning with respect to the collagen gel, complicating the physical microenvironments provided by the hydrogel matrix compared to those under static conditions.

The proliferative capacity of MSCs stimulated by perfusion culture allowed for more rapid and complete entry into an osteogenic differentiation. Expression of the relatively early-stage osteoblastic marker ALP was significantly enhanced after 14-day perfusion (Fig. 6), and that of key genes related to osteogenesis, such as *OPN*, *OCN*, and *BSP*, was significantly stimulated at 10 days by perfusion culture, although this was not the case at day 5 (Fig. 7). These results suggest that osteogenic differentiation commenced after 5–7 days in culture and became more pronounced in double that time. This apparent differentiation corresponded well to the proliferation assay results (Fig. 3), which indicated a slowing of proliferation roughly concomitant with our assessment of the initiation of differentiation. Positive immunofluorescent staining for these genes confirmed that differentiation occurred in the flow perfusion cultures after 10 days (Fig. 8). A large population of cells was strongly positive for all tested proteins, with more substantial staining detected for *OPN* and *BSP* than that for *OCN*, which is secreted during much later stages of osteogenic differentiation and mineralization³³; thus, it may require more prolonged culture time to see the effects on *OCN*. Protein secretion levels were subsequently confirmed by Western blot analysis after 20 days of extended culture, which permitted us to secure enough protein for assay. *OPN* and *OCN*, noncollagenous proteins characteristic of bone cells, demonstrated strong expression under flow perfusion culture (Fig. 9). Furthermore, the cellular mineralization was also significantly induced by the perfusion culture, particularly after prolonged cultures (4 weeks), as measured by the calcium quantification assay (Fig. 10). Overall, our *in vitro* results seem to demonstrate sequential differentiation and mineralization of MSCs through the osteogenic pathway within the combined collagen gel/PCL scaffolds.

Clearly, flow perfusion culture of MSCs within combined scaffolds significantly upregulated osteogenic differentiation and mineralization. We tried to elucidate the mechanism of

this stimulation based on the hypothesis that the mechanical/physical stresses associated with flow perfusion might play essential roles. We analyzed the gene expression of key factors downstream of mechanical signaling inputs, such as mechanotransduction, including *c-fos* and COX-2, by QPCR; these factors are believed to respond rapidly and accurately to mechanical loads sensed *in vitro* and *in vivo*.^{34,35} *c-fos*, an AP-1 subunit, is induced quickly in osteogenic cells upon mechanical stimulation. COX-2, an inducible isoform of cyclooxygenase, is a seminal enzyme in the production of prostaglandins in bone cells in response to mechanical stimuli *in vitro*.³⁶ Significant induction of *c-fos* and COX-2 mRNA, as well as their protein synthesis, has been reported with fluid shear stress or substrate-mediated tensile/compressive stress to osteoblast cell lines such as MG63 or MC3T3-E1.^{33,35,37,38} This stress-induced stimulation is linked to actin reorganization into stress fibers,³⁷ evidence of mechanotransmittance to major cellular physical compartments.³⁹ The stress-induced expression of COX-2 regulates MSC differentiation into the osteoblast lineage as well as the bone repair process.⁴⁰

Investigations into the cellular responses to mechanotransduction have principally been conducted in two-dimensional (2D) *in vitro* systems under conditions involving flow shear stress, substrate stretching, and hydraulic pressure.^{41–45} Very little research on cell mechanotransduction has been performed within 3D scaffold systems. Tanaka et al. investigated the effects of fluid flow together with mechanical load on osteoblast responses within 3D collagen and composite porous matrices.⁴⁶ As a result, fluid flow stimulated osteogenic development of MC3T3-E1 cells, including *c-fos* and COX-2 mRNA levels and COX-2 protein synthesis, when cells were under continuous loading.⁴⁶ Several other studies using 3D matrices such as Ti fiber mesh and human trabecular bone scaffolds also reported that hydrostatic shear stress enhances osteogenesis and bone matrix formation *in vitro*,^{47,48} underscoring the importance of the flow rate for the expression of key mechanotransduction genes, as this ultimately corresponds to stress levels meaningful for osteogenesis. It should also be noted that the stress applied to cells on the surface of 3D scaffolds varies widely depending on the scaffold pore configuration such as porosity, pore size, and pore connectivity. Therefore, flow rates presented in the literature as effective for osteogenesis are quite variable (0.1–3 mL/min). While the flow rate used in our study (0.6 mL/min) was well within this range, it is not directly comparable due to differences in scaffold design. McCoy et al. have reviewed the relevant shear stress levels for enhancing osteogenesis under 3D conditions and reported that they range between 1 and 10 mPa,

which were far lower than those observed under 2D conditions.⁴⁹ Computational shear stress modeling using the Lattice-Boltzmann equation or finite element analysis may be required in the future for our specific scaffold design to optimize culture conditions for tissue-engineered constructs.^{50,51} It is also essential to consider an additional element, particularly for our combined scaffold system. In contrast to cells directly seeded on a scaffold framework, the cells in our system were inside a collagen gel supported by the PCL scaffold. Therefore, fluid stress may not have acted directly on the cells, but instead was transmitted through the collagen gel matrix. The mechanical behaviors of the hydrogel, such as the compression and tension accompanying fluid flow, will influence cellular responses enormously. Stresses acting on cells within the collagen hydrogel may thus be somewhat lower than those applied directly. Additional research in this area will be required to determine optimal flow perfusion conditions specifically relevant to the current design of hydrogel-combined scaffolds for bone tissue engineering.

Conclusions

A novel bone tissue-engineering methodology was designed by constructing an MSC-collagen gel loaded within macrochanneled PCL scaffolds to improve cellular-loading efficiency into scaffolds and ensure a large population of cells. A dynamic flow perfusion system was then applied to provide microenvironments stimulating MSC proliferation and osteogenic differentiation. MSC-containing collagen gels were effectively combined with porous scaffolds, as cell-loading efficiency increased by 50–60% over direct cell seeding onto PCL scaffolds. Flow perfusion culture of the combined cell-scaffold constructs significantly improved proliferative potential of the MSCs. Moreover, osteogenic differentiation, as assessed by ALP activity, mRNA expression of bone-related genes (*OPN*, *OCN*, and *BSP*), and *OPN* and *OCN* protein levels, as well as cellular mineralization, were significantly upregulated by flow perfusion culture. We suggest that this was related to the mechanotransduction caused by perfusion flow, as revealed by the significant stimulation of *c-fos* and *COX-2* genes at the early culture stage. These results may be helpful in designs of bone tissue-engineering constructs using 3D porous scaffolds in concert with stem cells.

Acknowledgments

This study was supported by a Priority Research Centers Program (grant #: 2009-0093829) and the WCU (World Class University) Program (grant #: R31-10069) through the National Research Foundation (NRF) funded by the Ministry of Education, Science and Technology. The authors are thankful for the assistance of Dr. J.H. Lee with the cell assays.

Author Disclosure Statement

No competing financial interests exist.

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