J Mater Sci: Mater Med (2010) 21:2325–2330
DOI 10.1007/s10856-009-3966-z

Stimulation of osteoblasts using rest periods during bioreactor culture on collagen-glycosaminoglycan scaffolds

Sonia Partap · Niamh A. Plunkett · Daniel J. Kelly · Fergal J. O'Brien

Received: 29 August 2009 / Accepted: 2 December 2009 / Published online: 20 December 2009

Abstract Osteoblasts respond to mechanical signals which play a key role in the formation of bone however, after extended periods of stimulation they become desensitised. Mechanosensitivity has been shown to be restored by the introduction of resting periods between loadings. The aim of this study was to analyse the effect of rest periods on the response of osteoblast-like cells seeded on collagen-glycosaminoglycan (CG) scaffolds in a flow perfusion bioreactor up to 14 days. Short (10 s) and long (7 h) term rests were incorporated into stimulation patterns. Constructs cultured in the bioreactor had a more homogenous cell distribution albeit with lower cell numbers than the static group. Osteopontin expression was significantly higher on the rest-inserted group than on the steady flow and static control. These results indicate that the insertion of short term rests during flow improves cellular distribution and osteogenic responses on CG constructs cultured in a flow perfusion bioreactor.

S. Partap · N. A. Plunkett · F. J. O'Brien (✉)
Department of Anatomy, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland
e-mail: fjobrien@rcsi.ie

S. Partap
e-mail: soniapartap@rcsi.ie

N. A. Plunkett
e-mail: niamh.plunkett@gmail.com

S. Partap · N. A. Plunkett · D. J. Kelly · F. J. O'Brien
Department of Mechanical Engineering, School of Engineering, Trinity Centre for Bioengineering, Trinity College Dublin, Dublin 2, Ireland
e-mail: kellyd9@tcd.ie

1 Introduction

Bioreactors are used to influence biological processes by the application of a mechanical stimulus [1], as well as overcoming problems associated with static culture. These limitations include the tendency of cells to concentrate on the construct periphery leading to encapsulation and peripheral extracellular matrix formation which causes poor nutrient and waste exchange, and limited cell viability in the centre resulting in core degradation [2, 3]. In particular, flow perfusion bioreactors can be used to induce fluid flow throughout scaffolds; this allows for nutrient and waste exchange to occur to subsequently increase cell viability to deliver a more homogeneous construct [4, 5]. Simultaneously, they can be used to mechanically stimulate osteoblasts to accelerate the formation of increased bone matrix in scaffolds [6].

It is has been shown that osteoblasts respond to mechanical signals which plays a key role in the formation of bone [7, 8]; mechanical stimulation leads to an increase in proliferation and matrix synthesis [9, 10]. In vitro experiments in 2-D have shown fluid flow to have a number of effects on bone cells; the initiation of fluid flow induces a rapid and transient increase in cytosolic calcium, expression and regulation of numerous genes including COX-2, as well as the production and release of prostaglandin E_2 (PGE_2) into the culture medium [11]. Fluid flow has also been shown to stimulate expression of osteopontin (OPN) as well as increasing alkaline phosphatase (ALP) levels [2] ultimately enhancing mineralisation due to bioreactor culture [12].

However, it is reported that extended periods of continuous flow may hinder the development of an osteoblastic extracellular matrix because of (1) cell detachment [4], and (2) the loss of mechanosensitivity [13]. Firstly, fluid flow

reorganizes the actin cytoskeleton that may affect cell retention and cell viability, as well as blocking critical signaling pathways by depleting the cell microenvironment of secreted signaling factors (e.g. PGE_2 and ATP) [14]. Secondly, bone adapts to mechanical loading; in vivo studies have shown that bone can recover its responsiveness and is able to respond to mechanical stimuli with the same magnitude as earlier exposures to loading with the insertion of appropriate length rest periods [13]. Therefore, the aim of this study was to investigate the effect of rest periods during flow on the behaviour of osteoblast-like cells on collagen-glycosaminoglycan (CG) scaffolds up to 14 days using a flow perfusion bioreactor developed previously in our laboratory [15, 16].

2 Materials and methods

2.1 Scaffold fabrication

Collagen-GAG scaffolds were synthesised as described previously [17]. Briefly, type I collagen from bovine tendon (Integra Life Sciences, Plainsboro, NJ) and chondroitin-6-sulfate from shark cartilage (Sigma–Aldrich, Dublin, Ireland) were dispersed in an aqueous acetic acid solution (0.05 M). The slurry was lyophilised for 24 h using a final freezing temperature of −40°C. The scaffolds were sterilized using a dehydrothermal treatment for 24 h at 105°C. Discs (12.7 mm diameter × 3.5 mm thickness) were then crosslinked with an aqueous solution of 14 mM *N*-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC) and 5.5 mM *N*-Hydroxysuccinimide (NHS) for 2 h [18].

2.2 Construct pre-culture

Scaffolds (12.7 mm diameter × 3.5 mm thickness) were seeded with a total of 2×10^6 MC3T3-E1 pre-osteoblast cells. The cells were detached from T175 flasks with Trypsin–EDTA (Sigma–Aldrich, Dublin, Ireland) and suspended in α-MEM supplemented with 2% penicillin/streptomycin, 1% L-glutamine, 10% fetal bovine serum and 0.1% amphotericin (Sigma–Aldrich, Ireland, Dublin) at 10×10^6 cells/ml. In a 6-well plate, each scaffold was seeded drop-wise with 100 µl of the cell suspension. The 6-well plate was then placed in the incubator for 15 min to allow for cell attachment. After this time, the scaffolds were turned over and another 100 µl of the cell suspension was added drop-wise. The scaffolds were returned to the incubator for a further 15 min. After this second 15 min incubation period 5 ml of media was added to each well. The scaffolds were pre-cultured in static conditions (37°C and 5% CO_2) for 6 days after which point the constructs were cultured in the flow perfusion bioreactor or static culture conditions for 1 h, 49 h, 4, 7 or 14 days. For experiments carried out up to 49 h constructs ($n = 8$) were cultured in α-MEM supplemented with 2% penicillin/streptomycin, 1% L-glutamine, 10% fetal bovine serum and 0.1% amphotericin (Sigma–Aldrich Ireland, Dublin) to allow for cell attachment, infiltration and proliferation. For constructs cultured up to 14 days ($n = 6$) the media was replaced with osteogenic media on the 3rd day of the pre-culture period which was supplemented with 50 µg/ml ascorbic acid and 10 mM β-glycerol phosphate to encourage the cells to deposit matrix, and was used thereafter until the end of the experiment.

2.3 Bioreactor culture

Constructs were stimulated with flow patterns incorporating short and long term rest periods. Short term periods of no flow were incorporated into 1 h bouts of stimulation. They were of duration: 0 (steady flow group) and 10 s (rest-inserted group) and were inserted between bouts of 10 s of 1 ml/min flow. This hour of stimulation was followed by a 7 h long term rest period. This 8 h cycle was repeated for the duration of the culture period. Media in the reservoir was replaced with fresh osteogenic media every 2–3 days. Two different control groups were used; (1) a static control group, and (2) a bioreactor control in which constructs were placed into the bioreactor and immediately removed. This group was used to examine the effect of the process of setting up the bioreactor on cellular activity. After the culture period constructs were either flash frozen or fixed in formalin.

2.4 Analysis

2.4.1 DNA quantification

Cell number was determined by the quantification of the double stranded DNA from lysate obtained using the RNeasy mini kit (Qiagen) [15], 10 µl of the lysate was mixed with 200 µl of Hoechst 33258 dye (Sigma–Aldrich) and fluorescence was measured (excitation: 355 nm, emission: 460 nm; Wallac Victor^{2TM}, PerkinElmer, Waltham, MA) on samples in triplicate in a 96-well plate. Readings were converted into cell number using a standard curve.

2.4.2 Gene expression

RNA was extracted using a RNeasy mini kit (Qiagen, USA) according to manufacturer instructions. The quality and concentration of the RNA was quantified by measuring

absorbance at 260 nm (GeneQuant Pro RNA/DNA calculator, Biochrom Ltd., UK). Reverse transcription was performed on 400 ng of total RNA using the QuantiTect RT Kit (Qiagen) according to the manufacturer's instructions. RT–PCR was subsequently performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions with QuantiTect Primers (designed by Qiagen). Results were quantified for COL-1 α1, ALP and OPN via the relative quantification ($\Delta\Delta$Ct) method using 18-S rRNA as the endogenous reference. For each gene, results are expressed relative to the bioreactor control group. All PCR reactions were conducted in triplicate for each sample.

2.4.3 *Histological analysis*

Constructs were embedded in paraffin wax using an automatic tissue processor (ASP300, Leica, Wetzlar, Germany) and sectioned on a microtome (RM2255, Leica). 10 μm sections were processed and stained with Haematoxylin and Eosin (H&E) and digital images were captured on a microscope (Optimphot2, Nikon, Japan).

2.5 Statistical analysis

Results are expressed as mean ± standard deviations (SD). Statistics were done in SigmaStat 3.0 (SPSS, Chicago, IL) using a general linear model ANOVA with the Holm–Sidak post-hoc multiple comparison test. Statistical significance was taken at $P < 0.05$.

3 Results

Significantly higher cell numbers were observed on statically cultured constructs compared to the flow groups at all time points ($P \leq 0.025$). For static culture, all scaffolds retained more than 2×10^6 cells, whereas constructs cultured in the bioreactor maintained ca. $0.5–1 \times 10^6$ cells. There was no significant difference in cell number between the flow groups (Fig. 1).

Histological analysis revealed a difference in the cellular distribution of constructs that were cultured under static conditions compared to the bioreactor. H&E staining showed that there were more cells on the periphery of static constructs with few or no cells in the centre (encapsulation effect), compared to constructs exposed to flow where the cells appeared to have been detached from the edges and surfaces (Fig. 2a), which is the case for both of the bioreactor (steady and rest-inserted) groups. However, despite the loss of cells from the periphery, more cells had infiltrated further into the centre of constructs exposed to flow. At day 14 the encapsulation effect was more pronounced for the static constructs (Fig. 2b).

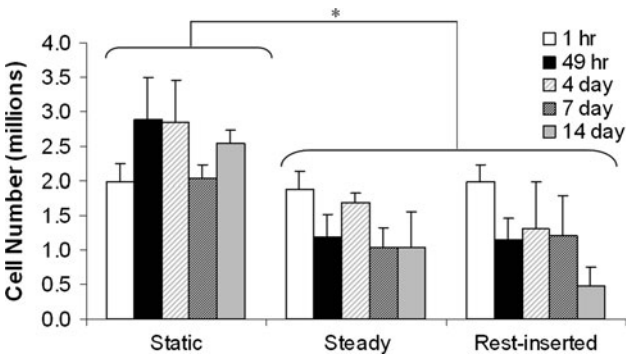

Fig. 1 Cell number on all groups, * represents $P \leq 0.025$. A decrease in cell number was observed on constructs cultured in the bioreactor

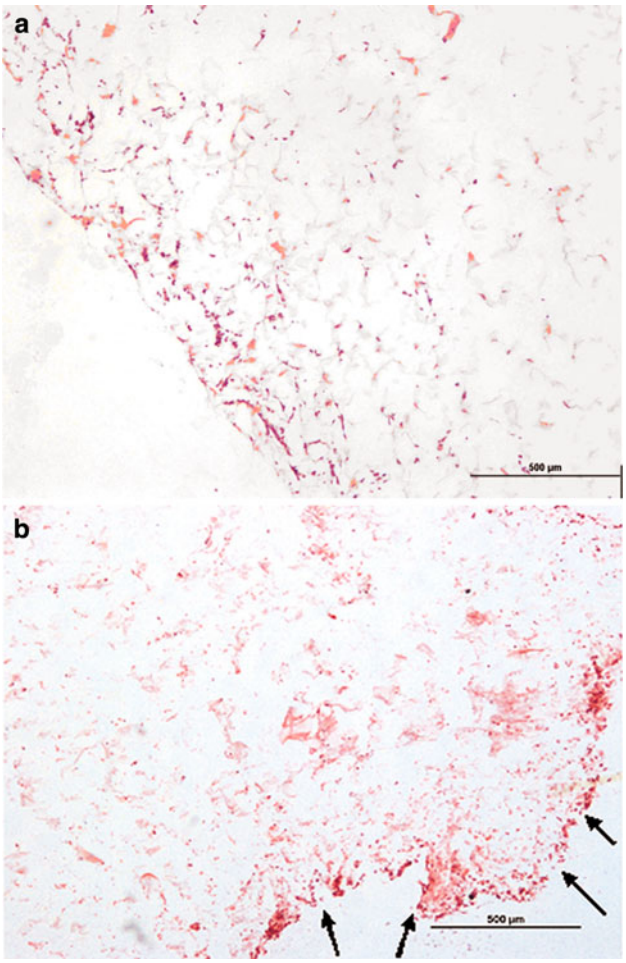

Fig. 2 Transverse Haematoxylin & Eosin images of constructs after 14 days of **a** bioreactor culture (rest-inserted flow) and **b** static culture. The scale bar is 500 μm in length. Note the formation of an external capsule on the static group which is not observed in the rest-inserted flow group as highlighted by the *arrows*

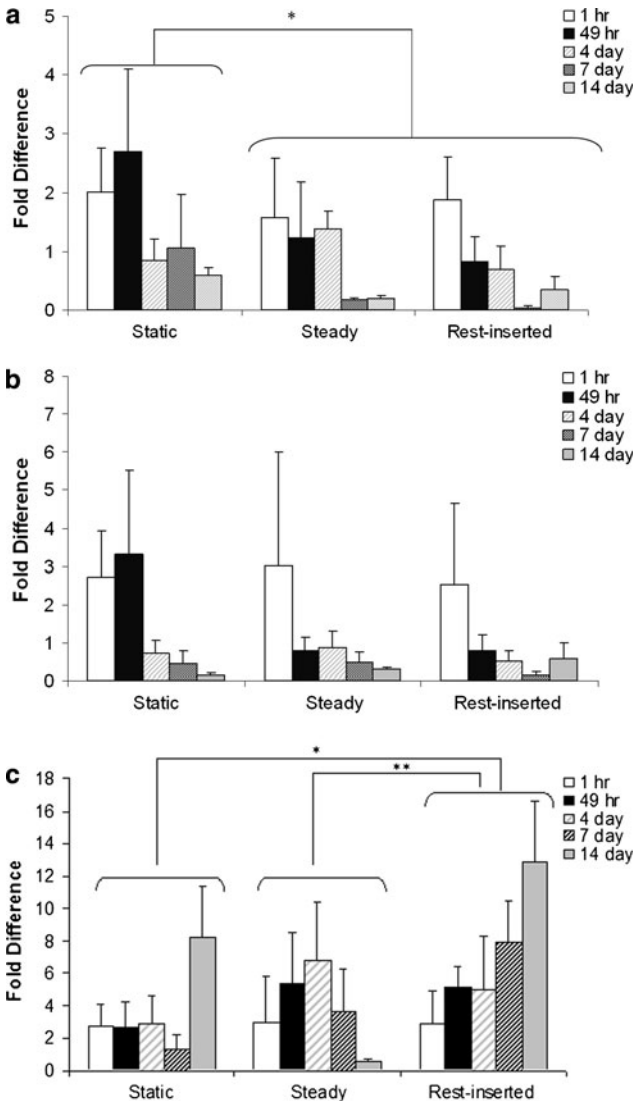

Fig. 3 Gene expression of COL-1. * Represents $P \leq 0.017$. Bioreactor groups showed significant decreases in COL-1 expression compared to the static group. **b** Gene expression of ALP. ALP expression decreased from 1 h to day 14 for all groups whilst there are no differences in its expression due to culture conditions. **c** Gene expression of OPN. * Represents $P \leq 0.017$ and ** represents $P \leq 0.025$. A significant increase in OPN expression was observed for the rest-inserted group in comparison to the static and steady groups. All groups are scaled to the bioreactor control group

The increase or decrease in gene expression was calculated as a fold change compared to the bioreactor control group (which was scaled to 1). COL-1 and ALP expression decreased from 1 h to day 14 for all groups ($P \leq 0.07$, Fig. 3a, b). However, the static control showed significantly higher expression of COL-1 than the flow groups ($P \leq 0.025$, Fig. 3a). Osteopontin (OPN) expression (Fig. 3c) peaked at day 14 for the static group (1 h vs. 14 days, $P \leq 0.005$); peaked at day 4 for the steady group, whereas the rest-inserted group showed a steady continuous increase up to day 14 (1 h vs. 14 days, $P \leq 0.005$). Overall, OPN levels were higher on the rest-inserted group than the steady group and static control ($P \leq 0.025$).

4 Discussion

The aim of this study was to investigate the response of osteoblasts to short term rest periods with regards to cell distribution, cell number and expression of a number of osteogenic genes on collagen-GAG constructs cultured up to 14 days in a flow perfusion bioreactor. The results show that bioreactor culture improved cell distribution compared to statically cultured constructs and enhanced expression of osteogenic markers with the rest-inserted group showing the most encouraging results with regards to osteopontin (OPN) expression.

In vivo studies have shown that mechanical loading of bone is a potent stimulus for new bone formation [13]. However, even though osteoblasts are stimulated by mechanical loading they also adapt to their mechanical environment. It has been shown that shorter more frequent loading sessions separated by adequate rest periods provide a greater osteogenic response than continuous loading [13, 19]. Therefore, we hypothesised that both short (10 s) and long (7 h) term recovery periods during which mechanical loading were halted would restore bones sensitivity to mechanical stimuli, prevent cell detachment and not deplete the cell microenvironment of the necessary regulatory molecules to preserve the signalling pathways, whilst flow would stimulate the cells and provide nutrient and waste exchange to occur.

Higher cell numbers were observed on statically cultured constructs compared to those constructs cultured in the bioreactor. This trend has also been observed by other groups [20, 21]. Despite the reduction in cell numbers seen for flow groups, cellular distribution was more homogeneous as has been seen in numerous other studies [4, 12, 15], and more cells had infiltrated further into the centre of constructs. In the statically cultured constructs cellular encapsulation of the scaffold was observed where the cells were concentrated along the periphery of the construct, a phenomenon that was lessened in bioreactor culture. This may be indicative of cells preferentially being sheared off the surfaces and edges of the constructs under flow conditions. However, it is worth noting that despite the significant decrease in cell number due to flow, there are still up to ca. 1 million cells on the constructs after the 14 day culture period. By removing cells concentrated on the periphery and increasing cell viability in the centre of the scaffold, it is envisaged that a more homogeneous scaffold may develop preventing core degradation occurring in vitro.

Therefore, the decrease in cell number seen under bioreactor culture may potentially be of benefit to tissue development.

Significant changes in the expression of genes associated with bone formation were observed in response to the different stimulation patterns used. Collagen I (COL-1) expression decreased for all groups by day 14 compared to 1 h. By day 14, both flow groups (steady and rest inserted) showed a significant decrease in COL-1 expression compared to the static group. COL-1 is expressed during proliferation in 2-D culture, is then gradually downregulated but is expressed at low levels throughout osteoblast differentiation and maturation [22]. The results suggests that culture in the bioreactor may either be downregulating this gene or accelerating osteoblast maturation. A similar trend for Alkaline Phosphatase (ALP) expression was observed as it also decreased by day 14 for all groups. ALP expression peaked at 49 h for the static group and 1 h for the flow groups. Including the 6 day pre-culture period, constructs were actually in culture for a period of 8 and 6 days for static and bioreactor culture respectively. ALP expression generally increases after the proliferation phase [22]. Proliferation may have been halted due to bioreactor culture and the reduced levels of ALP expression may be indicative of the end of the proliferative phase. Expression of osteopontin (OPN) increased from 1 h to day 14 for all groups, however, the rest-inserted group showed significant increases in expression compared to both the static and steady flow groups. This trend has also been observed in 2-D when rest-inserted flow was used [23]. OPN is a late stage marker in the mechanotransduction cascade, it regulates bone cell attachment and mineralisation [11] and is important in bone remodelling [24]. Taken together, increased OPN expression coupled with decreased COL-1 expression may indicate that bioreactor culture has enhanced expression of post proliferative genes at the expense of those found during proliferation.

In summary, a flow perfusion bioreactor (1) mechanically stimulated osteoblasts and (2) improved cell distribution throughout the construct. COL-1 expression decreased and OPN expression increased due to bioreactor culture, whilst ALP was downregulated on all groups. OPN was upregulated significantly on the rest-inserted group compared to the static control or steady flow group. These results indicate that the insertion of rest periods during flow improves osteogenic responses on CG constructs cultured in a flow perfusion bioreactor.

Acknowledgments Funding for this study was provided by Science Foundation Ireland (President of Ireland Young Researcher Award, 04/Yl1/B531), Research Frontiers Programme (07/RFP/ENMF142) and the Irish Research Council for Science, Engineering and Technology (RS/2005/173). Collagen materials were provided by Integra Life Sciences, Inc, through a Materials Transfer Agreement.

References

1. Darling EM, Athanasiou KA. Biomechanical strategies for articular cartilage regeneration. Ann Biomed Eng. 2003;31:1114–24.
2. Goldstein AS, Juarez TM, Helmke CD, Gustin MC, Mikos AG. Effect of convection on osteoblastic cell growth and function in biodegradable polymer foam scaffolds. Biomaterials. 2001;22:1279–88.
3. Yu X, Botchwey EA, Levine EM, Pollack SR, Laurencin CT. Bioreactor-based bone tissue engineering: the influence of dynamic flow on osteoblast phenotypic expression and matrix mineralization. Proc Natl Acad Sci USA. 2004;101:11203–8.
4. Cartmell SH, Porter BD, Garcia AJ, Guldberg RE. Effects of medium perfusion rate on cell-seeded three-dimensional bone constructs in vitro. Tissue Eng. 2003;9:1197–203.
5. Glowacki J, Mizuno S, Greenberger JS. Perfusion enhances functions of bone marrow stromal cells in three-dimensional culture. Cell Transplant. 1998;7:319–26.
6. Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA, et al. Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci USA. 2002;99:12600–5.
7. Rubin J, Rubin C, Jacobs CR. Molecular pathways mediating mechanical signaling in bone. Gene. 2006;367:1–16.
8. Papachroni KK, Karatzas DN, Papavassiliou KA, Basdra EK, Papavassiliou AG. Mechanotransduction in osteoblast regulation and bone disease. Trends Mol Med. 2009;15:208–16.
9. Klein-Nulend J, Bacabac RG, Mullender MG. Mechanobiology of bone tissue. Pathol Biol (Paris). 2005;53:576–80.
10. Sikavitsas VI, Bancroft GN, Holtorf HL, Jansen JA, Mikos AG. Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces. Proc Natl Acad Sci USA. 2003;100:14683–8.
11. You J, Reilly GC, Zhen X, Yellowley CE, Chen Q, Donahue HJ, et al. Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts. J Biol Chem. 2001;276:13365–71.
12. Janssen FW, Oostra J, Oorschot A, van Blitterswijk CA. A perfusion bioreactor system capable of producing clinically relevant volumes of tissue-engineered bone: in vivo bone formation showing proof of concept. Biomaterials. 2006;27:315–23.
13. Robling AG, Hinant FM, Burr DB, Turner CH. Improved bone structure and strength after long-term mechanical loading is greatest if loading is separated into short bouts. J Bone Miner Res. 2002;17:1545–54.
14. Kreke MR, Sharp LA, Lee YW, Goldstein AS. Effect of intermittent shear stress on mechanotransductive signaling and osteoblastic differentiation of bone marrow stromal cells. Tissue Eng Part A. 2008;14:529–37.
15. Jaasma MJ, O'Brien FJ. Mechanical stimulation of osteoblasts using steady and dynamic fluid flow. Tissue Eng Part A. 2008;14:1213–23.
16. Jaasma MJ, Plunkett NA, O'Brien FJ. Design and validation of a dynamic flow perfusion bioreactor for use with compliant tissue engineering scaffolds. J Biotechnol. 2008;133:490–6.
17. O'Brien FJ, Harley BA, Yannas IV, Gibson L. Influence of freezing rate on pore structure in freeze-dried collagen-GAG scaffolds. Biomaterials. 2004;25:1077–86.
18. Lee CR, Grodzinsky AJ, Spector M. The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. Biomaterials. 2001;22:3145–54.

19. Robling AG, Burr DB, Turner CH. Recovery periods restore mechanosensitivity to dynamically loaded bone. J Exp Biol. 2001;204:3389–99.
20. Vance J, Galley S, Liu DF, Donahue SW. Mechanical stimulation of MC3T3 osteoblastic cells in a bone tissue-engineering bioreactor enhances prostaglandin E2 release. Tissue Eng. 2005;11:1832–9.
21. Grayson WL, Bhumiratana S, Cannizzaro C, Chao PH, Lennon DP, Caplan AI, et al. Effects of initial seeding density and fluid perfusion rate on formation of tissue-engineered bone. Tissue Eng Part A. 2008;14:1809–20.
22. Stein GS, Lian JB. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev. 1993;14:424–42.
23. Batra NN, Li YJ, Yellowley CE, You L, Malone AM, Kim CH, et al. Effects of short-term recovery periods on fluid-induced signaling in osteoblastic cells. J Biomech. 2005;38:1909–17.
24. You J, Yellowley CE, Donahue HJ, Zhang Y, Chen Q, Jacobs CR. Substrate deformation levels associated with routine physical activity are less stimulatory to bone cells relative to load-induced oscillatory fluid flow. J Biomech Eng. 2000;122:387–93.