Mechanical Stimulation Promotes Osteogenic Differentiation of Human Bone Marrow Stromal Cells on 3-D Partially Demineralized Bone Scaffolds *In Vitro*

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Abstract. Bone is a dynamic tissue that is able to sense and adapt to mechanical stimuli by modulating its mass, geometry, and structure. Bone marrow stromal cells (BMSCs) are known to play an integral part in bone formation by providing an osteoprogenitor cell source capable of differentiating into mature osteoblasts in response to mechanical stresses. Characteristics of the *in vivo* bone environment including the three dimensional (3-D) lacunocanalicular structure and extracellular matrix composition have previously been shown to play major roles in influencing mechanotransduction processes within bone cells. To more accurately model this phenomenon *in vitro*, we cultured human BMSCs on 3-D, partially demineralized bone scaffolds in the presence of four-point bending loads within a novel bioreactor. The effect of mechanical loading and dexamethasone concentration on BMSC osteogenic differentiation and mineralized matrix production was studied for 8 and 16 days of culture. Mechanical stimulation after 16 days with 10 nM dexamethasone promoted osteogenic differentiation of BMSCs by significantly elevating alkaline phosphatase activity as well as alkaline phosphatase and osteopontin transcript levels over static controls. Mineralized matrix production also increased under these culture conditions. Dexamethasone concentration had a dramatic effect on the ability of mechanical stimulation to modulate these phenotypic and genotypic responses. These results provide increased insight into the role of mechanical stimulation on osteogenic differentiation of human BMSCs *in vitro* and may lead to improved strategies in bone tissue engineering.

Key words: Bioreactor — Tissue engineering — Biomaterial

Bone is a dynamic tissue that is able to sense and adapt to mechanical stimuli by modulating its mass, geometry, and structural strength [1, 2]. During skeletal loading, a variety of tissue level perturbations are generated including local deformations of the bone matrix [3], the creation of shear stresses due to load-induced fluid flow [4, 5], and the induction of electric fields caused by stress-generated streaming potentials [6, 7]. Each of these loading effects has been shown to influence various osteogenic responses of bone-derived cells (e.g., osteoblasts and osteocytes) and their progenitors *in vitro* [8–12].

A number of *in vitro* systems have been developed to simulate *in vivo* loading environments that have utilized primarily two dimensional (2-D) mechanical strains and/or fluid flows to stimulate bone cells in culture [13–20]. These systems may not, however, represent ideal environments for studying mechanotransduction responses of bone cells, since they lack both the 3-D cellular network and the lacunocanalicular structure of native bone, which in combination have been shown to play major roles in influencing these mechanisms [21]. In addition, bone cells are known to exhibit variable responses *in vitro* to mechanical stimuli depending on the type of material to which they are attached [22]. This behavior is due in part to the properties of the cell–material interface which can influence the ability of cells to transmit biochemical signals from the extracellular matrix to cell surface receptors in response to mechanical stimulation [22, 23]. Therefore, it is important that *in vitro* approaches for mechanical stimulation of bone cells or their progenitors utilize substrates that mimic the properties of native bone matrix in order to accurately model the *in vivo* responses of these cells.

Recently, the field of tissue engineering has utilized mechanical stimulation as a tool for promoting the development of a number of tissue types including cartilage [24], ligament [25], skeletal muscle [26], cardiac muscle [27], and bone [28, 29] *in vitro*. Bone marrow stromal cells (BMSCs) have been identified as an

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attractive cell source for a wide variety of tissue engineering strategies, since they contain a pluripotent population of cells capable of differentiating along multiple mesenchymal lineages (e.g., bone [30, 31], ligament [25, 32], adipose [33], cartilage [34], muscle [35] tissue), and can be easily expanded *ex vivo* utilizing routine cell culture techniques [36]. It has been established in previous studies that BMSCs can upregulate markers specific for osteogenic differentiation through mechanical stimulation *in vitro* [37–40]. These data suggest that this type of stimulation may be useful in tissue engineering of new osseous biomaterials that incorporate this cell source. However, since these previous studies utilized BMSCs in 2-D cell culture environments and/or cell scaffolds derived from nonosseous materials, they may not represent optimal environments for osteogenic differentiation by mechanical stimulation. Therefore, this phenomenon warrants further study in order to develop *in vitro* systems that more accurately simulate the *in vivo* environment of bone tissue.

The goal of the present study was to test the hypothesis that mechanical stimulation can promote osteogenic differentiation of human BMSCs within a 3-D partially demineralized bone (DMB) scaffold *in vitro*. We selected this 3-D matrix because it supports osteogenic differentiation of human BMSCs in medium supplemented with dexamethasone [41] and is derived from native bone matrix. Therefore, in conjunction with this model, we have developed a novel bioreactor capable of providing a four-point bending loading regimen during tissue cultivation, which in previous studies has been demonstrated to enhance *in vivo* bone formation [42–45]. Osteogenic differentiation was examined at the biochemical and molecular levels to characterize both phenotypic and genotypic responses to mechanical stimulation.

Materials and Methods

Tissue Culture

Primary Cell Culture

Human BMSCs were obtained from commercially available bone marrow aspirates from donors ≤ 25 years of age (Clonetics-Poietics) by using methods previously reported [25]. Whole bone marrow aspirates were plated at 8–10 μL/cm^2 on 185 cm^2 tissue culture plates and cultivated until confluency (~12–14 days) in 40 mL of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizone, and 1 ng/mL of basic fibroblast growth factor (bFGF) (Life Technologies, Rockville, MD). BMSCs were selected on the basis of their ability to adhere to the tissue culture plastic; nonadherent hematopoietic cells were removed during medium replacement after approximately 5 days in culture. Medium was changed twice per week thereafter. BMSCs were subsequently detached using 0.25% trypsin/1 mM ethylene diamine tetraacetic acid (EDTA) replated at 5×10^3 cells/cm^2, and cultured as first passage cells (P1) until confluency. First passage (P1) BMSCs were trypsinized and replated at 5×10^3 cells/cm^2 to yield second passage cells (P2), which were trypsinized near confluency and subsequently used for scaffold seeding.

#### Partially Demineralized Bone Scaffolds

Bovine cancellous bone was harvested from the femoral condyles of commercially obtained 2 to 3-year-old Angus steers (City Packing, Boston, MA). The samples were cleaned of residual tissue and sectioned into rectangular beams (length × width × height), (63 mm × 6 mm × 6 mm) The partially DMB scaffolds were prepared by using a modification of methods reported previously [41, 46]. Briefly, samples were incubated in anhydrous ethyl ether for 12 hours, partially demineralized in 0.6 N HCl for 15 minutes (50 mL/g of bone), and rinsed in distilled water for 2 hours. The scaffolds were then air-dried for 24 hours, sterilized in ethylene oxide for 16 hours at 4°C, and stored at 4°C until seeding.

Dynamic Cell Seeding

Partially DMB scaffolds were seeded with BMSCs in well-mixed spinner flasks by using a modification of methods reported previously [41, 47]. Scaffolds were threaded 1-mm deep onto 10-cm, 20-gauge stainless steel needles, embedded in a Fischer no 12 rubber stopper, and suspended in Bellco 250-mL spinner flasks. Four scaffolds per spinner flask were utilized. The flasks were filled with 350 mL, of α-MEM and placed into a vacuum chamber for 1 hour at 100 μm of Hg to remove residual air within the matrix. Spinner flasks were inoculated with 80×10^6 cells (2×10^5 cells/mL) in 400 mL of α-MEM supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone, and stirred with a 0.8-cm diameter × 4-cm long nonsuspended stir bar at 50 rpm in a humidified 37°C, 5% CO$_2$ incubator for 12 hours.

Bioreactor

A schematic view of the bioreactor is presented in Figure 1A. Mechanical loading of the partially DMB scaffold within the bioreactor is controlled with an Instron Model 8511 Material Tester, which consists of a computer-controlled, servomotor-driven linear actuator assembly with an interface controller that modulates vertical position and velocity. The linear actuator is coupled to a compression load cell that transmits a linear force through the external shaft to the transduction beam, which provides line contacts across the lateral face of the scaffold at axial distances of 19 mm from the center. The construct is supported by stationary bending supports positioned at 30 mm from its center in both positive and negative axial directions across the medial face. Prior to loading, the external shaft is allowed to contact the load cell by applying approximately 2 N of force; this relative position of the system is then defined as the minimum external displacement. The maximum external displacement of the linear actuator is then preset into the interface controller, and loading is accomplished by modulating between the maximum and minimum relative positions. During loading, the bioreactor translates the force of the linear actuator into four-point bending of the construct via the transduction beam and bending support contacts. The segment of the construct located within the transduction beam contacts is subjected to a uniform strain within each horizontal plane perpendicular to the applied load as predicted by theoretical four-point bending analysis [48]. However, the strain magnitude between these planes is predicted to increase proportionally from the central horizontal axis to maximum tensile and compressive stains at the external medial and lateral faces [48]. Gas and medium exchange are performed through sterile ports on the top of the bioreactor. An individual bioreactor vessel mechanically stimulated within the Instron Material Tester is displayed in Figure 1B.
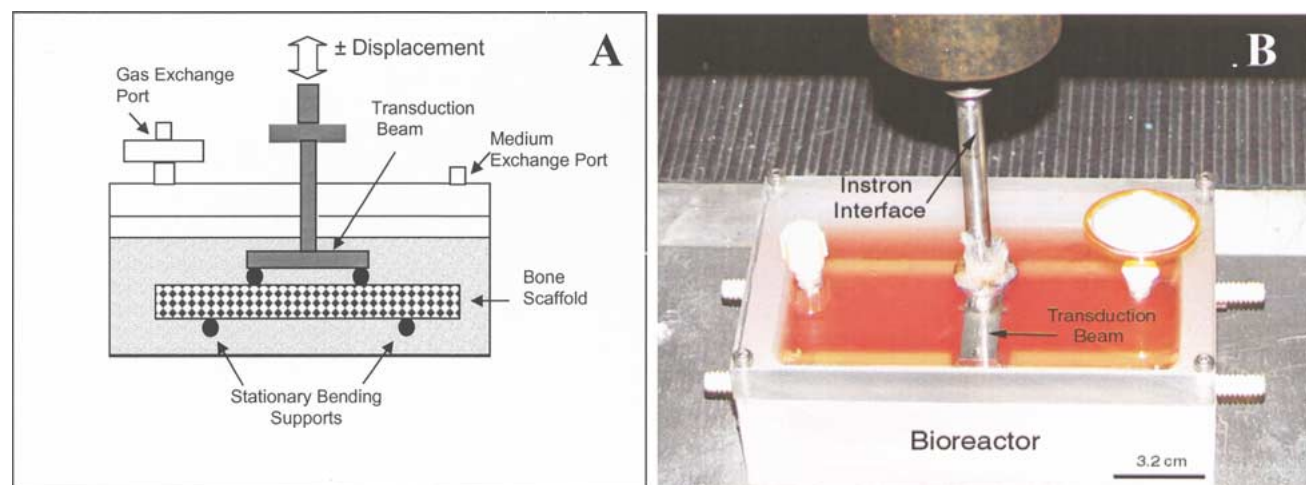
Fig. 1. (A) Schematic of bioreactor and internal components. (B) Photograph of bioreactor during mechanical stimulation within the Instron Material Tester.

Bioreactor Cultivation and Mechanical Loading

Seeded scaffolds were cultured for 8 and 16 days within independently maintained bioreactors containing 120 mL of α-MEM supplemented with 10% FCS, 100 u/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizone, 10 mM β-glycerophosphate (βGP), and 0.05 mM ascorbic acid-2-phosphate at 37°C in 5% CO_2 in a humidified incubator. The effects of various dexamethasone concentrations previously shown to induce human BMSC osteogenic differentiation were investigated with mechanical stimulation to determine the role of this biochemical factor within our system [41, 49]. Three experimental groups were established by using medium containing 0 nM (group I), 10 nM (group II), or 100 nM (group III) dexamethasone. Each group was further subdivided into samples subjected to mechanical stimulation and unloaded static controls. Medium exchange was accomplished every 4 days of culture. Loading was applied in a triangular waveform at a rate of 5 mm/minute with a maximum external displacement of 0.2 mm for 250 cycles every 24 hours throughout the entire culture period. Maximum external displacement and loading rate were preset within the external Instron controller and maintained during stimulation. Following cultivation, the uniform bending region of each scaffold located between the transduction beam line contacts was dissected along the central axis perpendicular to the applied load into five equivalent sections for analytical analysis as described in the next section.

Analytical Methods

Mechanical Properties

Strain magnitudes at the external medial and lateral planes of the partially DMB scaffolds were measured during bioreactor loading by using modifications of methods reported previously for strain characterization of animal femurs subjected to four-point bending loads [42, 43, 50]. The surfaces of the partially DMB scaffolds were cleaned with alcohol and then air-dried. Single-element strain gauges (Measurements Group, Raleigh, NC) were connected in a Wheatstone bridge and glued (M-bond AE-10) to the medial and lateral surfaces. The gauges were aligned directly in the center of the construct, equidistant from the transduction beam line contacts and parallel to the longitudinal axis. The scaffolds were placed in the bioreactor, and the external shaft was allowed to contact the load cell by applying force of approximately 2 N. Strains on the external medial and lateral surfaces of the scaffolds during external loading were then measured at five external displacements (0, 0.2, 0.4, 0.6, and 0.8 mm) at a rate of 5 mm/minute for three cycles. The average peak strains on the medial and lateral surfaces were then recorded with a Strain Indicator P-3500 Device (Measurements Group).

Cell Numbers

The relative number of metabolically active cells within each scaffold section was determined by a 3-4,5 dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [41]. Briefly, sections were rinsed with PBS immediately after harvest and incubated in 10 mL of 0.5 mg/mL MTT in culture media for 2 hours at 37°C in 5% CO_2. Sections were then immersed in 2 mL of 0.1 N HCl/isopropanol for 12 hours at 37°C for crystal dissolution. Aliquots (100-μL) were quantified spectrophotometrically for optical density in 96-well plates at 570 nm by a microplate reader (Spectramax 250, Molecular Devices). Relative cell numbers were expressed as the ratio of MTT absorbance units per scaffold section.

Alkaline Phosphatase Activity

Scaffold sections were assayed for the presence of alkaline phosphatase activity as described previously [41]. Sections were crushed and transferred to 1.5 mL of 0.2% Igepal CA 630 detergent with 5 mM $MgCl_2$. Homogenization was carried out in a Minibead beater (Biospec Inc.) with three stainless steel beads (3.1 mm in diameter) for 10-second intervals at 50,000 rpm for a total of 12 cycles. The samples were immersed in a 4°C water bath for 30 seconds between cycles to remove excess heat and to avoid enzyme denaturation. Following homogenization, samples were centrifuged at 13,000 rpm for 10 minutes 4°C. The supernatant was assayed for alkaline phosphatase using p-nitrophenyl phosphate as a substrate [51]. Briefly, 80 μL of the supernatant was added to 100 μL of 5 mM p-nitrophenylphosphate in 150 mM 2-amino-2-methlyl-1-propanol buffer solution (Sigma) and incubated for 1 hour at 37°C. A 100-μL aliquot of 0.2 N NaOH was added to stop the enzymatic reaction, and the absorption at 405 nm was measured spectrophotometrically by a spectramax 250 microplate reader (Molecular Devices). Alkaline phosphatase activity per sample was normalized to the relative cell number and displayed as relative activity.

Total RNA Isolation and Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was isolated from scaffold sections by first homogenizing samples in 1.5 mL of Trizol reagent (Life Technologies) and then by using the RNEasy Mini Kit system (Qiagen) as described previously [41, 52]. (RT-PCR) analysis of bone-specific protein expression for alkaline phosphatase, osteopontin, osteocalcin, and the housekeeping gene GAPDH was carried out using the Access RT-PCR system (Promega). PCR reaction mixtures consisted of 2 μL of total RNA (0.033–33 ng) combined with 31 μL distilled water of 5X AMV/Tfl reaction buffer, 1 μL of 10 mM dNTPs, 2 μL of 25 mM MgSO$_4$, 1 μL of 5U/μL of AMV reverse transcriptase, 1 μL of 5U/μL Tfl DNA polymerase, and 50 pm of the respective forward and reverse primers. Primers were designed with Primer Select software from genes with the following Genebank Accession numbers: osteocalcin, X04143; osteopontin, D14813; and GAPDH, J04038. Alkaline phosphatase primers were used as previously reported [53]. Primers were as follows. Osteocalcin, forward primer = 5′ AGG GCA GCG AGG TAG TGA AGA 3′, and reverse primer = 5′ AAG GGC AAG GGG AAG AGG AAA GAA 3′. Alkaline phosphatase, forward primer = 5′ TGC TCC CAC GCG CTT GTG CCT GGA 3′, and reverse primer = 5′ CTG GCA CTA AGG AGT TAG TAA G 3′. Osteopontin, forward primer = 5′ CAC TCC AGT TGT CCC CAC AGT AGA 3′, and reverse primer = 5′ GGC CTT GTA TGC ACC ATT CAA CTC 3′. GADPH, forward primer = 5′ GGG CAT CCT GGG CTA CAC TGA G 3′ and reverse primer = 5′ GGC CCC TCC CCT CTT CAA G 3′. PCR reactions were performed using a PTC-100 thermocycler (MJ Research).

The same reaction conditions were used for each primer set: first strand cDNA synthesis—48°C for 45 min, 94°C for 2 minutes; second strand cDNA synthesis and PCR amplification—94°C for 30 seconds, amplification temperature for 1 minute, 68°C for 2 minutes; and final extension—68°C for 7 minutes. Forty amplification cycles were used for all markers and the amplification temperature for osteocalcin, alkaline phosphatase, and GAPDH, was 58°C and 50°C for osteopontin. The number of cycles used was in the linear range of amplification for each specific gene product, as preliminary experiments were carried out with each primer set. The expected product size for each gene was as follows: osteocalcin, 266 bp; GAPDH, 345 bp; alkaline phosphatase, 215 bp; and osteopontin, 183 bp. The expression of all osteogenic markers was normalized to GAPDH. For each PCR reaction, 9 μL of the reaction product was combined with 1 μL of loading buffer. The products were electrophoresed in a 2-mm thick, 2.2% agarose gel at 1.3 V/cm^2 for 45 minutes in 1X Tris-borate-EDTA buffer. Molecular weight markers consisting of a 100-bp DNA ladder (Life Technologies) were run concurrently to estimate product length. The gels were then stained with 0.06 μg/mL ethidium bromide and visualized by using a Fluoro-S MultiImager (Biorad, Hercules, CA). Band intensities were then evaluated against background levels and quantified using MultiAnalyst software. Equivalent intensities of GAPDH between mechanical and control samples allowed semiquantitative comparison of the markers of interest expressed as a ratio to the reference gene (GAPDH) for each sample.

Mineralized Matrix Production

Alizarin Red S (sodium 1,2-dihydroxyanthraquinone-3-sulfonate) was used as a well-established colorimetric reagent for the determination of calcium salts [54]. Calcium intensity and distribution within the scaffold section were qualitatively determined by using modifications of procedures previously reported [55, 56]. Briefly, sections were fixed in 10% formalin for 24 hours and exposed to 40 mM alizarin red S solution (pH 4.2) for 5 minutes at room temperature. Sections were then washed in distilled water for 30 minutes to remove unreacted reagent and then air-dried, and photographed.

Statistical Analysis

All assays were repeated in two independent experiments with a minimum of $n = 3$ for each data-point. Data for RT-PCR and alkaline phosphatase activity were analyzed using a Student's two-tailed t test with Microsoft excel software (Microsoft Corp, Redmond, WA). Statistically significant values were defined as $P < 0.05$.
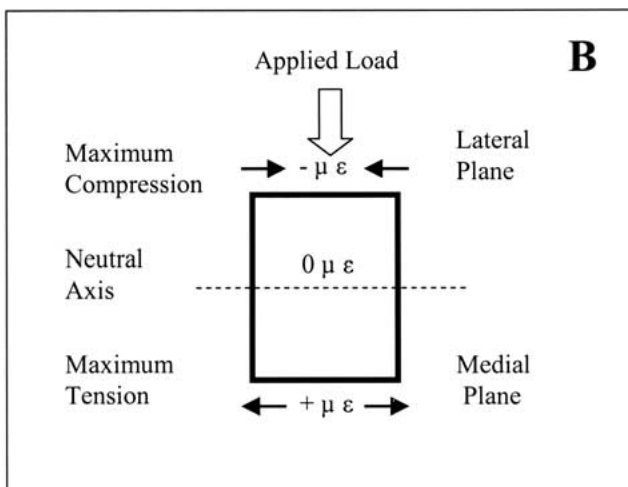
Results

There was a significant linear relationship between the external displacement and the applied tensile ($R^2 = 0.997$) and compressive ($R^2 = 0.998$) strains within the medial and lateral external planes, respectively, within the partially DMB scaffolds during bioreactor stimulation (Fig. 2A). The range of external strain magnitudes produced by the bioreactor during four-point bending was measured as 44 ± 32 to 2151 ± 672 tensile μ microstrain, $1 μ = 0.1\%$ strain within the external medial plane and -51 ± 41 to -2342 ± 400 compressive μ within the external lateral plane. The maximum external displacement (0.2 mm) utilized during BMSC stimulation generated an average strain magnitude of 505 ± 196 tensile μ within the external medial plane and -521 ± 216 compressive μ within the external lateral plane. A schematic view of the compressive and tensile strains within the cross-section of the partially DMB scaffold during loading is displayed in Fig. 2B.

Osteogenic differentiation of BMSCs on the partially DMB scaffolds was assessed by use of biochemical analysis of relative alkaline phosphatase activity at 8 and 16 days of cultivation) (Fig. 3). Relative enzyme activity was found to be a function of both dexamethasone concentration and mechanical stimulation at 16 days of cultivation. All culture conditions with dexamethasone treatment demonstrated significantly higher alkaline phosphatase activity when compared to non–dexamethasone-treated static samples following 16 days of cultivation. Alkaline phosphatase activity was significantly elevated in mechanically stimulated BMSCs by 80% and 92% over static controls in the presence of 0 and 10 nM dexamethasone, respectively. In contrast, mechanically stimulated samples containing 100 nM of dexamethasone displayed no significant difference in enzyme activity in comparison to static controls at this time-point. Mechanically stimulated BMSCs containing 10 nM dexamethasone displayed significantly higher levels of alkaline phosphatase activity compared to samples within all other tested culture conditions. No significant differences in enzyme activity between mechanical and static controls at day 8 of cultivation were observed at any experimental dexamethasone concentration (data not shown).

Analysis of transcripts specific for alkaline phosphatase and the bone matrix proteins, osteocalcin and os-
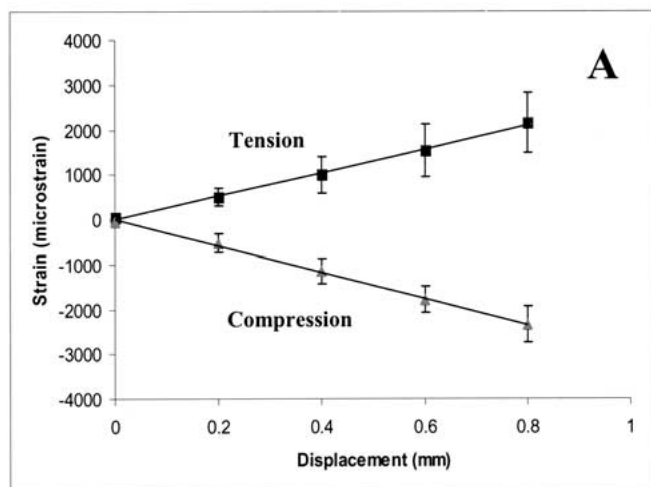
Fig. 2. (A) Relationship between the external displacement and strain within the external medial and lateral planes of the partially demineralized bone (DMB) scaffolds during four-point bending within the bioreactor. Mean ± SD for $n = 3$. (B) Axial cross-sectional view of the compressive and tensile strains in the uniform bending region of a partially DMB scaffold during four-point bending as predicted by theoretical four-point bending analysis. The magnitude of strain generated during bending increases from 0 μ in the central neutral axis to a maximal compressive strain (−μ) within the external lateral plane and a maximal tensile strain (+μ) within the external medial plane of the scaffold.
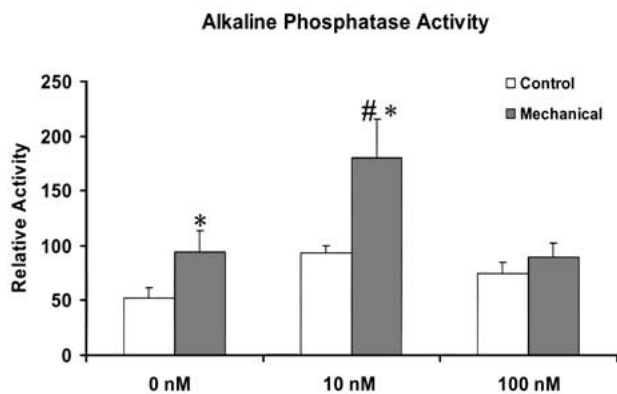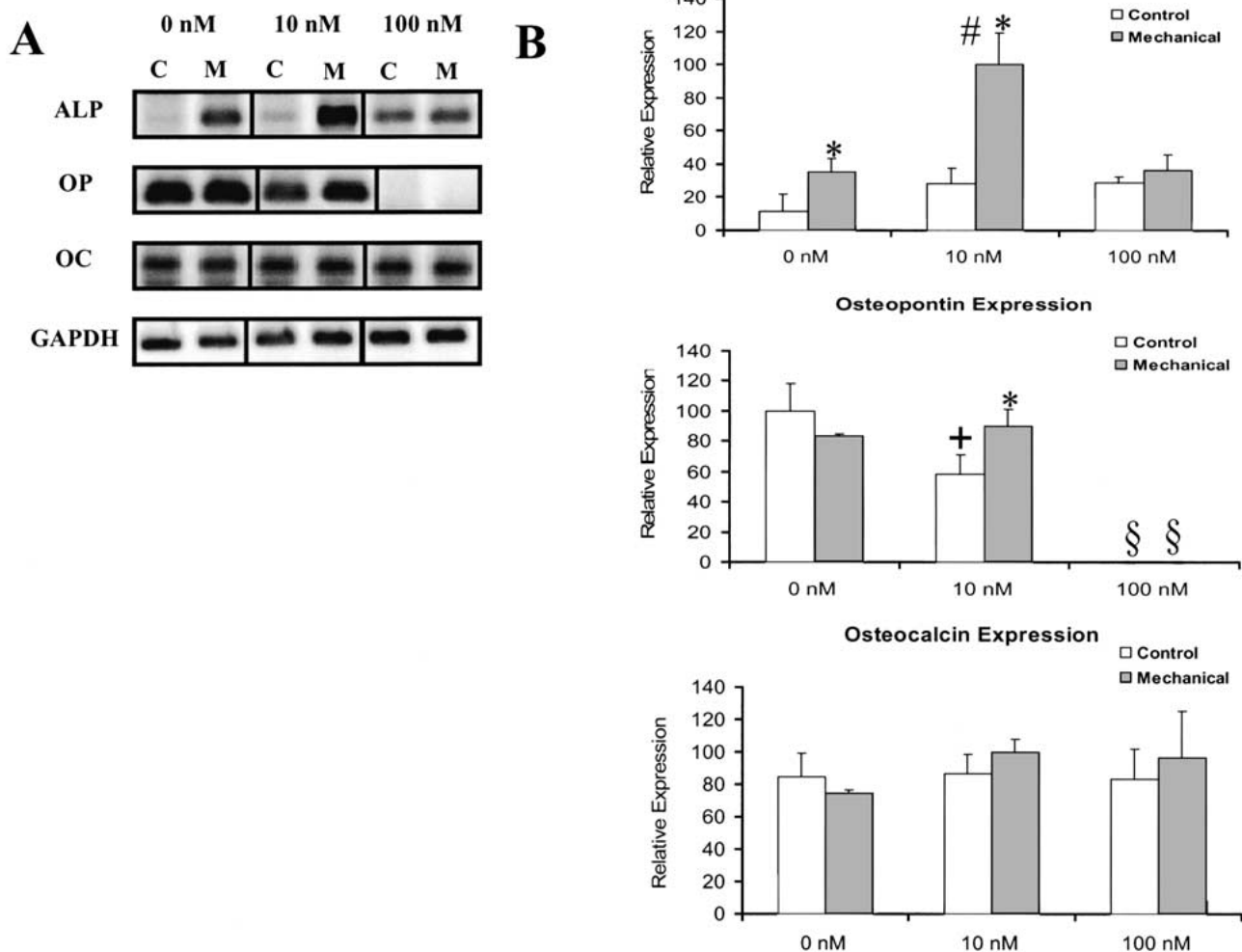

Fig. 3. Comparison of BMSC alkaline phosphatase activity in 0, 10, and 100-nM dexamethasone-treated groups after 16 days of bioreactor cultivation with mechanical stimulation or as static controls. Statistically significant difference (*) = compared to similar static control or (#) = compared to all other groups. Student's two tailed t test. $P < 0.05$. Mean ± SD for $n = 3$–4.

teopontin, were assessed by semiquantitative RT-PCR (Figs. 4A and B). The results demonstrated that alkaline phosphatase transcript levels paralleled the responses obtained for alkaline phosphatase activity at 16 days of cultivation. Alkaline phosphatase transcript levels were significantly elevated in mechanically stimulated BMSCs by 218% and 257% over static controls in the presence of 0 and 10 nM dexamethasone, respectively. In contrast, mechanically stimulated BMSCs containing 100 nM of dexamethasone displayed no significant difference in transcript levels in comparison to static controls. Transcript levels for alkaline phosphatase were significantly

higher in mechanically stimulated BMSCs with 10 nM dexamethasone in comparison to all other culture conditions after 16 days of cultivation.

BMSC osteopontin transcript levels were also a function of dexamethasone concentration and mechanical stimulation at 16 days of cultivation. Osteopontin transcript levels were shown to be negatively effected by dexamethasone with static samples containing 10 nM demonstrating significantly lower levels compared to static samples without dexamethasone. In addition, samples containing 100 nM displayed significantly lower transcript levels compared with samples at any other dexamethasone concentration. Osteopontin transcript levels were found to be significantly elevated by 55% in mechanically stimulated samples in comparison to static controls containing 10 nM dexamethasone. In addition, these levels were statistically similar to both mechanical and static samples containing 0 nM dexamethasone. In contrast, mechanical stimulation did not significantly increase osteopontin transcript levels in comparison to static controls at dexamethasone concentrations of 0 or 100 nM. Osteocalcin transcript levels showed no significant effects in response to mechanical stimulation over respective static controls at any dexamethasone concentration following 16 days of cultivation. Osteocalcin transcript levels were also not significantly effected by dexamethasone concentration following 16 days. No significant differences in osteopontin, alkaline phosphatase, or osteocalcin transcript levels were observed following 8 days of cultivation when comparing the mechanically stimulated and static controls at any experimental dexamethasone concentration (data not shown).

Fig. 4. RT-PCR analysis of osteogenic gene expression of BMSCs cultured in 0, 10, and 100-nM dexamethasone-treated groups after 16 days of bioreactor cultivation with mechanical stimulation or as static controls. (A) Agarose gel of representative ethidium bromide–stained products. M = mechanical stimulation, C = control groups. (B) Semiquantitative results of osteogenic transcript levels normalized to GAPDH expression. Statistically significant difference (*) = compared to similar static control, (+) = compared to 0 nM static control, and § = compared to all samples at other dexamethasone concentrations. Student's two-tailed t test. $P < 0.05$. Mean ± SD for n = 3-4. ALP = alkaline phosphatase; OP = osteopontin; OC = osteocalcin.

The production of calcified matrix by BMSCs within partially DMB was qualitatively analyzed following 16 days of cultivation by histochemical staining with alizarin red (Fig. 5). Relatively weak background staining was detected within the scaffold interior of all samples analyzed, due to the presence of residual bovine calcium prior to BMSC cultivation. Seeded scaffolds cultured in the presence of 10 nM dexamethasone and mechanical stimulation displayed substantially more intense alizarin red–positive staining along their external perimeters in comparison to internal background levels as well as to their respective static controls. No qualitative differences in staining intensity were observed between the mechanical and static controls at any other experimental
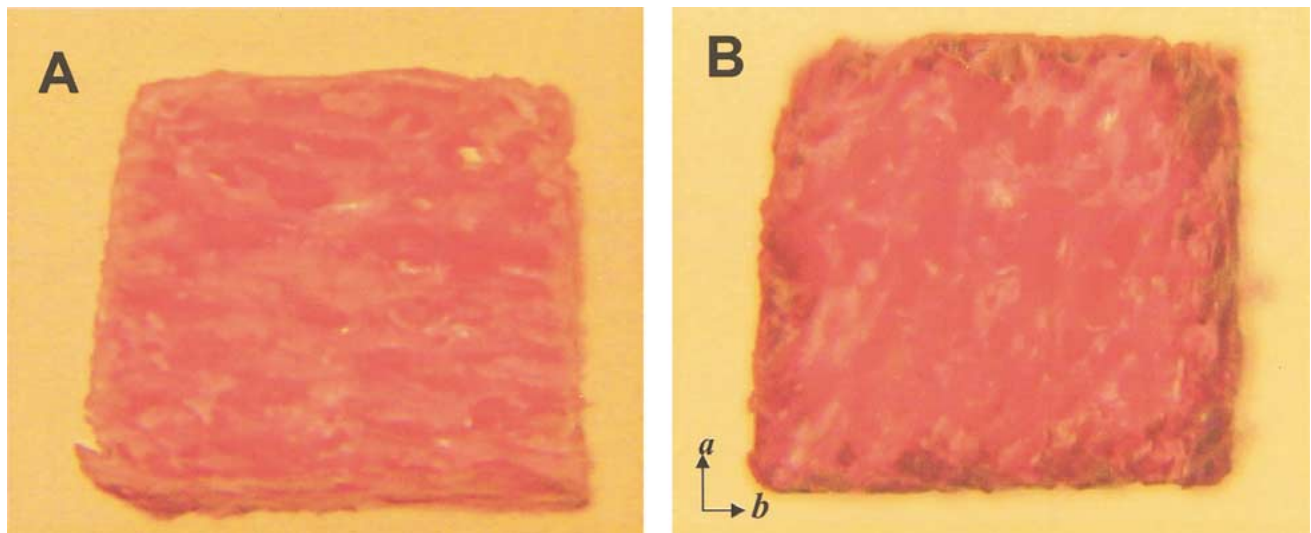
dexamethasone concentration following 16 days of cultivation (data not shown).

Discussion

In this present study, we have presented a novel *in vitro* bioreactor system capable of stimulating human bone marrow stromal cells mechanically within a 3-D partially DMB matrix. Our results show that cyclic mechanical stimulation within this culture system is capable of promoting osteogenic differentiation of BMSCs as well the formation of mineralized matrix, when cultured in the presence of specific dexamethasone concentrations. Mechanical stimulation *in vivo* has been

Fig. 5. Alizarin red–staining of axial cross-sections (6 × 6 cm) of seeded scaffolds cultured at 10-nM dexamethasone following 16 days of bioreactor cultivation. (A) mechanical and (B) static control. Mechanical samples are oriented similarly displaying their position during bioreactor stimulation. Arrows designate scaffold orientation: (a) = axis parallel to the di- rection of loading (b) = axis parallel to the external medial plane under maximum tensile strain (*) = qualitatively substantial increase in staining intensity at the external edges compared to internal background and respective static control (scale bar = 3 mm).

shown previously to play an integral role in bone formation by stimulating osteoprogenitor cells in the marrow stroma to differentiate into osteoblasts at cortical bone surfaces [57]. In addition, skeletal unloading has also been demonstrated to modulate the osteogenic differentiation capacity of BMSCs *ex vivo* and to effect their ability to produce mineralized matrix in culture [58–60]. These results together with our present study suggest that mechanical stimuli plays a significant role in influencing both the osteogenic differentiation process of BMSCs as well as their production of mineralized matrix *in vivo* and *in vitro*.

The bioreactor system utilized in this study incorporates a four-point bending loading regime capable of inducing average strain magnitudes of 44 to 2151 tensile $\mu\epsilon$ and −51 to −2342 compressive $\mu\epsilon$ along the external medial and lateral surfaces of the partially DMB scaffolds examined. This range includes strain magnitudes (50–2000 $\mu\epsilon$) routinely generated within the human skeletal system during vigorous activities [3] and, therefore, provides a useful 3-D *in vitro* system to mimic *in vivo* mechanical strains within bone tissue. Because the strain distribution during mechanical loading is nonuniform from the center to the periphery of the construct parallel to the applied load, the specific strain magnitude responsible for promoting osteogenic differentiation cannot be specified. However, since our results showed that the loading regimen utilized during BMSC stimulation generated maximum tensile and compressive strains of 505 ± 196 μ and −521 ± 216, respectively, it can be suggested that these cells respond to similar magnitudes of strain within the range of routine skeletal

loading *in vitro*. In addition, recent *in vivo* studies have shown that the application of bending forces within bone-loading systems significantly increased the ability of mechanical stimulation to promote bone formation in comparison to direct tissue compression (sham loading) [61–63]. This phenomenon is assumed to be caused by load-induced fluid flow effects (e.g., shear stresses and streaming potentials) generated as bending forces induce pressure gradients within the bone matrix [5, 57]. Although the characteristics of mechanical loading such as strain rate [18, 64], magnitude [65], and frequency [43, 66] have all been shown to play significant roles in osteogenic differentiation and bone formation, the incorporation of bending forces within our system may represent a critical feature in promoting osteogenic differentiation of BMSCs; however, this phenomenon warrants more study.

Characterization of the expression of several osteogenic markers was performed to provide an increased understanding of the events involved during osteogenic differentiation of BMSCs with mechanical stimulation *in vitro*. Alkaline phosphatase has been extensively used as a marker of osteogenic differentiation, with increases in enzymatic activity and protein expression corresponding to an osteoblastic phenotype within a number of cell culture systems [40, 50, 67, 68]. Alkaline phosphatase transcript levels were significantly elevated over static controls following 16 days of cultivation at dexamethasone concentrations of both 0 and 10 nM. In addition, transcript levels were significantly higher in mechanically stimulated samples containing 10 nM dexamethasone in comparison to all other culture con-

ditions. These results suggest that alkaline phosphatase expression can be elevated by mechanical stimulation independent of dexamethasone, but that relatively low concentrations (10 nM) can have a synergistic effect, producing significantly higher transcript levels than mechanical stimulation alone. It is interesting to note that this effect was abolished with a 100-nM concentration, suggesting that dexamethasone inhibits this mechanism at higher concentrations. These trends were also observed in levels of alkaline phosphatase activity at similar culture conditions, suggesting that these effects also play a role in modulating enzyme activity. Mechanical stimulation *in vitro* has been shown previously to upregulate alkaline phosphatase protein expression and activity within BMSC cultures in the presence, of dexamethasone concentrations of both 0 and 10 nM [37–39]. Therefore, these results together with those of the present study demonstrate that alkaline phosphatase activity and expression are load-responsive within BMSCs *in vitro*, and that this effect can be modulated by dexamethasone concentration.

Osteopontin (OPN) is an RGD (arginine-glycine-aspartate)–containing phosphorylated sialoprotein, which has been shown to be present within mineralized bone matrix and, in particular, to be localized at sites of bone formation and resorption [69]. OPN has been shown to play a role in both cell attachment and mechanotransduction responses of osteoblasts *in vitro* [12, 23]. The expression of OPN has also been identified as an early marker of osteoblast differentiation [68]. Our results demonstrate that transcript levels of OPN mRNA increased in BMSCs in response to mechanical stimulation following 16 days of cultivation in the presence of 10 nM dexamethasone. These data are consistent with those of other cell culture systems involving various osteogenic cell types that generated increases in OPN expression levels in response to mechanical stimulation [17, 18, 39, 70]. Another important observation of this study is the inability of mechanical stimulation to effect OPN transcript levels at dexamethasone concentrations of 0 or 100 nM at similar culture conditions. These results suggest that mechanically stimulated OPN expression within BMSCs is dexamethasone-dependent, and that an optimal concentration range exists where OPN expression is load-responsive within this system. It is also interesting to note that the addition of dexamethasone significantly decreased BMSC OPN transcript levels independent of mechanical stimulation. Dexamethasone has previously been reported to stimulate increases in OPN expression within BMSCs during osteogenic differentiation on various substrates [71–73]. However, recent studies have shown that biomaterial surface characteristics (e.g., surface roughness, composition, and charge) can play a significant role in influencing OPN expression during osteogenic differentiation *in vitro* [74, 75]. Therefore, one possible explanation for this

phenomenon may be the composition of DMB scaffolds, which are known to contain various matrix-incorporated factors (bone morphogenetic proteins and other noncollagenous proteins) capable of modulating osteogenic differentiation responses of BMSCs [76–80], and thus may have contributed to this effect of dexamethasone on OPN gene expression.

Transcript levels of osteocalcin, a bone matrix protein indicative of mature osteoblasts [81] were also analyzed and demonstrated not to be significantly effected by mechanical stimulation at any dexamethasone concentration within the 16 days of cultivation. Other related studies have shown that osteocalcin expression is sensitive to mechanical loading within BMSCs and osteoblastic cell lines *in vitro* [38, 82, 83]; however, these studies have reported varying responses, possibly because of the type of experimental system utilized. The lack of response within our system may also depend on the characteristics of our study, including loading regimen and culture duration. Further investigation is needed to determine the role of mechanical stimulation on osteocalcin expression within our model system. It is interesting to note, however, that the same mechanical regimen utilized in this study was able to differentially promote increases in the expression of some but not all bone-specific proteins investigated. This suggests that mechanical stimulation acts through specific mechanisms within BMSCs, which allow for the selective modification of particular osteogenic transcript levels during the differentiation process. Indeed, components of complex mechanotransduction pathways have been demonstrated within mature osteoblasts *in vitro* [83]; however, the role of these mechanisms in BMSCs has yet to be fully elucidated.

Mineralized matrix production was demonstrated to increase substantially in mechanically stimulated BMSCs cultured in 10 nM dexamethasone in comparison to respective static controls. We recently reported that within this culture system, BMSCs become localized to layers 200–300 μm from the scaffold exterior following a 14-day culture period [41]. Therefore, the observed region of increased matrix staining is demonstrated to correspond to this external cell layer. However, it should be noted that due to the presence of residual bovine hydroxyapatite within the partially DMB matrix, we are unable to conclude if the BMSC-mediated mineralization represents physiological calcification similar in crystal structure to hydroxyapatite or is dystrophic in nature. These data suggest that mechanical stimulation in combination with specific dexamethasone concentrations promotes increases in BMSC-mediated mineralization in comparison to static culture conditions. It is interesting to note that no apparent spatial differences in mineralization were detected along the exterior perimeter of the constructs, despite the nonuniform strain distribution parallel to the loading

axis. However, these data are consistent with the results of Wozniak and colleagues; they observed a spatially homogeneous increase in BMSC mineralization in response to a nonuniform mechanical strain distribution within a 2-D culture system [40]. These observations in conjunction with this current study provide further evidence of the previous notion that strain-induced factors that are independent of strain magnitude may exist, which promote matrix mineralization in human BMSCs. Such factors in our current system may include potential load-induced fluid-flow effects, which may have significantly contributed to the uniform effect of mechanical stimulation on matrix mineralization. The ability of mechanical stimulation to promote mineralization beyond that produced with dexamethasone alone may provide a new tool in bone tissue engineering for accelerating the development of mineralized tissues *in vitro* prior to clinical use.

In summary, this study describes a novel bioreactor for investigating osteogenic differentiation of BMSCs *in vitro* on 3-D partially DMB matrices. Mechanical stimulation induced increased levels of alkaline phosphatase activity, bone-specific protein transcript levels (alkaline phosphatase and osteopontin), and mineralized matrix production by BMSCs. Dexamethasone was also found to have a significant effect on the ability of mechanical stimulation to promote osteogenic differentiation within this system.

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