TISSUE ENGINEERING: Part A
Volume 17, Numbers 3 and 4, 2011

DOI: 10.1089/ten.tea.2010.0193

Ossifying Bone Marrow Explant Culture as a Three-Dimensional Mechanoresponsive *In Vitro* Model of Osteogenesis

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Mechanical cues play an important role in bone regeneration and affect production and secretion dynamics of growth factors (GFs) involved in osteogenesis. The *in vitro* models for investigating the mechanoresponsiveness of the involvement of GFs in osteogenesis are limited to two-dimensional monolayer cell culture studies, which do not effectively embody the physiological interactions with the neighboring cells of different types and the interactions with a natural extracellular matrix. Natural bone formation is a complex process that necessitates the contribution of multiple cell types, physical and chemical cues in a three-dimensional (3D) setting. There is a need for *in vitro* models that represent the physiological diversity and characteristics of bone formation to realistically study the effects of mechanical cues on this process. *In vitro* cultures of marrow explants inherently ossify and they embody the multicellular and 3D nature of osteogenesis. Therefore, the aim of this study was to assess the mechanoresponsiveness of the scaffold-free, multicellular, and 3D model of osteogenesis based on inherent marrow ossification and to investigate the effects of mechanical loading on the osteoinductive GF production dynamics of this model. These aims were achieved by (1) culturing rat bone marrow explants for 28 days under basal conditions that facilitate inherent ossification, (2) employing mechanical stimulation (compressive loading) between days 12 and 26, and (3) quantifying the final ossified volume (OV) and the production levels of bone morphogenetic protein-2, vascular endothelial growth factor, insulin-like growth factor-1, and transforming growth factor-β1. The results showed that the final OV of the marrow explants increased by about fourfold in mechanically stimulated samples. Further, mechanical stimulation sustained the production level of vascular endothelial growth factor (starting day 21), which otherwise declined temporally under static conditions. The production levels of insulin-like growth factor-1 and transforming growth factor-β1 were enhanced under the effect of loading after day 21. In addition, significant correlations were observed between the final OV and the levels of GFs analyzed. In conclusion, this study demonstrates that the scaffold-free, multicellular, and 3D model of bone formation based on inherent ossification of marrow tissue is mechanoresponsive and mechanical loading improves *in vitro* osteogenesis in this model with sustaining or enhancing osteoinductive GF production levels, which otherwise would decline with increasing time.

Introduction

Bone regeneration is a complex process that involves the direct contribution of multiple cell types, physical environment, and chemical and mechanical cues.[1–8] Numerous growth factors (GFs) are involved in osteogenesis in a sequential and interrelated manner.[1,9–12] However, the studies investigating the involvement of mechanical cues in osteogenesis-related GF expression, production, and secretion are limited to two-dimensional (2D) *in vitro* studies with particular cell types[13–24] or complex *in vivo* studies with associated experimental hurdles.[25–28] Gene expression of the cells in 2D monolayer cultures display significant differences compared to the cells of the native tissue origin and the cells cultured in 3D platforms.[29] These differences are possibly due to the limited presence of physiologically relevant interactions with the neighboring cells of different types and the absence of a natural extracellular matrix in 2D monolayer culture models.[29] The importance and potential effects of the 3D environment on bone cell response (i.e., osteocyte and osteoblast response to chemical and mechanical stimuli) have been well recognized; to address the need for mechanically active 3D culture environments, novel trabecular and cortical bone explant culture systems have been developed.[30–33] These explant culture models have been successfully used to study the mechanoresponsiveness of osteocyte cells together

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with osteoblasts in a 3D environment. To delineate the effects of mechanical cues on bone regeneration process, *in vitro* studies have been carried out on purified populations of cells with 2D monolayer culture models. The effect of mechanical cues on mesenchymal stem cells (MSCs)[13–18,34] and osteoblasts[19–24] have been investigated previously. These studies, almost without any exceptions, involve a purified (e.g., Ficoll purification and cell sorting) population of cells (e.g., excluding the nonadherent cells of marrow tissue), which do not fully represent the complex cellular and compositional characteristics of bone formation milieu and do not include all the cells that are normally present in bone regeneration process: hematopoietic stem cells (HSCs) and the osteopoietic accessory cells (OACs).[35,36] Differentiation of MSCs into osteoblasts (among many other connective tissue cell types) is essential for bone regeneration.[4,6,37,38] There is increasing evidence suggesting that in addition to the extracellular microenvironment of MSC niche, the presence of other cell types (i.e., HSCs and OACs) play a role in differentiation of MSCs to osteoprogenitors and osteoblasts. It has been suggested that a close interaction exists between the HCSs, OACs, MSCs, osteoprogenitors, and osteoblasts and that they regulate each other's functions.[35,36,39–42] Therefore, when the mechanoresponsiveness of MSCs during bone regeneration process is investigated, possible contribution of other cell types should also be considered and there is a need for *in vitro* osteogenesis models that reflect the physiological diversity of cell populations.

Bone marrow tissue houses OACs, HSCs, and MSCs[35,36,43–46] and hence partially reflects the physiological diversity of osteogenic milieu. Bone marrow is known to play a role in bone regeneration[3] and has been shown to have osteogenic potential.[47] Bone marrow explants inherently ossify *in vitro* without the addition of excipient osteoinductive factors.[12,48] Therefore, *in vitro* bone marrow explant cultures hold the potential to study bone regeneration in a more natural context. The scaffold-free, multicellular, and 3D model of osteogenesis based on self-inductive bone marrow ossification bridges the gap between the *in vitro* 2D monolayer culture systems employing single cell types and the complex *in vivo* animal models.

Some of the most potent osteoinductive factors involved in bone regeneration are bone morphogenetic protein (BMP)-2, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-1, and transforming growth factor (TGF)-β1.[1,8–10,49–51] BMPs play an important role in skeletal development and bone repair by means of their capacity to promote the differentiation of MSCs to osteoblastic phenotype.[1,52–58] The osteoinductive properties of BMPs have been investigated extensively and the most potent ones (BMP-2 and −7) have been introduced clinically.[54] VEGF is the most studied angiogenic GF that plays an important role in bone formation and healing[59–61] and in BMP induced osteogenesis.[57] On the other hand, IGF-1 is expressed in the fracture callus and there is evidence suggesting that the marrow stromal cells regulate osteoblast proliferation with the involvement of IGF-1.[1,51,62,63] In addition, IGF-1 has the potential to stimulate osteoblast mitogenesis and bone matrix synthesis *in vitro* and bone defect healing *in vivo*.[64,65] TGF-β is considered to enhance proliferation of osteoprogenitor cells at all stages of bone regeneration[1] and upregulated during embryogenesis as well as during bone regeneration.[50,66]

We have recently showed that throughout the ossification process of bone marrow tissue (under basal conditions) osteoinductive GFs are produced (BMP-2, VEGF, IGF-1, and TGF-β1) with a temporal pattern with highly correlating to the ossification level.[12] Therefore, by studying the mechanoresponsiveness of this natural *in vitro* ossification model, the effect of mechanical cues on the production dynamics of the key osteoinductive GFs can be elucidated. There are multiple GFs involved in bone regeneration, some of the most potent ones being BMP-2, VEGF, IGF-1, and TGF-β1.[1,9–11] The current study tested the hypotheses that *in vitro* ossifying bone marrow tissue is mechanoresponsive as reflected by greater amount of bone formation in mechanically loaded marrow explants, and that the mechanical stimulation will enhance the production levels of BMP-2, VEGF, IGF-1, and TGF-β1 by the ossifying marrow explants. To validate these hypotheses, rat bone marrow explants undergoing ossification were stimulated with compressive load in culture (starting day 12 up to day 26). The levels of BMP-2, VEGF, IGF-1, and TGF-β1 by the ossifying explants was measured with quantitative enzyme-linked immunosorbent assay (ELISA) throughout the culture period (at days 7, 14, 21, and 28) and compared to unloaded controls. The results of this study show that mechanical stimulation sustains and/or enhances the production levels of VEGF, IGF-1, and TGF-β1, but not of BMP-2 by inherently ossifying marrow explants *in vitro*.

Materials and Methods

In vitro *culture conditions*

Bone marrow was isolated from the femurs and tibiae of 80–90-day-old male Long-Evans rats (300–324 g) under the approval of Purdue Animal Care and Use Committee. Marrow extraction was performed with a centrifugation-based technique and transferred onto PET culture inserts (0.4 μm pore size, Transwell; Corning) at a 7 μL volume (Fig. 1A) and supplemented with sufficient amount of growth medium underneath the membrane. Culture medium was not added above the membrane, and therefore marrow explants were not in direct contact nor were they immersed in the medium, which prevented the nonadherent marrow cells from being washed away during medium changes. The complete details of the extraction and culture procedures were explained elsewhere.[12] The growth medium was composed of (modified from Luria *et al.*[48]): alpha minimum essential medium (α-MEM) (Sigma), 10% MSC-qualified-fetal bovine serum (FBS; Invitrogen), 60 U/mL Pen-Strep (Invitrogen), 2.5 μg/mL Fungizone (Sigma), 50 μg/mL ascorbic acid (Sigma), 5 mM Na-β-glycerophosphate (MP Biomedical), and 3.5 mg/mL glucose (Sigma). The cultures were kept at 37°C, 5% CO_2, and 95% relative humidity throughout the experiment (28 days). The unused culture medium was aliquoted in appropriate volumes and kept frozen till needed. The insert including the marrow explants were set aside and the culture medium in the well was changed three times a week and the spent (or conditioned) medium was collected and stored at −80°C.

Development and characterization of in vitro *mechanical loading system*

The mechanical stimulation was applied to the ossifying bone marrow nodules by means of a custom-made device

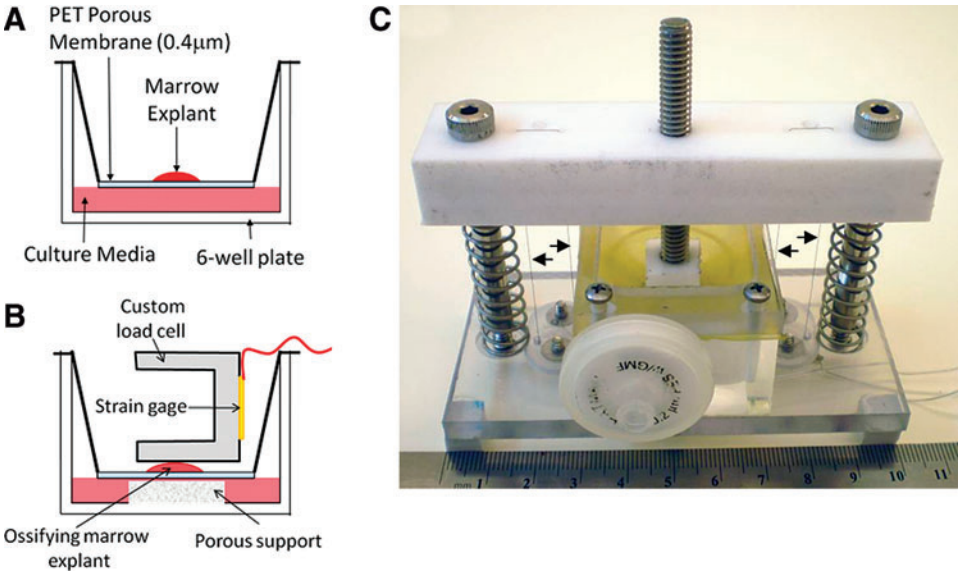

FIG. 1. **(A)** Cross-sectional view of air–medium interface culture system designed to preserve the adherent and nonadherent cellular composition of marrow tissue throughout the culture period. **(B)** The cross-sectional view of the custom-made *in vitro* loading chamber with an ossifying explant positioned inside. **(C)** A photograph of the fully functional custom loading setup. The force is generated by four Flexinol actuator wires running in parallel between the lower base and the upper loading bar (arrowheads). Color images available online at www.liebertonline.com/ten.

developed in our laboratory (Fig. 1B, C). The loading system is composed of a polycarbonate base with springs under compression and guide rods that are attached to the upper loading bar with the adjustable height loading rod (Fig. 1C). The actuation is provided by Flexinol actuator wires (arrowheads in Fig. 1C) and the frequency can be adjusted with a current control circuit driven by a 555 timer circuit. The loading chamber (sterile inside) is sealed from the outside with the elastic sealing membrane and a filtered (0.2 μm pore size) air vent. The adjustable height loading rod engages with the inside-chamber polytetrafluoroethylene (PTFE) loading tip with a custom-made load cell by means of magnetic coupling. The isolated chamber houses a PET membrane insert with the ossifying bone marrow nodule at its center. The elastic PET membrane is supported by a porous polyethylene polymeric block (100 μm pore size) that at the same time allows the flow of medium (Fig. 1B). The system operates under displacement control such that displacement occurs proportionally to the applied current. The displacement–current relation is linear and calibrated by a displacement gage before use. The displacement (Δ, μm) of the loading system in response to the applied current (I, mA) displayed a linear calibration curve ($\Delta = 3.9 \times I\text{-}499$, $R^2 = 0.997$). The error between the set displacement and the actual displacement was measured to be ranging between ±3.1% and ±5.6% for the minimum and maximum displacement set values, respectively. Prolonged tests of the system resulted in no detectable drift in the set displacement values.

Mechanical stimulation of ossifying marrow explants

The inserts were removed from the culture wells, transferred to the loading setup inside a laminar flow hood, and placed back in the incubator for mechanical loading under compression. The culture insert was located inside the loading chamber and a custom-made load cell (left facing C structure) was engaged, which is in contact with the partially ossified marrow explant (Fig. 1B). Marrow explant was sandwiched between the load cell and the membrane supported with a porous polymeric block underneath (Fig. 1B). Mechanical stimulation of the ossifying marrow explants was initiated concomitant with the appearance of a collagen-rich matrix formation, which was verified via Masson's trichrome-stained histology at earlier time points (i.e., day 14, Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/ten). Therefore, loading cycles were initiated at day 12 and mechanical stimulation was applied 900 cycles per day at 0.5 Hz up to day 26 for a total of 7 days (Fig. 2). Upon completion of loading, the inserts were placed back in culture wells and incubated until the next bout of mechanical stimulation. Five marrow explants were loaded as such longitudinally over time. There were also nine control explants that were also transferred to the loading setup, and the actuator tip was engaged; however, the loading was not performed. The maximum strain values were approximately determined by assuming an elastic modulus of woven bone tissue (4 GPa[67]) and estimating the ossifying area of the marrow explants by using the projected light microscope images. The inverted light microscope used in imaging cell cultures worked in the transmission mode. Therefore, any darkness indicated a greater absorption of light by the sample and hence considered to be corresponding to the ossified volume (OV) of the samples. The total peak load was measured by the custom-made load cell (Fig. 1B) and the maximum stress experienced by the explants was adjusted to $0.0313\,N/mm^2$ (31.3 kPa), which was estimated to induce a maximum apparent strain of about 5% in ossifying explants. The conditioned medium was collected 2 days after the application of each loading bout (Fig. 2) and stored at −80°C before being used in ELISAs.

Quantification of BMP-2, VEGF, IGF-1, and TGF-β1 levels produced by ossifying marrow explants

The quantification of GFs was performed on the medium conditioned by loaded and control groups of ossifying explants at four time points: at day 7 (5 days before the loading cycles started) and at days 14, 21, and 28 (after the loading cycles started; Fig. 2). The GF concentrations in the conditioned medium were measured by quantitative ELISA development kits (BMP-2: PeproTech; VEGF, IGF-1, and

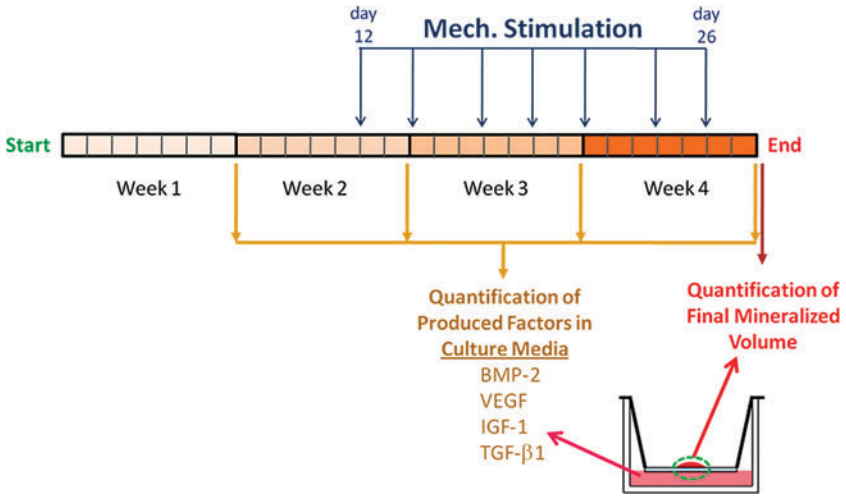

FIG. 2. Timeline of the experimental design. Mechanical stimulation was applied to the ossifying marrow explants starting day 12 up to day 26. The concentration levels of BMP-2, VEGF, IGF-1, and TGF-β1 in the conditioned culture medium were measured every 7 days starting day 7. The final OV of the marrow explants was quantified at the end of the experiment with microcomputed tomography (day 28). BMP, bone morphogenetic protein; IGF, insulin-like growth factor; OV, ossified volume; TGF, transforming growth factor; VEGF, vascular endothelial growth factor. Color images available online at www.liebertonline.com/ten.

TGF-β1: R&D Systems). TGF-β1 in the conditioned medium was acid-activated to make it immunoreactive and render it detectable by the immunoassay. Acid activation was carried out by incubating aliquots of the conditioned medium with 1N HCl followed by neutralization with 1.2N NaOH in 0.5 M HEPES buffer. Then, the standard ELISA protocols provided by the manufacturer of the kits were followed. Briefly, 96-well microplates (MaxiSorp; Nalge) were coated with capture antibody, and the wells were blocked for 1 h. Samples and standards were added to wells followed by incubation for 2–3 h at room temperature. After thorough washing, detection antibody was added at the specified concentration for each kit and the plates were incubated for 2 h at the room temperature. The peroxidase substrate solution was added (protected from direct light) and incubated at room temperature for 20 min. The enzyme reaction was stopped with 2 N hydrochloric acid solution. The color product was detected by a microplate reader set at 450 nm with wavelength correction set at 540 nm. The concentrations of GFs in the samples were calculated based on the standard curves obtained. The levels of the GFs in the nonconditioned growth medium (i.e., the complete growth medium including MSC-qualified FBS) were also measured to determine the baseline levels. The baseline levels of the factors in the nonconditioned growth medium (BMP-2, 71 pg/mL; VEGF, 5.7 pg/mL; IGF-1, 0 pg/mL; TGF-β1, 1099 pg/mL) were subtracted from the total concentrations to obtain the actual GF concentrations produced by the ossifying marrow explants at each time point.

The levels of solubilized BMP-2, VEGF, IGF-1, and TGF-β1 in the entire bone marrow explants at day 0 were quantified previously.[12] Briefly, marrow was extracted as described above and the same volume utilized in explant culture experiments (7 μL) was immediately dispersed in protein-LoBind tubes with growth medium ($n = 10$). Dispersed marrow extracts were incubated for 30 min at 37°C to allow the soluble factors to diffuse and dissolve in the medium. The suspension was then centrifuged and the supernatant was aspirated and filtered through a 0.2 μm filter to remove the remaining cells. The solubilized form of factors from bone marrow was then utilized in the quantitative ELISAs described above. The initial (day 0) concentration levels of the factors measured in marrow tissue was used to normalize the levels of the factors in loaded and control groups at each time point. Therefore, the concentration levels of the factors were reported as fold-change from day 0.

Microcomputed tomography of ossifying marrow explants

At the end of the 28-day-long experiment, the ossified marrow samples were fixed in 10% formalin and kept in the fixative before and throughout the microcomputed tomography (μCT) scans (μCT 40; SCANCO Medical AG). μCT scans were performed with a 16 μm voxel resolution ($I = 145$ μA, $E = 55$ kVp, integration time = 200 ms). The scanned images were reconstructed and analyzed with a commercial software (SCANCO evaluation software) and the standard segmentation parameters were used.[68–70] The total bone volume (mm^3) calculated by software was used and reported as the final OV of the marrow explants. The OV was normalized by the initial marrow explant volume per sample (7 μL or 7 mm^3) and reported as normalized OV (Fig. 3).

Histology of ossified marrow explants for matrix typification

At the end of the 28-day culture period (Fig. 2), the ossified marrow explants were fixed in 10% formalin.

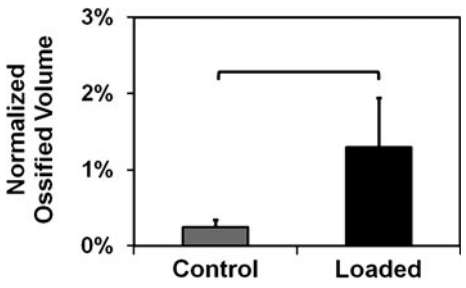

FIG. 3. The final normalized ossified (mineralized) volume of the loaded samples ($n = 5$) was significantly more compared to controls ($n = 9$). The OV of the marrow explants was normalized by the initial marrow explant volume per sample (7 μL or 7 mm^3). The bracket connecting the control and loaded groups indicates statistical significance ($p < 0.05$, Mann–Whitney *U*-test).

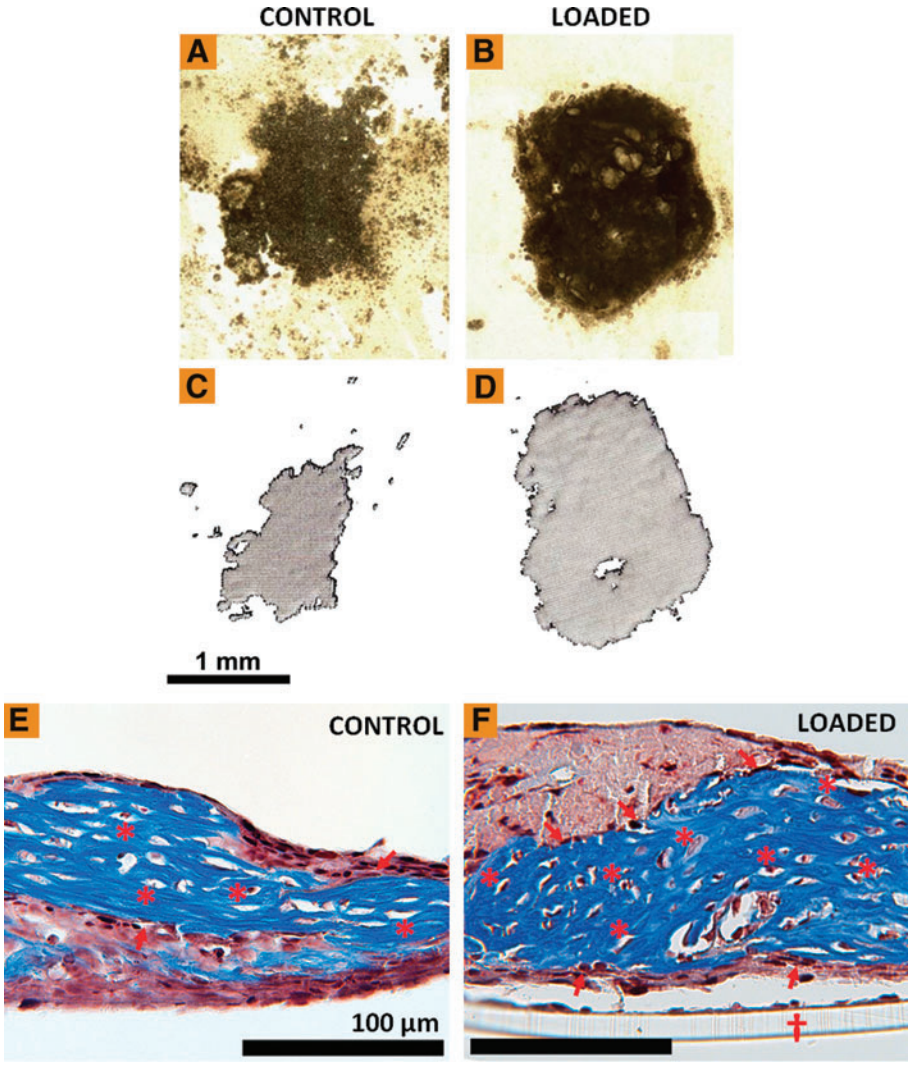

FIG. 4. Appearance of ossified marrow explants under light microscope (at day 28) that were cultured in the absence **(A)** and in the presence **(B)** of mechanical stimulation. Corresponding three-dimensional reconstructed images (from microcomputed tomography scans) of the ossified explants for a control sample **(C)** and a loaded sample **(D)**. Masson's trichrome-stained section of a control sample **(E)** and a loaded sample that underwent mechanical stimulation **(F)**. Blue color indicates the collagen-rich regions where ossification took place. Arrows indicate viable cells in both control and loaded samples with osteoblast-like morphology that are in the process of laying the ossified matrix and getting ready to be engulfed by the ossified matrix. *Viable cells with osteocyte-like morphology trapped in the ossified matrix. †The 0.4 µm pore-sized supporting membrane located below the explants. Color images available online at www.liebertonline.com/ten.

Decalcification of the samples was performed in formic acid solution (1:1 solution of 50% aqueous formic acid and 20% sodium citrate) for 12 h. Samples were then washed in tap water for 30 to 45 min, embedded in paraffin, sectioned, and dried overnight in 37°C oven. The sections were deparaffinized and hydrated in gradually decreasing percentages of alcohol solutions (100%, 95%, 70%, and water). The slides were then stained with Masson's trichrome method for observing the collagen-rich ossifying regions. The light microscope images were taken with Olympus Vanox microscope equipped with Qimaging Micropublisher 5.0 RTV 5 megapixel CCD camera.

Statistical analysis

The normalized OV in the loaded samples ($n = 5$) and the controls ($n = 9$) was compared statistically with Mann–Whitney *U*-test with a significance threshold set at 0.05 ($p < 0.05$). The normalized levels of GFs produced by control ($n = 4$) and loaded ($n = 4$) samples at various time points (days 7, 14, 21, and 28) were statistically analyzed by using General Linear Model with Tukey's *post hoc* test with statistical significance threshold set at 0.05. Relations between the GF concentrations and the final OV were analyzed by calculating the Pearson product moment correlation coefficient (PCC) with a significance threshold of 0.01 ($p < 0.01$). Error bars in the figures are displayed as standard error.

Results

The effect of mechanical stimulation on the final OV of marrow explants

The normalized OV in the loaded samples was significantly greater (about four times) than the control samples (Fig. 3). At the end of the 28-day culture period, the ossification of the bone marrow explants was visible through light microscopy (Fig. 4A, B). The ossified regions of the marrow explants appear darker under light microscope (Fig. 4A, B), which were observed to correspond to the mineralized volume detected by µCT (Fig. 4C, D). In addition, ossified center of the loaded samples (Fig. 4B) was observed to appear darker under light microscope compared to control samples (Fig. 4A). The OV was further observed and quantified by µCT. Three-dimensional reconstructed images obtained from µCT scans revealed a smaller ossified area and volume in the control samples (Fig. 4C, a well-ossified sample in the control group is shown) compared to loaded

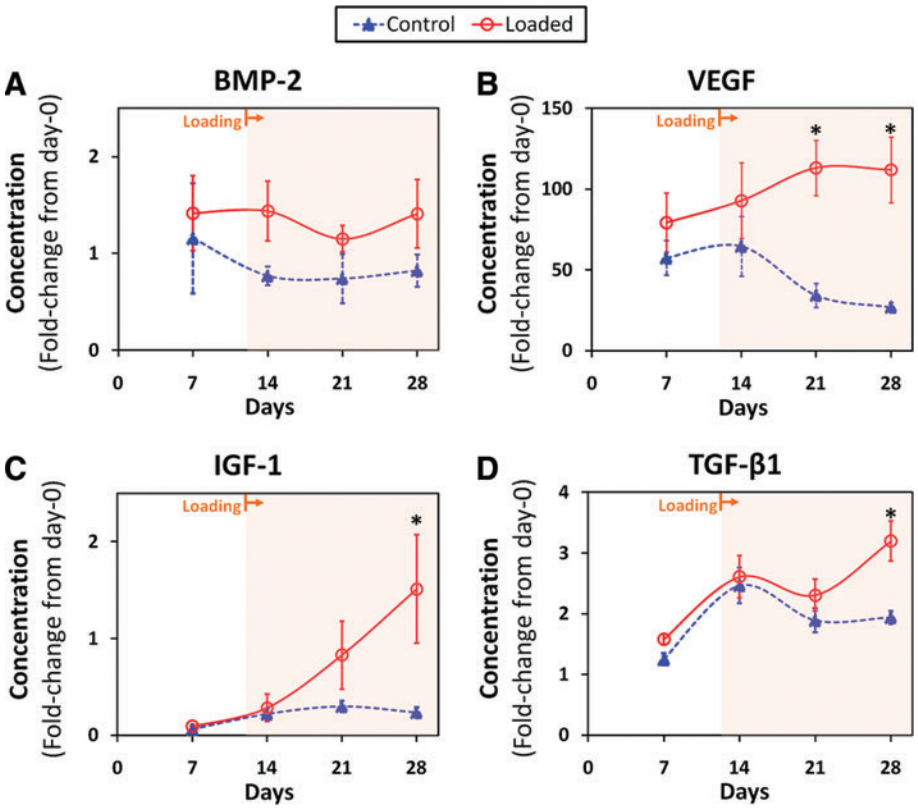

FIG. 5. The effect of mechanical stimulation on the production of BMP-2, VEGF, IGF-1, and TGF-β1 by ossifying marrow explants. The concentration levels of each factor at each time point were normalized by the initial (day 0) concentration levels of each factor in marrow tissue. **(A)** BMP-2 production was not affected significantly from mechanical stimulation. **(B)** VEGF production in the loaded samples was significantly higher at days 21 and 28. **(C)** IGF-1 production at day 28 was significantly greater in the loaded samples compared to controls. **(D)** TGF-β1 production was significantly higher in the loaded samples than the controls at day 28. *Statistical significance ($p < 0.05$) between the loaded samples and the controls at marked time points ($n = 4$ for each sample at each time point, General Linear Model with Tukey *post hoc* comparisons). Mechanical loading cycles were initiated at day 12 and continued till day 28 (shaded regions in the plots). Color images available online at www.liebertonline.com/ten.

samples (Fig. 4D, a well-ossified sample in the loaded group is shown).

Morphological characterization of ossified marrow explants

Histological assessment of marrow explants at day 28 (Fig. 4E, F) displayed a multicellular composition. Masson's trichrome stain revealed high-density collagen regions (blue color) starting day 14 at the bottom of the explants (the figure is not shown), which is the surface in contact with the porous membrane. The collagen density increased and covered a large portion of the sample by day 28 in loaded sample (Fig. 4F). However, lower collagen density regions were present in the lower sections of the control samples, which is the indication of ongoing active mineral deposition or ossification (Fig. 4E). Viable cells were present in both control and loaded samples with osteoblast-like morphology (arrows in Fig. 4E, F) above and below the collagen-rich regions were observed. In addition, viable cells with osteocyte-like morphology (asterisks in Fig. 4E, F) could be seen embedded in the collagen-rich sections.

The effect of mechanical stimulation on the production of BMP-2, VEGF, IGF-1, and TGF-β1 by ossifying marrow explants

The GFs in consideration were present in soluble form in the marrow tissue at day 0 (average concentrations per mm^3 of marrow: BMP-2, 8.6 pg; VEGF, 1.1 pg; IGF-1, 40.7 pg; TGF-β1, 60.9 pg), which were used to normalize the GF levels in both experimental groups at each time point as described in the Materials and Methods section. The difference between the levels of GFs in loaded and control groups before the loading cycles started (i.e., day 7 levels) was not statistically significant (Fig. 5). Even though BMP-2 was observed to be produced by both control and loaded groups, mechanical loading of the ossifying marrow explants did not have a significant effect on the production of BMP-2 at any time point (Fig. 5A). VEGF production by the nonloaded ossifying marrow explants declined after day 14. However, loaded marrow explants sustained VEGF production after day 14, which was significantly greater (Fig. 5B) on day 21 (about three times) and on day 28 (about four times) in the loaded samples compared to controls. IGF-1 production was increased about six times on day 28 by mechanical stimulation (Fig. 5C) in the loaded samples. Similarly, TGF-β1 production was also increased about 1.5 times on day 28 by mechanical loading (Fig. 5D).

Correlation between the levels of BMP-2, VEGF, IGF-1, TGF-β1, and the final OV of marrow explants

IGF-1 level at day 21 was observed to correlate significantly (PCC: 0.899, $p < 0.01$) with the final OV (Fig. 6A). Similarly, final OV correlated significantly with the day 28 levels of BMP-2 (Fig. 6B, PCC: 0.850, $p < 0.01$), and TGF-β1 (Fig. 6C, PCC: 0.907, $p < 0.01$).

Discussion

It was shown that *in vitro* ossifying marrow explants were mechanoresponsive since compressive mechanical stimulation induced significantly more bone formation in the loaded

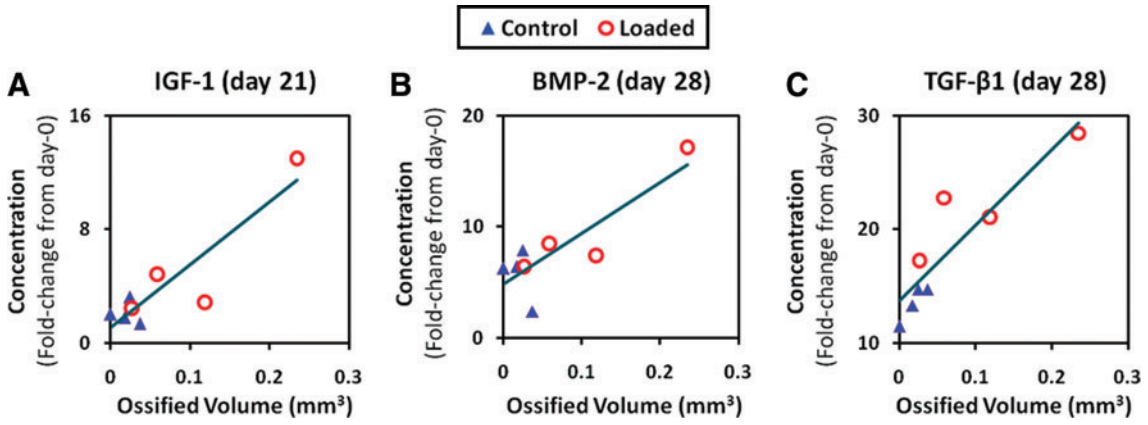

FIG. 6. Correlation between the normalized growth factor levels and the final OV of marrow explants. **(A)** IGF-1 level at day 21 correlated with OV (PCC: 0.899, $p < 0.01$). **(B)** BMP-2 level at day 28 correlated with OV (PCC: 0.850, $p < 0.01$). **(C)** TGF-β1 level at day 28 correlated with OV (PCC: 0.907, $p < 0.01$). Triangles indicate control (nonloaded) samples, and hollow circles indicate loaded samples. PCC, Pearson product moment correlation coefficient. Color images available online at www.liebertonline.com/ten.

samples. In addition, mechanical loading sustained the production level of VEGF between days 21 and 28 and enhanced production levels of IGF-1 and TGF-β1 after day 21 by *in vitro* ossifying bone marrow explants compared to nonloaded controls. However, mechanical stimulation did not induce a statistically significant effect on BMP-2 production level at any time point.

Naturally, bone marrow tissue resides in the confined cavities of bones, which provides a unique mechanical environment for the resident bone marrow cells.[71] Therefore, to replicate the natural mechanical environment of bone marrow tissue, mechanical stimulation in a confined loading chamber would be more appropriate. However, in the case of bone regeneration and fracture healing, structural integrity of bone tissue is compromised, and therefore bone marrow tissue does not reside in a strictly confined cavity any longer. In some cases of bone fracture, marrow may not be fully exposed or may be exposed but surrounded by other tissues around it, which would potentially act to filter out the physiological loads experienced by marrow tissue. The bone marrow explant culture model presented in this study is more relevant to the case in which bone marrow tissue is not confined in bone tissue or another type of tissue and exposed to a physiological environment, in which it naturally undergoes osteogenesis (i.e., similar to inherent ossification of marrow tissue after ectopic transplantation[72]). Therefore, the current study was based on the rationale that mechanical stimulation has osteogenic/anabolic effects on bone regeneration and fracture healing,[5,73–75] which generally involves physiologically exposed bone marrow tissue and the resident marrow cells. Therefore, in this study, strain values relevant to fracture healing (i.e., 5%–8% range, fracture callus stimulation) was utilized as opposed to anabolic strain values (0.5%–2% range) observed in healthy bone tissue.[26,27,76] Therefore, the rationale for the mechanical loading scheme employed in this study was based on the *in vivo* studies in the literature. Strains employed in fracture healing models are greater than those employed in anabolic stimulation of healthy bone and these studies commonly employ loading bouts with rest periods in between and frequency levels in the range of 0.5–2 Hz. A particular loading model for enhancing fracture healing demonstrated that the mechanical stimulation applied after about 10 days after fracture has an accelerating effect on bone healing, whereas the mechanical stimulation applied after 3 days of fracture has a deleterious effect on bone healing.[75] Therefore, in the light of the existing *in vivo* loading models, we have adopted a 5% strain and 0.5 Hz loading regime applied 900 cycles per day beginning from the 12th day after the initiation of culturing, which provided sufficient time for the early formation of a partially ossified matrix. In attestation, histological assessment (Masson's trichrome) of an earlier time point (ossifying marrow explant at day 14, Supplementary Fig. S1) indicated that collagen-rich extracellular matrix production was evident (emergence of blue color) by day 14, which can be considered as the indicator of earlier stages of ossification.

External mechanical stimulus in a 3D environment is experienced by the resident cells (i.e., bone and bone marrow cells in this case) in different forms: compression, tension, and fluid shear. Translation of external skeletal loading into different types of stimulation and their potential effects on bone and bone marrow cells have been discussed before in a detailed review article.[71] In this study, histological assessment of the Masson's trichrome-stained ossified marrow sections indeed revealed a 3D structure with coexistent presence of bone tissue and soft tissue with collagen-rich regions in the central regions of the explants (Fig. 4E, F) corresponding to the OV of the explants as detected by μCT. Viable cells with osteoblast-like morphology were observed surrounding the lower and upper surfaces of the ossification site, which were in the process of laying the mineralized matrix and getting trapped within the ossified matrix as cells with osteocyte-like morphology. Therefore, it can be suggested that the applied compressive mechanical load is experienced by the resident cells in different forms. Even though the *in vitro* loading model presented in this study is predominantly compression based, it would be reasonable to suggest that there may be other forms of stimulation experienced by the resident cells. A potential secondary stimulation mechanism induced by this loading system may be through fluid flow-induced shear due to compressive deformation of the ossifying matrix.[34,77] Unconfined compression of fibrous tissues resulted in loading-induced convection inside the tissue.[78] Therefore, the *in vitro* loading system combined with 3D marrow ossification

presented in this study may possibly result in a more complex stress–strain field (compression and fluid shear) for the resident cells. It can be suggested that the cells with osteoblast-like morphology surrounding the ossifying site may be experiencing predominantly compressive stress as they are either positioned at the interface of the ossifying site with the actuator tip, or between the ossifying site and the bottom support. On the other hand, the cells with osteocyte-like morphology encapsulated in the ossified matrix may be undergoing compression induced fluid flow based shear stress. This stress–strain field may be similar to the stress–strain field experienced by bone and bone marrow cells under natural conditions.[71,79] Therefore, the results of this study should be interpreted considering the possibly complex stress–strain field experienced by the resident cells.

Even though the GFs under consideration were present in the marrow tissue in soluble form at day 0, the secretion profiles after day 0 (i.e., between days 7 and 28) cannot be explained by sole diffusion of these soluble GFs from the marrow explants without any additional production by cells into the conditioned growth medium.[12] The initial concentrations of the GFs in the marrow tissue (day 0) are significantly lower than one (BMP-2 and IGF-1, Fig. 5A, C) or all (VEGF and TGF-β1, Fig. 5B, D) the following concentration levels produced by the ossifying marrow explants between days 7 and 28. Further, the concentration gradient (between the explant and the growth medium) is maintained over time since the medium underneath the marrow explant was changed every other day, and hence the medium below the explant acts as reservoir for collection of GFs. In other words, the levels of the GFs in the growth medium are always less than what is in the marrow explant (where the production is taking place), and hence the diffusion should be occurring from the explant to the medium. Therefore, it is clear that the concentration levels of these GFs in the conditioned medium result from the GF secretion dynamics of the resident cells in the marrow explants. However, the measured GF concentrations are a reflection of GF production dynamics/trends, but not the absolute amount of GFs in the explants. Since the measured concentration levels of the GFs in the conditioned medium are directly proportional to the levels of the GFs in the explants, this information can be used to infer the GF production dynamics explants and GF production response to mechanical stimulus.

The results indicated that the VEGF production was sustained over time due to mechanical loading. VEGF plays a critical role in BMP-induced osteogenesis.[57] In addition, the effectiveness of low level sustained VEGF release over burst-release for effective blood vessel formation in ischemic tissues was shown before.[80] Therefore, sustained production of VEGF in response to mechanical stimulation may be valuable for enhanced vascularization of newly forming bone tissue. In attestation, it was previously shown with an *in vivo* animal model (employing VEGF inhibitory antibody in the experimental animals) that VEGF signaling is essential for bone formation induced by mechanical strain.[81] In addition, VEGF gene expression was shown to be upregulated by pulsatile fluid shear stress in osteoblasts[82] and in bone marrow stromal cells.[83] Therefore, it can be suggested that the sustained VEGF production in the loaded ossifying explants was maintained by the resident marrow stromal cells and osteoblasts experiencing a complex stress–strain field of compression and fluid shear. In addition, VEGF expression has been observed in bone marrow environment[84] and it has been associated with endothelial cell recruitment (hematopoietic origin) and mobilization to the circulatory system.[85] Further, the bone marrow tissue houses cells of hematopoietic progenitor cells, and endothelial cells of hematopoietic origin[86] may be responsible for the upregulation of VEGF expression in mechanically stimulated samples.

There is evidence suggesting that marrow stromal cells regulate osteoblast proliferation with the involvement of IGF-1 and IGF-2.[62] Compressive loading enhanced IGF-1 gene expression in MSCs.[87] Similarly, tensile stretch increased mRNA expression of IGF-1 in human osteoblastic cell cultures.[19] The synergistic involvement of IGF-1 and mechanical loading was studied with an *in vivo* transgenic mouse study with osteoblasts selectively overexpressing IGF-1.[26] It was shown that bone formation in the transgenic mouse was elevated in response to mechanical loading in comparison to wild-type animals. The marrow platform presented here allowed quantification of IGF-1 production and indicated that IGF-1 was one of the most responsive GFs to mechanical loading. IGF-1 level increased substantially after day 21, during the second week of loading. Further, there was a correlation between the amount of IGF-1 production level at day 21 and the final ossified matrix volume. Therefore, mechanical anabolism in this model seems to occur through mediation of IGF-1; however, this assertion needs to be proven by targeted inhibition of IGF in this culture model.

TGF-β is considered to enhance proliferation of osteoprogenitor cells at all stages of bone regeneration.[1] Cyclic strain induced TGF-β1 production in human osteoblasts.[88] Similarly, fluid flow-induced shear increased gene expression of TGF-β1 in rat calvarial osteoblastic cultures[89] and in bone marrow stromal cells.[83] Further, equibiaxial strain enhanced expression of both TGF-β1 and VEGF (short term: 3 h) in calvarial osteoblasts.[90] The current study was able to investigate longer term response of GFs. Unlike VEGF, which readily responded to mechanical loading without delay, it took about 1 more week for TGF-β1 production to respond to mechanical stimulation. The level of TGF-β1 at day 28 was observed to be highly correlated with the final OV of the explants, which supports the importance of TGF-β1 in osteogenesis. TGF-β1 was present in the utilized growth medium at a relatively high concentration (1099 pg/mL), whereas the other GFs under consideration were present at low levels (BMP-2, 71 pg/mL; VEGF, 5.7 pg/mL; IGF-1, 0 pg/mL). Therefore, potential effects of TGF-β1 presence on the ossification of marrow explants should be considered, such as enhanced proliferation of MSCs and osteoprogenitors. In fact the FBS lot that was utilized throughout this study was specifically qualified for MSCs by the vendor (Invitrogen), and this condition might explain the relatively high level of TGF-β1, which would enhance the proliferation potential of MSCs. However, the same culture medium (i.e., same lot of FBS) was used for all the samples in both groups throughout the experiment. Therefore, the potential effects of TGF-β1 presence in the culture medium are expected to be the same on every sample in both experimental groups, which would not influence the experimental outcomes of this study.

BMP-2 has been shown to play an important role in bone regeneration by means of its capacity to promote the differentiation of MSCs to osteochondroblastic phenotype.[1,58]

Compressive stimulation of osteoblasts in 3D electrospun poly(e-caprolactone) scaffolds resulted in upregulation of BMP-2 mRNA at both 10% and 20% strain compression levels.[20] However, in this study, BMP-2 production by *in vitro* ossifying marrow explants was not observed to be affected significantly by an estimated 5% compressive strain level. This may be due to the presence of other cell types in the presented ossification model or the differences in loading regime and intensity employed. Even though BMP-2 production level was not significantly affected from mechanical stimulation, BMP-2 level at day 28 was observed to be highly correlating with the final OV of the explants.

The majority of the *in vitro* studies investigating the bone cell mechanoresponsiveness have used 2D monolayer cultures. There have been attempts to develop 3D *in vitro* models to better mimic the natural microenvironment of bone formation site by seeding cells in collagen-based matrices or scaffolds.[20,34,77,91] Even though these studies offer improvements over the traditional 2D models, they still neglect the coexistent multicellular composition of the bone formation environment (i.e., HSCs, MSCs, and OACs). It has long been considered that the microenvironment of the MSCs is the most critical parameter affecting the lineage decisions. However, HSCs are able to induce osteoblastic differentiation of MSCs under basal conditions.[35] Therefore, the inherent ossification of bone marrow explants under basal conditions presented in this study can be attributed to the coexistence of MSCs and HSCs in addition to the other resident cells of bone marrow. In attestation, we have previously shown that marrow tissue does not display self-ossification potential when marrow explants were dispersed and immersed in the culture medium (i.e., typical 2D culture conditions), which disrupts the cellular and structural integrity.[12] Dispersion and immersion of the marrow tissue allows the attachment of adherent cells onto the substrate (i.e., porous membrane) while suspending the nonadherent cells, which are then washed away with medium change. On the other hand, when the structural and cellular integrity of marrow is preserved in culture as described in this study (i.e., 3D marrow explant culture), marrow tissue ossifies inherently.

A limitation of the bone marrow explant culture is the variability of ossification in the samples, which, on the other hand, allows us to study the correlations between the ossification levels of the samples and the GF production levels (Fig. 6). It is possible to observe a range of ossification from low level to high level in the marrow samples obtained from the same animal and cultured under the same conditions. In fact, it is known that bone marrow displays spatial cellular composition gradients in both radial and longitudinal direction in long bones.[92] Since the marrow tissue is cultured after a minimal processing and disruption without a thorough homogenization, the variation in ossification potential is possibly due to the differences in the cellular compositions. The relationship between the cellular composition and the osteogenic potential of bone marrow tissue warrants further investigation.

In this study, the response of the GF production levels to mechanical stimulation is an accumulated response spread over a 2 day period (i.e., GF levels were quantified 2 days after mechanical stimulation; Fig. 2). Therefore, the results presented here should be considered accordingly and should not be confused with the short-term response of these factors. A detailed analysis of short-term responses of BMP-2, VEGF, IGF-1, and TGF-β1 to mechanical stimulation with this ossification model warrants further investigation. The correlations reported between the GF levels and the final OV can be used to study the temporal involvement of the GFs in bone formation. However, the correlations presented here do not necessarily imply causations, which require further targeted inhibition studies of the specific factors.

Mechanical stimulation was previously shown to enhance cellular proliferation in osteogenesis with *in vitro* studies.[15,16,18,21,88] Therefore, the increase in the final OV of marrow explants observed in this study in response to mechanical stimulation (Figs. 3 and 4) can be attributed to enhanced cellular proliferation and total collagenous matrix production by the increased cell population. On the other hand, the changes in the concentration profiles of the GFs can be attributed to the changes in the numbers and differentiation states of the resident cells in response to mechanical stimulation. Therefore, the effect of mechanical stimulation on cellular proliferation, differentiation, and nonmineralized matrix production (that was not detected by μCT scans in this study) dynamics in marrow ossification model warrants further investigation.

GFs control cell division, differentiation, and extracellular matrix synthesis. They are also known to play an important role in bone formation, and regeneration.[1,8,10,50] It has been suggested that there is a crosstalk between the GF signaling pathways and the overall osteogenic outcome may be a synergistic contribution.[1,9,10,50,55,57,93–96] Therefore, it is critical to investigate the effect of mechanical cues on the expression of multiple factors to better understand their individual and cooperative involvement in bone regeneration. GFs involved in osteogenesis are many and they are not limited to the ones studied here. A comprehensive analysis of other potent factors that are affected from mechanical stimulation can be investigated using the inherently ossifying marrow explant platform.

In conclusion, it was demonstrated that the *in vitro* ossifying marrow explants are mechanoresponsive and can be used to study the effect of mechanical stimulation on the production of various GFs. It was shown that the final OV increased in the mechanically stimulated marrow samples. The production levels of VEGF, IGF-1, and TGF-β1 were enhanced or sustained in response to compressive mechanical stimulation. The outcomes of this study are essential for understanding the nature's way of regenerating bone tissue in terms of the complex involvement of multiple GFs in a multicellular 3D environment and the effect of mechanical stimulation on this process.

Acknowledgment

This study was funded by a grant from the Musculoskeletal Transplant Foundation.

Disclosure Statement

No competing financial interests exist.

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Received: March 28, 2010
Accepted: August 30, 2010
Online Publication Date: November 3, 2010