The Effect of Mechanical Stimulation on Mineralization in Differentiating Osteoblasts in Collagen-I Scaffolds

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Developing a viable and functional bone scaffold *in vitro* that is capable of surviving and bearing mechanical load *in vivo* requires an understanding of the cell biology of osteoprogenitor cells, particularly how they are influenced by mechanical stimulation during cell differentiation and maturation. In this study, mechanical load was applied using a modified FlexCell plate to impart confined compression to collagen-I scaffolds seeded with undifferentiated murine embryonic stem cells. The activity, presence, and expression of osteoblast-cadherin (OB-Cad) and connexin-43, as well as various pluripotent and osteogenic markers were examined at 5–30 days of differentiation as cells were stimulated to differentiate to osteoblasts with and without applied mechanical load. Fluorescence recovery after photobleaching, immunofluorescence, viability, von Kossa, and real-time polymerase chain reaction assessments revealed that mechanical prestimulation of this cell-seeded scaffold altered the expression of OB-Cad and connexin-43 and resulted in significant differences in the structure and organization of mineralization present in the collagen matrix. Specifically, cells in gels that were loaded for 40 h after 5 days of differentiation and then left to fully differentiate for 30 days produced a highly structured honeycomb-shaped mineralization in the matrix; an outcome that was previously shown to be indicative of late osteoblast/early osteocyte activity. This study highlights the potential of mechanical load to accelerate differentiation and enhance osteoblast communication and function during the differentiation process, and highlights a time point of cell differentiation within this scaffold to apply load in order to most effectively transduce a mechanical signal.

Introduction

VARIOUS TISSUE ENGINEERING strategies have been developed to promote bone healing. However, a design that fully satisfies the biological and mechanical requirements of an effective bone substitute has yet to be developed. To meet the clinical demand for effective strategies to treat acute and chronic bone defects, a fundamental understanding of the biology and mechanobiology of stem cells and bone formation is necessary.^{1,2} The process of bone healing requires the complex coordination of osteoprogenitors, osteoblasts, osteoclasts, and osteocytes.² Few studies of bone formation or healing characterize the three-dimensional (3D) mechanical environment experienced by cells *in situ*, and even fewer characterize the biological factors involved in detecting or responding to a mechanical stimulus.² To translate classical two-dimensional (2D) *in vitro* tissue engineering platforms into 3D requires optimization of mechanically sensitive bone formation processes *in situ*.^{2–4} Ideally, a tissue-engineered construct that promotes bone

formation would be a self-sustaining milieu in which osteogenic cells within a 3D scaffold perceive and respond to mechanical signals without the need for exogenous drugs or growth factors.⁵ A previous group showed that osteoblast differentiation and nodules of mineralization could be enhanced by periods of cyclic mechanical strain *in vitro*.⁶ This study used a triple-supplement technique on cultured human embryonic stem cells (hESCs) for driving osteogenesis (using ascorbic acid, vitamin D, and beta-glycerol phosphate [BGP]).⁶ Our group reported that murine embryonic stem cells (mESCs) seeded in 3D collagen-I scaffolds cross-linked with BGP provides a milieu permissive for osteoblast differentiation *in vitro* while reducing formation of teratomas upon transplantation *in vivo*.⁷ The present study extends this work using mESCs seeded in 3D type-I collagen scaffolds to promote bone formation *in vitro*, and presents a method to generate a 3D tissue-engineered construct capable of responding to mechanical load.

It has been shown that intercellular communication is crucial for the differentiation of functional populations of

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cells, including bone cells.⁸ Specifically, connexin-43 and osteoblast-cadherin (OB-Cad) have been implicated as major regulators of *in vitro* and *in vivo* osteoblast differentiation.^{9–11}

In addition to regulating osteoblast differentiation, connexin-43 and OB-Cad have also been shown to have an essential role in the ability of bone cells to induce a biochemical response to mechanical stress.^{12–14} Previous reports show that mechanical compression of mesenchymal stem cells increases the expression of *Sox-9*, collagen-II, and aggrecan, all molecules highly expressed by chondrocytes, whereas compression loading of ESCs downregulates these cartilage-specific markers.^{15,16} These seemingly paradoxical findings imply a fundamental shift in the mechanobiology of stem cells that accompanies the transition from pluripotency to multipotency, and seems to favor chondrogenesis. Hence, the present study characterizes the mechanobiology of ESCs in a 3D scaffold exposed to mechanical compression and aims to optimize conditions that promote bone formation—in particular, intercellular communication and matrix mineralization. Specifically, this study explores the effects of confined compression on scaffold matrix mineralization and identifies time points during differentiation when mechanical stimulation influences cells and affects the development of functional osteoblasts.

Materials and Methods

The treatment groups in this study were defined as follows and will be used to discuss results in this article (Fig. 1). Gels that were not subjected to any mechanical load at different time points of differentiation were named day 5, 7, 15, 20, and 30. For gels that were loaded and then immediately stained or analyzed, gels were named day 5 loaded, day 15 loaded, day 20 loaded, and day 30 loaded. For day 5 gels that were loaded and then left to differentiate for 30 days prior to staining or analysis, gels were named day 5 long-term.

Cell preparation

mESCs (ES-D3 cell line) were maintained in their pluripotent state in media containing high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum, 1% nonessential amino acids, 50 U/mL penicillin, 50 mg/mL streptomycin, 0.1 mM β -mercaptoethanol (all supplied from Invitrogen, Life Technologies), and 1000 U/mL leukemia inhibitory factor (Millipore). The mESCs were passaged in T-75 flasks prepared with 0.1%

gelatin, and after three to four passages, cells were counted using a hemocytometer, spun down, and resuspended in $5 \times$ DMEM containing 10 mM pro-osteoblastic BGP (Sigma). No ascorbic acid, 1,25-OH₂ VD₃, or dexamethasone was added to the media as per the pro-osteoblastic differentiation protocol determined by a previous group.⁷ The cells in BGP media were then combined with purified bovine collagen-I (Advanced Biomatrix) with a final volume composed of 80% collagen-I, and 20% cells+BGP. The final cell density in each gel consisting of cells+BGP+collagen-I was 1 million cells/mL. The mixture was pipetted in 1-mL volumes into a 24-well plate and left in a 37°C incubator to gel. Once the gels were pipetted into the 24-well plate, cells were considered to be at a day 0 time point for differentiation. Four differentiation time points were investigated in this study: day 5, 15, 20, and 30.

Mechanical loading regime

After 5, 15, 20, and 30 days of differentiation, gels were transferred from the 24-well plate to a customized BioPress™ FlexCell FX-4000™ (FlexCell International Corp.) 6-well loading plate. Confined compression of the gels was performed at these time points with a loading regime of two cycles of 4 h of loading followed by 16 h in a resting state, for a total of 40 h.¹⁷ The loading plate has been previously characterized by our group and calibrated to subject the gels to a pressure of 5 kPa with an overall compressive strain of 5% and frequency of 1 Hz (Fig. 2). This cyclic loading regime has been widely used to investigate mechanically sensitive cell responses and has been shown to be representative of physiological mechanical strain and frequency in bone during locomotion.^{17–20} The rest states in this regime allow media to be drawn back into the collagen matrix and thus enables recovery following loading.¹⁷ Further, it has been shown that these loading parameters are sufficient to produce measurable differences in osteoblast response when cells are loaded in the FlexCell system,^{19,20} which is widely used to study the effect of mechanical loading on osteoblast differentiation in both 2D and 3D scaffolds.^{21–23}

Fluorescence recovery after photobleaching

At 5, 15, 20, and 30 days of differentiation, gels were loaded (total $n=32$) or left unloaded (total $n=31$) and then incubated at 37°C with 500 μ M calcein-AM for 2 h. A Zeiss LSM510 microscope was used for imaging (63×1.2 NA water immersion objective, 488 nm argon laser, 1.3 μ m

FIG. 1. Experimental design and treatment groups for this study. FRAP, fluorescence recovery after photobleaching; RT-PCR, real time polymerase chain reaction. Color images available online at www.liebertpub.com/tea

FIG. 2. Cross-sectional diagram of the modifications made to the Flex-Cell loading plate to apply confined compression to the gels (image courtesy of Hazenbiller¹⁷). Color images available online at www.liebertpub.com/tea

optical slice).^{24,25} An initial image was recorded (t_{ini}) at 20% laser intensity. An oval region of interest (ROI) was then fit around the cell body and any visible cell processes. This ROI was then photobleached at 100% laser intensity for 15 s. At least three regions within each construct were imaged. Images were obtained immediately after photobleaching (t_0), after 5 min (t_5), 10 min (t_{10}), 20 min (t_{20}), and 30 min (t_{30}) of recovery. The mean pixel intensity within cells was determined using NIH ImageJ 1.43 software and normalized to an uninvolved cell in the image field.^{24,25} Percent recovery for each image obtained after photobleaching gave an indication of gap junction functionality and was calculated using the following equation^{24,25}:

$$\% \text{Recovery} = \frac{t_i - t_0}{t_{ini}} \times 100$$

Five images with a 10×0.3 objective were taken of each treatment group to view overall cell density and perform automated supplementary cell counts using ImageJ.²⁶

Immunofluorescence staining

At 5, 15, 20, and 30 days of differentiation, unloaded ($n=12$ for each time point) and loaded ($n=12$ for each time point) gels were prepared for immunofluorescence staining. An additional day 7 unloaded time point ($n=6$) was investigated to represent the approximate time point of day 5 loaded cells following the 40-h loading regime described earlier. Each gel was fixed in filtered 4% paraformaldehyde/PBS overnight at 4°C. The gels were then washed three times in 1× PBS and incubated overnight at 4°C in 0.5% saponin/PBS. The gels were washed again three times in 1× PBS and blocked overnight at 4°C with 3% bovine serum albumin/phosphate-buffered saline (BSA/PBS). After the blocking step, gels were incubated with goat OB-Cad primary antibody, or mouse connexin-43 primary antibody at a dilution of 1:50 in 3% BSA/PBS. The gels were then washed three times in 1× PBS and blocked again overnight at 4°C in 3% BSA/PBS. Gels were then incubated with anti-goat Alexa488 secondary antibody, or in anti-mouse Alexa488

secondary antibody at a dilution of 1:50 in 3% BSA/PBS (all antibodies supplied from Life Technologies). Finally, gels were washed three times in 1× PBS and visualized using a Zeiss LSM510 microscope. Gels that were not incubated in primary antibody served as a negative control ($n=4$). A preosteoblast cell line known to have OB-Cad and connexin-43 was used as a positive control ($n=4$).

Viability study

At 5, 15, 20, and 30 days of differentiation, viability of cells within unloaded (total $n=22$) and loaded (total $n=24$) collagen gels was determined. To determine viability, gels were incubated in 4 μM calcein-ethidium homodimer (Molecular Probes LIVE-DEAD Kit; Life Technologies) for 45 min prior to visualization on a Zeiss LSM510 microscope. Cell counts were made using NIH ImageJ 1.43 software.

von Kossa staining

At 5, 15, 20, and 30 days of differentiation, unloaded (total $n=20$) and loaded (total $n=20$) gels were embedded, frozen, and cut using a cryostat in 10-μm sections. One group of day 5 loaded gels ($n=9$) was returned to the incubator to allow cells to differentiate until 30 days. von Kossa staining of gels was performed by incubating sections for 1 h under UV light in 1% silver nitrate, neutralizing the reaction with 5% sodium thiosulfate, and counterstaining with nuclear fast red. Images were taken with a Zeiss Axioptan 2 stereology microscope. Negative controls for von Kossa staining were prepared by treating gels with formic acid prior to incubation with silver nitrate. Sections of 16–18-day-old mouse embryos were prepared as positive controls. To test for nonspecific mineralization of the matrix due to BGP present in the gels, von Kossa staining was performed on gels containing BGP without seeded cells ($n=3$). To examine the effect of hypoxia or pH changes in the incubator during loading, von Kossa staining was performed on gels left in an incubator with a porous plug placed for 40 h on top of the gels ($n=3$).

Gene expression study for OB-Cad and connexin-43

At 5, 15, 20, and 30 days of differentiation, quantitative real-time polymerase chain reaction (RT-PCR) for connexin-43 (*Gjal*) and OB-Cad (*Cdh11*) was performed on unloaded ($n=5$ for each time point) and loaded ($n=5$ for each time point) gels using the iCycler IQ Multi Color Real Time PCR detection system (Bio Rad Laboratories, Inc). RT-PCR was also performed on unloaded day 7 differentiated gels ($n=5$) to compare the gene expression of day 5 cells following the 40-h loading regime ($n=5$). RT-PCR was also performed on day 5 loaded gels left to differentiate to 30 days ($n=5$). The following genes were also considered for all groups described previously: *Oct-4*, *Runx2*, *collagen-II* (*Col2a1*), and *osteocalcin* (*OCN*). A protocol previously demonstrated to be sufficient in isolating RNA and generating complementary DNA (cDNA) for gene expression studies on these gels was used.⁷ RNA isolation of gels was performed using Trizol (Invitrogen) with the addition of glycogen for complete dissociation. Thirty microliters of ultrapure water was used to dissolve RNA pellets and RNA was stored at -70°C until cDNA extraction was performed. RNA was quantified using a NanoVue spectrometer and bioanalyzer (Bio-Rad). For the spectrometric analysis, the spectrometer was blanked using the same solution that was used to dissolve RNA. Then, 2 μL of total RNA was pipetted in the center and values for RNA yield, 260/280, and 260/230 ratios were observed. RNA integrity was determined using an ExperionTM RNA StdSens Analysis Kit (Bio-Rad). The RNA yield obtained using the NanoVue spectrometer was only used for cDNA analysis if 260/280 ratios were ≥ 1.7 and if 260/230 ratios were ≥ 1.5 . cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies). RT-PCR was performed using the TaqMan[®] Universal PCR Master Mix with no AmpErase (Applied Biosystems, Life Technologies) to ensure that PCR products could not be reamplified and therefore result in false positive results. All PCR samples were run in triplicate for each gene using a 96-well plate. All

gene expression data were analyzed using the comparative C_T method, which involves normalization to a reference gene followed by normalization to a cell control.²⁷ In this study, gene expression data were normalized to 18S rRNA, as this has been found to be a stable reference gene for normalizing PCR data for *ex vivo* studies.^{28–32} Following normalization, samples were then compared against undifferentiated mESC gene expression data.

Statistical analyses

For the methods described previously, the indicated sample sizes denote the number of individual gels that were tested. For fluorescence recovery after photobleaching (FRAP) and viability measures, all statistical evaluations at each time point between mean fluorescence recovery values and average viability of unloaded and loaded samples were performed using independent two-sample *t*-tests with a significance level of 0.05. For RT-PCR measures, one-way ANOVA was performed between time points of differentiation for fold induction in unloaded and loaded samples (GraphPad Prism 6.0) with a significance level of 0.05. If differences within groups were significant, then Bonferroni's multiple-comparison test was performed.

Results

The different treatment groups and experiments performed on each group are summarized in Figure 1.

In this study, FRAP demonstrated the functionality of gap junctions within the gels (Fig. 3A–C). Cell clusters for FRAP experiments ranged from two to five cells per cluster (Fig. 3D). FRAP experiments of gels treated for 40 h with octanol (a general cell-to-cell communication blocker) or 18- α -glycyrhethinic acid (AGA) (a specific gap junction blocker) showed that communication inhibition significantly decreased fluorescence recovery to $\sim 2.8\%$ and 4.9% for octanol-treated and AGA-treated cells, respectively, at both early and late stages of differentiation (Fig. 4). FRAP experiments also

FIG. 3. Fluorescence recovery due to selective photobleaching in day 5 differentiated gels; red circle indicates photobleached cell, arrow indicates reference cell used for normalization. (A) Cells before 15-s photobleach, (B) cells immediately after 15-s photobleach, (C) cells 30 min after 15-s photobleach. Scale bar is 25 μm . (D) Three-dimensional volume reconstruction of a stack of images (thickness: 150 μm) taken in the z-plane of day 5 cells stained with calcein. Scale bar is 200 μm . Color images available online at www.liebertpub.com/tea

FIG. 4. Effect of communication inhibition on maximum percent fluorescence recovery $\pm$ standard deviation in early (day 5) and late (days 15–30) differentiated gels. Asterisks signify significant differences between groups at $\alpha=0.05$.

showed that there was a significant increase in gap junction function in day 5 differentiated gels that were loaded compared with unloaded gels (Fig. 5). At an early stage of differentiation (day 5), average fluorescence recovery values were 6.6%, whereas in day 5 loaded gels, this recovery significantly increased ($p=0.0012$) to an average of 12.9% (Fig. 5). Supplementary cell counting experiments at each time point within

the scaffold were performed and revealed no significant differences in cell numbers as differentiation progressed.

Viability results indicated that 46.9% of cells survived up to day 30 in unloaded gels (Table 1). Importantly, there was no significant detrimental effect of loading on viability of cells at all time points of differentiation (Table 1).

Immunofluorescence staining for OB-Cad and connexin-43 showed greater presence of both proteins in cells differentiated for 15, 20, and 30 days compared with cells differentiated for 5 days (Fig. 6A, B, E, F). Immunofluorescence staining of day 7 gels showed a slight increase in presence of OB-Cad and connexin-43 compared with day 5 gels (Fig. 6C, G). Immunofluorescence staining for OB-Cad and connexin-43 in loaded gels showed greater localized presence of both proteins in day 5 loaded gels (Fig. 6D, H). At all other time points, no increase in presence of OB-Cad or connexin-43 was observed due to loading. Images of the negative controls did not show any presence of OB-Cad or connexin-43, while images of the positive controls demonstrated that the protocol was sufficient in revealing OB-Cad and connexin-43 presence in the gels (Fig. 7).

Staining of day 5 gels resulted in no visual positive staining, while day 30 gels showed positive staining for mineralization of the matrix (Fig. 8A, B). Immediate von Kossa staining of day 5 loaded gels resulted in no positive staining. Staining of day 30 loaded gels showed similar

FIG. 5. Effect of mechanical stimulation of gels on maximum percent fluorescence recovery $\pm$ standard deviation in (A) day 5, (B) day 15, (C) day 20, and (D) day 30. Asterisks signify significant differences between groups at $\alpha=0.05$. Color images available online at www.liebertpub.com/tea

TABLE 1. AVERAGE VIABILITY $\pm$ STANDARD DEVIATION OF CELLS WITHIN COLLAGEN-I CONSTRUCTS IN THE PRESENCE AND ABSENCE OF LOADING AT DAYS 5, 15, 20, AND 30

Treatment group	Average viability $\pm$ standard deviation (%)
Day 5	66.0 $\pm$ 5.7
Day 5 loaded	57.8 $\pm$ 7.9
Day 15	54.3 $\pm$ 3.6
Day 15 loaded	42.2 $\pm$ 4.7
Day 20	48.9 $\pm$ 8.0
Day 20 loaded	54.3 $\pm$ 3.7
Day 30	46.9 $\pm$ 4.8
Day 30 loaded	36.0 $\pm$ 4.8

$n \geq 5$ for each time point.

mineralization concentration as in day 30 unloaded gels (Fig. 8D), but cells within day 30 loaded gels appeared to be connected by cell processes ranging between 100 and 150 μ m in length (Fig. 8D). Staining of day 5 long-term gels showed the greatest concentration of mineralization where a distinct honeycomb structure was observed (Fig. 8C). von Kossa staining of day 5 unloaded gels treated for 40 h with either octanol or AGA showed minimal mineralization after communication inhibitors were removed. von Kossa staining of day 30 gels treated with formic acid (negative controls) resulted in decalcification of mineralization in the extracellular matrix (Fig. 9A). Staining of 16–18-day-old mouse embryos (positive controls) showed positive staining for matrix mineralization (Fig. 9B). Staining of gels with BGP but without cells, and gels with porous plugs placed on top for 40 h resulted in no appreciable von Kossa staining (Fig. 9C, D).

RT-PCR for OB-Cad did not result in any measurable expression at any time point. In contrast, connexin-43 expression was detected at all time points. Connexin-43 was upregulated significantly at day 7, followed by a significant decrease in expression at days 15 and 30 (Fig. 10). RT-PCR in loaded gels for OB-Cad also did not result in any measurable expression at each time point. A significant 122-fold increase in connexin-43 expression was present in day 5 loaded gels, and this increase was sustained in day 5 long-term gels (Fig. 10). In day 30 loaded gels, the increase in connexin-43 expression was approximately fivefold compared with day 30 gels that were unloaded (Fig. 10). Collagen-II was not detected at any time points. However, as differentiation progressed within the scaffolds, expression of *Oct-4* decreased, *Runx2* expression was highest at day 7, and osteocalcin expression was highest at day 30 (Fig. 11). In loaded gels, *Oct-4* expression was higher in day 5 loaded gels compared with other time points (Fig. 11) and all loaded gels did not express *Runx2* as highly as day 7 gels (Fig. 11). However, day 30 loaded gels and day 5 long-term gels showed significant upregulation of osteocalcin (Fig. 11).

Discussion

The goal of this investigation was to determine whether bone formation could be augmented during osteoblast differentiation in a 3D tissue-engineered scaffold, lending in-

FIG. 6. Immunofluorescence of osteoblast-cadherin (OB-Cad) as differentiation progressed in (A) day 5, (B) day 30, (C) day 7, and (D) day 5 loaded gels. Immunofluorescence of connexin-43 as differentiation progressed in (E) day 5, (F) day 30, (G) day 7, and (H) day 5 loaded gels. Scale bars are 200 μ m. Color images available online at www.liebertpub.com/tea

sight into the relationship between osteoblast differentiation state and ability to respond to a mechanical stimulus. A recent study has shown that cyclic mechanical strain can enhance triple-supplement-driven osteogenesis in day 21 differentiated hESCs, and can also enhance the presence of nodules of mineralization.⁶ This study showed a precise role

FIG. 7. Immunofluorescence staining for OB-Cad in (A) negative controls and (B) positive controls. Immunofluorescence staining for connexin-43 in (C) negative controls and (D) positive controls. Scale bars are 100 μ m. Color images available online at www.liebertpub.com/tea

FIG. 8. (A) von Kossa staining of day 5 gels, (B) day 30 gels, (C) day 5 long-term gels, and (D) day 30 loaded gels. Black arrows indicate counterstained cells; brown/black staining indicates positive staining for mineralization. Scale bar is 250 μ m. Color images available online at www.liebertpub.com/tea

FIG. 9. von Kossa staining of (A) negative control (day 30 gel treated with formic acid), (B) positive control (16–18-day-old mouse embryo), (C) day 30 gel without cells with beta-glycerol phosphate (BGP), and (D) day 5 gel left with a porous plug for 40 h in a 37°C incubator. Scale bar is 250 μ m. Red stain indicates counter-stained cells; brown/black stain indicates areas of positive staining for mineralization. Color images available online at www.liebertpub.com/tea

FIG. 10. (A) Connexin-43 expression in cell gels at days 5, 7, 15, and 30. (B) Connexin-43 expression in loaded gels at days 5, 15, and 30. (C) Connexin-43 expression of day 5 loaded, day 7, and day 5 long-term gels. (D) Connexin-43 expression in day 30 and day 30 loaded gels. Asterisks signify significant differences between groups at $\alpha=0.05$. Color images available online at www.liebertpub.com/tea

FIG. 11. (A, C, and E) *Oct-4*, *Runx2*, and osteocalcin expression, respectively, in day 5, day 7, day 15, and day 30 gels. (B, D, and F) *Oct-4*, *Runx2*, and osteocalcin expression, respectively, in day 5 loaded, day 7, day 15 loaded, day 30 loaded, and day 5 long-term gels. Asterisks signify significant differences between groups at $\alpha=0.05$. Color images available online at www.liebertpub.com/tea

of mechanical stimulation in guiding or modifying stem cell lineage and function.⁶ Further investigation of the specific interaction between cells and the matrix is challenging, as the mechanical environment that is perceived by cells *in vivo* is complex and not fully characterized.^{33,34}

In the present study, we have demonstrated that mechanical prestimulation via confined compression of a gel at an early stage of cell differentiation (day 5) can accelerate and enhance the amount and organization of mineralization that is initiated by differentiating osteoblasts within a collagen-I scaffold. The results from this study indicate that the BGP technique for driving osteogenesis within a collagen-I

scaffold is a preparation that is responsive to mechanical loading, and produces significant increases in the amount of mineralization present in the matrix following loading. The BGP technique for promoting osteogenesis within this scaffold has reduced teratogenic potential.⁷ This study builds on the osteogenic capability of this scaffold preparation by introducing a loading regime and highlighting a differentiation time point when cells within this scaffold are most responsive to loading. Therefore, we present an optimization factor (confined mechanical compression at day 5) that can be used to prime this scaffold prior to implantation in bone healing applications.

As the cells differentiated to osteoblasts within this scaffold, intercellular communication structures, such as OB-Cad and connexin-43, increased in presence, and overall gap junction activity increased. It has been previously shown that interference with gap junction function results in delayed maturation of osteoblasts, and reduced mineralization.^{10,35–37} The effect of interfering with cell communication structures within this scaffold preparation can be investigated to lend further insight on the role of gap junctions in downstream osteoblast function.

The majority of tissue engineering strategies that incorporate stem cells for bone healing focus on using bone-marrow-derived stromal cells.^{38,39} The ability of differentiating osteoblasts to initiate mineralization of the matrix in collagen gels has been previously reported with murine mesenchymal stem cells.⁴⁰ In bone, collagen-I is the primary component of the organic matrix, and therefore makes it a favorable candidate as a scaffold for bone tissue engineering.⁴¹ Collagen-I is known to be a favorable material for cell adhesion, and provides a permeable matrix through which nutrients, oxygen, and mechanically transduced molecules can be transferred.³⁸ Using ESCs within a collagen-I scaffold creates an environment where it has been previously demonstrated that cells can be driven to an osteoblast fate, and the resulting construct has a reduced teratogenic potential.⁷ In this study we have shown that this scaffold preparation can be controlled and modified via mechanical prestimulation to augment stem cell fate and function. The methods used in this study to evaluate the effectiveness of mechanical loading on altering cell differentiation and activity can be transferred for other healing applications in addition to bone formation, and most importantly highlights a loading regime that can be used to augment osteoblast activity within this scaffold.

In this study, *Runx2* and *OCN* were expressed in late-differentiated gels, indicating that by day 30, cells had differentiated to osteoblasts. The timeline of expression of early and late osteoblast markers in this scaffold preparation, and the characterization of these cells have been previously performed.⁷ For this reason we believe that in this study, the measured gene expression of *Runx2* and osteocalcin as cell differentiation progresses is sufficient to support that these cells are driven to an osteoblast fate.

Under prestimulation with confined compression, this study showed that cells in an earlier state of differentiation are more sensitive to mechanical loading and respond by significantly increasing the activity of gap-junction-mediated cellular communication. As differentiation to osteoblasts progressed within the gels, connexin-43 expression was greatest at day 7, suggesting that the loading regime initially applied on day 5 gels (for a total time period of 40 h) specifically focused on a time point when these cells were not only responsive to mechanical stimulation but also already highly expressing connexin-43.

Day 5 long-term gels showed the greatest concentration and structure of mineralization present following loading. Specifically, a distinct honeycomb structure of mineral deposition was observed. This honeycomb structure resembles the structure of mineralization attributed to late osteoblast/early osteocyte activity.^{42,43} Therefore, the loading results from this study suggest that between days 5 and 7, changes in activity of gap junctions, protein presence, and gene ex-

pression of connexin-43 occur to guide the development of functional osteoblasts within this scaffold. It is possible that prestimulation with mechanical loading during this time period may be most advantageous for bone healing applications.

Several studies have shown that prestimulation of cells generally improves proliferative potential and enhances osteogenic differentiation.^{44,45} This study provides a novel combination of a cell-based scaffold with mechanical prestimulation at a specific time point of differentiation to provide targeted acceleration of differentiation of mESCs to an osteoblast fate, and to optimize osteoblast activity within a collagen-I scaffold. The necessity of intercellular communication in the ability of osteoblasts within this scaffold to perceive this mechanical stimulus, and the extent to which cell communication is required to drive cells in this scaffold to an osteoblast fate is yet to be determined.

Overall, this study demonstrates a loading protocol that can be used to modify the activity of ESCs stimulated to differentiate to osteoblasts within a collagen-I scaffold, and can augment the concentration and structure of mineralization present in the matrix following mechanical prestimulation. The findings from this study will contribute to an understanding of the coupling of mechanical stimulation and intercellular communication in the determination of the lineage and function of differentiating cells, and enables exploration to optimize this scaffold for different healing applications.

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Disclosure Statement

No competing financial interests exist.

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