Response of a Preosteoblastic Cell Line to Cyclic Tensile Stress Conditioning and Growth Factors for Bone Tissue Engineering

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Bone regeneration can be accelerated by utilizing mechanical stress and growth factors (GFs). However, a limited understanding exists regarding the response of preosteoblasts to tensile stress alone or with GFs. We measured cell proliferation and expression of heat-shock proteins (HSPs) and other bone-related proteins by preosteoblasts following cyclic tensile stress (1%–10% magnitude) alone or in combination with bone morphogenetic protein-2 (BMP-2) and transforming growth factor- β 1 (TGF- β 1). Tensile stress (3%) with GFs induced greater gene upregulation of osteoprotegerin (3.3 relative fold induction [RFI] compared to sham-treated samples), prostaglandin E synthase 2 (2.1 RFI), and vascular endothelial growth factor (VEGF) (11.5 RFI), compared with samples treated with stimuli alone or sham-treated samples. The most significant increases in messenger RNA expression occurred with GF addition to either static-cultured or tensile-loaded (1% elongation) cells for the following genes: HSP47 (RFI=2.53), cyclooxygenase-2 (RFI=72.52), bone sialoprotein (RFI=11.56), and TGF- β 1 (RFI=8.05). Following 5% strain with GFs, VEGF secretion increased 64% (days 3–6) compared with GF alone and cell proliferation increased 23% compared with the sham-treated group. GF addition increased osteocalcin secretion but decreased matrix metalloproteinase-9 significantly (days 3–6). Tensile stress and GFs in combination may enhance bone regeneration by initiating angiogenic and anti-osteoclastic effects and promote cell growth.

Introduction

MECHANOSIGNALS GENERATED by strain modulation can control diverse cell activities such as proliferation,¹ migration,¹ and differentiation.² *In vitro* strain modulation has been investigated as a promising mechanical cue for regeneration of bone,³ tendon,⁴ and blood vessels.⁵ The Flexcell[®] tensile stress system is a commercial bioreactor that has been utilized to explore the response of bone cells to supraphysiological levels of mechanical stimulation (6%–18% elongation with 0.05–2 Hz).^{3,6,7} This bioreactor has applied 5%–10% elongation with 0.2–1 Hz frequency to 3D scaffolds including polyurethane nanofibrous scaffolds,⁸ and fibrin,⁹ and collagen gels.¹⁰ Tensile stress of 3%–20% magnitude modulated expression of bone-related molecules such as collagen,^{7,11} osteocalcin (OCN),^{1,7} osteopontin (OPN),^{1,7} matrix metalloproteinase-13 (MMP-13),¹² osteoprotegerin (OPG),⁶ prostaglandin E₂ (PGE₂),¹³ cyclooxygenase-2 (COX-2),¹⁴ vascular endothelial growth factor (VEGF),^{1,15} and transforming growth factor- β 1 (TGF- β 1).¹⁶

Other external stimulating factors for bone regeneration are osteoinductive growth factors (GFs) such as TGF, bone morphogenetic protein (BMP), and platelet-derived GF.¹⁷

Diverse delivery methodologies for these GFs have been developed for *in vitro* or *in vivo* bone tissue engineering research and clinical studies.^{18–22} *In vitro* studies using preosteoblasts and stem cells suggest that osteoinductive GFs can induce increased cell proliferation and osteogenic differentiation.¹⁷ Exogenous BMP-2 delivery has been shown to enhance bone regeneration,²³ and TGF- β 1 can induce higher alkaline phosphate (ALP) production and proliferation in bone marrow cells.²⁴

In response to external mechanical stresses such as tensile stress, compression, hydrostatic pressure, and shear stress and exogenous addition of GFs,^{25–30} cells produce elevated heat-shock proteins (HSPs). HSPs act as molecular chaperones and have pivotal roles in anti-apoptotic protection, proliferation, and differentiation.^{31,32} The histological analysis by Leonardi *et al.*³³ demonstrated strong expression of HSP27 in osteoblasts and dental laminar/epithelium undergoing the development process in craniofacial bones and teeth, respectively, suggesting HSP27 may be a key modulator in controlling bone formation. HSP47 is instrumental in the collagen synthesis process as a procollagen-binding molecular chaperone residing in the endoplasmic reticulum.³⁴ Deletion of the HSP47 gene in murine models resulted in abnormal collagen

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production and triple helix formation.^{35,36} Furthermore, HSP27 and HSP47 can be induced by TGF- β .^{30,37} HSP27 can also be promoted by bone-related molecules such as estrogen or prostaglandin in osteoblasts.^{38,39} HSPs (e.g., HSP27, HSP47, and HSP70) and type I collagen are highly expressed in osteoblasts of rat tibia near the bone-forming region.⁴⁰ Therefore, HSP expression by bone cells may modulate osteoblast activity and matrix production in response to stress.^{30,37–40}

Although prior studies have explored the response of osteoblasts to tensile stress and GFs independently, few studies have investigated the combined effect of tensile stress with exogenous GFs. No previous studies have explored the effect of adding both BMP-2 and TGF- β 1 to a tensile stress conditioning system simultaneously. Furthermore, prior bone tissue engineering studies measuring the response of osteoblasts to tensile stress and GFs have considered only a limited number of osteogenic markers and have not characterized the response of HSPs, which could be potentially beneficial in bone formation. The purpose of this study was to investigate whether combinatorial stress conditioning with tensile stress and GFs could (1) synergistically upregulate three HSPs (i.e., HSP27, HSP47, and HSP70) and potentially enhance (2) mitogenic, (3) osteogenic, (4) angiogenic, and (5) anti-osteoclastic activity of preosteoblasts, considering a wide range of markers representative of these processes. The effect of supraphysiological cyclic tensile stress (equibiaxial 1%–10% elongation) using the Flexcell® Tensile stress System in combination with two GFs (BMP-2 and TGF- β 1) on MC3T3-E1 cell proliferation and expression of HSPs, pivotal bone-related proteins (e.g., matrix proteins and enzymes), angiogenic factors (VEGF), and anti-osteoclastic proteins (e.g., OPG and MMP-9) was investigated in this study.

Materials and Methods

Cell culture and preparation for stress treatment

A murine preosteoblastic cell line, MC3T3-E1 (American Type Culture Collection), was cultured as a monolayer with growth media composed of alpha minimum essential medium (α MEM; Mediatech), 10% fetal bovine serum (FBS; Sigma), and 1% penicillin–streptomycin (PS; Invitrogen) in a 5% CO₂ incubator at 37°C. Cells were seeded in a six-well BioFlex® plate (Flexcell International) coated with type I collagen and then cultured further for 16 h before stress treatment. Cells were seeded at a concentration of 5×10^4 cells/well and cultured in 10% FBS osteogenic media for the following studies: cell morphology and measurement of gene expression with PCR following 1% tensile stress, and cell proliferation following 5% tensile stress with GFs after 3 or 6 days of treatment. For PCR (measured following 24 h stress conditioning) and enzyme-linked immunosorbent assay (ELISA) analysis (measured at 3 and 6 days of treatment), cells were plated at a concentration of 2×10^5 cells/well and cultured in 1% FBS osteogenic media during stress conditioning. Osteogenic media was composed of α MEM, 50 μ g/mL L-ascorbic acid, 10 mM β -glycerol phosphate, 1% PS, and 1% or 10% FBS.

Stress treatment and GF addition

The Flexcell® Tensile stress Plus™ System (Flexcell International) was utilized to apply a range of cyclic tensile stress

conditioning protocols of 1%, 3%, 5%, and 10% maximum elongation with 0.2 Hz frequency (10 s cyclic sinusoidal tensile stress, 10 s rest phase inserted, intermittent mode) for varying durations depending on analysis. Maximum tensile elongations were represented as a percentage, which can be converted into corresponding strain (ϵ) and associated applied negative pressures (kPa) as follows: 1%, 3%, 5%, and 10% tensile elongations denote 0.01, 0.03, 0.05, and 0.1 strain (ϵ), respectively, which were generated by corresponding negative pressures of 6.13, 16.74, 25.52, and 42.50 kPa. A circular loading post (diameter = 25 mm) was used to apply equibiaxial tensile stress as shown in Figure 1. This system generates tensile stress by modulating the flexible bottoms of multiwell plates with negative (vacuum) pressure. Immediately before tensile stress, growth media was replaced with osteogenic media to induce osteogenesis during tensile stress. To investigate the effect of GF treatment alone or the combination of tensile stress and GFs, cells were cultured during the application of tensile stress in osteogenic media supplemented with BMP-2 and TGF- β 1 at 50 ng/mL and 2 ng/mL, respectively. These concentrations were shown to cause differentiation in the MC3T3-E1 cell line as previously reported by Chung *et al.*⁴¹

Morphology analysis

Cell morphology following cyclic tensile stress (1% magnitude) for 6 days was visualized by fixing the cells immediately after stress and staining for F-actin, a cellular skeleton protein, using rhodamine phalloidin (Invitrogen). Cells were fixed with 3.7% paraformaldehyde in a phosphate-buffered solution (PBS; Fisher Scientific) and permeabilized using 0.1% Triton X-100 (Sigma)/PBS. For blocking, samples were incubated in 1% bovine serum albumin (Amersham) dissolved in PBS for 30 min at room temperature followed by 20 min incubation in rhodamine phalloidin solution in the dark. For nucleolus counterstaining, cells were mounted with VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Stained images were acquired using a fluorescent inverted microscope (CTR6500; Leica Microsystems).

Quantitative real-time RT-PCR

Gene expression of markers listed in Table 1 was measured following 24-h (1 day) short-term and 3- and 6-day long-term tensile stress conditioning alone or in combination with GFs. RNA was isolated by spin protocol using RNeasy Mini kit (Qiagen) and QIAshredder (Qiagen) immediately following stress conditioning according to the manufacturer's protocol. Isolated RNA was converted to cDNA using Reverse Transcription System (Promega) by continuous reactions at 25°C for 10 min and 42°C for 45 min followed by heating at 99°C for 5 min. After reverse transcription, cDNA samples were mixed with Taqman PCR Master Mix (Applied Biosystems) and each specific primer and polymerized in a 7300 Real-Time PCR System (Applied Biosystems). The PCR was performed at 50°C for 2 min followed by 95°C for 10 min. For each polymerization (a total of 45 cycles), temperature was set at 95°C for 15 s and 60°C for 1 min. Taqman® Gene Expression Assay (Applied Biosystems) for specific gene detection was used, and primers and probes are listed in Table 1. Relative fold induction (RFI) of

FIG. 1. Illustration of a computer-controlled Flexcell® tension bioreactor. Color images available online at www.liebertonline.com/tea

TABLE 1. SPECIFIC GENES MEASURED WITH REAL-TIME RT-PCR WITH CORRESPONDING ASSAY ID FROM APPLIED BIOSYSTEMS, SEQUENCE, AND SIZE OF MARKER

Target protein name	Assay ID	Sequence (5' → 3')	Size
HSP27	Heat-shock protein 2	Mm00517908_ml	TCGGAGAAGGCCTCCTGCCAGAAGA 115
HSP47	Serine (or cysteine) peptidase inhibitor, clade H, member 1	Mm00438056_ml	TGGTAAACCCTCACAGGTCCTCTGT 76
HSP70	Heat-shock protein 1B	Mm03038954_sl	GTAAAGGTTTTGTGGTATAACCACT 141
OPN	Secreted phosphoprotein 1	Mm01611440_mH	GAACAGTATCCTGATGCCACAGATG 102
OCN	Bone gamma-carboxyglutamate protein, related sequence 1	Mm00649782_gH	CCTTGGAGCTTCAGTCCCCAGCCCA 89
OPG	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Mm01205928_ml	AGTGTGGAATAGATGTCACCCTGTG 75
BSP	Integrin binding sialoprotein	Mm00492555_ml	GGTTCCAGTCCAGGGAGGCAGTGA 98
ALP	Alkaline phosphatase, liver/bone/kidney	Mm01187113_gl	TCCTGGGAGATGGTATGGGCGTCTC 71
Col 1	Collagen, type 1, alpha 1	Mm00801666_gl	CGATGGATTCCCGTTCGAGTACGGA 89
MMP-13	Matrix metalloproteinase 13	Mm01168713_ml	CTTTAGAGGGAGAAAATTCTGGGCT 124
MMP-9	Matrix metalloproteinase 9	Mm00600164_gl	TCTTCAAGGACGGTTGGTACTGGAA 72
Runx2	Runt-related transcription factor 2	Mm00501580_ml	GACGAGGCAAGAGTTTCACCTTGAC 129
VEGF	Vascular endothelial growth factor A	Mm00437308_ml	CAAAGCCAGAAAATCACTGTGAGCC 66
TGF-β1	Transforming growth factor, beta 1	Mm00441724_ml	TGGTGGACCGCAACAACGCCATCTA 99
COX-2	Prostaglandin-endoperoxide synthase 2	Mm01307330_gl	TGTACTACACCTGAATTTCTGACAA 73
PGES-2	Prostaglandin E synthase 2	Mm00460181_ml	CAGGAAGGAGACAGCTTGCAACAGC 73
BMPR-2	Bone morphogenetic protein receptor type II (serine/threonine kinase)	Mm03023976_ml	TGGAGTATTATCCCAATGGATCTCT 94
Angiopoietin 1	Angiopoietin 1	Mm00456498_ml	AAAAAACAGTTTACTAGAGCACAAA 118
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_gl	GTGAACGGATTTGGCCGTATTGGGC 107

COX-2, cyclooxygenase-2; MMP, matrix metalloproteinase; OCN, osteocalcin; OPN, osteopontin.

each messenger RNA (mRNA) expression was calculated according to the $2^{-\Delta\Delta C_T}$ method used by Lee *et al.*⁴² Threshold cycle (C_T), derived using SDS v1.2×system software of 7300 Real-Time PCR System, denotes the fractional cycle number at threshold polymerized gene and $\Delta\Delta C_T$ was derived from the following formula: (C_T of target gene - C_T of GAPDH)_{treated group} - (C_T of target gene - C_T of GAPDH)_{control group}.⁴² Treated groups denote samples exposed to tensile stress or GFs alone or in combination, and control groups indicate static-cultured cells without GF addition. Based on this method, the RFI level of all markers was normalized relative to the control as well as the house-keeping gene, giving a mean basal level of expression close to 1. In addition, RFI_{MAX} and RFI_{MIM} were denoted, respectively, as a maximum and minimum level among all normalized RFIs derived from four independent tests, which included treatment with tensile stress (magnitudes of 1%, 3%, 5%, and 10%) alone or in combination with GFs. These terms were used in the Results section to describe the data trends for the response to individual or combinatorial tensile stress and GFs on gene expression.

Enzyme-linked immunosorbent assay

For the analysis of protein secretion, osteogenic conditioned media was collected at days 3 and 6 following 5% cyclic tensile stress alone or in combination with GFs. As the osteogenic media was replaced once at day 3, the osteogenic media collected at day 6 included the protein secreted during the stress conditioning periods of days 3–6. OPG, VEGF, OPN, and MMP-9 protein expressions were measured using Quantikine® enzyme-linked immunosorbent assay (ELISA) (R&D Systems) and OCN levels were determined by Mouse Osteocalcin EIA kit (Biomedical Technologies) according to the manufacturer's protocol. Cell culture supernatant was added to a 96-well microplate coated with antibodies specific for the previously mentioned markers and incubated further with detection solutions at room temperature. The optical absorbance of the solution was measured at 450 nm by a microplate reader and compared with a standard curve.

MTS assay

Proliferation of MC3T3-E1 cells immediately following 5% cyclic tensile stress alone or in combination with GFs at 3 and 6 days was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega). MTS working solution composed of MTS stock solution and basal culture media (w/o FBS and PS; volume ratio of MTS and media = 1:5) was added to cells. After 4 h of incubation at 37°C, the optical density of the MTS solution was measured at 490 nm by a microplate reader (SpectraMax M2e; Molecular Devices).

Statistical analysis

All data are presented as mean ± standard deviation. Experimental groups with a minimum of three repetitions were tested and analyzed independently. To demonstrate statistical significance of tensile stress and GF treatment, two-way ANOVA supplemented by a Tukey multiple comparison test was performed to compare the means be-

tween each group. This method was used to determine whether there was a statistically significant interaction between tensile stress and GFs based on measurement of alterations in mRNA or protein expression of bone-related proteins. For tests involving tensile stress alone, one-way ANOVA supplemented by a Tukey multiple comparison test was applied. The statistical significance was defined as a *p*-value lower than 0.05.

Results

Morphological analysis of tensile stress conditioning

F-actin filaments were stained immediately after cyclic tensile stress (1% elongation) for 6 days to determine its effect on cellular morphology. Static-cultured cells possessed an irregular shape with randomly oriented actin filaments as observed in Figure 2A. In contrast, cells located on the edge of the dish exposed to tensile stress exhibited an elongated morphology with aligned actin in a circular direction according to the edge of the loading post (Fig. 2B). In the center of the dish for samples exposed to tensile stress, the morphology was nearly identical to static-cultured cells (Fig. 2C).

Effect of long-term cyclic tensile stress conditioning without GF treatment on mRNA of HSPs and bone-related proteins

mRNA from MC3T3-E1 cells was measured by real-time RT-PCR following 3 and 6 days of tensile stress (1% elongation). Tensile stress caused statistically significant increases in HSP27 and HSP70 mRNA at day 3 but decreases in HSP27 and HSP47 at day 6 (HSP27, RFI = 1.82 and 0.68; HSP47, RFI = 1.12 and 0.47; HSP70, RFI = 1.53 and 0.90 at days 3 and 6, respectively) (Fig. 3A–C). Tensile stress induced significant upregulation in OPN (RFI = 2.21) and COX-2 (RFI = 4.60) mRNA on day 6 (Fig. 3D, E). However, ALP (RFI = 0.06), BSP (RFI = 0.04), type I collagen (RFI = 0.40), and MMP-9 (RFI = 0.07) mRNA were significantly suppressed in response to 6 days of tensile stress (Fig. 3F, G, H, I). There were no significant alterations in the genes of other bone-related proteins such as Runx2, VEGF, prostaglandin E synthase 2 (PGES-2), TGF-β1, MMP-13, and angiotensin I (data not shown).

Combined conditioning effect of short-term (24 h) cyclic tensile stress and GFs on mRNA expression of HSPs

The combinatorial effect of tensile stress and GFs was investigated by exposing confluent cell monolayers to varying magnitudes of cyclic tensile stress (1%, 3%, 5%, and 10%) and supplementing culture media with GFs (BMP-2 and TGF-β1) for 24 h (Fig. 4). Cells exposed to tensile stress alone possessed significantly higher HSP27 (RFI = 1.57 and 1.46 for 1% and 3% magnitude tests) and HSP70 mRNA (RFI = 1.76, 1.24, and 1.42 at 1%, 3%, and 10% magnitudes, respectively) RFIs when compared with the unstressed control group; however, HSP47 mRNA experienced no significant change in response to tensile stress. GFs with tensile stress inhibited HSP27 and HSP70 mRNA expression compared with tensile stress alone (43% and 33% decrease in HSP27 for 3% and 10% magnitude tests; 58%, 51%, and 61% decrease in HSP70 at 1%, 3%, and 5% tensile magnitudes; *p* < 0.05). HSP47 mRNA RFI

FIG. 2. Cell morphology measured following 6 days of cyclic tension (1% elongation) for cells exposed to static conditions (A) and tension located at the edge (B) and center (C) of a six-well BioFlex[®] culture plate with a flexible culture substrate coated with type I collagen (scale bar=100 µm). The arrow in (B) indicates the direction of actin according to the shape of the loading post. The images in B show different cell morphology depending on the area of the BioFlex[®] culture plate. Color images available online at www.liebertonline.com/tea

(RFI_{MAX}=2.53) was increased because of inclusion of GFs alone ($p<0.05$) and also in combination with tensile stress ($p<0.05$ for 1%, 5%, and 10% tests). However, GFs alone showed significantly increased expression of HSP47 mRNA (RFI=2.30 and 2.47 at 3% and 10% tests, respectively) compared with tensile stress with GFs (RFI=1.59 for both tests). Two-way ANOVA demonstrated that the interaction between tensile stress and GFs significantly affected the regulation of the following genes for the corresponding % strain: HSP27 (3% and 10% tests), HSP47 (10%), and HSP70 (1%).

Combined conditioning effect of short-term cyclic tensile stress and GF treatment on mRNA expression of bone-related proteins

Identical methods as described in the previous section were used to explore the effect of combined conditioning with tensile stress (applied for 24h) and GFs on bone-related protein mRNA (Figs. 5–7). OPN mRNA expression (RFI_{MIM}=0.14) was suppressed significantly by GFs in both static-cultured and tensile-loaded cells. OPN mRNA was significantly increased (RFI=1.97) following 1% tensile stress, but no significant difference was observed following other magnitudes of tensile stress (Fig. 5A). There was no apparent impact on OCN mRNA expression by either tensile stress or GFs (Fig. 5B). BSP mRNA was significantly upregulated (RFI_{MAX}=9.75) in response to GF addition alone, but 3%–10% tensile stress with GFs (RFI_{MAX}=3.43 RFI) diminished the induction effect of GF alone on BSP mRNA (RFI_{MAX}=8.33 RFI) (Fig. 5C). Similarly, type I collagen mRNA was induced by GFs alone (RFI_{MAX}=1.83), but tensile stress (RFI=1.13 and 1.42 at 3% and 10%; $p<0.05$) in combination with GFs reduced the stimulatory effect that was observed

by the addition of GFs (RFI=1.83 and 1.72) (Fig. 5D). OPG mRNA was induced more significantly by cyclic tensile stress (3%) and GFs (RFI_{MAX}=3.55) compared with either tensile stress or GFs alone (Fig. 6A). MMP-9 mRNA was increased significantly (RFI=1.83) in response to 1% tensile stress alone but decreased with GF addition (RFI=0.17 and 0.21 for samples experiencing static conditions or tensile stress, respectively) (Fig. 6B). However, MMP-9 mRNA expression was inhibited significantly by 5% and 10% tensile stress alone (RFI=0.58 and 0.48, respectively) and more significantly suppressed with GF addition (RFI_{MIM}=0.13) in both static and tensile-stressed cells.

The effect of exogenous GF treatment (BMP-2 and TGF-β1) on endogenous production of TGF-β1 mRNA in combination with tensile stress was measured (Fig. 6C). Addition of both GFs exogenously promoted significant endogenous induction of TGF-β1 mRNA (RFI_{MAX}=8.05) in static-cultured and tensile-loaded cells. Without GF treatment, endogenous induction of TGF-β1 mRNA by tensile stress was minimal. Bone morphogenic protein receptor type II (BMPR-2) mRNA expression was promoted by the addition of GFs to static or tensile stress-treated cells (RFI_{MAX}=2.04 at 1%, 5%, and 10%) (Fig. 6D). Further, COX-2 mRNA expression was significantly induced by GF addition in both static-cultured and tensile-loaded cells (RFI_{MAX}=72.52) (Fig. 7A). Similar to OPG, PGES-2 mRNA was significantly increased (RFI=2.08 RFI) in the cells exposed to combinatorial cyclic tensile stress (3%) and GFs, suggesting additional benefit when both stimuli are provided compared with treatment with tensile stress (RFI=1.58) or GFs (RFI=1.49) independently (Fig. 7B). Statistical analysis confirmed significant interaction between tensile stress and GFs in the regulation of the genes for the following proteins: OPN (1% magnitude test), PGES-2 (10%),

FIG. 3. Expression of messenger RNA (mRNA) for heat-shock proteins (HSPs) and bone-related proteins on days 3 and 6 following cyclic tension alone (1% elongation). HSP27 (A); HSP47 (B); HSP70 (C); osteopontin (OPN) (D); cyclooxygenase-2 (COX-2) (E); type I collagen (F); alkaline phosphate (ALP) (G); bone sialoprotein (BSP) (H); matrix metalloproteinase-13 (MMP-13) (I). *Statistically significant difference between the tension-treated groups and control ($p < 0.05$).

BSP (3%, 5%, and 10%), OCN (5%), type I collagen (5% and 10%), COX-2 (3%, 5%, and 10%), MMP-9 (1%, 5%, and 10%), and TGF- β 1 (3%).

Induction of VEGF gene and protein by combined stress conditioning with cyclic tensile stress and GFs

All magnitudes (1%, 3%, 5%, and 10%) of cyclic tensile stress in combination with GFs ($\text{RFI}_{\text{MAX}} = 11.48 \text{ RFI}$) for 24 h

caused the greatest level of induction of VEGF mRNA expression among all test groups (significance at 1%, 3%, and 10% elongation) (Fig. 8A). However, GF addition to both static and tensile-stressed groups increased VEGF mRNA expression significantly. Similarly, the addition of tensile stress (5%) and GFs caused significant VEGF protein secretion for 0–3 and 3–6-day durations compared with static-cultured and tensile stress alone. VEGF expression was undetectable by ELISA for cells untreated with GFs or tensile

FIG. 4. Expression of mRNA for HSPs following 24 h cyclic tension (1%, 3%, 5%, and 10% elongation) and growth factors (GFs). HSP27 (A); HSP47 (B); HSP70 (C). *Statistical significance between the control group and the treated groups (GF alone; tension alone for all protocols; GF and tension protocols in combination). **Statistically significant difference between groups experiencing static with GFs and tension with GFs. #Statistically significant difference between groups exposed to tension alone and with GFs ($p < 0.05$).

FIG. 5. Expression of mRNA for bone matrix proteins following 24 h conditioning with cyclic tension (1%, 3%, 5%, and 10% elongation) and GFs. OPN (A); osteocalcin (OCN) (B); BSP (C); type I collagen (D). *Statistical significance between the control group and the treated groups (GF alone; tension alone for all protocols; GF and tension protocols in combination). **Statistically significant difference between groups experiencing static with GFs and tension with GFs. #Statistically significant difference between groups exposed to tension alone and with GFs ($p < 0.05$).

stress. VEGF levels following tensile stress with GFs were significantly higher at 74% and 64% for days 0–3 and 3–6, respectively, compared with GF addition alone (Fig. 8B). Statistical analysis confirmed that the interaction between tensile stress and GFs enhanced both VEGF mRNA expression (3% and 10% test) and protein secretion.

Protein release following combined conditioning with cyclic tensile stress and GFs

Bone-related proteins (OPN, OPG, OCN, and MMP-9) in the media at days 0–3 and 3–6 were measured by ELISA to determine the level of protein secretion for combinatorial preconditioning with 5% (magnitude) tensile stress and GFs (Fig. 9). The addition of GFs to static-cultured and tensile-cultured cells diminished OPN secretion by 39% and 102% respectively for initial cultivation periods of days 0–3, but enhanced expression by 123% and 240% (Fig. 9A) at day 6. GF-treated cells exhibited greater levels of OPN secretion with increasing time (53% in GF addition alone; 106% in tensile stress with GFs), but cells without GF addition demonstrated decreasing OPN secretion with increasing time. On day 6, tensile stress without GFs stimulated 135% higher secretion of OPG compared with the static-cultured cells ($p < 0.05$) (Fig. 9B). There was no detection of OCN without the addition of GFs, suggesting that GFs significantly promoted secretion (Fig. 9C). In contrast, with GFs for both tensile stress and static-cultured groups, MMP-9 secretion was downregulated to an undetectable amount in response to GFs. However, without GFs, secreted MMP-9 levels for

longer cultivation time (days 3–6) increased more significantly (56%) under static conditions but decreased (97%) in response to tensile stress (Fig. 9D). Statistical analysis showed that significant interaction between tensile stress and GFs caused secretion of the following proteins: OPN, OCN, and MMP-9 at days 0–3, and OPN and MMP-9 at days 3–6.

Cell proliferation in response to combined conditioning with cyclic tensile stress and GFs

Cell proliferation following tensile stress (5% elongation) with GFs was measured using MTS assay based on mitochondrial metabolic activity following 3 and 6 days of stress conditioning (5%) (Fig. 10). Viability of all test groups on day 3 was not statistically different. However, only cells treated with tensile stress and GFs on day 6 exhibited significantly greater levels of viability of 23% compared with the static-cultured cells.

Discussion

Although native bone experiences multiple types of stress and combinations of GFs, few studies have explored the response of bone to dual-mechanical stress^{43,44} or combinatorial treatment with mechanical stress and GFs.^{45,46} This study investigated whether tensile stress alone or in combination with GFs could positively impact pivotal markers suggestive of mitogenic, osteogenic, angiogenic, and anti-osteoclastic activities of preosteoblasts. Our chosen tensile stress protocols (1%–10%) fall within the typical magnitudes of 1%–20%

FIG. 6. Expression of mRNA for osteoprotegerin (OPG) (A), MMP-9 (B), TGF- β 1 (C), and bone morphogenetic protein receptor type II (BMPR-2) (D) following 24 h conditioning with cyclic tension (1%, 3%, 5%, and 10% elongation) and GFs. *Statistical significance between the control group and the treated groups (GF alone; tension alone for all protocols; GF and tension protocols in combination). **Statistically significant difference between groups experiencing static with GFs and tension with GFs. #Statistically significant difference between groups exposed to tension alone and with GFs ($p < 0.05$). TGF- β 1, transforming growth factor β 1.

and frequencies of 0.1–1 Hz previously applied by other researchers for bone-related studies.^{1,6,12,14,15,47–49} Studies by Simmoms *et al.* have shown that mesenchymal stem cells produced increased calcium accumulation in response to 3% strain (0.25 Hz).⁵⁰ Other studies have demonstrated that a 1% magnitude of cyclic uniaxial tensile stress with 1 Hz frequency increased OPN, ALP, and OCN mRNA⁴⁷ and 3% strain (1 Hz) induced osteonectin mRNA.¹ Other studies have shown that VEGF, TGF- β 1, and type I collagen in-

creased following 10% equibiaxial tensile stress.⁴⁹ In other studies using MC3T3-E1 cells, OPG, COX-2, and PGE₂ were induced in response to 6%–18% cyclic strain (0.1–0.2 Hz).^{6,12,14} The type of GFs (BMP-2 and TGF- β 1) were chosen based on previous studies that have shown BMP-2 to improve bone regeneration²³ and TGF- β to be involved in HSP expression.^{38,39} TGF- β can induce bone-related proteins such as ALP,²⁴ collagen,⁵¹ and OPG,⁵² suggesting a promising dual-application protocol using BMP-2 and TGF- β 1.

FIG. 7. Expression of mRNA for COX-2 (A) and prostaglandin E synthase 2 (PGES-2) (B) following 24 h conditioning with cyclic tension (1%, 3%, 5%, and 10% elongation) and GFs. *Statistical significance between the control group and the treated groups (GF alone; tension alone for all protocols; GF and tension protocols in combination). **Statistically significant difference between groups experiencing static with GFs and tension with GFs. #Statistically significant difference between groups exposed to tension alone and with GFs ($p < 0.05$).

FIG. 8. Vascular endothelial growth factor (VEGF) mRNA and protein secretion following conditioning with cyclic tension and GFs. PCR (A) was performed after 24 h cyclic tension (1%, 3%, 5%, and 10% elongation) with GFs and secreted concentration (B) was acquired from the culture media collected for two durations: days 0–3 and 3–6 of tensile stress (5% elongation) with GFs. (A) *Statistical significance between the control group and the treated groups (GF alone; tension alone for all protocols; GF and tension protocols in combination). (B) *Statistically significant difference between each control group and the treated groups (GF alone; tension alone for all protocols; GF and tension protocols in combination) measured on days 3 and 6; δ statistically significant difference between tension for 3 and 6 days. N.D. denotes no detection level by measuring the protein using the ELISA kit ($p < 0.05$). (A, B) **Statistically significant difference between groups experiencing static conditions with GFs and tension with GFs. #Statistically significant difference between groups experiencing tension alone and tension with GFs.

However, this is the first study utilizing simultaneous addition of BMP-2 and TGF- β 1.

We confirmed that morphological modifications of MC3T3-E1 cells could be induced in response to a low level of 1% strain using a Flexcell[®] tensile bioreactor for 6 days (Fig. 2). In our study, cells in the center of collagen-coated BioFlex[®] plates appeared equibiaxially stretched, which is analogous to U937 cell morphology following tensile stress described in the study by Matheson *et al.*⁵³ However, cells close to the edge of the circular loading post boundary in our study were aligned and appeared uniaxially stretched. This is similar to the study by Hasty *et al.*,⁵⁴ in which articular chondrocytes exhibited variations in cell morphology following 10% cyclic equibiaxial tensile stress (0.5 Hz) for 24 h. Spatial variations in stress and associated morphology across the culture plate may induce a wide array of differences in gene and protein expression throughout the dish. In this study, we measured the total gene expression averaged across the entire cell population and total protein secretion.

To our knowledge, we have shown for the first time induction of several HSPs by preosteoblasts following short durations of tensile stress alone or in combination with GFs (Figs. 3 and 4). HSP induction following tensile stress alone was observed to be transient (Fig. 3), similar to heating-inductive trends observed for HSP70 following water bath and incubator heating.^{55,56} Short-term tensile conditioning with GFs suggested varying levels of induction of each HSP mRNA (Fig. 4). HSP47 mRNA increased in response to GF inclusion in static-cultured and tensile-loaded cells unlike HSP27 and HSP70. These differences may be caused by the fact that HSP27 and HSP70 are involved in apoptosis or differentiation in several intracellular or nuclear locations,³² distinct from the role of HSP47. In addition, it may indicate

that cells possess or lose initial anti-apoptotic capacity following tensile stress under exogenous TGF- β 1/BMP-2 by suppressing HSP27 and HSP70, suggesting potential conflicts between cellular responses due to tensile stress and GFs.

Regulation of bone matrix proteins such as OPN, OCN, BSP, and type I collagen following tensile stress and GFs was demonstrated (Figs. 5 and 9). Gene and protein expression of OPN was inhibited by GFs during the initial periods (24 h to day 3) of tensile stress. However, GF inclusion in combination with tensile stress increased OPN secretion for later cultivation periods (days 3–6), indicating the need for longer periods of stress conditioning. The slow induction of OPN by tensile stress correlates with mRNA data showing higher mRNA expression for the period of 3–6 days compared with 0–3 days. Significant induction of BSP mRNA (greater than 5 RFI in only GF-treated cells) by GFs was reduced with the application of tensile stress (3%–10%), indicating that tensile stress may conflict with the cellular action of GFs for early durations (24 h). The induction trends of OPN (maximum increase in protein secretion=240%) and BSP (maximum mRNA RFI=9.75) in response to GFs in our study are consistent with the work of Lai *et al.*,⁵⁷ which demonstrated 2.4- and 2-fold increased protein expressions of BSP and OPN, respectively, in human osteoblasts by BMP-2 addition (100 ng/mL). However, our study demonstrated that BSP is more sensitive to GFs than OPN, and mechanical stress could impair BSP induction in MC3T3-E1 cells. In previous literature, type I collagen was shown to be induced by TGF- β 1 in MC3T3-E1 cells,³⁷ whereas cyclic tensile stress (5%–12.5%, 0.5 Hz, 24 h) only slightly promoted type 1 collagen mRNA compared with static-cultivated SaOs-2 cells.¹¹ However, we observed that type I collagen mRNA induction by GFs was

FIG. 9. Secretion of bone-related proteins following cyclic tension (5% elongation) in combination with GFs for two cultivation durations: days 0–3 and 3–6. OPN (**A**); OPG (**B**); OCN (**C**); MMP-9 (**D**) secretion. *Statistically significant difference between each control group and groups following tension measured on days 3 and 6. **Statistically significant difference between groups exposed to static conditions with GFs and those treated with tension and GFs. ™Statistically significant difference between response for 3 and 6 days of tension ($p < 0.05$). #Statistically significant difference between groups experiencing tension alone and tension with GFs. N.D. denotes no detection level by measuring the protein using ELISA.

inhibited when tensile stress was used in combination with GFs. One possible explanation is that mechanical stress may not significantly affect the regulation of type I collagen or hamper the stimulatory effect of GFs in the early duration. In our study, OCN mRNA was unaffected by tensile stress or GFs at 24 h. However, GFs promoted OCN secretion significantly on day 6 similar to the study by Lai *et al.*, showing OCN induction by BMP-2.⁵⁷ Kaspar *et al.* reported uniaxial cyclic stretching (0.1%, 1 Hz, 1800 cycles, 2 days) by a 4-point-bending apparatus and showed ~31% enhancement in cell growth, 18% increase in procollagen I propeptide release, 20% decrease in ALP activity, and 11% increase in OCN release compared with unstressed cells.⁵⁸ However, in the study by Ignatius *et al.*,⁴⁷ uniaxial mechanical strain (1%, 1 Hz, 30 min/day, 21 days) in 3D collagen gels promoted proliferation and slightly affected mRNA expression of OCN, OPN, and ALP.

Our individual or combined strategy utilizing cyclic tensile stress and GFs suggested anti-osteoclastic effects by inducing OPG and suppressing MMP-9 (Figs. 6 and 9). OPG is known to inhibit osteoclastogenesis by intervening in the association between receptor activator nuclear factor κ B (RANK) and its ligand.⁵⁹ Greater induction of OPG mRNA

following 24 h tensile stress with GFs compared with tensile stress or GFs alone suggests rapid anti-osteoclastogenic effects of our combinatorial stress conditioning. However, OPG secretion increased significantly by tensile stress on days 3–6 (later durations). Our results can expand previous understanding regarding OPG induction in response to GF addition and tensile stress. In MC3T3-E1 cells, BMP-2 can upregulate OPG through Wnt/ β -catenin pathway⁶⁰ and TGF- β 1 also can increase OPG/OCIF (osteoclastogenesis inhibitory factor) induction.⁶¹ Tang *et al.*⁶ described that OPG was induced in a magnitude-dependant manner following 6%, 12%, and 18% elongation by cyclic tensile stress. Our data suggested that OPG regulation may be influenced more significantly by tensile stress than GFs.

Suppression of MMP-9 by tensile stress alone or significantly by GF treatment in our study suggested that these stimuli may provide anti-osteoclastic benefit to stress-preconditioned preosteoblasts by decreasing overexpressed MMP-9. We demonstrated that MMP-9 was suppressed by tensile stress, which is similar to trends in prior work showing that MMP-9 in mouse monocyte macrophages decreased following 10% cyclic tensile stress.⁴⁸ We observed that GFs prevented MMP-9 production and that OCN and

FIG. 10. MC3T3-E1 cell proliferation following combinatorial stress conditioning with cyclic tension (5% elongation) and GFs measured with MTS assay following 3 and 6 days of tension. *Statistically significant difference between the control group and the treated groups after 3 and 6 days of tension. &Statistically significant difference between cellular response for 3 and 6 days of tension. **Statistically significant difference between groups treated with tension and GFs and groups exposed to static conditions with GFs ($p < 0.05$).

MMP-9 secretion exhibited opposite expression trends following treatment with GFs (Fig. 9). MMP-9, known as 92-kDa gelatinase, is an osteoclast-phenotyping protein whose regulation in the bone microenvironment can be potentially an important target to cure bone abnormalities such as osteoporosis caused by overactivated osteoclasts.^{62,63} Further, the regulation of MMP-9 secreted by MC3T3-E1 cells⁶⁴ is critical in bone formation by dissociating collagen in endochondral ossification with MMP-13.⁶⁵ However, significant increase in MMP-9-specific genes in osteoclasts occurred because of RANKL treatment, suggesting that osteoclastogenesis may be related to MMP-9.⁶⁶ Possibly, overexpression of MMP-9 by osteoblasts in bone could be pathogenic for osteoporosis. However, MMP-9 levels in osteoporotic disease is still controversial, because bone of osteoporotic women showed low levels of MMP-9 gene,⁶⁷ but it is highly expressed in rat osteoporotic bone-specific tissue.⁶⁸ Therefore, the optimal MMP-9 levels for therapeutic usage still need to be further clarified.

We measured the regulation of two enzymes (COX-2 and PGES-2) (Figs. 3 and 7) involved in the synthesis of PGE₂, a fatty acid-derived hormone, derived from arachidonic acid.⁶⁹ We observed COX-2 mRNA to increase significantly in response to 1% cyclic tensile stress alone at day 6 with minimal alteration of PGES-2. With GF addition, COX-2 mRNA was induced more significantly by GFs compared to PGES-2 at 24 h. Similarly, the induction of COX-2 mRNA by tensile stress in our study, compared with other studies that have shown shear stress loading or ultrasound exposure to preosteoblasts, resulted in COX-2 gene expression.^{70,71} Other studies have shown that 3–24 h equibiaxial stretching (5% and 10%, 1 Hz) increased COX-2-specific gene expression *ca.* 2.5-fold in endothelial cells.⁷² Prior studies have also shown that TGF- β 1 (2 ng/mL) can promote COX-2 protein expression in benign keratinocytes.⁷³ BMP-2 (100 ng/mL) increased expression of COX-2 mRNA in primary osteoblasts

rapidly within several hours, and the induction of PGE₂ was 45% higher in the wild type compared with COX-2 knockout cells.⁷⁴ It can be expected that COX-2 upregulation by mechanical stress or biochemical cues may positively influence upregulation of PGE₂ based on a prior study that COX-2 induction can induce PGE₂ synthesis by *in vitro* cyclic tensile stress (9%, 3 h, 0.1 Hz).¹⁴ To our knowledge, no prior study has investigated the effects of dual osteoinductive GFs alone or in combination with mechanical stress on COX-2. Zhang *et al.*⁷⁵ suggested that COX-2 may be a critical factor in bone healing and bone marrow stromal cell differentiation through a mice model with COX-2-specific gene deletion, and bone nodule formation may proceed with involvement with BMP-2 and PGE₂. Therefore, we expect that our results showing significant increase in COX-2 mRNA by GF addition may provide a means to enhance bone healing or osteoblastogenesis.

Our results demonstrate greater VEGF induction following tensile stress combined with GFs compared with tensile stress or GFs alone (Fig. 8). Mechanical signals and osteoinductive GFs appear to trigger the rapid induction of the angiogenic factor, VEGF, from various cells including preosteoblasts.^{3,76–78} Deckers *et al.* reported that BMP-2 induced VEGF-A secretion by preosteoblasts and promoted angiogenesis.⁷⁶ However, TGF- β is known to impair angiogenesis, demonstrated by fetal metatarsal assay.⁷⁶ In the study by Singh *et al.*, VEGF was elevated in response to cyclic tensile stress (12%, 0.1 and 0.2 Hz for 24 and 48 h) in osteoblasts using the Flexcell® bioreactor.³ Similarly, cyclic tensile stress (20%, 1 Hz) induced 2–3.5-fold VEGF mRNA by endothelial cells in 2–5 h. However, there was no significant change in VEGF mRNA expression in our study in response to tensile stress alone.⁷⁷ In the study by Thi *et al.*, MC3T3-E1 cells rapidly expressed 5.8-fold increased level of VEGF-A cDNA following 1 Hz fluid shear stress (5 dyn/cm²) for 5 h, also suggesting autocrine and paracrine action of VEGF.⁷⁸ However, even though these studies demonstrated that GF or mechanical cue individually induced VEGF, our results demonstrated that dual GFs of TGF- β 1 and BMP-2 may be a more powerful angiogenic inducer than tensile stress. Our PCR data for gene expression also suggested that dual GF treatment can promote endogenous induction of TGF- β 1 and expression of HSP47, COX-2, BSP, and BMP-2 in preosteoblasts. Our observation of BMP-2 mRNA induction by GF addition suggests that GFs may be a more powerful stimulus compared with tensile stress for osteoblastogenesis.⁷⁸

Only cells treated by GFs in combination with cyclic tensile stress (5%) showed higher MC3T3-E1 proliferation of 23% on day 6 compared with the unstressed cells (Fig. 10), but no difference was observed on day 3. This suggests that sufficient time is necessary to demonstrate positive mitogenic stimulation effects. Individual doses of TGF- β 1^{17,24} and BMP-2^{17,79} have been reported to promote proliferation of bone-related stem or progenitor cells in bone repair and mediate bone healing in an autocrine or paracrine manner.¹⁷ However, the osteoinductive effect of exogenous TGF- β 1 treatment is controversial when compared with the bone regenerative capacity of BMP-2.^{80,81} However, we utilized TGF- β 1 with BMP-2, because TGF- β 1 is associated with regulation of several HSPs as well as bone physiology.^{30,37–39,82} Further, in previous literature, tensile stress has been shown

to have varying effects on bone cell proliferation, including promotion^{1,83} or inhibition,^{3,47} possibly because of differences in tensile stress conditioning factors, cell type, and culture system. Therefore, our results provide important evidence confirming the beneficial effect of dual GFs and tensile stress on cell proliferation.

In conclusion, we have shown that tensile stress and GFs can promote cell proliferation and diverse regulations of HSPs and bone-related molecules. This study has demonstrated correlation between protocols using tensile stress and GFs in the *in vitro* response of preosteoblasts, providing a foundation for the development of an effective stress conditioning protocol for bone tissue engineering.

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Disclosure Statement

No competing financial interests exist.

References

- Bhatt, K.A., Chang, E.I., Warren, S.M., Lin, S.E., Bastidas, N., Ghali, S., *et al.* Uniaxial mechanical strain: an *in vitro* correlate to distraction osteogenesis. *J Surg Res* **143**, 329, 2007.
- Kanno, T., Takahashi, T., Tsujisawa, T., Ariyoshi, W., and Nishihara, T. Mechanical stress-mediated Runx2 activation is dependent on Ras/ERK1/2 MAPK signaling in osteoblasts. *J Cell Biochem* **101**, 1266, 2007.
- Singh, S.P., Chang, E.I., Gossain, A.K., Mehara, B.J., Galiano, R.D., Jensen, J., *et al.* Cyclic mechanical strain increases production of regulators of bone healing in cultured murine osteoblasts. *J Am Coll Surg* **204**, 426, 2007.
- Garvin, J., Qi, J., Maloney, M., and Banes, A.J. Novel system for engineering bioartificial tendons and application of mechanical load. *Tissue Eng* **9**, 967, 2003.
- Stegemann, J.P., and Nerem, R.M. Phenotype modulation in vascular tissue engineering using biochemical and mechanical stimulation. *Ann Biomed Eng* **31**, 391, 2003.
- Tang, L., Lin, Z., and Li, Y.M. Effects of different magnitudes of mechanical strain on Osteoblasts *in vitro*. *Biochem Biophys Res Commun* **344**, 122, 2006.
- Harter, L.V., Hruska, K.A., and Duncan, R.L. Human osteoblast-like cells respond to mechanical strain with increased bone matrix protein production independent of hormonal regulation. *Endocrinology* **136**, 528, 1995.
- Lee, C.H., Shin, H.J., Cho, I.H., Kang, Y.M., Kim, I.A., Park, K.D., *et al.* Nanofiber alignment and direction of mechanical strain affect the ECM production of human ACL fibroblast. *Biomaterials* **26**, 1261, 2005.
- Nieponice, A., Maul, T.M., Cumer, J.M., Soletti, L., and Vorp, D.A. Mechanical stimulation induces morphological and phenotypic changes in bone marrow-derived progenitor cells within a three-dimensional fibrin matrix. *J Biomed Mater Res A* **81**, 523, 2007.
- Ferdous, Z., Lazaro, L.D., Iozzo, R.V., Hook, M., and Grande-Allen, K.J. Influence of cyclic strain and decorin deficiency on 3D cellularized collagen matrices. *Biomaterials* **29**, 2740, 2008.
- Liu, X., Zhang, X., and Luo, Z.P. Strain-related collagen gene expression in human osteoblast-like cells. *Cell Tissue Res* **322**, 331, 2005.
- Yang, C.M., Chien, C.S., Yao, C.C., Hsiao, L.D., Huang, Y.C., and Wu, C.B. Mechanical strain induces collagenase-3 (MMP-13) expression in MC3T3-E1 osteoblastic cells. *J Biol Chem* **279**, 22158, 2004.
- Grimston, S.K., Screen, J., Haskell, J.H., Chung, D.J., Brodt, M.D., Silva, M.J., *et al.* Role of connexin43 in osteoblast response to physical load. *Ann N Y Acad Sci* **1068**, 214, 2006.
- Narutomi, M., Nishiura, T., Sakai, T., Abe, K., and Ishikawa, H. Cyclic mechanical strain induces interleukin-6 expression via prostaglandin E2 production by cyclooxygenase-2 in MC3T3-E1 osteoblast-like cells. *J Oral Biosci* **49**, 65, 2007.
- Motokawa, M., Kaku, M., Tohma, Y., Kawata, T., Fujita, T., Kohno, S., *et al.* Effects of cyclic tensile forces on the expression of vascular endothelial growth factor (VEGF) and macrophage-colony-stimulating factor (M-CSF) in murine osteoblastic MC3T3-E1 cells. *J Dent Res* **84**, 422, 2005.
- Cillo, J.E., Jr., Gassner, R., Koepsel, R.R., and Buckley, M.J. Growth factor and cytokine gene expression in mechanically strained human osteoblast-like cells: implications for distraction osteogenesis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **90**, 147, 2000.
- Devescovi, V., Leonardi, E., Ciapetti, G., and Cenni, E. Growth factors in bone repair. *Chir Organi Mov* **92**, 161, 2008.
- DeFail, A.J., Chu, C.R., Izzo, N., and Marra, K.G. Controlled release of bioactive TGF-beta 1 from microspheres embedded within biodegradable hydrogels. *Biomaterials* **27**, 1579, 2006.
- Lin, Y., Tang, W., Wu, L., Jing, W., Li, X., Wu, Y., *et al.* Bone regeneration by BMP-2 enhanced adipose stem cells loading on alginate gel. *Histochem Cell Biol* **129**, 203, 2008.
- Burkus, J.K., Sandhu, H.S., and Gornet, M.F. Influence of rhBMP-2 on the healing patterns associated with allograft interbody constructs in comparison with autograft. *Spine (Phila Pa 1976)* **31**, 775, 2006.
- Govender, S., Csimma, C., Genant, H.K., Valentin-Opran, A., Amit, Y., Arbel, R., *et al.* Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. *J Bone Joint Surg Am* **84-A**, 2123, 2002.
- Klein, G.L., Wolf, S.E., Langman, C.B., Rosen, C.J., Mohan, S., Keenan, B.S., *et al.* Effects of therapy with recombinant human growth hormone on insulin-like growth factor system components and serum levels of biochemical markers of bone formation in children after severe burn injury. *J Clin Endocrinol Metab* **83**, 21, 1998.
- Wang, C.K., Ho, M.L., Wang, G.J., Chang, J.K., Chen, C.H., Fu, Y.C., *et al.* Controlled-release of rhBMP-2 carriers in the regeneration of osteonecrotic bone. *Biomaterials* **30**, 4178, 2009.
- Lu, L., Yaszemski, M.J., and Mikos, A.G. TGF-beta1 release from biodegradable polymer microparticles: its effects on marrow stromal osteoblast function. *J Bone Joint Surg Am* **83-A Suppl 1**, S82, 2001.
- Westerheide, S.D., and Morimoto, R.I. Heat shock response modulators as therapeutic tools for diseases of protein conformation. *J Biol Chem* **280**, 33097, 2005.
- Luna, C., Li, G., Liton, P.B., Epstein, D.L., and Gonzalez, P. Alterations in gene expression induced by cyclic mechanical stress in trabecular meshwork cells. *Mol Vis* **15**, 534, 2009.

27. Tsuruoka, M., Ishizaki, K., Sakurai, K., Matsuzaka, K., and Inoue, T. Morphological and molecular changes in denture-supporting tissues under persistent mechanical stress in rats. *J Oral Rehabil* **35**, 889, 2008.
28. Kaarniranta, K., Elo, M.A., Sironen, R.K., Karjalainen, H.M., Helminen, H.J., and Lammi, M.J. Stress responses of mammalian cells to high hydrostatic pressure. *Biorheology* **40**, 87, 2003.
29. Azuma, N., Akasaka, N., Kito, H., Ikeda, M., Gahtan, V., Sasajima, T., *et al.* Role of p38 MAP kinase in endothelial cell alignment induced by fluid shear stress. *Am J Physiol Heart Circ Physiol* **280**, H189, 2001.
30. Hatakeyama, D., Kozawa, O., Niwa, M., Matsuno, H., Ito, H., Kato, K., *et al.* Upregulation by retinoic acid of transforming growth factor-beta-stimulated heat shock protein 27 induction in osteoblasts: involvement of mitogen-activated protein kinases. *Biochim Biophys Acta* **1589**, 15, 2002.
31. Soti, C., Nagy, E., Giricz, Z., Vigh, L., Csermely, P., and Ferdinandy, P. Heat shock proteins as emerging therapeutic targets. *Br J Pharmacol* **146**, 769, 2005.
32. Lanneau, D., de Thonel, A., Maurel, S., Didelot, C., and Garrido, C. Apoptosis versus cell differentiation: role of heat shock proteins HSP90, HSP70 and HSP27. *Prion* **1**, 53, 2007.
33. Leonardi, R., Barbato, E., Paganelli, C., and Lo Muzio, L. Immunolocalization of heat shock protein 27 in developing jaw bones and tooth germs of human fetuses. *Calcif Tissue Int* **75**, 509, 2004.
34. Nagata, K. Expression and function of heat shock protein 47: a collagen-specific molecular chaperone in the endoplasmic reticulum. *Matrix Biol* **16**, 379, 1998.
35. Nagai, N., Hosokawa, M., Itohara, S., Adachi, E., Matsushita, T., Hosokawa, N., *et al.* Embryonic lethality of molecular chaperone hsp47 knockout mice is associated with defects in collagen biosynthesis. *J Cell Biol* **150**, 1499, 2000.
36. Ishida, Y., Kubota, H., Yamamoto, A., Kitamura, A., Bachinger, H.P., and Nagata, K. Type I collagen in Hsp47-null cells is aggregated in endoplasmic reticulum and deficient in N-propeptide processing and fibrillogenesis. *Mol Biol Cell* **17**, 2346, 2006.
37. Yamamura, I., Hirata, H., Hosokawa, N., and Nagata, K. Transcriptional activation of the mouse HSP47 gene in mouse osteoblast MC3T3-E1 cells by TGF-beta 1. *Biochem Biophys Res Commun* **244**, 68, 1998.
38. Cooper, L.F., Tiffée, J.C., Griffin, J.P., Hamano, H., and Guo, Z. Estrogen-induced resistance to osteoblast apoptosis is associated with increased hsp27 expression. *J Cell Physiol* **185**, 401, 2000.
39. Tokuda, H., Kozawa, O., Niwa, M., Matsuno, H., Kato, K., and Uematsu, T. Mechanism of prostaglandin E2-stimulated heat shock protein 27 induction in osteoblast-like MC3T3-E1 cells. *J Endocrinol* **172**, 271, 2002.
40. Tiffée, J.C., Griffin, J.P., and Cooper, L.F. Immunolocalization of stress proteins and extracellular matrix proteins in the rat tibia. *Tissue Cell* **32**, 141, 2000.
41. Chung, C.Y., Iida-Klein, A., Wyatt, L.E., Rudkin, G.H., Ishida, K., Yamaguchi, D.T., *et al.* Serial passage of MC3T3-E1 cells alters osteoblastic function and responsiveness to transforming growth factor-beta1 and bone morphogenetic protein-2. *Biochem Biophys Res Commun* **265**, 246, 1999.
42. Lee, Y.W., Eum, S.Y., Chen, K.C., Hennig, B., and Toborek, M. Gene expression profile in interleukin-4-stimulated human vascular endothelial cells. *Mol Med (Cambridge, MA)* **10**, 19, 2004.
43. Mauney, J.R., Sjøstorm, S., Blumberg, J., Horan, R., O'Leary, J.P., Vunjak-Novakovic, G., *et al.* Mechanical stimulation promotes osteogenic differentiation of human bone marrow stromal cells on 3-D partially demineralized bone scaffolds *in vitro*. *Calcif Tissue Int* **74**, 458, 2004.
44. Jackson, R.A., Kumarasuriyar, A., Nurcombe, V., and Cool, S.M. Long-term loading inhibits ERK1/2 phosphorylation and increases FGFR3 expression in MC3T3-E1 osteoblast cells. *J Cell Physiol* **209**, 894, 2006.
45. Kapur, S., Mohan, S., Baylink, D.J., and Lau, K.H. Fluid shear stress synergizes with insulin-like growth factor-I (IGF-I) on osteoblast proliferation through integrin-dependent activation of IGF-I mitogenic signaling pathway. *J Biol Chem* **280**, 20163, 2005.
46. Gosain, A.K., Song, L.S., Santoro, T., Weihrauch, D., Bosi, B.O., Corrao, M.A., *et al.* Effects of transforming growth factor-beta and mechanical strain on osteoblast cell counts: an *in vitro* model for distraction osteogenesis. *Plast Reconstr Surg* **105**, 130; discussion 7-9, 2000.
47. Ignatius, A., Blessing, H., Liedert, A., Schmidt, C., Neidlinger-Wilke, C., Kaspar, D., *et al.* Tissue engineering of bone: effects of mechanical strain on osteoblastic cells in type I collagen matrices. *Biomaterials* **26**, 311, 2005.
48. Suzuki, N., Yoshimura, Y., Deyama, Y., Suzuki, K., and Kitagawa, Y. Mechanical stress directly suppresses osteoclast differentiation in RAW264.7 cells. *Int J Mol Med* **21**, 291, 2008.
49. Fong, K.D., Nacamuli, R.P., Lobo, E.G., Henderson, J.H., Fang, T.D., Song, H.M., *et al.* Equibiaxial tensile strain affects calvarial osteoblast biology. *J Craniofac Surg* **14**, 348, 2003.
50. Simmons, C.A., Matlis, S., Thornton, A.J., Chen, S., Wang, C.Y., and Mooney, D.J. Cyclic strain enhances matrix mineralization by adult human mesenchymal stem cells via the extracellular signal-regulated kinase (ERK1/2) signaling pathway. *J Biomech* **36**, 1087, 2003.
51. Lieb, E., Milz, S., Vogel, T., Hacker, M., Dauner, M., and Schulz, M.B. Effects of transforming growth factor beta1 on bonelike tissue formation in three-dimensional cell culture. I. Culture conditions and tissue formation. *Tissue Eng* **10**, 1399, 2004.
52. Thirunavukkarasu, K., Miles, R.R., Halladay, D.L., Yang, X., Galvin, R.J., Chandrasekhar, S., *et al.* Stimulation of osteoprotegerin (OPG) gene expression by transforming growth factor-beta (TGF-beta). Mapping of the OPG promoter region that mediates TGF-beta effects. *J Biol Chem* **276**, 36241, 2001.
53. Matheson, L.A., Maksym, G.N., Santerre, J.P., and Labow, R.S. Differential effects of uniaxial and biaxial strain on U937 macrophage-like cell morphology: influence of extracellular matrix type proteins. *J Biomed Mater Res A* **81**, 971, 2007.
54. Huang, J., Ballou, L.R., and Hasty, K.A. Cyclic equibiaxial tensile strain induces both anabolic and catabolic responses in articular chondrocytes. *Gene* **404**, 101, 2007.
55. Rylander, M.N., Diller, K.R., Wang, S., and Aggarwal, S.J. Correlation of HSP70 expression and cell viability following thermal stimulation of bovine aortic endothelial cells. *J Biomech Eng* **127**, 751, 2005.
56. Wang, S., Xie, W., Rylander, M.N., Tucker, P.W., Aggarwal, S., and Diller, K.R. HSP70 kinetics study by continuous observation of HSP-GFP fusion protein expression on a perfusion heating stage. *Biotechnol Bioeng* **99**, 146, 2008.
57. Lai, C.F., and Cheng, S.L. Alphasbeta integrins play an essential role in BMP-2 induction of osteoblast differentiation. *J Bone Miner Res* **20**, 330, 2005.

58. Kaspar, D., Seidl, W., Neidlinger-Wilke, C., and Claes, L. *In vitro* effects of dynamic strain on the proliferative and metabolic activity of human osteoblasts. *J Musculoskelet Neuronal Interact* **1**, 161, 2000.
59. Aubin, J.E., and Bonnellye, E. Osteoprotegerin and its ligand: a new paradigm for regulation of osteoclastogenesis and bone resorption. *Osteoporos Int* **11**, 905, 2000.
60. Sato, M.M., Nakashima, A., Nashimoto, M., Yawaka, Y., and Tamura, M. Bone morphogenetic protein-2 enhances Wnt/ β -catenin signaling-induced osteoprotegerin expression. *Genes Cells* **14**, 141, 2009.
61. Takai, H., Kanematsu, M., Yano, K., Tsuda, E., Higashio, K., Ikeda, K., *et al.* Transforming growth factor- β stimulates the production of osteoprotegerin/osteoclastogenesis inhibitory factor by bone marrow stromal cells. *J Biol Chem* **273**, 27091, 1998.
62. Vu, T.H., and Werb, Z. Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev* **14**, 2123, 2000.
63. Rodan, G.A., and Martin, T.J. Therapeutic approaches to bone diseases. *Science* **289**, 1508, 2000.
64. Fornoni, A., Cornacchia, F., Howard, G.A., Roos, B.A., Striker, G.E., and Striker, L.J. Cyclosporin A affects extracellular matrix synthesis and degradation by mouse MC3T3-E1 osteoblasts *in vitro*. *Nephrol Dial Transplant* **16**, 500, 2001.
65. Stickens, D., Behonick, D.J., Ortega, N., Heyer, B., Hartenstein, B., Yu, Y., *et al.* Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. *Development* **131**, 5883, 2004.
66. Song, I., Kim, J.H., Kim, K., Jin, H.M., Youn, B.U., and Kim, N. Regulatory mechanism of NFATc1 in RANKL-induced osteoclast activation. *FEBS Lett* **583**, 2435, 2009.
67. Balla, B., Kosa, J.P., Kiss, J., Borsy, A., Podani, J., Takacs, I., *et al.* Different gene expression patterns in the bone tissue of aging postmenopausal osteoporotic and non-osteoporotic women. *Calcif Tissue Int* **82**, 12, 2008.
68. Zhao, H., Cai, G., Du, J., Xia, Z., Wang, L., and Zhu, T. Expression of matrix metalloproteinase-9 mRNA in osteoporotic bone tissues. *J Tongji Med Univ* **17**, 28, 1997.
69. Samuelsson, B., Morgenstern, R., and Jakobsson, P.J. Membrane prostaglandin synthase-1: a novel therapeutic target. *Pharmacol Rev* **59**, 207, 2007.
70. Wadhwa, S., Godwin, S.L., Peterson, D.R., Epstein, M.A., Raisz, L.G., and Pilbeam, C.C. Fluid flow induction of cyclooxygenase 2 gene expression in osteoblasts is dependent on an extracellular signal-regulated kinase signaling pathway. *J Bone Miner Res* **17**, 266, 2002.
71. Tang, C.H., Yang, R.S., Huang, T.H., Lu, D.Y., Chuang, W.J., Huang, T.F., *et al.* Ultrasound stimulates cyclooxygenase-2 expression and increases bone formation through integrin, focal adhesion kinase, phosphatidylinositol 3-kinase, and Akt pathway in osteoblasts. *Mol Pharmacol* **69**, 2047, 2006.
72. Zhao, H., Hiroi, T., Hansen, B.S., and Rade, J.J. Cyclic stretch induces cyclooxygenase-2 gene expression in vascular endothelial cells via activation of nuclear factor κ -B. *Biochem Biophys Res Commun* **389**, 599, 2009.
73. Rasanen, K., and Vaheri, A. TGF- β 1 causes epithelial-mesenchymal transition in HaCaT derivatives, but induces expression of COX-2 and migration only in benign, not in malignant keratinocytes. *J Dermatol Sci* **58**, 97, 2010.
74. Chikazu, D., Li, X., Kawaguchi, H., Sakuma, Y., Voznesensky, O.S., Adams, D.J., *et al.* Bone morphogenetic protein 2 induces cyclo-oxygenase 2 in osteoblasts via a Cbfa1 binding site: role in effects of bone morphogenetic protein 2 *in vitro* and *in vivo*. *J Bone Miner Res* **17**, 1430, 2002.
75. Zhang, X., Schwarz, E.M., Young, D.A., Puzas, J.E., Rosier, R.N., and O'Keefe, R.J. Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. *J Clin Invest* **109**, 1405, 2002.
76. Deckers, M.M., van Bezooijen, R.L., van der Horst, G., Hoogendam, J., van Der Bent, C., Papapoulos, S.E., *et al.* Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. *Endocrinology* **143**, 1545, 2002.
77. Hurley, N.E., Schildmeyer, L.A., Bosworth, K.A., Sakurai, Y., Eskin, S.G., Hurley, L.H., *et al.* Modulating the functional contributions of c-Myc to the human endothelial cell cyclic strain response. *J Vasc Res* **47**, 80, 2010.
78. Thi, M.M., Jacobas, D.A., Jacobas, S., and Spray, D.C. Fluid shear stress upregulates vascular endothelial growth factor gene expression in osteoblasts. *Ann N Y Acad Sci* **1117**, 73, 2007.
79. Liu, S.B., Hu, P.Z., Hou, Y., Li, P., Cao, W., and Tian, Q. Recombinant human bone morphogenetic protein-2 promotes the proliferation of mesenchymal stem cells *in vivo* and *in vitro*. *Chin Med J (Engl)* **122**, 839, 2009.
80. Spinella-Jaegle, S., Roman-Roman, S., Faucheu, C., Dunn, F.W., Kawai, S., Gallea, S., *et al.* Opposite effects of bone morphogenetic protein-2 and transforming growth factor- β 1 on osteoblast differentiation. *Bone* **29**, 323, 2001.
81. Heckman, J.D., Ehler, W., Brooks, B.P., Aufdemorte, T.B., Lohmann, C.H., Morgan, T., *et al.* Bone morphogenetic protein but not transforming growth factor- β enhances bone formation in canine diaphyseal nonunions implanted with a biodegradable composite polymer. *J Bone Joint Surg Am* **81**, 1717, 1999.
82. Tokuda, H., Kato, K., Oiso, Y., and Kozawa, O. Contrasting effects of triiodothyronine on heat shock protein 27 induction and vascular endothelial growth factor synthesis stimulated by TGF- β in osteoblasts. *Mol Cell Endocrinol* **201**, 33, 2003.
83. Zhuang, H., Wang, W., Tahernia, A.D., Levitz, C.L., Luchetti, W.T., and Brighton, C.T. Mechanical strain-induced proliferation of osteoblastic cells parallels increased TGF- β 1 mRNA. *Biochem Biophys Res Commun* **229**, 449, 1996.

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