

Tensile Strain as a Regulator of Mesenchymal Stem Cell Osteogenesis

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Abstract—A role for mechanical stimulation in the control of cell fate has been proposed and mechanical conditioning of mesenchymal stem cells (MSCs) is of interest in directing MSC behavior for tissue engineering applications. This study investigates strain-induced differentiation and proliferation of MSCs, and investigates the cellular mechanisms of mechanotransduction. MSCs were seeded onto a collagen-coated silicone substrate and exposed to cyclic tensile mechanical strain of 2.5% at 0.17 Hz for 1–14 days. To examine mechanotransduction, cells were strained in the presence of the stretch-activated cation channel (SACC) blocker, gadolinium chloride (GdCl₃); the extracellular regulated kinase (ERK) inhibitor, U0126; the p38 inhibitor, SB203580; and the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor, LY294002. Following exposure to strain, the osteogenic markers Cbfa1, collagen type I, osteocalcin, and BMP2 were temporally expressed. Exposure to strain in the presence of GdCl₃ (10 μM) reduced the induction of collagen I expression, thus identifying a role for SACC, at least in part, as mechanosensors in strain-induced MSC differentiation. The strain-induced synthesis of BMP2 was found to be reduced by inhibitors of the kinases, ERK, p38, and PI3 kinase. Additionally, mechanical strain reduced the rate of MSC proliferation. The identification of the mechanical control of MSC proliferation and the molecular link between mechanical stimulation and osteogenic differentiation has consequences for regenerative medicine through the development of a functional tissue engineering approach.

Keywords—Mesenchymal stem cells, Mechanical strain, Osteogenic differentiation, Proliferation, Stretch-activated cation channels, Mitogen-activated protein kinase, PI3-kinase.

INTRODUCTION

Mesenchymal stem cells (MSCs) from the bone marrow are multipotent cells that can differentiate toward the bone, cartilage, muscle, and fat lineages.⁴⁵

They are of clinical interest for their potential use as a cell source for tissue engineering of skeletal tissue, although much remains to be investigated about the precise mechanisms that control their differentiation. Physiological osteoblast development during skeletogenesis and fracture repair follows a defined sequence of events that begins with the proliferation of MSCs.^{26,36} The differentiation that follows is particularly responsive to the mechanical composition of the microenvironment.^{7,39} The requirement of mechanical loading for bone remodeling to maintain bone turnover is well established³⁸ and mechanical stimulation is favorable for the promotion of bone and MSC proliferation. For example, progenitor cells lose their *in vitro* proliferative capacity following the withdrawal of *in vivo* mechanical stimulation by hind-limb suspension³² and osteoblasts demonstrate enhanced proliferation in response to uniaxial and biaxial strain.^{3,27} Theoretical models relating biophysical stimuli to stem cell fate have been proposed⁴⁷ and there is increasing evidence to support a relationship between mechanical stimulation and osteogenic differentiation.^{1,23,43} However, the signaling mechanisms involved in the strain-induced regulation of differentiation remain to be fully defined.

Mechanical strain influences the differentiation of precursor cells by increasing the expression of the osteoblast-specific core binding factor alpha-1 (Cbfa1) transcription factor which is required for the induction of most genes that are required for the synthesis of bone extracellular matrix.¹¹ Following *in vitro* and *in vivo* mechanical loading, there is an upregulation of Cbfa1 expression in response to the application of tensile strain.^{28,65} During differentiation, osteoblasts express a characteristic pattern of genes^{5,48} including collagen type I, which is expressed from the beginning of osteoblast differentiation during the active proliferation phase and osteocalcin, which is upregulated in the late stages of osteoblast differentiation. Maximum

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elevation of osteocalcin marks the transition from matrix maturation to mineralization and thus osteocalcin plays an important role in the mineralization of the matrix.^{10,20,42} Bone morphogenic proteins (BMPs), members of a family of secreted growth factors, provide specific signals that are essential for full osteogenic differentiation. BMPs are upregulated in response to differentiation signals in allograft bone fracture healing, bone regeneration, and heterotopic bone formation, and stimulate osteogenic differentiation in mesenchymal progenitor cells via the extracellular-regulated kinase (ERK) pathway.^{34,64}

The precise cellular mechanisms underlying the conversion of mechanical signals to cellular responses such as new bone formation remain to be fully resolved; however, stretch-activated cation channels (SACC) are present on osteoblasts⁸ and have been identified as transducers of mechanical strain in osteoblasts.⁹

Signaling through mitogen-activated protein kinases (MAPKs) is essential for the early stages of osteoblast differentiation.^{13,15,22} An upregulation of phosphorylated extracellular regulated kinase (ERK)1/2 is often concomitant with strain-induced Cbfx1 activity^{28,65} and it has been shown that ERK and p38 MAPK signaling are involved in the stimulation of osteoblast-related gene expression.^{33,52,58} Mechanical stimulation of MSCs also initiate MAPK responses⁵⁵ and mechanical stretch activates the PI3-kinase pathway in osteoblastic cells, as well as other cell types.^{9,44,59}

The aim of this study was to assess the effect of cyclic tensile mechanical stimulation on MSC proliferation and differentiation, and to identify the mechanotransduction mechanism underpinning the strain-mediated induction of osteogenic protein expression. This study investigated the hypothesis that mesenchymal cell fate, in particular proliferation and osteogenic differentiation, could be regulated by mechanical strain via SACC and intracellular signaling associated with the MAPK members, ERK and p38, and the PI3-kinase enzyme.

METHODS

Cell Culture

MSCs were isolated from rat bone marrow, as described in Farrell *et al.*¹² Briefly, bone marrow was harvested by flushing the femurs and tibiae of 12-week-old male Wistar rats with Dulbecco's modified Eagles medium (Sigma-Aldrich, Poole, England) supplemented with 100 U/mL penicillin/streptomycin (Gibco BRL, Dublin), 10% fetal bovine serum (Gibco BRL, Dublin), 1 mM L-glutamine (Gibco BRL, Dublin),

2 mM Glutamax (Gibco BRL, Dublin), and 1% non-essential amino acids (Gibco BRL, Dublin). After incubation for 30 min in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C, the supernatant was removed and plated at approximately 5×10^7 nucleated cells per 75 cm³ in T75 culture flasks (Sarstedt, Leicester, England). Nonadherent cells were removed after 24 h. Culture medium was replaced every 3–4 days. Cells were passaged upon reaching 80–90% confluency using trypsin EDTA (Sigma-Aldrich, England). Cells were passaged at a 1:2 subcultivation ratio and were used at passage 3–5. Fluorescence-activated cell sorter analysis on cells at passage 3 verified that the cell population expressed high levels of the MSC surface marker CD90 ($96.9 \pm 0.4\%$) but not CD45, in agreement with previously published data on rat MSC cell surface markers.⁵¹

Application of Mechanical Strain and Culture Conditions

Silicone strips (10 mm × 60 mm; Specialty Manufacturing, Saginaw, USA) were coated with rat-tail collagen type I (1%; Sigma-Aldrich, Dublin, Ireland). Cells were suspended in 100 μL liquid cell culture medium and were applied to a defined surface area of 600 mm² at a density of 208 cells/mm². Following 30 min to allow attachment, the cell culture dish was filled with culture medium. No osteoinductive agents were included in the culture medium, and the collagen coating on the silicone strip had no effect on osteogenesis (results not shown). After 2 days, cyclic uniaxial tensile strains of 0 and 2.5% were applied at 0.17 Hz for 3 days using a custom-made single station stretching device, as previously described.³⁷ Non-strained control cells were grown on collagen-coated silicone membranes placed in a tissue-culture dish. Our previous studies have demonstrated that strains greater than 5% evoke apoptosis of MSCs²⁹; thus, we elected to apply strains at the lower magnitude of 2.5% in this study. In some experiments, cells were exposed to the SACC blocker, GdCl₃ (Sigma-Aldrich, Dublin, Ireland), stored as a 2 mM stock solution in dH₂O at –20 °C, and diluted to a final concentration of 10 μM in culture medium; an inhibitor of ERK, U0126 (Calbiochem, Nottingham, UK), stored as a 1 mM stock solution in dimethylsulfoxide (DMSO; LGC Promochem, Middlesex, UK) at –20 °C, and diluted to a final concentration of 2 μM in culture medium; an inhibitor of p38, SB 203580 (Calbiochem, Nottingham, UK), stored as a 1 mM stock solution in DMSO at –20 °C, and diluted to a final concentration of 10 μM in culture medium; or an inhibitor of PI3-kinase, LY294002 (Cell Signalling Technology, Danvers, USA), stored as a 10 mM stock solution in DMSO

at -20°C , and diluted to a final concentration of $2\ \mu\text{M}$ in culture medium.

In Vitro MSC Proliferation Assay

Proliferation was examined by assessing the synthesis of DNA through ^3H -thymidine uptake. During the last 24 h of the experiment, strained and control unstrained cells were pulsed with $2.0\ \mu\text{Ci}/\text{mL}$ methyl-tritiated thymidine (Amersham, UK). Cells were scraped from the substrate and digested in $150\ \mu\text{L}$ of a 0.1% papain/buffer solution [$5\ \text{mM}$ EDTA, pH 8; $5\ \text{mM}$ cysteine-HCl (Sigma-Aldrich, Dublin, Ireland)]. Cells ($120\ \mu\text{L}$) were harvested onto a filtermat (PerkinElmer, Dublin, Ireland) using a TomTech cell harvester. The filtermat was placed in a beta-plate scintillation counter and DNA synthesis through ^3H -thymidine uptake was measured. Counts per sample were normalized to the total cell number in each sample, measured by the Hoechst DNA assay.

DNA Assay

To determine cell number from quantity of DNA in each sample, $10\ \mu\text{L}$ was taken from each papain-digested cell sample in triplicate.³¹ From a stock solution of $1\ \text{mg}/\text{mL}$ (dH_2O ; 4°C ; protected from light), a $0.1\ \mu\text{g}/\text{mL}$ solution of Hoechst 33258 dye was prepared in Hoechst buffer ($10\ \text{mM}$ Tris, $1\ \text{mM}$ Na_2EDTA , $1\ \text{M}$ NaCl, pH 7.4; 4°C ; protected from light) and $200\ \mu\text{L}$ was added to each sample. Following a gentle vortex, samples were transferred to a black 96-well plate, which was read on a fluorescent plate reader with the excitation wavelength at $365\ \text{nm}$ and emission of $458\ \text{nm}$. A standard calibration curve was generated using a range of MSC digests of known concentration.

Assessment of Cbfx1, Collagen Type I, and Osteocalcin Expression by Fluorescence Immunocytochemistry

To determine the stimulation of osteogenic markers in response to tensile strain, cells were examined for Cbfx1, collagen type I, or osteocalcin expression. Fixed cells were incubated with blocking buffer (2% BSA in TBS with 0.1% Triton-X100 and 20% heat-inactivated horse serum; Gibco BRL, Dublin), and incubated at 4°C overnight with a collagen type I goat polyclonal antibody (Santa Cruz Biotechnology), Cbfx1 rabbit polyclonal (Alpha Diagnostic International), or osteocalcin goat polyclonal (Diagnostic Systems Laboratories) primary antibody. Cells were then incubated for 2 h at room temperature with an appropriate biotinylated secondary IgG (Vector) followed by incubation with ExtrAvidin-FITC (Sigma). Samples were

thoroughly washed, mounted in Vectashield (Vector Laboratories), and viewed by fluorescence microscopy (excitation $490\ \text{nm}$; emission $520\ \text{nm}$) using a Leica fluorescence microscope and Improvision software. Immunoreactivity was expressed as arbitrary fluorescence units (AFU). When comparing between treatment groups, the samples were exposed to the fluorescence lamp for the same duration and the gain setting was kept constant. Approximately, 200 cells were analyzed per treatment group. The data reported are expressed as the mean $\text{AFU} \pm \text{SEM}$ for six independent culture preparations. The coverslips were randomly coded so that the cells could be assessed by the user in a blind manner to avoid user bias. Background fluorescence is the staining observed following omission of the primary antibody and represents nonspecific binding of the secondary antibody and fluorophore.

Western Immunoblot for Analysis of BMP2

To assess BMP2 expression, cells were lysed in buffer ($25\ \text{mM}$ HEPES, $5\ \text{mM}$ MgCl_2 , $5\ \text{mM}$ EDTA, $5\ \text{mM}$ dithiothreitol, $0.1\ \text{mM}$ PMSF, $5\ \mu\text{g}/\text{mL}$ pepstatin A, $2\ \mu\text{g}/\text{mL}$ leupeptin, $2\ \mu\text{g}/\text{mL}$ aprotinin, $2\ \mu\text{g}/\text{mL}$ sodium orthovanadate, pH 7.4). Cells were centrifuged ($15,000 \times g$ for 20 min at 4°C) and the supernatant diluted by a factor of 2 with sample buffer [$150\ \text{mM}$ Tris-HCl, pH 6.8; 10% glycerol (v/v); 4% SDS (w/v); 5% β -mercaptoethanol (v/v); 0.002% bromophenol blue (w/v)]. Protein concentration was determined using the PIERCE BCA assay in accordance with the manufacturer's instructions. Samples were then heated to 100°C for 3 min. Proteins ($10\ \mu\text{g}$ per lane) were separated by electrophoresis on a 12% polyacrylamide gel, transferred to nitrocellulose membrane, and immunoblotted with a rabbit polyclonal antibody raised against the synthetic peptide corresponding to amino acids 45–60 of BMP2 protein and secondary antibody (anti-rabbit IgG; 1:1000) conjugated to horseradish peroxidase. Bands were visualized by chemiluminescence (Supersignal, Pierce, Leiden, the Netherlands). Blots were stripped with antibody stripping solution (1:10 dH_2O ; Reblot Plus Strong antibody stripping solution; Chemicon, California, USA) and re-probed for GAPDH to normalize protein loading. Band widths were quantified by densitometry (D-Scan PC software) and data is expressed as a ratio to GAPDH.

Statistical Analysis

Data are reported as the mean \pm SEM of the number of experiments indicated in each case. Each n -number represents an individual culture preparation

prepared from one rat. Statistical analysis to compare controls and treatments was carried out by a paired Student's *t*-test to determine whether significant differences existed between the populations. For Western blot data sets, the Shapiro–Wilk normality test was applied and subsequently the Wilcoxon matched pairs test to test for significance. In all cases, the alpha level was set to 0.05. All statistical analysis was carried out using Graphpad Prism 4 software (Graphpad Software Inc.).

RESULTS

Tensile Mechanical Strain of 2.5% Reduces MSC Proliferation

To determine the influence of a 2.5% uniaxial strain on MSC proliferation dynamics, the rate of mitosis was monitored through quantification of new DNA formation detected by the uptake of tritiated (^3H -) thymidine (Fig. 1). Following 1 day of 2.5% uniaxial strain, ^3H -thymidine uptake was comparable to that in control cells that had not been exposed to stretch. The mean cell retrieval after 1 day was 1.5×10^5 for control samples and 0.9×10^5 for strained. However, following 2 days of 2.5% strain, ^3H -thymidine uptake was significantly decreased from 0.09 ± 0.02 cpm/cell (mean \pm SEM) in the control group to 0.04 ± 0.01 cpm/cell in the strained group ($p < 0.05$, Student's paired *t*-test; $n = 11$ cultures). The mean cell retrieval after 2 days was 1.8×10^5 for control samples and 1.2×10^5 for strained. Similarly, after a 3-day 2.5%

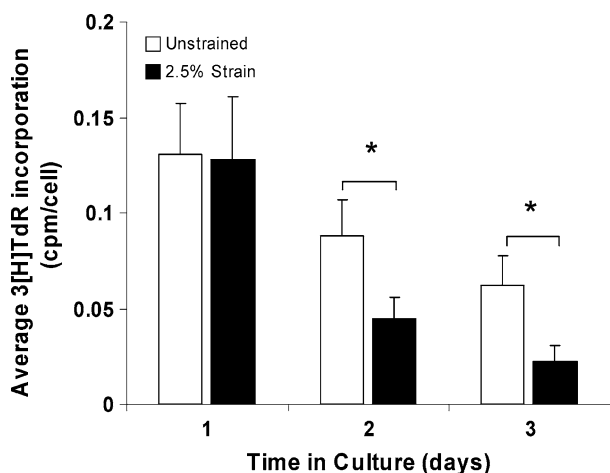


FIGURE 1. Mechanical strain reduces MSC proliferation. Mechanical strain of 2.5% for 1 day had no effect on MSC proliferation; however, 2.5% strain for 2 and 3 days significantly reduced MSC proliferation in MSCs, compared to unstrained controls. Results are expressed as mean \pm SEM for 8–11 independent culture preparations. Student's paired *t*-test, * $p < 0.05$ strain vs. control.

strain stimulation, ^3H -thymidine uptake was significantly decreased from 0.06 ± 0.02 cpm/cell in the control unstrained group to 0.02 ± 0.007 cpm/cell in cells exposed to mechanical strain ($p < 0.05$, Student's paired *t*-test; $n = 8$ cultures). The mean cell retrieval after 3 days was 1.7×10^5 for control samples and 1.0×10^5 for strained. This finding demonstrates that mechanical stimulation of 2.5% significantly reduces the rate of MSC proliferation. This reduction in proliferation does not correlate with an increase in cell death as we have previously demonstrated that the application of 2.5% tensile strain does not induce cell loss in this cell type, although tensile strains greater than 5% do evoke engagement of the apoptotic cascade.²⁹

Tensile Mechanical Strain of 2.5% Induces Osteogenic Differentiation

Strain-induced differentiation toward the osteogenic lineage was assessed by examining changes in bone-related protein expression following cyclic tensile stimulation of 2.5% at 0.17 Hz for 3–14 days using immunofluorescence and Western immunoblot (Table 1). Representative images of Cbfa1, collagen type 1, and osteocalcin immunoreactivity following 6 days of tensile strain are displayed in Fig. 2. Expression of the transcription factor Cbfa1 was increased following a 3- and 6-day strain when compared to unstrained controls (Table 1; Fig. 2ai, ii). Quantification of fluorescence intensity demonstrated a significant increase in Cbfa1 immunoreactivity from 15.91 ± 1.04 mean arbitrary fluorescent units (AFU; mean \pm SEM) to 20.11 ± 1.04 AFU in cells exposed to a 6-day mechanical strain ($p < 0.05$, Student's paired *t*-test; $n = 4$ cultures; Fig. 2aiii). Following 3, 6, and 9 days of cyclic tensile strain, expression of collagen type I was increased compared to unstrained controls (Table 1; Fig. 2bi, ii). Collagen type 1 immunoreactivity increased significantly from 12.53 ± 1.29 AFU (mean \pm SEM) in control cells to 21.53 ± 1.44 AFU in cells exposed to 6 days of mechanical strain ($p < 0.05$, Student's paired *t*-test; $n = 4$ cultures; Fig. 2biii). Osteocalcin expression was increased in mechanically stimulated cells following 6, 9, and 12 days of strain when compared to controls (Table 1; Fig. 2ci, ii). Osteocalcin immunoreactivity increased significantly from 13.83 ± 1.36 AFU (mean \pm SEM) in control cells to 20.60 ± 1.60 AFU in cells exposed to 6 days of mechanical strain ($p < 0.05$, Student's paired *t*-test; $n = 4$ cultures; Fig. 2ciii).

Expression levels of BMP2 following a 14-day 2.5% strain were measured by Western immunoblot and normalized to expression levels of the housekeeping protein GAPDH. Following a 14-day stimulus, BMP2

TABLE 1. Protein expression patterns following a continuous 2.5% tensile cyclic strain (0.17 Hz) for 3–14 days.

Time under strain (days)	Cbfx1	Collagen type 1	Osteocalcin	BMP2
3	Significant increase $p < 0.05$, 13.66 ± 1.15 to 27.07 ± 1.84 AFU, $n = 320$ cells	Increase $n = 480$ cells	No change	n.a.
6	Significant increase $p < 0.05$, 15.91 ± 1.04 to 20.11 ± 1.04 AFU, $n = 320$ cells	Significant increase $p < 0.05$, 2.53 ± 1.29 to 21.53 ± 1.44 AFU, $n = 320$ cells	Significant increase $p < 0.05$, 13.83 ± 1.36 to 20.60 ± 1.60 AFU, $n = 320$ cells	n.a.
9	n.a.	Significant increase $p < 0.05$, 10.02 ± 0.97 to 20.75 ± 2.12 AFU, $n = 320$ cells	Significant increase $p < 0.05$, 9.52 ± 0.76 to 19.73 ± 1.94 AFU, $n = 320$ cells	n.a.
12	n.a.	No change	Significant increase $p < 0.05$, 10.65 ± 1.86 to 28.47 ± 2.42 AFU, $n = 320$ cells	n.a.
14	n.a.	n.a.	n.a.	Significant increase $p < 0.05$, 0.79 ± 0.15 to 4.34 ± 1.88 mean arbitrary units, $n = 10$

MSCs were exposed to 2.5% mechanical strain and probed for osteogenic protein marker expression to determine strain-induced differentiation. Following 3 and 6 days of strain, Cbfx1 immunoreactivity significantly increased (Student's paired *t*-test, $p < 0.05$; $n = 4$ independent cultures with at least 320 cells analyzed in total). Collagen type 1 expression significantly increased following 9 days of strain (Student's paired *t*-test, $p < 0.05$; $n = 4$ independent cultures with at least 320 cells analyzed in total) but was unchanged compared to unstrained cells by 12 days. Osteocalcin expression in strained cells was comparable to unstrained controls following 3 days of strain; however, osteocalcin immunoreactivity in strained cells was significantly increased following 6, 9, and 12 days of strain (Student's paired *t*-test, $p < 0.05$; $n = 4$ independent cultures, with at least 320 cells analyzed in total). BMP2 expression was significantly increased following 14 days of strain (Wilcoxon matched pairs test, $p < 0.05$; $n = 10$ independent culture preparations). n.a., not assessed. AFU, arbitrary fluorescence units per cell.

expression was significantly increased from 0.79 ± 0.15 arbitrary units (normalized to GAPDH; mean \pm SEM) in unstrained control cells to 4.34 ± 1.88 arbitrary units ($p < 0.05$, Wilcoxon signed rank test, $n = 10$; Fig. 2d). These findings indicate that 2.5% tensile strain induces osteogenic protein expression in MSCs within a temporal timeframe.

Stretch-Activated Ion Channels are Involved in Strain-Induced Differentiation

To examine the role of SACC in strain-induced osteogenic protein expression, cells were cultured under nonstrained or strained conditions for 6 days in the absence and presence of the SACC blocker, gadolinium chloride (GdCl_3 ; $10 \mu\text{M}$). Cbfx1, collagen type I, and osteocalcin expression were assessed by fluorescence microscopy. An increase in Cbfx1 immunoreactivity is demonstrated following mechanical stimulation for 6 days; however, this increase is less marked in cells that were strained in the presence of Gd^{3+} (Fig. 3a), although the result fails to reach statistical significance. Thus, Cbfx1 fluorescence intensity significantly increases from 15.91 ± 1.04 AFU (mean \pm SEM) in

unstrained controls to 20.11 ± 1.04 AFU following a 6-day 2.5% strain stimulation ($p < 0.05$, Student's paired *t*-test; $n = 4$); however, in the presence of Gd^{3+} , fluorescence intensity was comparable to control levels (Fig. 3a). An increase in collagen type I immunoreactivity is demonstrated following mechanical stimulation for 6 days and this is significantly decreased in cells that were strained in the presence of Gd^{3+} (Fig. 3b). Thus, collagen type I immunoreactivity significantly increases from 17.44 ± 1.65 AFU (mean \pm SEM) in unstrained controls to 22.90 ± 0.40 AFU following exposure to a 2.5% strain ($p < 0.05$, Student's paired *t*-test; $n = 4$ cultures; 320 cells). This induction of collagen type I was significantly decreased when cells were strained in the presence of Gd^{3+} whereby immunoreactivity decreased from 22.9 ± 0.4 to 20.9 ± 0.8 AFU ($p < 0.05$, Student's paired *t*-test; $n = 4$ cultures; 320 cells; Fig. 3b). Osteocalcin immunoreactivity increases following 2.5% strain for 6 days and this increase is less marked in cells that were strained in the presence of Gd^{3+} (Fig. 3c), although the result fails to reach statistical significance. In the nonstrained samples, GdCl_3 had no significant effect on expression of osteogenic markers. Overall, these

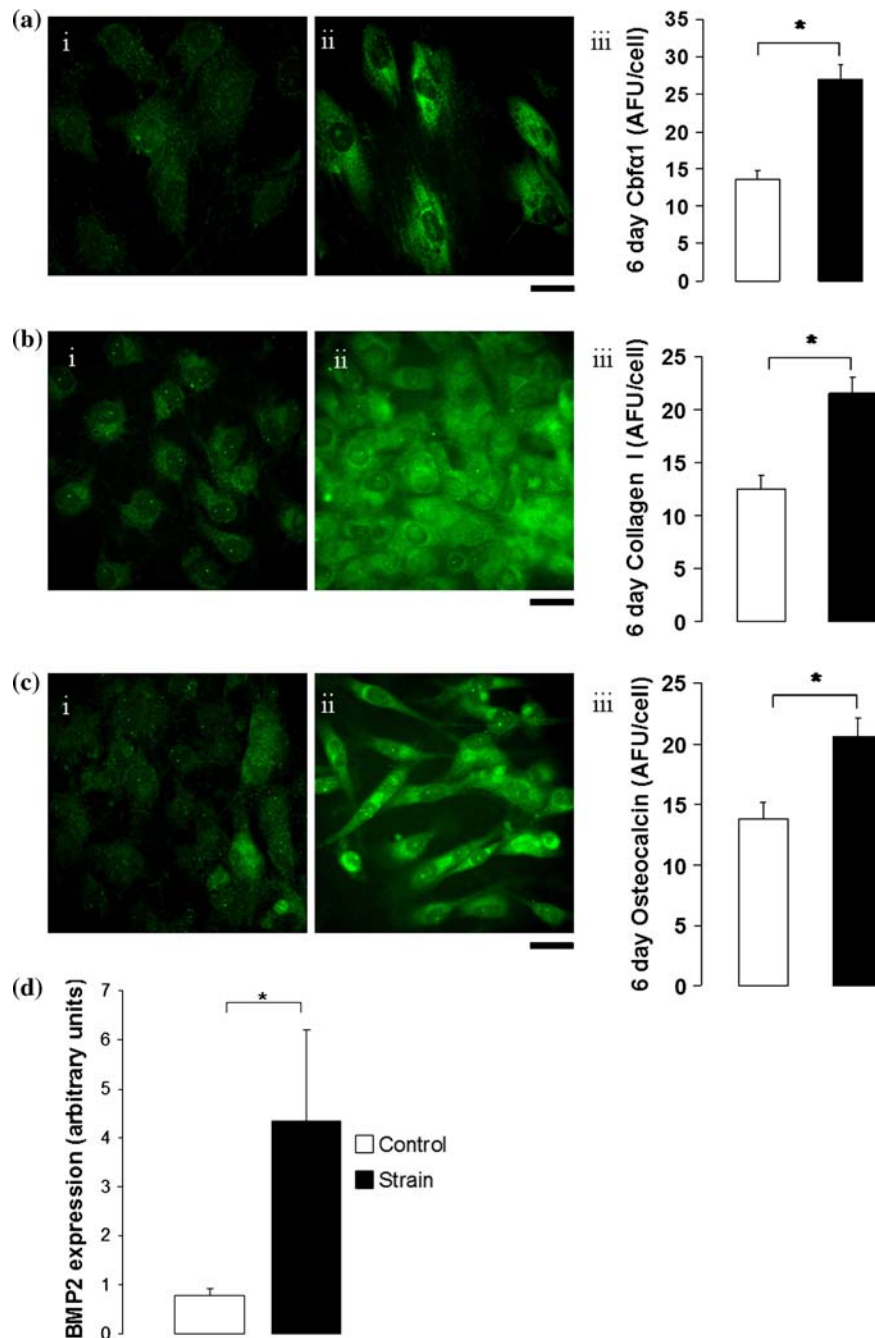


FIGURE 2. Strain induces the expression of the osteogenic markers Cbfa1, collagen type I, and osteocalcin. (a)—(i) MSCs in static culture for 6 days display only background staining for Cbfa1; however, MSCs exposed to mechanical strain (2.5%; ii) display intense Cbfa1 immunoreactivity. (iii) Cbfa1 immunoreactivity significantly increased in cells exposed to mechanical strain after 6 days compared to unstrained controls (Student's paired *t*-test $*p < 0.05$; $n = 4$ independent culture preparations. Results are expressed as mean AFU per cell \pm SEM. Scale bar = 100 μ m). (b)—(i) MSCs in static culture for 6 days display only background staining for collagen type I; however, MSCs exposed to mechanical strain (2.5%; ii) display intense collagen type I immunoreactivity. (iii) Collagen type I immunoreactivity significantly increased in cells exposed to mechanical strain (Student's paired *t*-test $*p < 0.05$; $n = 4$ independent culture preparations. Results are expressed as a mean AFU per cell \pm SEM. Scale bar = 100 μ m). (c)—(i) MSCs in static culture for 6 days display only background staining for osteocalcin; however, MSCs exposed to mechanical strain (2.5%; ii) display intense osteocalcin immunoreactivity. (iii) Osteocalcin immunoreactivity significantly increased in cells exposed to mechanical strain compared to unstrained controls (Student's paired *t*-test $*p < 0.05$; $n = 4$ independent culture preparations. Results are expressed as a mean AFU per cell \pm SEM. Scale bar = 100 μ m). (d) Following 14 days in static culture or exposed to 2.5% tensile mechanical strain, MSCs were harvested and analyzed for expression of BMP2 using Western immunoblotting. BMP2 expression was normalized to the housekeeping protein GAPDH expression. Strain of 2.5% significantly increased BMP2 expression following a 14-day stimulation (Wilcoxon signed rank test; $*p < 0.05$; $n = 10$ independent culture preparations. Results are expressed as a mean \pm SEM. Scale bar = 100 μ m).

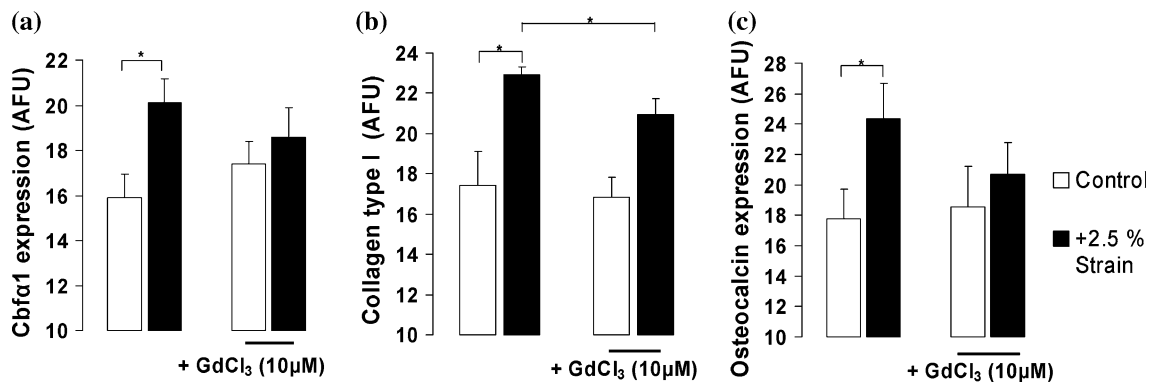


FIGURE 3. Role of SACCs in the strain-induced expression of osteogenic markers, Cbfa1, collagen type I, and osteocalcin. MSCs were exposed to mechanical strain (2.5%) for 6 days in the absence or presence of GdCl₃ (10 μM) and probed for osteogenic protein expression to determine the involvement of SACC in the osteogenic differentiation of MSCs. (a) Cbfa1 immunoreactivity significantly increases in MSCs exposed to mechanical strain compared to unstrained controls (Student's paired *t*-test, **p* < 0.05; *n* = 5 culture preparations) and the induction of Cbfa1 is abolished in the presence of GdCl₃ although that result failed to reach statistical significance. (b) MSCs exposed to strain display a significant increase in collagen type I immunoreactivity compared to unstrained cells. The strain-induced collagen type I immunoreactivity was significantly decreased when cells were stimulated in the presence of GdCl₃ (Student's paired *t*-test, **p* < 0.05; *n* = 6 culture preparations). (c) Osteocalcin immunoreactivity significantly increased in MSCs exposed to mechanical strain compared to unstrained controls (Student's paired *t*-test, **p* < 0.05; *n* = 7 culture preparations). In the presence of GdCl₃, the induction of osteocalcin was reduced but that result failed to reach statistical significance. Results are expressed as a mean AFU per cell ± SEM for 5–7 independent culture preparations, Student's paired *t*-test **p* < 0.05.

findings provide evidence that SACCs are involved in the mechanotransduction of tensile strain that induces collagen type I expression after 6 days.

Strain-Induced BMP2 Synthesis Occurs Via ERK and PI3-kinase but not p38

To examine the role of ERK, PI3-kinase, and p38 on the strain-mediated induction of BMP2, cells were strained in the absence or presence of the ERK inhibitor, U0126 (2 μM); the PI3-kinase inhibitor, LY 294002 (2 μM); or the p38 inhibitor, SB 203580 (10 μM), for 14 days. Expression levels of BMP2 protein was measured by Western immunoblot and BMP2 expression was normalized to GAPDH. Figure 4a demonstrates that following exposure to a 2.5% strain stimulus for 14 days, BMP2 expression was increased 9.09 ± 1.42 -fold (mean ± SEM) over control levels; however, in the presence of the ERK inhibitor, U0126 (2 μM), the strain-mediated induction of BMP2 was significantly decreased (*p* < 0.05, Student's paired *t*-test, *n* = 5, Fig. 4a). Following a 14-day exposure to the 2.5% strain stimulus, BMP2 expression was increased 7.89 ± 1.67 -fold (mean ± SEM) over control levels; however, in the presence of the PI3-kinase inhibitor, LY 294002 (2 μM), the strain-mediated induction of BMP2 was significantly decreased (*p* < 0.05, Student's paired *t*-test, *n* = 6, Fig. 4b). In Fig. 4c, following a 14-day exposure to the 2.5% strain stimulus, BMP2 expression was increased 4.51 ± 1.69 -fold (mean ± SEM) over control levels. The p38 inhibitor, SB 203580 (10 μM), substantially

reduced the strain-mediated induction of BMP2, but this failed to reach statistical significance, possibly due to the high standard error in the control culture preparation. These findings identify a definite role for ERK and PI3-kinase signaling in strain-induced BMP2 expression in MSCs.

DISCUSSION

This study tested the hypothesis that mechanical strain influences mesenchymal cell proliferation and differentiation in the absence of osteogenic factors and that strain-induced osteogenic tissue differentiation involves SACCs. Additionally, we investigated the role of the MAPK family members, ERK and p38, and the PI3-kinase enzyme in the strain-induced osteogenic response. Continuous cyclic tensile strain of 2.5% at 0.17 Hz for 24–72 h reduced MSC proliferation. Coupled with the reduced proliferation, this mechanical stimulation paradigm also induced the temporal expression of the osteogenic-specific transcription factor Cbfa1, collagen type I, and osteocalcin, and initiated an autocrine stimulation of BMP2 in a manner that involved ERK and PI3-kinase. Using the SACC blocker, GdCl₃, we found an involvement of SACC in the strain-induced expression of bone-related collagen I. Although the sensitivity of MSCs to mechanical strain has been established,¹⁴ and that mechanical conditioning enhances osteogenic factor-induced MSC differentiation, this study supports the argument that biomechanical stimulation alone (i.e., in the absence of

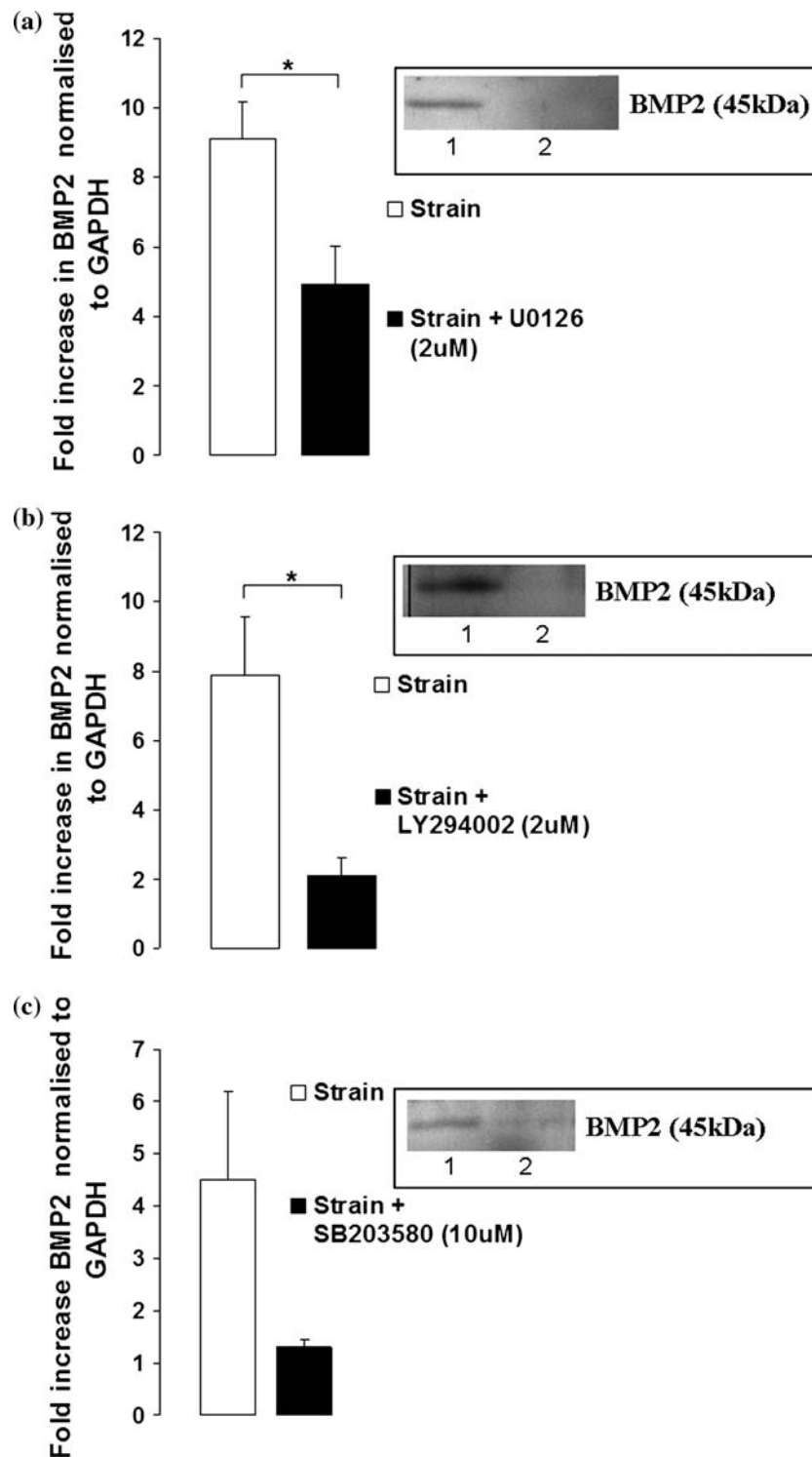


FIGURE 4. Strain induces BMP2 synthesis in MSCs under the regulation of ERK and PI3-kinase, but not p38. Cells were exposed to 2.5% strain for 14 days in the presence or absence of U0126 (2 μ M), LY 294002 (2 μ M), or SB 203580 (10 μ M) and probed for BMP2. In the presence of U0126 (2 μ M; a) and LY 294002 (2 μ M; b), the strain-induced fold increase in BMP2 expression was significantly reduced. (c) In the presence of SB 203580 (10 μ M), there was a marked reduction in the strain-induced fold increase in BMP2 expression; however, this failed to reach statistical significance. Inset, sample BMP2 immunoblot in strained samples in the absence (lane 1) and presence (lane 2) of (a) ERK inhibitor, (b) PI3-Kinase inhibitor, and (c) p38 inhibitor. Results are expressed as mean \pm SEM for 6–10 independent culture preparations, Wilcoxon matched pairs test, * p < 0.05. Protein expression is normalized to GAPDH. Representative Western blots of BMP2 are shown.

osteogenic growth factors) is sufficient to initiate events that promote osteogenic differentiation, partly through parallel signaling mechanisms that regulate osteogenic differentiation.^{16,24}

Our finding that strain reduces MSC proliferation may occur as a consequence of lineage commitment and osteogenic differentiation since in skeletogenesis and fracture repair, the cessation of progenitor cell proliferation marks the onset of tissue-specific development.^{3,4,6} In mesenchymal bone cell progenitors, *Cbfa1* gene activity marks a key developmental transition to osteoblast maturation by functionally supporting an exit from the cell cycle and activating genes that support bone development.^{11,46} Therefore, in our study, the proliferation dynamics coupled with the onset of *Cbfa1* expression represents the early stages of MSC osteogenic lineage commitment. Our observation that a level of proliferation persists concomitant to the induction of *Cbfa1* may indicate that a subpopulation of the cells have commenced differentiation at this early time point, with the remainder of cells still engaging in proliferative activity. We also noted that after 3 days of culture on the silicone substrate, the proliferation of the unstrained cells was reduced, albeit to a lesser extent than those cells exposed to 2.5% tensile strain. The observation that the unstrained samples displayed reduced proliferation over the 3-day period may reflect an effect of the relatively soft silicone substrate on the cells' proliferative capacity. The number of cells retrieved from the silicone was similar between treatment groups, despite reductions in proliferation, and this may reflect variations in initial seeding density or cell attachment characteristics.

In previous investigations on the mitotic response following mechanical strain, both positive and negative regulation has been reported. Short-term intermittent loading regimens are associated with an increase in cell number and an increase in proliferation gene activity^{56,61}; whereas, the response to longer mechanical stimulation reduces cell proliferation.⁶¹ Simmons *et al.*⁵⁵ report a decrease in proliferation in response to a continuous equibiaxial strain of similar magnitude, frequency, and duration to that used in the current study. Additionally, a series of recent studies that use continuous long-term cyclic strain report reduced MSC proliferation in both 2D and 3D environments^{21,35,40} with simultaneous detection of lineage-specific protein expression, indicating commitment of MSCs. Thus, MSC proliferation is affected by mechanical stimulation, and the response is sensitive to strain duration. In agreement with previous studies, the strain durations applied in this study favor a suppression of MSC proliferation and this is concomitant with the promotion of MSC differentiation.

The temporal expression of bone-specific protein induction suggests commitment of the MSCs toward an osteochondral lineage since during embryonic development *Cbfa1* expression precedes osteoblast differentiation. The sustained increase in collagen type I expression supports the evidence for commitment to the osteogenic lineage. The detection of osteocalcin marks late-stage osteoblast development and represents the differentiated state of the osteoblast⁵⁴ and functionally marks matrix mineralization.^{10,42}

A number of studies have linked the upregulation of *Cbfa1* following mechanical stimulation to early osteogenic events.^{23,28,65} The results in this study demonstrate that mechanical stimulation promotes the osteogenic differentiation of MSCs by targeting the crucial *Cbfa1* transcription factor. *Cbfa1* upregulation has been coupled with an ERK dependency,^{28,62,65} and it has been suggested that the mechanical stimulation may be an epigenetic factor in the posttranslational modification of *Cbfa1*.¹³ Since *Cbfa1* can be phosphorylated and activated by the MAPK pathway,¹³ it is possible that the *Cbfa1* activity observed in response to mechanical stimulation occurs through strain-induced phosphorylation of ERK, and this is supported by our data demonstrating that inhibition of ERK activity reduces the autocrine BMP2 response, which is intricately linked with *Cbfa1*.^{2,11}

The expression of collagen type I was observed in MSCs exposed to tensile strain for up to 9 days of continuous loading, but not beyond this. It is known that bone remodeling is driven by dynamic rather than static loading and bone cells desensitize rapidly to mechanical signals.⁴⁹ A recent long-term *in vivo* study determined that mechanical loading is more effective in enhancing bone biomechanical and structural properties if the loads are applied in discrete bouts, separated by recovery periods, than if the loads are applied in a single session.⁵⁰ Other studies describe a desensitization of osteoblasts to mechanical stimulation following 1 h as reflected in the pattern of ERK phosphorylation.^{25,52} Thus, the collagen type I expression pattern following continuous tensile stimulation observed in this study may reflect the development of a strain tolerance or a desensitization of MSCs, where a saturation of the osteogenic response occurs as the duration of the loading increases without interruption. This has been described by Turner,⁶⁰ for bone cells, in accommodation to a customary mechanical loading environment, making them less responsive to routine loading signals, particularly when the loading stimulus is of low magnitude.^{19,57}

In this study, the increase in BMP2 levels following 14 days of stimulation represents an autocrine osteogenic growth factor response to uniaxial strain. The ERK and PI3-kinase pathways are responsible for

mediating this response, which is in agreement with other studies.^{15,18} In this study, the role of p38 signaling in the induction of BMP2 was inconclusive due to the high standard error in the control groups. However, other studies have demonstrated p38 to be associated with the regulation of other BMP2-induced osteogenic responses, such as alkaline phosphatase activity and osteocalcin expression.^{15,41} While the mechanical stimulation paradigm used in this study favored osteogenic differentiation, alternative stimulation regimes using strain of different magnitudes and duration may be used to support differentiation toward other lineages. For example, cyclic stretch has been shown to support the differentiation of MSCs to smooth muscle cells without the addition of growth factors.¹⁷ It would be of interest to examine whether the application of tensile strain using our custom-designed device can evoke differentiation of MSCs along other connective tissue lineages. While this study concentrated on the application of a 2.5% strain to modulate MSC osteogenic differentiation, we also found that 5% strain induced osteogenesis (data not shown), whereas application of 10% evoked apoptosis.²⁹ Thus, identification of the effective range of tensile strains that successfully control differentiation along specific lineages, as opposed to induction of programmed cell death, is another important factor for consideration in MSC mechanoregulation.

SACCs are mechanosensitive and respond to membrane tension.⁸ In this study, we found that SACCs were involved in the strain-induced expression of bone-related proteins, notably collagen I, following a 6-day strain stimulus. The presence of SACCs on primary bone cells has been confirmed,⁸ and upon investigating mechanotransduction in a bone cell line, Danciu *et al.*⁹ report the involvement of SACCs in strain-induced early signal transduction responses, including activation of the PI3-kinase pathway, which we also found to be involved in MSC mechanotransduction.

Studies on cellular mechanotransduction in terminally differentiated cells report a role for SACCs in coordinating many physiological events; however, this is less well understood in undifferentiated primary progenitor cells. Therefore, our finding represents a role of SACCs in the mechanotransduction processes that are necessary, at least in part, for MSC differentiation. SACCs contribute to a wide array of cellular activities, and stretch-induced gene expression via SACCs has been reported in mammalian myocardium.³⁰ Since the strain-induced increase in collagen I was reduced when SACCs were blocked, this suggests that these channels link alterations in membrane tension to changes in gene expression, although other

proteins, such as integrins, may also be involved.⁵³ Focal adhesion kinase signaling has also been identified as an important pathway in linking tensile strain to osteogenic differentiation of MSCs.⁶³

It can be concluded from this study that mechanical forces play a significant role in regulating proliferation and the osteogenic differentiation pathway. Although our findings suggest that short-term mechanical stimulation induces differentiation toward an osteogenic phenotype in 2D culture, long-term stimulation evokes a desensitization to tensile strain in bone cells.^{50,60} Furthermore, cells can reorientate perpendicular to the axis of strain,³⁷ and it is important to note that this may modify cellular responses to continual strain such that the impact of strain on the recruitment of specific signaling pathways and subsequent differentiation may change over time. Through the application of a continuous, low magnitude, low frequency tensile mechanical strain to MSCs seeded on a 2D collagen-coated silicone membrane, this study demonstrates that MSCs are capable of detecting, transducing, and responding phenotypically to a biomechanical stimulus. The expression of Cbfa1, collagen type I, osteocalcin, and BMP-2 regulation strongly suggests the directed differentiation toward the osteogenic lineage and can be compared to osteoblast differentiation that has been described in a number of studies on bone development.⁴²

The increasing evidence for mechanical stimulus alone (i.e., in the absence of osteoinductive growth factors) as a regulator of MSC osteogenic differentiation, and the identification of the associated mechanotransduction pathways, holds important consequences for the development of orthopedic tissue engineering solutions by circumventing the need for the addition of growth factors, and using mechanically primed precursor cells to promote osteogenic differentiation.

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