

# Cyclic strain enhances matrix mineralization by adult human mesenchymal stem cells via the extracellular signal-regulated kinase (ERK1/2) signaling pathway

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## Abstract

Physical stimuli play critical roles in the development, regeneration, and pathology of many mesenchymal tissues, most notably bone. While mature bone cells, such as osteoblasts and osteocytes, are clearly involved in these processes, the role of their progenitors in mechanically mediated tissue responses is unknown. In this study, we investigated the effect of cyclic substrate deformation on the proliferation and osteogenic differentiation of human mesenchymal stem cells (hMSCs). Application of equibiaxial cyclic strain (3%, 0.25 Hz) to hMSCs cultured in osteogenic media inhibited proliferation and stimulated a 2.3-fold increase in matrix mineralization over unstrained cells. The strain stimulus activated the extracellular signal-regulated kinase (ERK1/2) and p38 mitogen-activated protein kinase pathways, but had no effect on c-Jun N-terminal kinase phosphorylation or activity. Strain-induced mineralization was largely mediated by ERK1/2 signaling, as inhibition of ERK1/2 attenuated calcium deposition by 55%. Inhibition of the p38 pathway resulted in a more mature osteogenic phenotype, suggesting an inhibitory role for p38 signaling in the modulation of strain-induced osteogenic differentiation. These results demonstrate that mechanical signals regulate hMSC function, suggesting a critical role for physical stimulation of this specific cell population in mesenchymal tissue formation.

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## 1. Introduction

Mesenchymal stem cells (MSCs) are a small sub-population of the heterogeneous mixture of cells found in adult bone marrow stroma. MSCs are characterized by their ability to replicate as undifferentiated cells *ex vivo* and by their potential to differentiate to several lineages of mesenchymal tissues, including bone, cartilage, fat, and muscle (Pittenger et al., 1999). Accordingly, there is

significant interest in identifying the factors that regulate MSC growth and differentiation, with the goals of determining the natural role of MSCs in regeneration, disease, and aging, and using them as a cell source for therapeutic interventions such as tissue engineering.

Among the factors that regulate MSC growth and differentiation are soluble factors (Ferrari et al., 1998; Pittenger et al., 1999; Sanchez-Ramos et al., 2000; Wakitani et al., 1995; Woodbury et al., 2000; Yoo et al., 1998), and cell–substrate interactions (Ohgushi and Caplan, 1999; Yang et al., 2001). Although little is known about the molecular mechanisms by which soluble and substrate signals regulate MSC function, Jaiswal et al. (2000) recently showed that the commitment of human MSCs (hMSCs) to the

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osteogenic and adipogenic lineages in vitro involves signaling by mitogen-activated protein kinase (MAPK) pathways. In particular, they found that induction of hMSC differentiation to osteoblasts by dexamethasone, ascorbic acid, and  $\beta$ -glycerophosphate is regulated by the extracellular signal-regulated kinase (ERK1/2) cascade. Furthermore, blocking the ERK1/2 pathway inhibits osteogenic differentiation of hMSCs and leads to adipogenesis. Thus MAPK pathways, which are generally activated by growth factors/cytokines (Robinson and Cobb, 1997) and integrin-mediated cell adhesion (Morino et al., 1995), play critical roles in directing MSC commitment.

MAPK pathways are also activated by physical stimuli to regulate the function of a variety of cell types, including bone cells (Gebken et al., 1999; Granet et al., 2001, 2002; Jessop et al., 2002; Kletsas et al., 2002; Matsuda et al., 1998; Pommerenke et al., 2002; Schmidt et al., 1998; Weyts et al., 2002; You et al., 2001; Ziros et al., 2002). In bone, it has been proposed that mature cells such as osteocytes (Ajubi et al., 1999; Huiskes et al., 2000; Kawata and Mikuni-Takagaki, 1998) and osteoblasts (Jessop et al., 2002; Smalt et al., 1997; Weyts et al., 2002; Wozniak et al., 2000; You et al., 2001) are responsible for sensing and responding to mechanical stimuli. Whether the progenitors that give rise to these cells are responsive to mechanical signals and if so, by what mechanisms remain open and important questions, however. Bone marrow stromal cell populations have been shown to be mechanically responsive (Altman et al., 2002; Keila et al., 1994; Rubin et al., 2000, 2002; Thomas and el Haj, 1996; Wang et al., 2001, 2002; Zhang et al., 1995), but the procedures traditionally used to isolate stromal cells from bone marrow result in heterogeneous populations comprised of a mixture of undifferentiated to fully committed cells (Friedenstein et al., 1982; Phinney et al., 1999). Thus, it is unclear whether the stromal cells that are responsive to mechanical signals are fully differentiated cells, committed progenitors, or undifferentiated stem cells.

In the present study, we hypothesized that the mesenchymal stem cell subpopulation of the bone marrow stroma is responsive to mechanical stimuli through MAPK signaling. To test this hypothesis, we used a relatively homogeneous population of bone marrow-derived hMSCs and determined the effect of cyclic mechanical strain on the proliferation and differentiation of this specific cell population under osteogenic conditions. We show that cyclic deformation of the cell substrate inhibits the growth of hMSCs and increases deposition of mineralized matrix. Further, we demonstrate that this strain-induced mineralization is mediated by the ERK1/2 pathway. These results demonstrate that mechanical strain is

a potent regulator of hMSC function, suggesting a mechanism by which mechanical signals might regulate bone tissue development, regeneration, and pathology.

## 2. Methods

### 2.1. Cell culture

Primary hMSCs isolated from adult human bone marrow aspirates by density gradient centrifugation were obtained from Cambrex Bio Science (Walkersville, MD). The cells are characterized by their ability to attach and proliferate in culture, by uniform expression of approximately 50 surface antigens (with 98% homogeneity at passage 2), and by their consistent differentiation to multiple mesenchymal lineages under controlled conditions in vitro (Pittenger et al., 1999). The cells were expanded in non-differentiating growth medium consisting of MSC Basal Medium (Cambrex) supplemented with 10% fetal bovine serum (FBS; Cambrex), 4 mM L-glutamine (Cambrex), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (both from Invitrogen, Carlsbad, CA) to passage four to seven. In any particular experiment, cells for the control and experimental conditions were at the same passage number. For all experiments, the hMSCs were maintained in osteogenic supplemented (OS) medium (serum-containing medium supplemented with 50  $\mu$ g/ml ascorbic acid, 10 mM  $\beta$ -glycerophosphate, and 0.1  $\mu$ M dexamethasone (all from Sigma-Aldrich, St. Louis, MO)).

### 2.2. Application of mechanical strain to cultured cells

Cells were plated on six-well culture dishes with flexible silicone rubber bottoms (Flexcell, Hillsborough, NC). The plates were pre-coated with 1  $\mu$ g/cm<sup>2</sup> type I collagen by adsorption in a carbonate/bicarbonate buffer (15 mM Na<sub>2</sub>CO<sub>3</sub> and 35 mM NaHCO<sub>3</sub> in PBS, pH 9.4). Cells were seeded at various densities (see below) in the center of the wells (~5 cm<sup>2</sup> area) using Teflon o-rings as physical barriers (Cunningham et al., 2002). The cells were allowed to attach and spread for ~24 h, after which the o-rings were removed, the OS medium was added, and the cells were immediately subjected to mechanical stimulation. Using a modified Flexercell system (FX-2000, Flexcell), a spatially uniform 3% equibiaxial cyclic strain was applied to the cells continuously at 0.25 Hz (2 s on, 2 s off) for the duration of the experiment, with OS media changes every 3 days. Unstrained (control) plates were maintained identically except for the application of strain.

### 2.3. Proliferation studies

To assess the effect of mechanical strain on cell proliferation, the hMSCs were plated at an initial density of 1000 cells/cm<sup>2</sup> to ensure cell growth would not be contact inhibited and differentiation would not occur (Jaiswal et al., 1997). Net proliferation was determined at specified time points by harvesting adherent cells with 0.05% trypsin/0.02% EDTA (Invitrogen) and counting using a Coulter Counter.

### 2.4. Osteogenic differentiation studies

To assess the effect of mechanical strain on the osteogenic differentiation of hMSCs, cells were plated with an initial density of 10,000 cells/cm<sup>2</sup> and strained for up to 16 days while maintained in OS medium. Alkaline phosphatase (ALP) activity, an early but non-specific marker of osteogenesis, and mineral deposition, a late differentiation marker, were assessed qualitatively by histochemical staining. Cells were fixed in 70% ethanol and stained for ALP activity using Sigma Kit #85 or for mineral deposition by the von Kossa method (Halvorsen et al., 2001). To assess differentiation to other lineages, certain wells were stained for evidence of chondrogenesis with Safranin (0.1% Safranin in 0.1% acetic acid) or of adipogenesis with Oil Red O (0.3% Oil Red O in isopropanol).

To measure ALP activity quantitatively, cell layers were washed twice with PBS and then harvested using a passive lysis buffer (Promega, Madison, WI). The lysate was sonicated and centrifuged. The supernatant was assayed for ALP activity by incubating with 50 mM *p*-nitrophenyl phosphate in an assay buffer (100 mM glycine, 1 mM MgCl<sub>2</sub>, pH 10.5) at 37°C for 10–20 min. The absorbance was measured at 405 nm and converted to *p*-nitrophenol concentration based on standard solutions prepared in parallel. To determine the amount of DNA in each well, the cell nuclei were disrupted by addition of a lysis buffer (0.025 M *tris*HCl, 0.4 M NaCl, 0.5% SDS, pH=7.4), followed by sonication and centrifugation. The DNA content was determined from the supernatant using the Hoescht 33258 assay (Cesarone et al., 1979) and standard solutions of calf thymus DNA prepared in parallel.

To measure matrix mineralization quantitatively, the cell layers were rinsed twice with calcium-free PBS and the accumulated matrix-deposited calcium was solubilized in 0.6 N HCl overnight at 4°C. After centrifugation, the supernatant was assayed for calcium content using the *o*-cresolphthalein reaction (Sigma Kit #587), with the absorbance read at 575 nm. Total calcium in each well was determined based on standard solutions prepared in parallel.

### 2.5. MAPK activation studies

Activation of MAPK pathways by mechanical strain was assessed by immunoblotting analysis. Consistent with the differentiation experiments, hMSCs were seeded at a density of 10,000 cells/cm<sup>2</sup> and allowed to adhere for 24 h in serum-containing medium. To synchronize the cells and reduce basal MAPK activity, the cells were then maintained for 48 h in basal medium containing 0.5% FBS, and finally serum-starved for 4 h prior to and during the application of strain. The hMSCs were strained for periods of 8, 15, 30, or 60 min, as strain-induced activation of MAPK pathways has been observed on these time scales in osteoblastic cells (Ziros et al., 2002). After the cells were strained, they were washed once with ice cold PBS and incubated on ice in cell lysis buffer (Cell Signaling Technology, Beverly, MA). The DNA content in the sonicated lysates was measured using the Hoescht 33258 assay, and the results confirmed that there were equal numbers of cells in each sample. Lysate supernatants were retained for selective immunoprecipitation and chemiluminescent detection of ERK1/2, p38 MAPK (p38), or c-Jun *N*-terminal kinase (JNK) activity using MAPK Activity Kits (Cell Signaling Technology) according to the manufacturer's protocols. In some cases, we used anti-phospho-JNK antibody in Western blots to examine the presence of phospho-JNK in the lysates directly (i.e., without selective pulldown).

### 2.6. MAPK inhibition studies

The role of individual MAPK pathways in the response of hMSCs to strain was assessed by blocking the pathways using specific inhibitors. The ERK1/2 pathway was blocked with U0126 (Cell Signaling), an MEK1/2 inhibitor (Favata et al., 1998). The p38 MAPK was specifically inhibited with SB203580 (Calbiochem, San Diego, CA) (Cuenda et al., 1995). We found that JNK was not phosphorylated or activated by the mechanical stimulus applied (see Section 3), and therefore JNK inhibition experiments were not pursued. Preliminary experiments showed that the optimal concentrations for inhibition of MEK1/2 and p38 without cytotoxicity were 10 μM U0126 and 15 μM SB203580, respectively. To assess the effects of the inhibitors on MAPK activity, inhibitors were added to the serum-free cell culture media 1 h prior to application of the strain stimulus, the cells were then strained for 15 min, and the lysates were collected for immunoblot analysis.

To determine the effects of MAPK pathway inhibition on osteogenic differentiation, differentiation experiments were performed as described above, but in the presence of the MAPK inhibitors. In contrast to the immunoblotting experiments in which serum-free media

were used to reduce basal MAPK activity, OS media were used in these experiments to promote differentiation. At the conclusion of the experiments, ALP activity and matrix calcium deposition were measured to assess osteogenic differentiation.

### 3. Results

We first examined the effect of mechanical strain on the proliferation of hMSCs cultured in OS media. While both control (unstrained) and strained cells proliferated in an exponential manner, the strain stimulus slowed the rate of proliferation (Fig. 1A), resulting in significant inhibition of net proliferation over a 10 day period ( $P < 0.02$ ; Fig. 1B). This effect appeared to be due to inhibition of proliferation, as cell counts from the aspirated media were similar for the control and strained conditions (data not shown), indicating that the differences in net proliferation were not due to greater cell detachment from the strained substrate.

The effect of mechanical strain on osteogenic differentiation was assessed by examining ALP activity and mineral deposition. The ALP levels expressed by the hMSCs increased with time cultured in the OS medium (Figs. 2A–D), but the application of strain did not have a detectable effect on ALP activity at the time points examined (Figs. 2A–E). Application of strain did not induce differentiation to the chondrogenic and adipogenic lineages (assessed histochemically; data not shown). Straining the hMSCs did result, however, in greater matrix mineral deposition (Fig. 3A and B), with a significant  $2.3 \pm 1.0$  (SD) fold increase in matrix-deposited calcium with strain after 16 days in culture ( $P < 0.05$ ; Fig. 3C).

We next examined the involvement of MAPK pathways in mechanically regulated mineralization by hMSCs. With the application of strain, both ERK1/2 and p38 activity peaked after 15 min of strain, whereas there was no detectable JNK activity in response to strain (Fig. 4A). To confirm this result was due to low JNK activity and not an inability to detect active JNK, we also measured JNK phosphorylation in response to treatment with anisomycin, a JNK activator (Cano et al., 1994), and in response to strain. Treatment for 30 min with  $25 \mu\text{g/ml}$  anisomycin resulted in JNK phosphorylation, whereas no phosphorylation was evident with application of strain for 15 min (Fig. 4B), consistent with the JNK activity assay. Treatment of the cells 1 h prior to strain application with  $10 \mu\text{M}$  U0126 or  $15 \mu\text{M}$  SB203580 inhibited the strain-induced activity of ERK1/2 (Fig. 4C) and p38 (Fig. 4D), respectively.

When ERK1/2 activity was blocked by U0126, we observed a significant decrease in the expression of osteogenic markers by unstrained cells after 16 days in culture, with both decreased ALP activity ( $-55 \pm 21\%$ ;  $P < 0.001$ ) (Fig. 5A) and decreased mineral production ( $-31 \pm 18\%$ ; NS) (Fig. 5B). Furthermore, with ERK1/2 inhibited there were no statistically significant differences in ALP activity (Fig. 5A) or mineralization (Fig. 5B) between control and strained cells. The degree of mineralization with strain in ERK 1/2 inhibited cells was significantly attenuated from that seen in strained cells without MAPK inhibition ( $-55 \pm 27\%$ ;  $P < 0.05$ ; Fig. 5B), further demonstrating the involvement of ERK1/2 signaling in strain-induced mineralization.

Blocking p38 activity with SB203580 in the absence of strain had little effect on the deposition of matrix calcium (Fig. 5B), but did result in a  $2.1 \pm 0.5$  fold increase in ALP activity, compared with uninhibited control cells ( $P < 0.01$ ; Fig. 5A). When the cells were

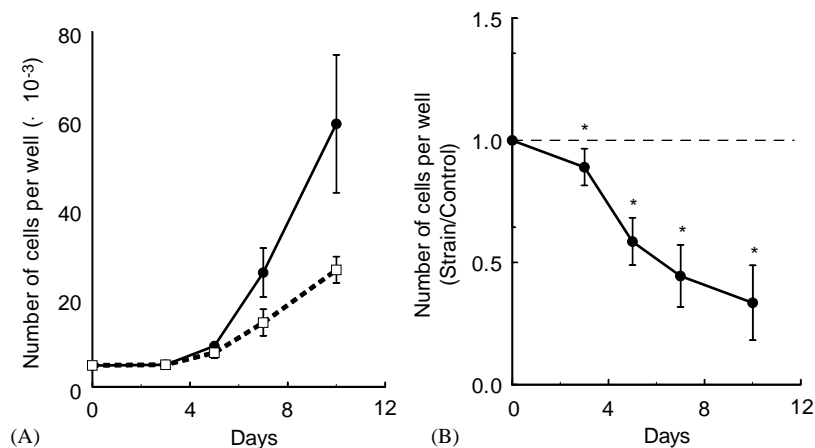


Fig. 1. Mechanical strain inhibits the growth of hMSCs. (A) Unstrained cells ( $\bullet$ , solid line) proliferated at a greater rate than did cells subjected to strain ( $\square$ , dotted line). Data represent means  $\pm$  SD of a typical experiment with  $n = 3$  samples for each condition. (B) Cell counts of strained cells normalized to unstrained cells demonstrated significant inhibition of proliferation of hMSCs when subjected to strain. Data represent means  $\pm$  SD of pooled data from two independent experiments in which there were three samples for each condition.  $*P < 0.02$  vs. unstrained control.

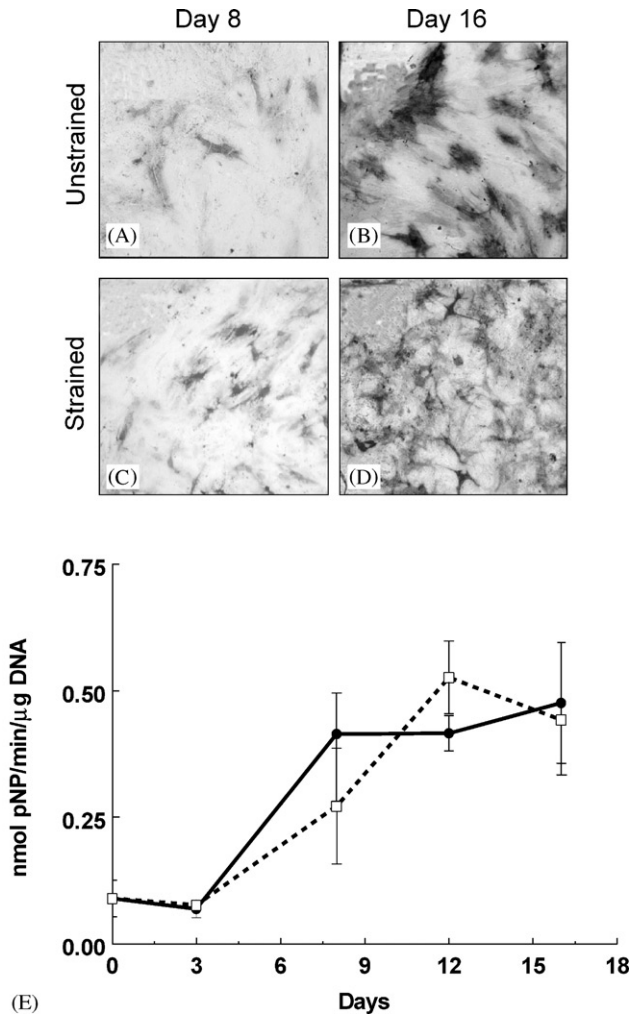


Fig. 2. ALP activity increased with time in culture, but was not detectably affected by application of strain. Histochemical staining of ALP activity demonstrated an increase in activity with time in OS culture for both unstrained (A vs. B) and strained (C vs. D) cells, but no difference between unstrained and strained cells at day 8 (A vs. C) or day 16 (B vs. D). Original magnification 100X. (E) Quantitative measurement of ALP activity confirmed there were no differences in activity between unstrained (●, solid line) and strained cells (□, dotted line) measured at various time points throughout the duration of the experiment. Data represent means  $\pm$  SD of a typical experiment with  $n = 3$  samples for each condition. An additional independent experiment showed similar results.

strained with the p38 pathway inhibited, there was a significant  $81 \pm 46\%$  decrease in ALP activity compared to p38-inhibited control cells ( $P = 0.02$ ; Fig. 5A) coupled with a significant  $4.6 \pm 2.4$  fold increase in calcium deposition ( $P = 0.02$ ; Fig. 5B). These findings suggest the SB203580-treated strained cells were in a more differentiated state, as indicated by higher expression of later markers with decreased expression of early markers. Additionally, relative to the uninhibited strained cells, the p38-inhibited strained cells had decreased ALP activity ( $-62 \pm 35\%$ ;  $P < 0.02$ ; Fig. 5A) but greater mineralization ( $1.4 \pm 0.7$  fold; NS; Fig. 5B),

again suggesting more differentiated cells with p38 inhibited.

#### 4. Discussion

We demonstrated that hMSCs, cultured in osteogenic medium and stretched by cyclic deformation of the cell substrate, inhibited their growth and increased their production of mineralized matrix in response to the mechanical stimulus. This strain-induced response was largely mediated by ERK1/2 signaling. The p38 MAPK pathway was also activated by strain and its inhibition resulted in a more mature osteogenic phenotype, suggesting an inhibitory role for the p38 pathway in the modulation of strain-induced osteogenic differentiation. These novel results demonstrate the potent effect of mechanical signals on mesenchymal stem cell function, suggesting a mechanism by which mechanical forces might regulate developmental, regenerative, and disease processes in mesenchymal tissues.

Mechanically regulated mineralization in vitro has been demonstrated with mature osteoblasts (Wozniak et al., 2000) and with stromal cells isolated from bone marrow aspirates by selecting cells that were adherent and formed colonies in culture (Keila et al., 1994; Wang et al., 2002; Zhang et al., 1995). This isolation method, pioneered by Friedenstein et al. (1970), results in a heterogeneous cell population (Friedenstein et al., 1982; Phinney et al., 1999) with significant possibility of “contamination” by committed cells, thus making it difficult to identify whether the strain-responsive cells were fully differentiated cells, committed progenitors, or undifferentiated stem cells. We used a purified post-natal stem cell population selected by density centrifugation (Pittenger et al., 1999) to isolate the response of primary mesenchymal stem cells to mechanical stimulation. To our knowledge, this is the first demonstration that this specific cell population is responsive to strain, with functional consequences in terms of matrix mineralization.

The mechanical stimulus we applied to the hMSCs was 0.25 Hz, 3% equibiaxial cyclic strain generated by deformation of the cell substrate. Spatially uniform substrate deformation was achieved by using a modified Flexcell system and confining cell seeding to the center of the wells, thus eliminating the variability typically observed in other systems (Brown, 2000). Because the membranes were deformed minimally at low frequency, it is unlikely that reactive fluid stresses would stimulate the cells in our system significantly (Brown et al., 1998). Thus the primary stimulus was matrix deformation, likely transduced to the cell by surface receptors, such as integrins (Ingber, 1991; Shyy and Chien, 1997). The strain magnitude may be critical to the specific response of cells to this stimulus, as mature osteoblasts have been

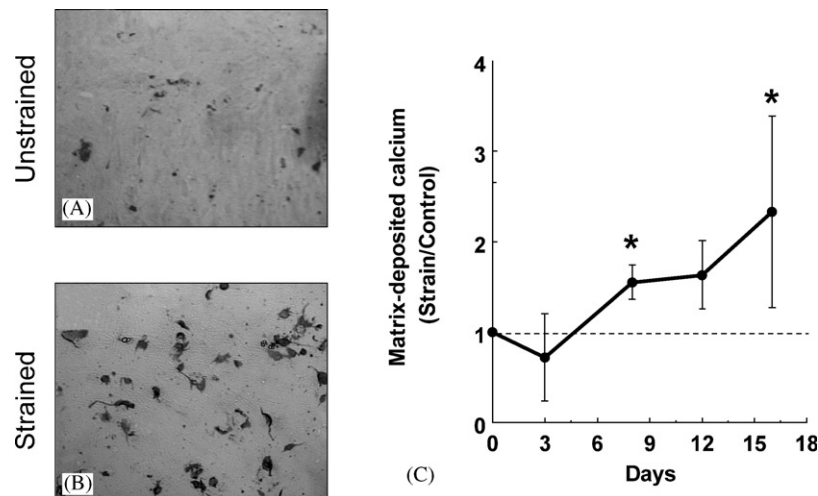


Fig. 3. Mechanical strain induced greater matrix mineralization by hMSCs. Von Kossa staining of cell layers after 16 days in culture demonstrated less evidence of matrix mineralization in the unstrained condition (A) than in the strained condition (B). Original magnification 100X. (C) Measurement of matrix-deposited calcium by strained cells (normalized to calcium deposition by unstrained, control cells) confirmed that there was significantly more mineral formed by the strained cells after 16 days. Data represent means  $\pm$  SD of pooled data from two experiments in which there were three samples for each condition. \* $P < 0.05$  vs. unstrained control.

reported to up-regulate proliferation and down-regulate differentiation in response to 1% uniaxial strain (Kaspar et al., 2000). However, it is unclear whether these effects, which differ from those we obtained, are due to the different cell types, mechanical stimulus, or both. Due to the rigidity of mineralized bone tissue, maximum continuum-level matrix deformations in situ are approximately an order of magnitude lower than that applied in the current study ( $\sim 3000 \mu\text{strain}$ ) (Burr et al., 1996). Thus, the strain magnitudes applied in the current study are supra-physiological for mature bone cells (i.e., osteocytes and osteoblasts) that are embedded in or adherent to mineralized substrates. However, the putative role of MSCs in bone regeneration implies these cells reside in collagenous matrices that are significantly more compliant than mineralized bone tissue. Therefore, the magnitude of physiologically relevant strains for MSCs may be greater than that for mature bone cells, and the stimulus we applied in the current study was selected accordingly. Our results are consistent with computational models of fracture healing (Claes and Heigele, 1999) and of bone formation around implants (Simmons et al., 2001) that predict tissue strains as high as 5% are permissive for intramembranous bone formation. Although the cellular-level functional loads experienced by MSCs in the stroma and at regenerative sites require further investigation, for therapeutic applications such as bone tissue engineering, the identification of a beneficial stimulus, regardless of its nature, is an important finding.

The substrate deformation stimulus we applied activated the ERK1/2 pathway in the hMSCs. This novel finding is consistent with observations in osteo-

blasts, in which ERK1/2 is activated in response to various mechanical stimuli, including substrate deformation (Granet et al., 2001, 2002; Kletsas et al., 2002; Ziros et al., 2002), forces applied directly to integrins (Pommerenke et al., 2002; Schmidt et al., 1998), high energy shock waves (Wang et al., 2001, 2002), and fluid flow (Jessop et al., 2002; Weys et al., 2002; You et al., 2001). The activation of ERK1/2 enhanced mineralization by the hMSCs, a functional consequence that was largely inhibited with the addition of U0126. We did not observe an effect of strain alone on ALP activity, suggesting that strain-enhanced mineralization was not mediated through stimulation of ALP. The molecular target of ERK1/2 activation in hMSCs and its link to mineralization have not been reported. One possibility is that, similar to osteoblastic cells, physical stimulation leads to increased expression and DNA binding activity of core-binding factor A1 (Cbfa1), a transcription factor that is the “master” regulator of osteoblast differentiation (Ducy et al., 1997), by phosphorylation of Cbfa1 by ERK1/2 (Wang et al., 2002; Ziros et al., 2002). The induction of osteoblast differentiation by soluble signals (Gallea et al., 2001; Jaiswal et al., 2000; Lai et al., 2001; Xiao et al., 2002) and cell–matrix interactions (Lai et al., 2001; Takeuchi et al., 1997; Xiao et al., 2000, 2002) also involves ERK1/2 activation, indicating that the ERK1/2 pathway may be not only a common mediator for a variety of mechanical stimuli in MSCs and bone cells, but also a common mediator of mechanical and non-mechanical signals.

In addition to ERK1/2 activation, the matrix deformation stimulus activated p38 MAPK. This pathway has been shown previously to be activated during

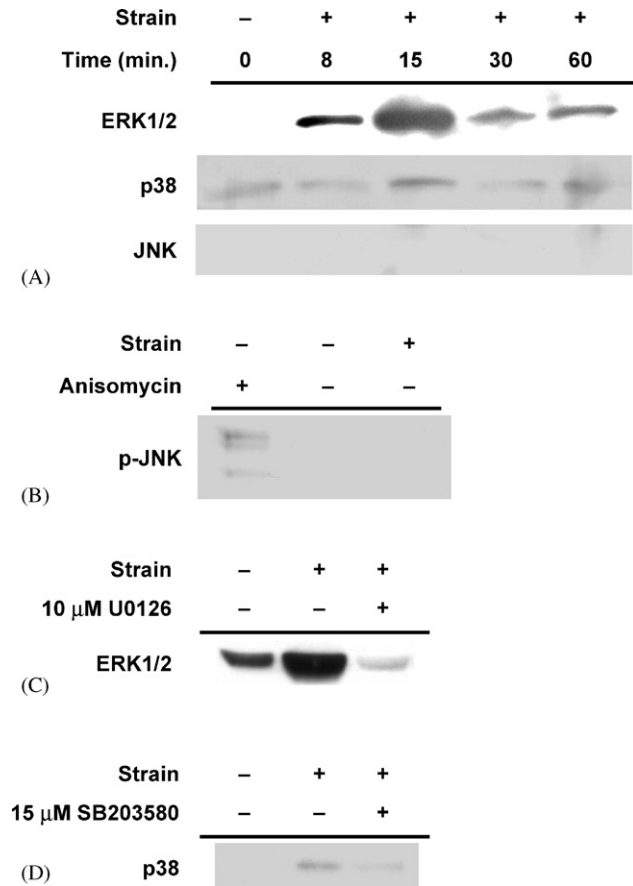


Fig. 4. ERK1/2 and p38, but not JNK, are activated by mechanical strain in a time-dependent manner and can be inhibited. hMSCs were strained and, at the times indicated, cell lysates were collected, and the activity of ERK1/2 (Elk-1 phosphorylation), p38 (ATF-2 phosphorylation), and JNK (c-Jun phosphorylation) were analyzed. (A) Activity of both ERK1/2 and p38 increased with up to 15 min of strain application, and then decreased with longer periods of strain application, whereas JNK activity was not induced by the strain stimulus. (B) Phosphorylated JNK was detectable in anisomycin-treated hMSCs, but not in strained cells, thus supporting the results of the activity assay. The activity of ERK1/2 (C) and p38 (D) after 15 min of strain application were inhibited by 10 μM U0126 and by 15 μM SB203580, respectively. These concentrations were determined in preliminary experiments to be optimal for inhibiting kinase activity and minimizing cytotoxicity. While baseline activity of ERK1/2 in (A) and of p38 in (D) is not apparent in the figure, low intensity bands were visible for these conditions on the original blots.

osteogenic differentiation of hMSCs induced by OS media (Jaiswal et al., 2000). Inhibition of the p38 pathway increased ALP activity in unstrained cells and increased expression of late osteogenic markers in strained p38-inhibited cells. Together these results suggest that in contrast to the positive osteogenic effect of ERK1/2 activation, the p38 pathway plays an inhibitory role in MSC osteogenic differentiation. A similar inhibitory role for p38 signaling in osteoblast differentiation was observed with multipotent mouse C2C12 cells in response to induction by BMP-2 (Vinals

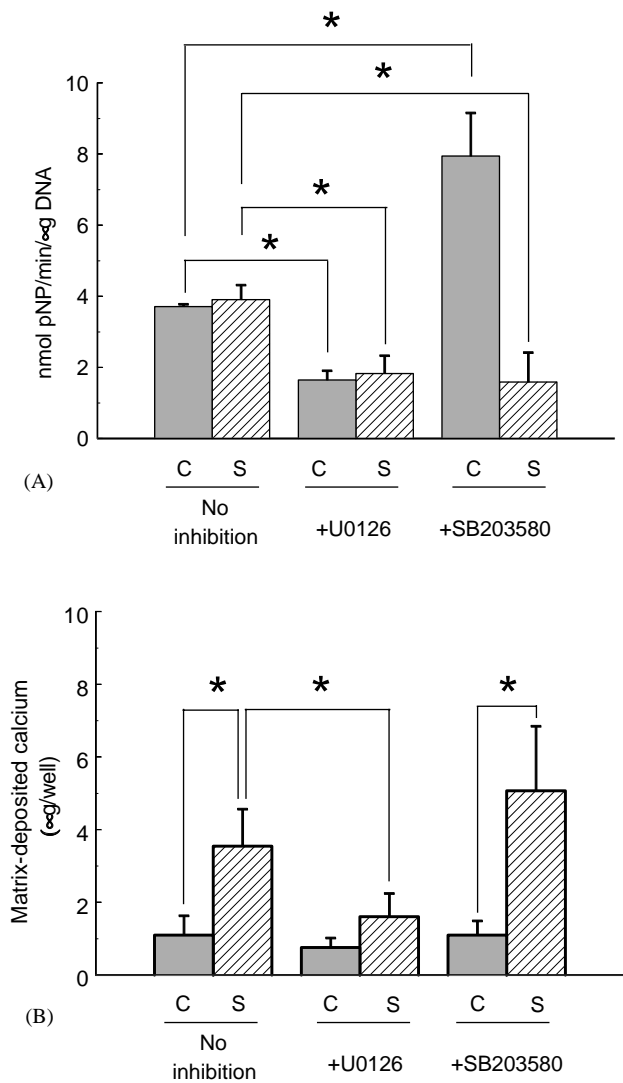


Fig. 5. Mechanical strain regulates mineralization by hMSCs through MAPK pathways. hMSCs were cultured in OS media with or without the MAPK inhibitors and with application of strain (S; hatched bars) or without application of strain (C; shaded bars) for 16 days. (A) ALP activity of hMSCs after 16 days in culture. There was no difference in ALP activity between uninhibited control and strained cells. Blocking the ERK1/2 pathway with 10 μM U0126 inhibited ALP activity in the absence or presence of strain. Blocking p38 with 15 μM SB203580 resulted in elevated ALP activity in unstrained cells, but reduced activity in strained cells, relative to both uninhibited and p38-inhibited unstrained cells. Data represent means ± SD of a single experiment with  $n = 3$  samples for each condition. \* $P < 0.02$ . (B) Matrix-deposited calcium after 16 days in culture. Application of strain increased mineral production in the absence of MAPK inhibitors. Blocking the ERK1/2 pathway with 10 μM U0126 inhibited mineral production in the absence of strain, and attenuated the strain-induced mineralization. Blocking p38 with 15 μM SB203580 had no effect on the mineral produced by unstrained cells. However, with p38 inhibited, application of strain resulted in a significant increase in deposited calcium, relative to unstrained cells, and an increase, although not significant, relative to strained, uninhibited cells. Data represent means ± SD of a single experiment with  $n = 3$  samples for each condition. \* $P < 0.05$ .

et al., 2002), suggesting similarities in signaling between different types of epigenetic stimuli.

In conclusion, we have shown that mechanical strain mediated by matrix deformation stimulates mineralization by hMSCs through MAPK signaling. The ability of mechanical signals to regulate bone formation by MSCs could potentially be exploited in bone tissue engineering through application of external loading, as has been attempted with other cells and tissues (Altman et al., 2002; Bancroft et al., 2002; Carver and Heath, 1999; Guilak, 2001, 2002; Kim et al., 1999; Niklason et al., 1999), or by appropriate matrix design (Simmons and Mooney, 2003). More generally, our results suggest that the MSC subpopulation of the stroma and the mechanical signals to which it is subjected might play critical roles in the development and regeneration of mesenchymal tissues, and in diseases in which there are deficiencies in the regulation of bone formation.

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