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Mechanical Stress-Mediated Runx2 Activation is Dependent on Ras/ERK1/2 MAPK Signaling in Osteoblasts

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Abstract The sequence of biochemical events involved in mechanical stress-induced signaling in osteoblastic cells remains unclear. Runx2, a transcription factor involved in the control of osteoblast differentiation, has been identified as a target of mechanical stress-induced signaling in osteoblastic cells. In this study, uniaxial sinusoidal stretching (15% strain, 115% peak-to-peak, at 1/12 Hz) stimulated the differentiation of osteoblast-like MC3T3-E1 cells and rat primary osteoblastic cells by activating Runx2. We examined the involvement of diverse mitogen-activated protein kinase (MAPK) pathways in the activation of Runx2 during mechanical stress. Mechanical stress increased alkaline phosphatase activity, a marker of osteoblast differentiation, increased the expression of the osteoblast-specific extracellular matrix (ECM) protein osteocalcin, and induced Runx2 activation, along with increased osterix expression. Furthermore, activation of ERK1/2 and p38 MAPKs increased significantly. U0126, a selective inhibitor of ERK1/2, completely blocked Runx2 activation during periods of mechanical stress, but the p38 MAPK-selective inhibitor SB203580 did not alter nuclear phosphorylation of Runx2. Small interfering RNA (siRNA) targeting Rous sarcoma kinase (RAS), an upstream regulator of both ERK1/2 and p38 MAPKs, inhibited stretch-induced ERK1/2 activation, but not mechanically induced p38 MAPK activity. Furthermore, mechanically induced Runx2 activation was inhibited by Ras depletion, using siRNA. These findings indicate that mechanical stress regulates Runx2 activation and favors osteoblast differentiation through the activation of MAPK signal transduction pathways and Ras/Raf-dependent ERK1/2 activation, independent of p38 MAPK signaling. J. Cell. Biochem. 101: 1266–1277, 2007.

Key words: mechanical stress; mitogen-activated protein kinase (MAPK) pathway; osteoblast; Runx2; signal transduction

Mechanical stress plays a fundamental role in the regulation of bone homeostasis and in skeletal morphology during development [Oxlund et al., 1998; Franceschi and Xiao, 2003; Lian et al., 2004; Liedert et al., 2006a]; a reduction in mechanical stress results in a loss of bone mass through the inhibition of bone formation and an increase in bone resorption [Wang et al., 2002; Ontiveros and McCabe, 2003; Meyers et al., 2004]. Mechanical stresses stretch the surfaces of osteoblastic cells, generating biological signals, which include phenomena as diverse as lipid bilayer stretching, Ca^{2+} channel activation, and ECM-integrin-cytoskeleton signaling [Ziros et al., 2002; Yang et al., 2004; Liedert et al., 2006a]. However, the precise signal transduction pathway that links mechanical stress with the regulation of gene expression has not been determined. Moreover, the nature of the cellular-level mechanical signal may be dependent on the kind of stress applied, such as mechanical stretching, fluid-induced shear stress, or compressive stress [Franceschi and Xiao, 2003; Lian et al., 2004; Tang et al., 2006].

Currently, osteocytes are believed to be the primary mechanosensory cells in bone [Kapur

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et al., 2004; Mullender et al., 2004; Liedert et al., 2006a], and osteoblastic cells also play a central role in the response of bone to mechanical stimuli [Ziros et al., 2002; Costessi et al., 2005; Liedert et al., 2006a]. Although the benefits of mechanical stress have been demonstrated in a clinical setting, experiments have only begun to examine the underlying molecular mechanism in bone that transduces mechanical stress into a cellular response [Franceschi et al., 2003; Liedert et al., 2006a].

In a previous study, we showed that uniaxial sinusoidal stretching initiated the differentiation of preosteoblastic cells into osteogenic cells by inducing Runx2 expression in vitro, suggesting a possible clinical application for this event in bone regenerative therapy, such as distraction osteogenesis [Kanno et al., 2005a]. From a clinical point of view, distraction histogenesis is a recent regenerative technique. It requires application of a large, continuous mechanical stretching force on the osteotomized distraction gaps, consisting of newly promoted osteogenic and osteoblastic cells, using rigid distraction devices, to promote better bone regeneration [Ryoyama et al., 2004; Kanno et al., 2005a; Singare et al., 2006].

Runx2, a Runt domain transcription factor family member, is a master regulator of osteoblast differentiation that binds to osteoblast-specific *cis*-acting element 2 (OSE2) sites, found in the promoter regions of all major osteoblast-specific genes, including osteocalcin (OCN), osteopontin, type 1 collagen, and bone sialoprotein [Schinke and Karsenty, 1999; Komori, 2003; Lian et al., 2004]. Runx2 forms a heterodimeric complex with its partner cbf-β and binds to DNA directly, while cbf-β modulates the affinity of the complex for DNA [Schinke and Karsenty, 1999; Mengshol et al., 2001; Lian et al., 2004]. In addition, osterix (Osx) has been identified as a key transcription factor in osteoblast differentiation, which appears to be activated via a Runx2-independent pathway [Nakashima et al., 2002; Jadlowiec et al., 2004; Rubin et al., 2006]. Both of these transcriptional regulators, Runx2 and Osx, seem to be involved early in the cellular response in mechanically strained osteoblastic cells [Fan et al., 2006; Salingcarnboriboon et al., 2006].

In developmental regulation, Runx2 acts in many diverse signaling pathways [Liedert et al., 2006a]. Runx2 activation may occur via several routes: through the interaction between the extracellular matrix (ECM) and cell surface integrins; through BMPs; via the mitogen-activated protein kinase (MAPK), FGF-2, and PTH signaling pathways; and as a result of ATP modulation of intracellular Ca^{2+} mobilization by multiple P2 receptors [Costessi et al., 2005; Rubin et al., 2006; Liedert et al., 2006a]. MAPK pathways play important regulatory roles during early osteoblast differentiation in response to mechanical stress, environmental stress, and growth factors [Furthauer et al., 2002; Franceschi and Xiao, 2003; Meyers et al., 2004; Fan et al., 2006].

The role of MAPK signaling in the differentiation of osteoblasts has been disputed, although several MAPKs, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (p38), have been shown to favor osteoblastic cell differentiation [Xiao et al., 2000; Sowa et al., 2002; Ziros et al., 2002]. In particular, ERK1/2 is involved in cell transformation, proliferation, and survival, whereas p38 participates in many cellular process, such as cell cycle control, inflammatory responses, apoptosis, and the differentiation of several cell types, including osteoblasts [Katz et al., 2006]. Recently, an ERK1/2-dependent pathway was reported to be responsible for Runx2 activation in mechanically loaded osteoblastic cells, similar to our previous study [Meyers et al., 2004; Kanno et al., 2005a; Liedert et al., 2006a].

The upstream target of the MAPK signal transduction pathways involved in osteoblastic cell differentiation and function was recently elucidated [Wang et al., 2001; Boutahar et al., 2004; Jadlowiec et al., 2004; Costessi et al., 2005; Fan et al., 2006]. Specifically, the common target of MAPKs induced by mechanical stress during osteoblast differentiation and function is believed to be the ERK pathway, which is mediated by ATP-dependent Ca^{2+} influx at calcium channels via protein kinase C (PKC), Src family kinase, and protein kinase A signaling (PKA), and via phosphorylation of focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (PYK2). These far-upstream MAPK mediators are in turn activated by mechanoreceptors, such as integrins, cadherins, and stretch-activated Ca^{2+} channels [Wang et al., 2001, 2002; Franceschi and Xiao, 2003; Fan et al., 2006; Katz et al., 2006; Liedert et al., 2006a].

These observations, combined with our present data, indicate that MAPK pathways contribute to the activation of Runx2 [Jadlowiec et al., 2004; Costessi et al., 2005; Fan et al., 2006]. Using two osteoblastic cell lines, we thus examined the effects of mechanical stress on the expression of critical osteogenic transcription factors and interactions between these key regulators. We also examined the downstream molecules involved in Rous sarcoma kinase (Ras) signaling and the downregulation of mechanical stress-mediated MAPK pathways, associated with the induction of osteogenic osteoblastic differentiation [Boutahar et al., 2004; Liedert et al., 2006a]. Ras signaling has attracted attention because it may represent a target in MAPK pathways that may be involved in the induction of osteoblastic cell responses [Meyers et al., 2004; Chang et al., 2005; Zreiqat et al., 2005; Liedert et al., 2006a]. Our experimental results suggest a molecular link between mechanical stress and osteoblastic cell responses, which may have clinical relevance in bone regenerative therapy.

MATERIALS AND METHODS

Cell Culture

The murine pre-osteoblastic cell line MC3T3-E1 and rat primary osteoblast-like cells were cultured in alpha-minimum essential medium (α-MEM; Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS; Gibco), supplemented with penicillin G (100 U/ml) and streptomycin (100 μg/ml). Rat primary osteoblast-like cells were isolated from three-day-old Wistar rat calvarias as described previously, and were used in the experiments after the third passage [Hu et al., 2003]. All cells were maintained at 37°C in 5% CO_2.

Mechanical Stretching of Cultured Osteoblastic Cells and Preparation of Cell Extracts

Approximately 5×10^5 cells were seeded into 10 cm^2 fibronectin-coated silicone chambers and cultured as previously described [Ziros et al., 2002; Iwasaki et al., 2004]. The medium was then changed to α-MEM supplemented with 0.1% FCS to maintain quiescence [Ziros et al., 2002]. After 24 h, the chambers were attached to a stretching apparatus that was used to apply a uniaxial sinusoidal stretch (15% strain, 115% peak-to-peak, at 1/12 Hz), for the times indicated, as described previously [Iwasaki et al., 2004; Kanno et al., 2005a]. Control (unstretched) cultures were incubated under the same conditions for the maximum stretching period. Immediately after the stretching was completed, whole cell extracts or nuclear lysates were prepared as previously described [Wang et al., 2002; Ziros et al., 2002]. Nuclear extracts were prepared using a Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA). Total cell extracts were prepared in SDS sample buffer, as described elsewhere [Ariyoshi et al., 2005].

Alkaline Phosphatase (ALP) Activity

ALP activity of the cell lysate was measured using *p*-nitrophenylphosphate (pNPP), as described previously [Wang et al., 2002; Kanno et al., 2005b].

RT-PCR

Total RNA was extracted using the Total RNA Extraction Miniprep System (Viogene Co., Sunnyvale, CA). DNase-treated total RNA was first reverse transcribed using oligo-dT primers. PCR reactions were performed as follows: 9 min at 95°C, followed by 30–38 cycles of 40 s at 94°C, 40 s at the gene-specific annealing temperature, and 9 min at 72°C. RT-PCR reactions were carried out according to standard procedures using the following primer sets for osteocalcin, Runx2, and Osx: OCN: 5′-CAGACCTAGCAGACACCATGAG-3′ and 5′-CGTCCATACTTTCGAGGCAG-3′; Runx2: 5′-CCAGATGGGACTGTGGTTACC-3′ and 5′-ACTTGGTGCAGAGTTCAGGG-3′; and Osx: 5′-CTGGGGAAAGGAGGCACAAAGAAG-3′ and 5′-GGGTTAAGGGGAGCAAAGTCAGAT-3′. Total RNA isolated from cells treated with 50 ng/ml bone morphogenetic protein (BMP)-2, kindly provided by Seikagaku Kogyo Corporation, Tokyo, Japan, was included as a positive control. The PCR products were electrophoresed in 2% agarose gels and visualized with ethidium bromide. The results were analyzed using the NIH Image 1.61 Software (Scion Inc., Frederick, MD).

Western Blotting and Immunoprecipitation

An equal amount of protein extract per sample was subjected to SDS–PAGE and transferred to polyvinylidene fluoride membranes (Millipore Co., Bedford, MA), as previously described [Ariyoshi et al., 2005].

Immunoprecipitation was performed as previously described [Wang et al., 2001, 2002]. Briefly, the nuclear extracts were incubated with anti-Runx2 antibody, after which the immune complexes were precipitated with protein A-agarose beads (Sigma Chemical Co., St. Louis, MO). The beads were then washed and subjected to SDS–PAGE and immunoblotting. The total Runx2 on the blot was recognized by a rabbit anti-Runx2 antibody, followed by goat anti-rabbit horseradish peroxidase-conjugated IgG as the second antibody. The phosphorylated Runx2 on the blot was further recognized by a specific mouse anti-phosphotyrosine antibody, followed by goat anti-mouse horseradish peroxidase-conjugated IgG as the second antibody. Antibodies for immunoblotting included anti-Runx2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphotyrosine (Oncogene, Boston, MA), anti-c-Jun, anti-phospho-c-Jun, anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38 (all from Cell Signaling, Beverly, MA), horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham Pharmacia Biotech, Uppsala, Sweden), and horseradish peroxidase-conjugated anti-goat IgG secondary antibody (Chemicon International Inc., Temecula, CA). Bands were detected using the ECL chemiluminescence detection method (Amersham Pharmacia Biotech). The ERK1/2 (MEK1/2)-selective inhibitor, U0126, and the p38 (MEK3/6)-selective inhibitor, SB203580, were purchased from Calbiochem (San Diego, CA). Nuclear extracts of cells treated with 50 ng/ml BMP-2 were used as a positive control.

Small Interfering (si)RNA Experiments

Ras small interfering RNA (siRNA) assays were performed using a commercially available Ras siRNA/siAb assay kit from Dharmacom (Upstate, Lake Placid, NY). MC3T3-E1 cells were grown to 50% confluence before transfection, according to the manufacturer's protocol. Cells were transfected with SMARTpool Ras and non-specific Control Pool (negative control) siRNA oligo mixtures in each chamber, using Lipofectamine (Invitrogen, San Diego, CA), according to the manufacturer's protocol. Transfection of Ras and non-specific control siRNA was confirmed using an anti-Ras antibody (clone RAS10), with non-stimulated A431 cell lysate as a positive control, according to the manufacturer's protocol. Seventy two hours after siRNA transfection, cells were subjected to mechanical stretching, according to the protocols described above.

Statistical Analysis

Significant differences were determined using Student's t-test. Data are expressed as the mean $\pm$ SEM.

RESULTS

Effect of Mechanical Stress on Osteoblast Differentiation

To examine the initiation of osteogenesis by mechanical stress, we assessed the induction of the osteoblast differentiation markers ALP and OCN at the protein and mRNA levels. Osteoblastic cells subjected to constant mechanical stress for 6 h showed the largest increase in ALP activity (Fig. 1A,B), and a time-course experiment showed that mechanical stress also upregulated ALP protein expression (data not shown). ALP activity was dramatically enhanced by 6 h of mechanical stress loading in a post-loading time course (Fig. 1A,B). MC3T3-E1 cells also exhibited a dramatic increase in OCN mRNA expression following 3 h of mechanical stress loading (Fig. 1C), while OCN mRNA expression increased in rat primary osteoblastic cells in a time-dependent manner (Fig. 1D).

Mechanical Stress Activates Runx2 Expression

We next examined whether mechanical stress induced the expression of the osteoblast-specific transcription factors Runx2 and Osx at the mRNA and/or protein levels. Runx2 and Osx mRNA levels were dramatically increased by mechanical stress loading (Fig. 2A,B,E,F). Furthermore, Western blotting using nuclear extracts revealed a slight increase in the amount of Runx2 protein after 3 h of mechanical stress (Fig. 2C,D).

Involvement of MAPK Pathways in Mechanically Stretched Osteoblastic Cells

Next, we examined stretch-mediated MAPK activation. Whole cell extracts were isolated from osteoblastic cells that had been subjected to mechanical stress for the duration of stretch loading, 0–90 min, and the presence of active ERK1/2, JNK, and p38, and their phosphorylated forms (p-ERK1/2, p-JNK, and p-p38, respectively) was monitored by Western blotting.

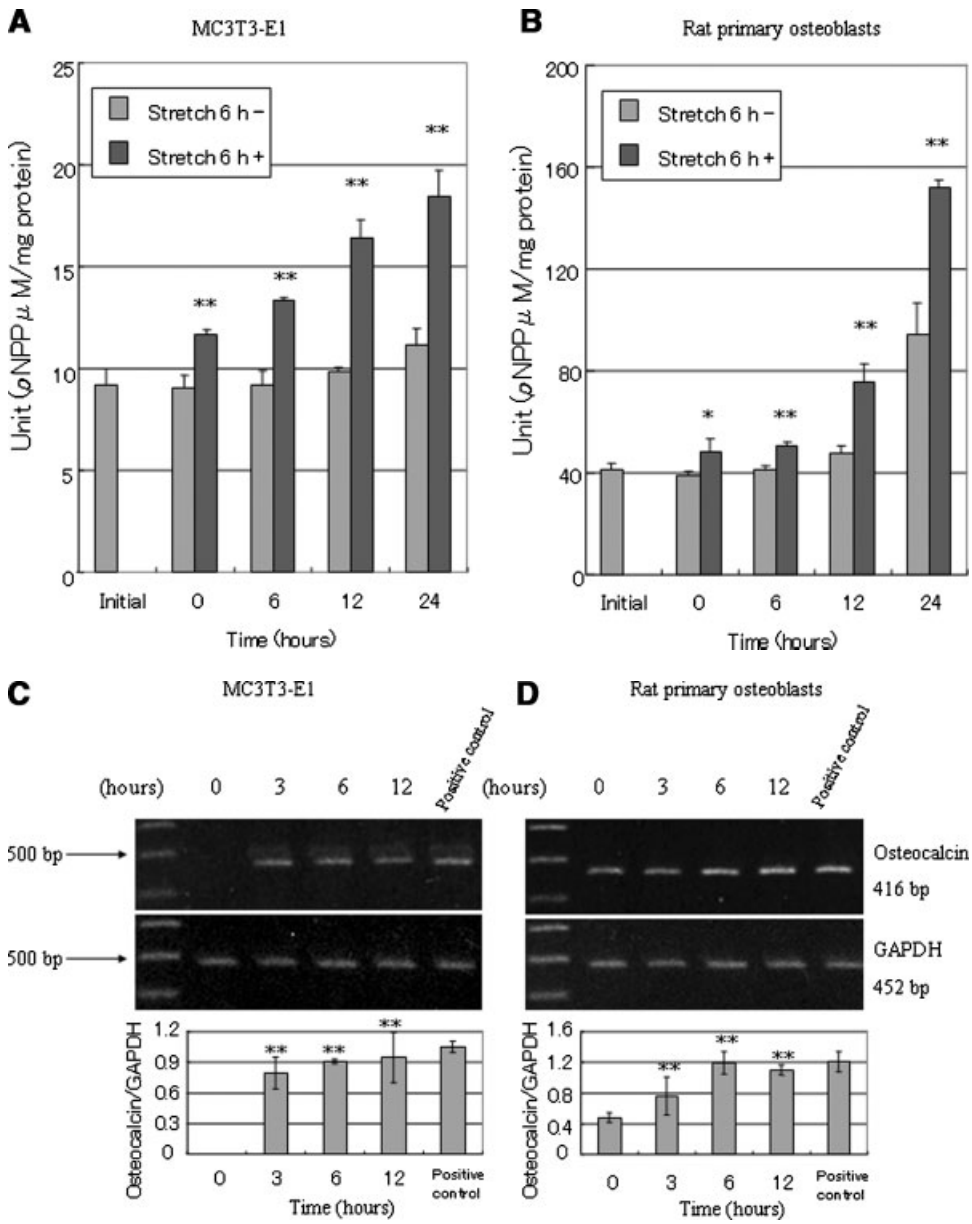

Fig. 1. Osteoblast differentiation and ALP activity in mechanically stretched osteoblastic cells. Quiescent MC3T3-E1 (**A**) and rat primary osteoblastic cells (**B**) underwent mechanical stretching (MS) for 6 h. Samples were prepared before the application of stress for the baseline measurement and after 6 h of stress treatment (0 h), and then at 6, 12, and 24 h after the cessation of MS. Control samples, without MS, were prepared at the same time points. The specific activity of ALP (U/mg protein) was calculated. Results are presented as the means ± SEM (*$P < 0.05$, **$P < 0.01$, compared to each control; n = 4). Osteocalcin (OCN) mRNA expression in mechanically stretched osteoblastic cells. RT-PCR analysis of OCN mRNA expression in quiescent MC3T3-E1 (**C**) and rat primary osteoblastic cells (**D**) at different time points (0–12 h) following MS treatment. A sample treated with 50 ng/ml BMP-2 was used as a positive control. OCN/GAPDH ratios were also analyzed and are shown. Representative results are given. Results are presented as the mean ± SEM (**$P < 0.01$ compared to 0 h; n = 3).

Induction of both ERK1/2 and p38 was detected, and the intensity of the bands increased steadily up to 60 min after the cessation of stretching (Fig. 3A). No substantial or rapid enhancement of p-JNK occurred compared to JNK, the internal control expressed in both groups of osteoblastic cells (data not shown).

MAPK Activation is Correlated With Runx2 Activation and Phosphorylation in Mechanically Stretched Osteoblastic Cells

To address the roles of specific MAPKs in stretch-induced Runx2 activation, we used two specific membrane-permeable protein kinase

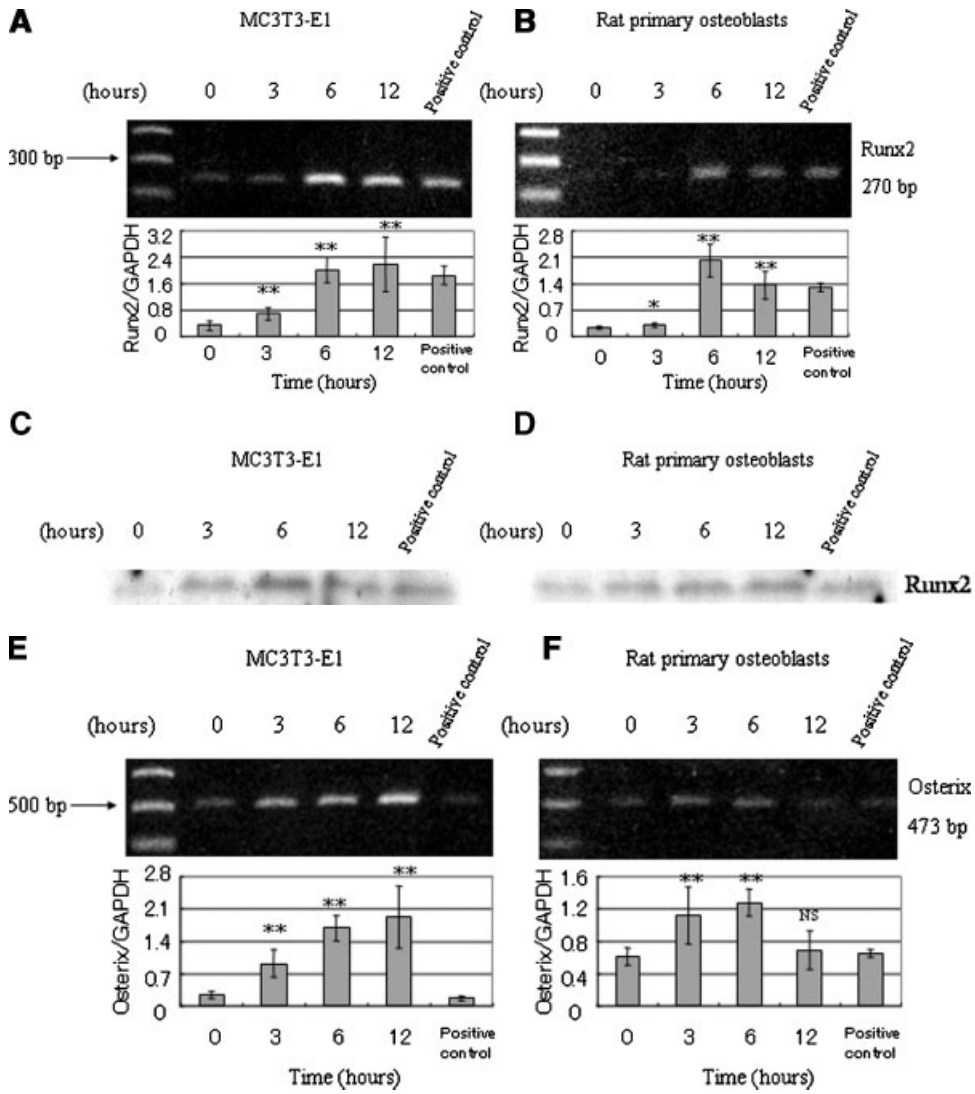

Fig. 2. Mechanical stress upregulates Runx2 mRNA and protein levels. RT-PCR analysis of Runx2 mRNA expression in quiescent MC3T3-E1 (**A**) and rat primary osteoblastic cells (**B**) at different time points (0–12 h) following mechanical stress treatment from the same samples shown in Fig. 1. Runx2/GAPDH ratios were analyzed and are shown. Representative results are given. Results are presented as the mean ± SEM (*$P < 0.05$, **$P < 0.01$, compared to 0 h; n = 3). Western blotting of nuclear extracts (20 μg/lane) from quiescent MC3T3-E1 (**C**) and rat primary osteoblastic cells (**D**) exposed to mechanical stress for the same time period as indicated above using anti-Runx2 antibodies. A sample treated with 50 ng/ml BMP-2 was used as a positive control. The experiment was performed three times; similar results were obtained each time. Mechanical stress upregulates Osterix (Osx) mRNA expression. RT-PCR analysis of Osx mRNA expression in quiescent MC3T3-E1 (**E**) and rat primary osteoblastic cells (**F**) at different time points (0–12 h) following MS treatment of the same samples used above. Osx/GAPDH ratios were also analyzed and are shown. Representative results are given. Results are presented as the mean ± SEM (**$P < 0.01$; NS: not statistically significant compared to 0 h, $P > 0.05$; n = 3).

inhibitors: U0126 (10 μM), a potent and selective inhibitor of ERK1/2 kinase (MEK1/2), and SB203580 (10 μM), which selectively inhibits p38 (MEK3/6). A MTT viability assay demonstrated that the inhibitors had no cytotoxic effects over the range of concentrations used (data not shown) [Liedert et al., 2006b]. Runx2 activation was examined by immunoprecipitation, as previously described. Our findings showed that the ERK1/2-selective inhibitor completely blocked Runx2 activation; however, the p38-selective inhibitor had no effect on Runx2 phosphorylation (Fig. 4A). We hypothesized that p38 would behave in a manner similar to other transcription factors, such as AP-1 and its family members, which showed a radical

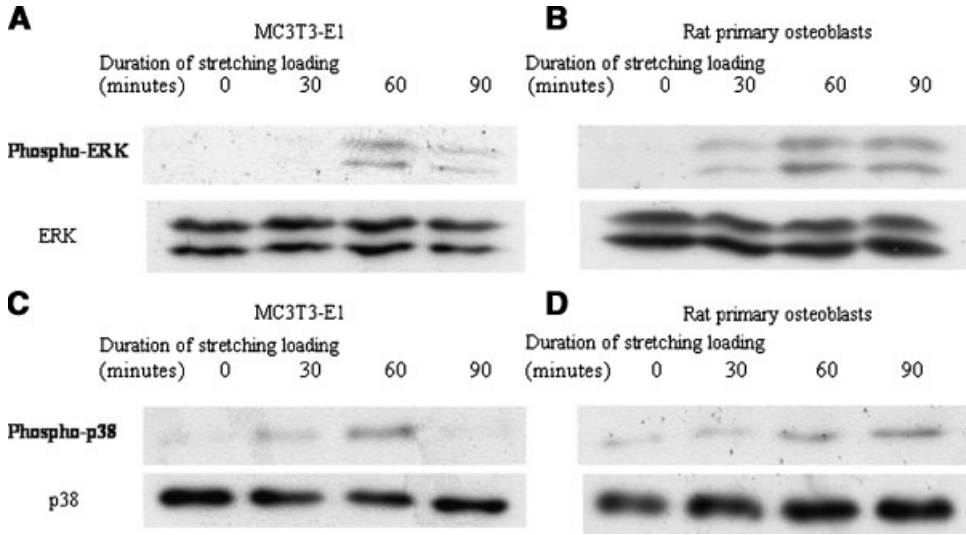

Fig. 3. MAPK signaling pathways involved in mechanical stress-induced osteoblast differentiation and osteogenic activity. **A–D**: Kinetics of MAPK phosphorylation (ERK1/2, JNK, and p38) were examined by Western blotting using mechanically stressed quiescent MC3T3-E1 and rat primary osteoblastic cells. Whole cell extracts (20 μg/lane for ERK1/2, JNK, and p38 species; 50 μg/lane for phospho-ERK1/2, phospho-JNK, and phospho-p38 species) were prepared at the indicated time points (0–90 min). The experiment was performed three times; similar results were obtained each time.

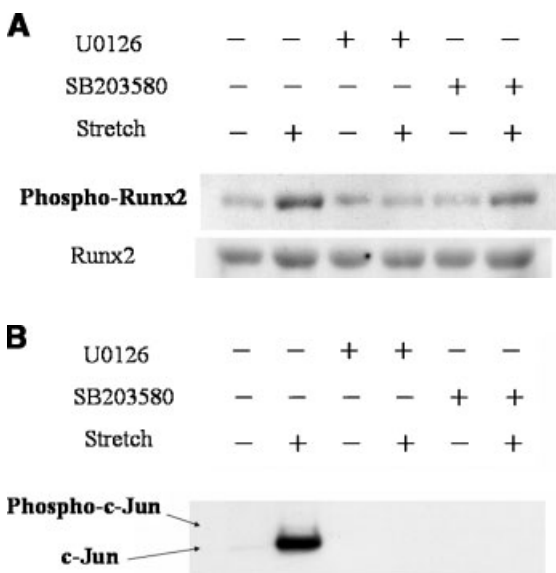

Fig. 4. Effects of MAPK inhibitors on mechanical stress-induced Runx2 activation in osteoblastic cells. Quiescent MC3T3-E1 cells were treated with the MAPK inhibitors U0126 (10 μM) and SB203580 (10 μM) for 1 h. **A**: After 6 h of mechanical stress, Runx2 activity in the immunoprecipitated complexes (15 μg) was measured as phosphorylated Runx2 and visualized by Western blotting. **B**: Effects of MAPK inhibitors on mechanical stress-induced c-Jun nuclear translocation were also measured by Western blotting of nuclear extracts (10 μg/lane) using anti-c-Jun and anti-phospho-c-Jun double-band antibodies. The experiment was performed three times; similar results were obtained each time.

nuclear translocation of c-Jun (a major component of AP-1) in both MC3T3-E1 and rat primary osteoblastic cells upon exposure to nuclear extracts of cells treated with mechanical stress for various time periods (data not shown). Our findings showed that both the ERK1/2 (MEK1/2)- and p38 (MEK3/6)-selective inhibitors completely blocked mechanical stress-induced nuclear translocation of c-Jun, while nuclear translocation and phosphorylation were observed in the non-treated control sample (Fig. 4B).

Effects of Ras Depletion (by Ras siRNA) on the Activation of MAPK and Runx2 in Mechanically Stretched Osteoblastic Cells

Finally, we investigated whether Ras, an upstream mediator of ERK1/2 and p38 activation via the Ras pathway, is critical for MAPK signaling by studying ERK1/2- and p38-induced Runx2 activation and cooperation in mechanically stretched osteoblasts. We transfected MC3T3-E1 cells with Ras siRNA, and examined the effect of Ras downregulation (Fig. 5A). Western blotting revealed that Ras knockdown inhibited the ERK1/2 signal pathway (Fig. 5B) but did not affect the p38 pathway (Fig. 5C). Furthermore, Runx2 activation was further

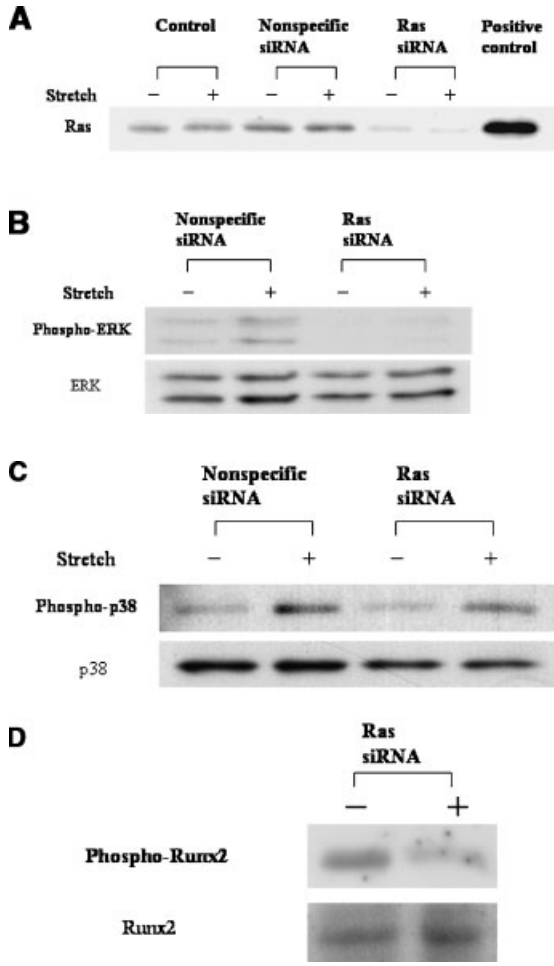

Fig. 5. Effect of Ras depletion on the activation of MAPK signaling in mechanically stretched osteoblastic cells. The effects of Ras depletion on the activation of MAPK signaling were examined using equal amounts of whole cell extracts from either non-specific siRNA-treated MC3T3-E1 or Ras siRNA-treated cells. **A**: Following 60 min of mechanical stress treatment, Ras knockdown was determined by Western blotting in the untreated control, non-specific siRNA-treated, and Ras siRNA-treated samples, using anti-Ras antibodies. Western blotting of mechanical stress-induced MAPK activity from MC3T3-E1 cells upon knockdown of Ras. **B**: Measurements of ERK and (**C**) p38 were also performed using whole cell extracts. The effect of Ras depletion on the activation of Runx2 was further examined using equal amounts of nuclear extract from Ras siRNA-treated MC3T3-E1 cells. (**D**) After 6 h of mechanical stress, the same time as in the previous experiments using MAPK inhibitors, Runx2 activity in the immunoprecipitated complexes (15 μg) was measured as phosphorylated Runx2 and visualized by Western blotting. The image is representative of two separate experiments.

examined by immunoprecipitation, as previously described. The Ras knockdown clearly inhibited the mechanically stretching-induced activation of Runx2 in osteoblasts (Fig. 5D).

DISCUSSION

Mechanical stress has long been recognized as a critical regulatory factor in bone biology and a postnatal determinant of bone homeostasis and skeletal morphology [Franceschi et al., 2003; Komori, 2003; Lian et al., 2004; Liedert et al., 2006a]. Mechanical stress is converted into a cellular/molecular response that involves rapid, kinase-mediated changes in gene expression [Boutahar et al., 2004; Katz et al., 2006]. To date, several studies have reported responses of bone cells to various mechanical stresses, such as stretch, fluid flow, and hydrostatic pressure [Wang et al., 2002; Franceschi and Xiao, 2003; Katz et al., 2006; Liedert et al., 2006b], and the magnitude of the applied strain influences the cellular response at the tissue level. It has been reported that elongation strains on the order of 1–3% (10,000–30,000 μstrain) are sufficient to elicit a cellular response in vitro, although physiological strains recorded in human long bones during strenuous activity are on the order of 2,000–4,000 μstrain, and 1,500 μstrain is the minimum effective strain at which bones undergo remodeling [Frost, 1992; Tang et al., 2006].

In this study, we used an in vitro cell straining system that allowed the mechanical stimulus to be quantified at one fixed magnitude over time (15% strain, 115% peak-to-peak, at 1/12 Hz), which is similar to our previous report on the clinical application of distraction histogenesis in which large magnitude straining was shown to affect osteoblastic cell function [Kanno et al., 2005a]. Although different magnitudes of strain might produce different osteoblastic cell responses, it is very difficult to compare in vitro-applied strains with those applied in vivo and ex vivo because the characteristics of the strains are different. In this experiment, however, we sought to mimic clinical applications, such as the distraction histogenesis technique. This is often used in maxillofacial surgery as a tissue regenerative therapy and requires a very large mechanical force on distracted bony segments, using rigorous devices [Ryoyama et al., 2004; Kanno et al., 2005a; Singare et al., 2006].

The results of this study demonstrate that tensile mechanical strain in osteoblasts stimulates ERK1/2-dependent activation of the transcription factor Runx2. Moreover, ERK1/2-mediated activation of Runx2 is dependent on

Ras, which is an upstream mediator of MAPKs, ERK1/2, and p38 [Boutahar et al., 2004; Wang et al., 2004; Liedert et al., 2006a]. The mechanical stress-mediated activation of p38 is independent of Ras signaling and Runx2 activation, while the role of p38 appears to be linked to the nuclear translocation of c-Jun, a major component of the AP-1 family. Furthermore, ERK1/2 might also be involved in c-Jun translocation.

The expression of ALP and OCN at the protein and mRNA levels was elevated in osteoblastic cells following the application of steady mechanical stress, which is consistent with several lines of evidence suggesting that mechanical stress enhances osteogenesis via upregulation of several osteoblast genes, such as ALP, type 1 collagen, OCN, osteopontin, and collagenase-3, which are under the regulation of Runx2 and Osx [D'Alonzo et al., 2002; Franceschi and Xiao, 2003; Rubin et al., 2006]. Our data are also consistent with previous reports stating that mechanical stress induced the expression of the osteogenic transcription factors Runx2 and Osx at the mRNA and/or protein levels [Fan et al., 2006; Salingcarnboriboon et al., 2006]. However, to date, no conclusive evidence exists indicating that Osx is a direct target of Runx2, and any link between these transcriptional factors in the induction of osteoblast differentiation remains unclear, especially under the condition of mechanical stimulation [Jadlowiec et al., 2004; Fan et al., 2006]. Furthermore, a slight increase in nuclear Runx2 and a marked elevation in Runx2 phosphorylation were detected after several hours of mechanical stress similar to the previous report in hPDL cells [Ziros et al., 2002].

MAPK activity plays a pivotal role in a variety of cellular functions and has been shown to be modulated by diverse external stimuli, such as growth factors, cytokines, and physical stresses (e.g., mechanical stress, ultraviolet radiation, or hyperosmolarity), which are communicated to the intracellular environment by mechanoreceptors [Fan et al., 2006; Katz et al., 2006]. Mechanoreceptors, such as integrins, cadherins, and stretch-activated Ca^{2+} channels, together with various signal transduction pathways, comprise mechanotransduction pathways that ultimately regulate nuclear gene expression, but these systems remain largely uncharacterized in osteoblastic cells [Fan et al., 2006; Liedert et al., 2006a]. Runx2 augmentation by mechanical stress is believed to be mediated by specific MAPK pathways [Wang et al., 2002; Ziros et al., 2002; Costessi et al., 2005; Liedert et al., 2006b]. In particular, MEK/ERK signaling provides a plausible link between cell surface integrin activation and subsequent stimulation of Runx2-dependent transcription [Franceschi and Xiao, 2003; Meyers et al., 2004; Fan et al., 2006]. However, some recent studies have indicated the involvement of MAPKs (e.g., ERK1/2 and p38) in osteoblast differentiation in HOBIT cells through regulation of Runx2 nuclear function. ATP-dependent mechanical stress-activated Ca^{2+} influx at calcium channels via Ser/Thr kinase pathways, such as PKC-c-Src signaling, or via FAK and PYK2 phosphorylation, have been implicated as far-upstream mediators of MAPK cascade activation in osteoblastic cells, including ROS 17/2.8 cells [Boutahar et al., 2004; Katz et al., 2006], CIMC-4 cells [Fan et al., 2006], and HOBIT cells [Costessi et al., 2005] (Fig. 6). In addition, the activation of P2Y receptors (P2Y1 and P2Y2 subtype receptors) has attracted attention as mechanical stress-activated channels in ROS-A 17/2.8 osteoblastic cells, leading to calcium influx and activation of ERK1/2 and p38 by PKC and Src family kinases [Boutahar et al., 2004; Katz et al., 2006; Liedert et al., 2006a] (Fig. 6). Our Western blot data revealed that the maximum levels of ERK1/2 and p38 occurred 60 min after the cessation of stretching, which confirms the involvement of the MAPK pathway; however, no substantial or rapid enhancement of p-JNK was observed compared to JNK, similar to the results of a previous study (data not shown) [You et al., 2001; Brancho et al., 2003; Lian et al., 2004]. Finally, our siRNA-mediated knockdown of Ras signaling, which downregulates PKC-Src family kinase function and FAK signaling, showed that this factor mediates ERK1/2, but not p38, signaling.

Extracellular ATP and UTP were recently identified as regulators of osteoblast-specific Runx2 activation in an ERK1/2- (but not p38-) dependent manner, suggesting that PKC acts upstream of ERK1/2 in HOBIT osteoblastic cells, upon application of mechanical stress [Fan et al., 2006; Katz et al., 2006]. Runx2 activity is controlled by phosphorylation, and Runx2 is phosphorylated in vivo by ERK/MAPK in response to mechanical stress [Ziros et al., 2002]. Our data involving the selective inhibitors U0126 and SB203580 are consistent with a

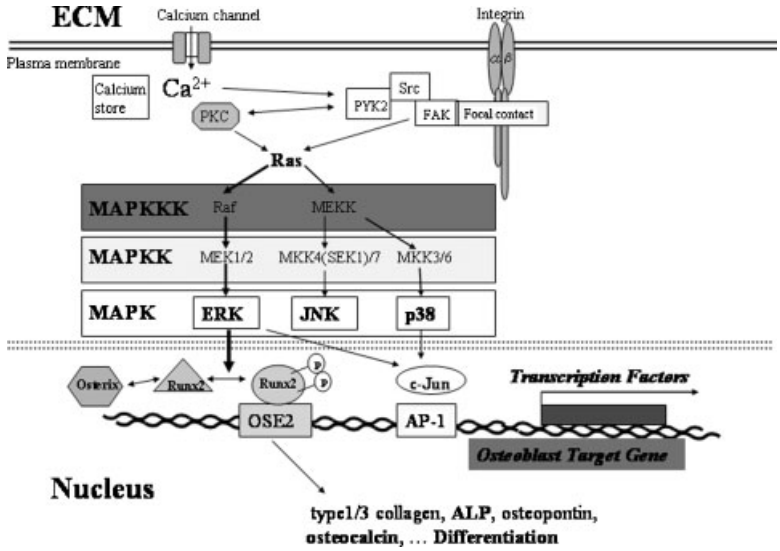

Fig. 6. Model of signal transduction pathways involved in the effects of Ras reduction on stretch-mediated MAPKs and Runx2 activation in osteoblastic cells. Our data, combined with previous studies on mechanical stress-related signaling pathways and key regulatory molecules [Franceschi et al., 2003; Meyers et al., 2004; Katz et al., 2006; Liedert et al., 2006a], suggest fundamental information regarding Runx2 nuclear activation, which is regulated in a Ras/ERK1/2-dependent manner, indicating that intracellular signaling activates the osteogenic program.

previous report showing the modulation of nuclear Runx2 activation by mechanical stress-induced ERK1/2 and p38 in MC3T3-E1 cells [Ziros et al., 2002; Inoue et al., 2004; Liedert et al., 2006b]. Runx2 activation was examined using nuclear extracts from mechanically stretched osteoblast MC3T3-E1 cells, by immunoprecipitation using a specific mouse anti-phosphotyrosine antibody, as described previously [Wang et al., 2002]. Our findings showed that the ERK1/2-selective inhibitor completely blocked Runx2 activation, but the p38-selective inhibitor had no effect on Runx2 phosphorylation, similar to previous reports [Fan et al., 2006; Liedert et al., 2006a]. Taken together, these data suggest that extracellular receptor signaling regulates ERK1/2 induction of Runx2 nuclear activation through PKC-Src signaling or FAK-upstream mediators [Meyers et al., 2004; Katz et al., 2006; Liedert et al., 2006a]. ERK1/2 activation may be essential for the mechanical stress-induced differentiation and proliferation of osteoblasts, independent of p38 signaling. In this respect, p38 has no association with nuclear Runx2 activation; however, U0126 and SB203580 inhibited the nuclear translocation of c-Jun, a major constituent of AP-1, upon the application of mechanical stress, which leads us to hypothesize that p38 might be involved with other transcription factors. A previous report presented similar data for mechanical stress-induced c-fos (another constituent of AP-1, and a transcription factor present in diverse cells), which is also regulated by both ERK1/2 and/or p38 in osteoblasts and periodontal ligament osteoblastic cells [Kletsas et al., 2002; Ziros et al., 2002], as well as in human bone-derived cells [Zreiqat et al., 2005]. Thus, interestingly, the role of both ERK1/2 and p38 appears to be closely linked with nuclear translocation of c-Jun in mechanically stressed osteoblasts.

Finally, we showed that Ras induction is a critical feature of ERK- and p38-associated Runx2 activation in mechanically stretched osteoblasts, although the Ras/Raf pathway was thought to be a common downstream mediator of the PKC-c-Src and FAK signaling pathways during mechanotransduction [Boutahar et al., 2004; Wang et al., 2004; Katz et al., 2006]. Conversely, our previous report suggested reduced downstream signaling via the integrin-Ras/MAPK signaling pathway, as evidenced by a reduction in Ras and ERK activation, through reduced expression of FAK and PYK2 during osteoblastgenesis in human mesenchymal stem cells under modeled microgravity conditions [Meyers et al., 2004]. It has

also been reported that integrin-mediated Ras-related signal transduction cascades provide the main route for MAPK-related intracellular events initiated by ECM–integrin interactions, as the signal enters the Ras-MEK-ERK route during Runx2 activation [Xiao et al., 2000; Franceschi et al., 2003; Boutahar et al., 2004]. Western blot analyses revealed that abrogation of the Ras signal inhibited ERK1/2 signaling, but that the p38 pathway was unaffected. Furthermore, Ras depletion, using siRNA knockdown, inhibited the mechanical stretching-induced activation of Runx2. These results suggest that mechanical stress activates p38-associated osteoblast differentiation; however, its mechanism of downregulation may be independent of the ERK1/2-Runx2 osteogenic differentiation mechanism, and independent of the previously reported signaling pathways, suggesting different mediators for its activation under conditions of mechanical stress [Fan et al., 2006; Katz et al., 2006; Liedert et al., 2006a]. For example, it might be mediated by a different signaling mechanism, such as apoptosis signal-regulating kinase 1 (ASK1) [Mizumura et al., 2006]. These results demonstrate that the differentiation of osteoblasts in response to mechanical stress may be associated with various MAPKs and might be determined by additional regulatory mechanisms other than the selective activation of a MAPK.

In conclusion, our results provide fundamental information regarding Runx2 nuclear activation, which is regulated in a Ras/ERK1/2 dependent-manner, indicating that intracellular signaling activates the osteogenic program, while p38 upregulation is independent of Runx2 activation in mechanically stressed osteoblasts.

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