Journal of Cellular Biochemistry 102:1202–1213 (2007)

Nmp4/CIZ Contributes to Fluid Shear Stress Induced *MMP-13* Gene Induction in Osteoblasts

Kanokwan Charoonpatrapong-Panyayong,[1] Rita Shah,[1] Jieping Yang,[1] Marta Alvarez,[1] Fredrick M. Pavalko,[2] Rita Gerard-O'Riley,[2] Alexander G. Robling,[1] Evan Templeton,[1] and Joseph P. Bidwell[1]*

[1]Department of Anatomy & Cell Biology, Indiana University School of Medicine (IUSM), Indianapolis, Indiana 46202

[2]Department of Cellular and Integrative Physiology, Indiana University School of Medicine (IUSM), Indianapolis, Indiana 46202

Abstract The expression of matrix metalloproteinase-13 (MMP-13), involved in bone turnover, is elevated in stretched MC3T3-E1 osteoblast-like cells. Strain-mediated forces impact bone remodeling due in large part to the movement of fluid through the canalicular-lacunar network. The resulting fluid shear stress (FSS) over the surface membranes of bone cells initiates bone remodeling. Although the nuclear events mediating putative FSS-induced changes in osteoblast *MMP-13* transcription are unknown, previous studies with bone cells suggest an overlap between osteoblast FSS- and PTH-induced signal response pathways. *MMP-13* PTH response is regulated by a 110 bp 5′ regulatory region, conserved across the mouse, rat, and human genes, that supports the binding of numerous transcription factors including Runx2, c-fos/c-jun, Ets-1, and nuclear matrix protein 4/cas interacting zinc finger protein (Nmp4/CIZ) a nucleocytoplasmic shuttling trans-acting protein that attenuates PTH-driven transcription. Nmp4/CIZ also binds $p130^{cas}$, an adaptor protein implicated in mechanotransduction. Here we sought to determine whether Nmp4/CIZ contributes to FSS-induced changes in *MMP-13* transcription. FSS (12 dynes/cm^2, 3–5 h) increased *MMP-13* promoter-reporter activity approximately two-fold in MC3T3-E1 osteoblast-like cells attended by a comparable increase in mRNA expression. This was accompanied by a decrease in Nmp4/CIZ binding to its cis-element within the PTH response region, the mutation of which abrogated the *MMP-13* response to FSS. Interestingly, FSS enhanced Nmp4/CIZ promoter activity and induced $p130^{cas}$ nuclear translocation. We conclude that the PTH regulatory region of *MMP-13* also contributes to FSS response and that Nmp4/CIZ plays similar but distinct roles in mediating hormone- and FSS-driven induction of *MMP-13* in bone cells. J. Cell. Biochem. 102: 1202–1213, 2007.

Key words: bone; mechanical load; mechanosome; mechanotransduction; $p130^{cas}$

Mechanical force, e.g. gravity and load-bearing exercise, influences bone remodeling, the turnover of bone matrix and mineral involving osteoclast-mediated resorption and osteoblast-mediated formation. The mechanisms underlying this regulation remain to be fully elucidated [Ehrlich and Lanyon, 2002; Turner and Robling, 2005]. Strain-mediated compressive and tensile forces on bone move fluid through the canalicular-lacunar network resulting in fluid shear stress (FSS) on the membranes of osteocytes, lining cells, and osteoblasts, ultimately initiating the cellular activity involved in bone remodeling [Basso and Heersche, 2002; Cowin, 2002]. The intracellular signaling pathways that transduce mechanical stimuli in bone cells likely involve the activation of focal adhesions, release of prostaglandin E_2 (PGE_2), nitric oxide, and adenosine triphosphate (ATP) as autocrine/paracrine factors, up-regulation of

Kanokwan Charoonpatrapong-Panyayong's present address is Department of Oral Biology and Occlusion, Faculty of Dentistry, Prince of Songkla University, Hatyai, Songkhla 90110, Thailand.

Rita Shah's present address is Eli Lilly and Company, Indianapolis 46221.

Evan Templeton's present address is Dickinson College, Carlisle, Pennsylvania 17013.

*Correspondence to: Joseph P. Bidwell, Department of Anatomy & Cell Biology, Indiana University School of Medicine, Medical Science Bldg 5035, 635 Barnhill Drive, Indianapolis, IN 46202. E-mail: jbidwell@iupui.edu

Received 12 January 2007; Accepted 2 March 2007

DOI 10.1002/jcb.21349

the immediate early gene c-fos and other transcription factors necessary for target gene regulation [Mullender et al., 2004; Pavalko et al., 2003a; Iqbal and Zaidi 2005; Li et al., 2005].

Matrix metalloproteinase-13 (MMP-13) is a load-responsive collagenase involved in the normal and pathological turnover of connective tissue including bone [Wu et al., 2001; Kurz et al., 2005]. Mechanical strain or FSS regulates its expression in synovial fibroblasts and intervertebral disc cells from the nucleus pulposus and annulus fibrosus [Sun and Yokota, 2001; Sun and Yokota, 2002; Sun et al., 2003; MacLean et al., 2005]. Furthermore, application of a supraphysiological 8% biaxial stretch to MC3T3-E1 osteoblast-like cells enhanced *MMP-13* mRNA levels approximately 2.8-fold [Yang et al., 2004].

The nuclear events that mediate *MMP-13* response to load are unknown but recent evidence suggests that mechanical stimulation and parathyroid hormone (PTH) stimulation of osteoblasts share some common signaling mechanisms [Ryder and Duncan, 2000; Li et al., 2003]. Accordingly, the load-induced increase in *MMP-13* mRNA was accompanied by MC3T3-E1 expression of c-fos [Yang et al., 2004], and c-fos plays a significant role in PTH-induced *MMP-13* transcription [Selvamurugan et al., 1998; Hess et al., 2001], raising the likelihood of additional parallel nuclear and signaling events.

A 110 bp region within the first 140 nucleotides of the 5′ regulatory region regulates *MMP-13* basal and PTH-induced transcription. This region supports the binding of numerous transcription factors including Nmp4/CIZ, Runx2, c-fos/c-jun, and Ets-1, and is conserved across mouse, rat, and human [Selvamurugan et al., 1998; Hess et al., 2001; Shah et al., 2004]. Within the context of the *MMP-13* PTH regulatory region, Nmp4/CIZ attenuates hormone-driven as well as PGE_2-driven transcription [Shah et al., 2004]. Given the role of this region in mediating the gene's response to several anabolic stimuli, we hypothesized that it might also regulate load-induced changes in *MMP-13* expression. Additionally, the interaction between the nucleocytoplasmic shuttling Nmp4/CIZ and the adaptor protein $p130^{cas}$ [Nakamoto et al., 2000] provides another potential element of the mechanotransduction pathway mediating load-induced *MMP-13* transcription. A component of multiple integrin signaling casacades [Schlaepfer et al., 1997; Turner, 2000; Webb et al., 2004], $p130^{cas}$ itself is mechano-responsive [Okuda et al., 1999; Zaidel-Bar et al., 2005; Geiger, 2006; Sawada et al., 2006].

In the present study we determined that physiologic levels of FSS, an active component of the load profile [Mullender et al., 2004], increases *MMP-13* promoter activity and mRNA expression comparable in magnitude to that observed using supraphysiological stretch. Furthermore, our data show that Nmp4/CIZ plays a role in FSS-driven *MMP-13* transcription induction similar but distinct from the function it plays in mediating PTH-induced changes in *MMP-13* transcription.

MATERIALS AND METHODS

Cell Culture

The murine osteoblast-like cell line MC3T3-E1 (a generous gift from Dr. Laurie McCauley, University of Michigan, Ann Arbor, MI) was cultured in α-MEM medium (GibcoBRL, Grand Island, NY) supplemented with 2.2 g/L $NaHCO_3$, 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin, 2 mM L-glutamine (GibcoBRL), and 10% fetal bovine serum (Sigma, St. Louis, MO). Cells were maintained at pH 7.3, in humidified 95% air/5% CO_2 at 37°C. The culture medium was replenished every 2–3 days. Cells were subcultured once a week using 0.05% Trypsin-EDTA (GibcoBRL) to detach the cells.

MMP-13 and Nmp4/CIZ Promoter-Reporter Constructs

The promoter-reporter constructs used in the present study have been previously described. The rat *MMP-13* promoter-reporter construct (wild type collagenase 3-chloramphenicol acetyltransferase [^{WT}CL3-CAT]) contains the +34 nucleotide (nt) to −1329 nt of 5′ regulatory region [Quinn et al., 1990; Rajakumar and Quinn 1996; Shah et al., 2004]. The ^{MUTN}CL3-CAT promoter-reporter is the ^{WT}CL3-CAT construct containing a 3 A-to-C mutation of the Nmp4/CIZ binding element [−119/−110 nt; Shah et al., 2004]. The mouse Nmp4/CIZ gene has two adjacent promoters in the 5′ regulatory region P_1 (−2521 nt/−597 nt) and P_2 (−2521 nt/+1 nt) and we linked both of these promoters to the luciferase reporter [Alvarez et al., 2005].

Cell Transfection

Cells were seeded at 30,000 cells/cm^2 24 h before transfection and then transiently transfected with the promoter-reporter constructs using Lipofectamine^{TM} and Plus^{TM} reagents (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. To normalize for transfection efficiency, the pSV-β-galactosidase (βgal) control vector (Promega, Madison, WI) was cotransfected with each reporter gene construct. For *MMP-13*-promotor activity studies, the culture medium was supplemented with 50 μg/ml of ascorbic acid and replenished daily for 4 days before exposure to FSS. For Nmp4-promotor activity studies, cells were exposed to FSS 48 h post-transfection. Typically, cells were harvested immediately upon cessation of FSS, the lysates frozen and stored at −80°C until assayed for CAT or luciferase activity. MC3T3-E1 cells stably transfected with the ^{WT}CL3-CAT or ^{MUTN}CL3-CAT constructs as previously described [Shah et al., 2004] were also evaluated for their response to FSS.

Fluid Flow Experiment

The FSS studies were performed in parallel-plate chambers using a flow loop [Frangos et al., 1988; marketed by Cytodyne, San Diego, CA]. This system produces steady unidirectional flow resulting in well-characterized shear stress over a cell monolayer. The flow chamber containing the glass slide with attached cell monolayer forms a uniformed channel depth. All loop parts were autoclaved and assembled under a laminar flow hood.

Before starting the FSS experiments, medium containing 1% serum were added into the lower reservoirs. The system was maintained at 37°C in 95% air/5% CO_2 during the flow experiments. A flow sensor (SWF-5 Flowmeter, Zepeda Instruments, Seattle, WA) incorporated into the flow loop monitored flow rate. The flow rate was maintained to yield a shear stress of 12 dynes/cm^2. The value of 12 dynes/cm^2 was chosen because it is within the estimated physiological range in bone [Weinbaum et al., 1994]. Cells were seeded onto sterile glass slides 75 mm × 38 mm (Fisher Scientific, Pittsburgh, PA) in culture medium and subjected to FSS at confluence. Cells were subjected to FSS for the times indicated and harvested. Control (static) treatments consisted of cells plated on the sterile glass slides (monolayer) transferred to medium containing 1% serum at 37°C in 95% air/5% CO_2 for the flow times indicated.

Promotor-Reporter Assay

Cells were harvested with CAT Enzyme Assay System With Reporter Lysis Buffer (Promega) and analyzed for CAT activity according to the manufacturer's instructions. Assay grade [^{14}C] Chloramphenicol, D-Threo-[*dichoroacetyl*-1,2-^{14}C] CAT (PerkinElmer, Boston MA) and n-butyryl coenzyme A were used as a substrate. Total protein concentrations were determined using Coomassie Plus^{TM}—The Better Bradford Assay kit (Pierce, Rockford, IL). The results for CAT activity were reported in disintegrations per minutes per μg per hour. For transient transfection experiments, cell lysates were aliquoted before heating and transferred to a new tube for the β-galactosidase assay the results of which were used to normalize CAT activity. The β-galactosidase activities were measured using β-galactosidase Enzyme Assay System With Reporter Lysis Buffer (Promega). Equal volume of cell lysates and Assay 2× buffer (1.33 mg/ml *o*-nitrophenyl β-D-galactopyranoside, 200 mM sodium phosphate buffer pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol) were mixed and incubated overnight at 37°C. The reactions were terminated by adding 1 M sodium carbonate (for CAT lysis buffer) or 1 M Tris–HCl pH 7.4 (for luciferase buffer, see below) and the absorbance read at 420 nm.

For evaluation of luciferase activity, cells were harvested using Glo Lysis Buffer and Bright-Glo^{TM} Luciferase Assay System (Promega) according to the manufacturer's instructions. The luciferase expression was measured in microtiter plates using a Packard Bioscience Fusion Luminometer (Packard Instrument, Meriden, CT). Total protein concentrations were determined using the Bradford Assay kit (Pierce). For transient transfection experiments, scraped cell lysates were aliquoted and transferred to a new tube for β-galactosidase assay. Results for the luciferase assay were reported as relative light units/βgal activity/μg protein.

Quantitative Real-Time PCR Analysis

MC3T3-E1 cells were exposed to FSS for 5 h and then allowed to recover under static conditions for 24 h. At the end of the recovery period, total cellular RNA was harvested for

quantitative real time RT-PCR analysis using RNeasy Mini Kit (QIAGEN Inc. Valencia, CA) followed by DNase treatment with RNase-Free DNase Set (QIAGEN Inc.) according to the manufacturers' instructions. First-strand cDNA synthesis was performed with First-Strand cDNA Synthesis Kit (Amersham Bioscience, Piscataway, NJ). For amplification in an ABI PRISM 7000 system (Applied Biosystems, Foster City, CA), the reactions were performed using the TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), Assay-on-Demand mixtures, and diluted cDNA. The following Assays-on-Demand™ (Applied Biosystems) were used: MMP-13 (Mm00439491-m1) and GAPDH (Mm99999915-g1). The reaction mixes were preincubated for 10 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The CT values were normalized against GAPDH. We observed no changes in GAPDH expression in cells challenged with FSS. The reactions were analyzed using the ABI PRISM sequence detection application software SDS V1.2.

Nuclear and Cytoplasmic Fractionation

Isolated nuclear and cytoplasmic fractions were obtained using a slightly modified version of a previously described protocol [Andrews and Faller, 1991]. Briefly, cells were gently washed and scraped into cold phosphate buffer saline (PBS). The cell pellet was resuspended in cold buffer A (10 mM HEPES-KOH pH 7.9 at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl, plus freshly added 0.5 mM DTT, and 0.5 mM PMSF) and incubated on ice for 10 min. The lysates were centrifuged at 14,000 rpm for 5 min and supernatants were removed and stored at −80°C as the cytoplasmic fraction. The pellets were subsequently resuspended in cold buffer C (20 mM HEPES-KOH pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, plus freshly added 0.5 mM DTT, and 0.5 mM PMSF) and incubated on ice for 15 min. These preparations were then centrifuged at 14,000 rpm for 15 min and supernatants were removed and stored at −80°C as the nuclear fraction. The pellets were subjected to second nuclear extraction with freshly added cold buffer C and this fraction was also analyzed as a nuclear fraction.

In a second protocol separate cytoplasmic, nuclear, and membrane fractions were obtained. Differential detergent fractionation was performed according to Ramsby et al. [1994] with slight modification. Briefly, cells were washed and scraped with cold phosphate buffer saline. Washed cells were then re-suspended by gently swirling in 5 volumes of ice-cold digitonin buffer (0.01% digitonin, 10 mM PIPES pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, and 5 mM EDTA, 1.2 mM PMSF) and incubated on ice with gentle agitation for 10 min. The extraction mix was centrifuged at $480 \times g$ for 5 min at 4°C. The cytoplasmic fraction was transferred to a new tube and stored at −80°C. The digitonin-insoluble pellet was then resuspended in a volume of ice-cold Triton extraction buffer (0.5% Triton X-100, 10 mM PIPES pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 3 mM EDTA, 1.2 mM PMSF) equivalent to that utilized for digitonin extraction. Care was taken to ensure a homogenous suspension. The extraction mix was incubated on ice for 30 min with gentle agitation and centrifuged at $5{,}000 \times g$ for 10 min at 4°C. The supernatants (membrane-orgenelle fraction) were transferred to a new tube and stored at −80°C. Subsequently, the Triton-insoluble pellet was resuspended using glass homogenizer (five strokes, medium speed) in ice-cold low salt buffer without sucrose (1% Tween-40, 0.5% deocycholate, 10 mM PIPES pH 7.4, 10 mM NaCl, 1 mM $MgCl_2$, 1.2 mM PMSF) at half the volume used for Triton extraction. This extract was centrifuged at $6{,}780 \times g$ for 15 min at 4°C and the supernatant was kept as a nuclear fraction and stored at −80°C.

Electrophoretic Mobility Shift Assay (EMSA)

Protein-DNA interactions were characterized using electrophoretic mobility shift assay (EMSA) as previously described [Alvarez et al., 1998]. The second nuclear fraction from the modified Andrews and Faller [1991] protocol was used for EMSAs. Total protein concentrations were measured using the Bradford Assay kit (Pierce). Rat *MMP-13* oligo (sequence CCACAAACCACACGTACGAAAAAAAAAATAC between −137 to −107 region) were used as a probe. Sense strands of the probe were labeled with γ^{32}P ATP (PerkinElmer) using T4 polynucleotide kinase (Invitrogen). Binding reactions included 40,000 cpm of labeled probe, 2 μg of nuclear proteins, 2 μl rabbit polyclonal antibody to full length of Nmp4 or control normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA), and binding buffer (100 mM KCl, 20% glycerol, 0.2 mM EDTA, 25 mM HEPES-KOH pH 7.5, 0.01% NP-40, 0.7 μg of poly(dI)·poly(dC),

0.1 mM DTT) in the total volume of 20 μl. Unlabelled specific and nonspecific competitor probes were added to some binding reactions at 200 M excess. Binding reactions were incubated for 30 min at room temperature before running on pre-cooled 8% nondenaturing polyacrylamide gels (20% of 80:1 acrylamide:bis-acrylamide solution) at 250 V at 4°C. Gels were dried under vacuum for 1 h at 80°C and for 30 min at room temperature. The gels were exposed to Kodak X-Omat AR film with intensifying screen at −80°C.

Western blot analysis

Western blot analysis was performed as previously described [Torrungruang et al., 2002]. Briefly, protein fractions were diluted into sodium dodecylsulfate (SDS) sample buffer, heated at 95°C for 5 min, electrophoresed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels (10%), and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). The blots were incubated with rabbit polyclonal antibody to C-terminus of $p130^{Cas}$ (Santa Cruz Biotechnology Inc) at 1:100 dilution, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Labs) and subsequently developed for chemiluminescence with ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK). The chemiluminescence signals were acquired with the LAS-1000 Plus Luminescent Image Analyzer (Fujifilm, Sunnyvale, CA) and further processed for densitometric analysis using Image Reader LAS-1000 software (Fujifilm).

Statistical Analysis

We used a one-way ANOVA incorporating comparison tests as our statistical treatment of the data or *t*-test (JMP Statistical Software, Version 4.0.4, Copyright 2001 by SAS Institute, Cary, NC). P values <0.05 were considered statistically significant whereas values greater than 0.05 were considered not significant (NS).

RESULTS

FSS Induces an Approximate Two-Fold Increase in Rat MMP-13 Promoter Activity and an Increase in MMP-13 mRNA

The observation that biaxial stretch induces an increase in MC3T3-E1 *MMP-13* expression [Yang et al., 2004] raises the questions as to whether FSS can effect a similar response from these cells since fluid forces induced by extracellular fluid flow within the bone matrix play a significant role in the bone remodeling response to mechanical loading [Owan et al., 1997]. Therefore we transiently transfected the *MMP-13* promoter-reporter (^{WT}CL3-CAT) into MC3T3-E1 cells and then exposed them to FSS or static conditions for 1 h, 3 h, or 5 h. Cells subjected to 3 h or 5 h FSS exhibited a 1.2- to 2.5-fold increase in *MMP-13* promoter activity as compared to static controls (Fig. 1A), whereas cells exposed to FSS for 1 h and harvested 4 h post-flow showed no significant change in

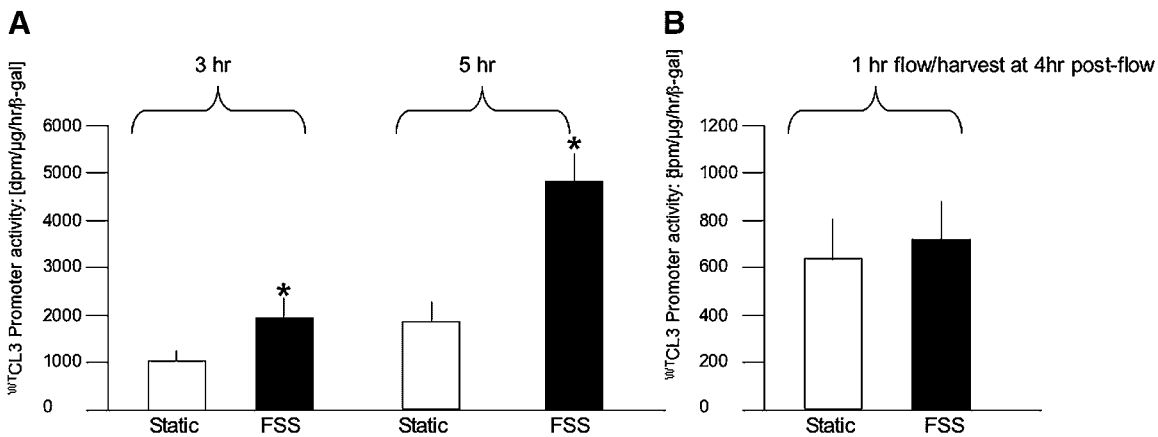

Fig. 1. FSS induces an increase in *MMP-13* promoter activity. **A**: MC3T3-E1 cells transiently transfected with the ^{WT}CL3 promoter-reporter were exposed to static or FSS (12 dynes/cm^2) for 3 or 5 h, harvested, and the promoter activity measured as described in Materials and Methods (average ± SE; 3 h static 1066.44 ± 164.14, n = 8; 3 h flow 1963.46 ± 302.13, n = 8, asterisk $P < 0.05$; 5 h static 1897.12 ± 369.24, n = 4; 5 h flow 4796.86 ± 685.68, n = 4, asterisk $P < 0.05$). **B**: Cells were exposed to FSS for 1 h and harvested 4 h post-flow (static 619.08 ± 183.38, n = 3; flow 697.20 ± 181.66, n = 3; NS, not significant).

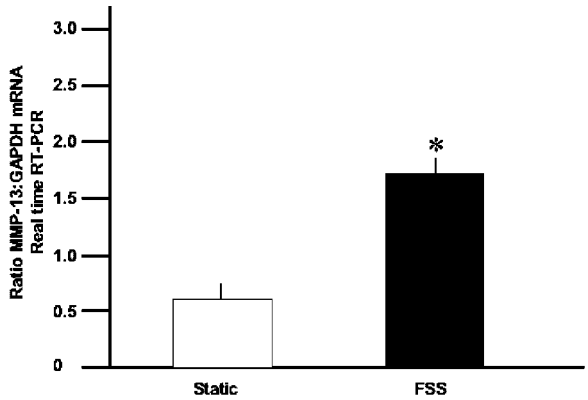

Fig. 2. FSS induces an increase in MMP-13 mRNA expression in MC3T3-E1 cells. Cells were exposed to FSS for 5 h and then allowed to recover under static conditions for 24 h. At the end of the recovery period, total cellular RNA was harvested for quantitative real time RT-PCR analysis. The ratio of MMP-13/GAPDH increased approximately three-fold in response to FSS (average ± SE, static 0.57 ± 0.15, $n = 4$; flow 1.73 ± 0.19, $n = 3$, asterisk $P < 0.05$).

promoter activity (Fig. 1B). FSS stimulated only a modest increase in *MMP-13* promoter-reporter activity in those cells that had been stably transfected and collected as pools (300–600 colonies) [data not shown].

We next determined whether the observed FSS-induced changes in the activity of the promoter-reporter construct reflected a change in the expression of the native gene. Using real-time RT-PCR we observed that MC3T3-E1 cells challenged with FSS for 5 h exhibited an approximate three-fold increase in *MMP-13* mRNA 24 h post-flow as compared to static controls ($P < 0.01$, Fig. 2). We did not analyze mRNA response to the 3 h FSS exposure or for shorter time periods of post-flow recovery.

FSS Alters Nmp4/CIZ Binding Activity Within the PTH Response Region of the MMP-13 Gene

Nmp4/CIZ governs PTH-driven *MMP-13* transcription induction, in part, through its interaction with its cis-element within the PTH response region of the *MMP-13* gene [Fig. 3A, Shah et al., 2004]. Therefore, to determine whether Nmp4/CIZ plays a similar role in FSS-driven *MMP-13* transcription we first established whether FSS alters Nmp4/CIZ association within this region of the gene. EMSA revealed that exposure of MC3T3-E1 cells to FSS modestly attenuated total nuclear binding to the PTH response region (Fig. 3B). This appeared to be due, at least in part, to a decrease in Nmp4/CIZ binding within this region of the gene. Supershift analysis using an antibody to Nmp4/CIZ confirmed that FSS specifically decreased Nmp4/CIZ binding within the PTH response region but had no effect on Runx2 binding (Fig. 3B). Addition of an antibody to $p130^{cas}$ did not alter the EMSA profiles (data not shown).

Mutation of the Nmp4/CIZ cis-Element Within the MMP-13 PTH Response Region Abrogates Gene Response to FSS

To establish whether the Nmp4/CIZ cis-element within the PTH response region plays a functional role in mediating *MMP-13* response to FSS, we mutated this site in *MMP-13* so as to abolish Nmp4/CIZ binding. We have previously demonstrated that this mutation specifically abrogates Nmp4/CIZ association with its cis-element without attenuating the adjacent Runx2 binding [Shah et al., 2004]. We then transiently transfected the promoter-reporter containing this mutation (^{MUTN}CL3-CAT) into MC3T3-E1 cells. We observed no consistent significant difference in the basal promoter activity between the ^{WT}CL3-CAT and ^{MUTN}CL3-CAT constructs in the MC3T3-E1 cells (data not shown); however, FSS did not induce a response from the ^{MUTN}CL3-CAT promoter-reporter (Fig. 4).

FSS Enhances the Activity of the P_2 but not the P_1 Nmp4/CIZ Promoter

The murine *Nmp4/CIZ* gene has two promoters (P_1 and P_2, Fig. 5A) both of which are positively auto-regulated; additionally PTH negatively regulates both of these promoters [Alvarez et al., 2005]. To determine whether FSS regulates the expression of Nmp4/CIZ at the transcriptional level, we transiently transfected MC3T3-E1 cells with either the P_1 or P_2 promoter-reporter constructs and exposed them to FSS or static conditions. FSS induced a moderate but significant increase (30–38%) in P_2 promoter-reporter activity but the P_1 promoter was unresponsive to FSS (Fig. 5B).

FSS Induces $p130^{cas}$ Translocation to the Nucleus of MC3T3-E1 Cells

We next characterized the effects of FSS on $p130^{cas}$ tyrosine phosphorylation and subcellular

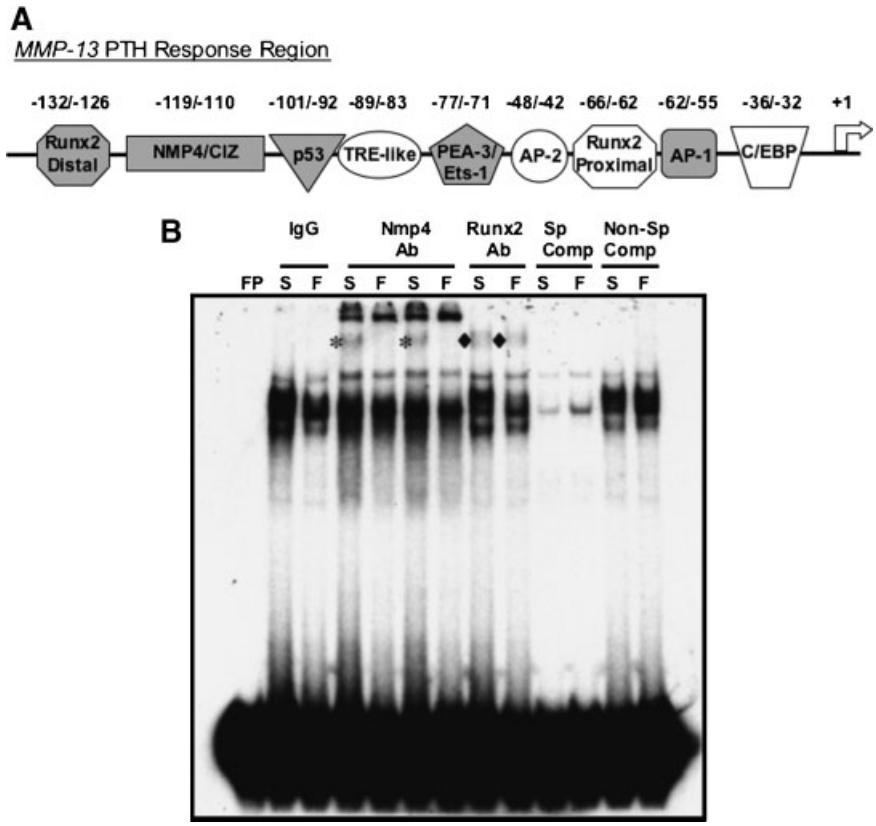

Fig. 3. FSS abrogates Nmp4/CIZ binding to the *MMP-13* PTH response region. **A**: Schematic diagram of the rat PTH response region (−132/+1 nt). The elements shown in gray are conserved across the rat, mouse, and human genes. **B**: EMSA of nuclear extracts derived from MC3T3-E1 cells exposed to static (S) or FSS (F). Addition of an Nmp4 antibody (Ab) to the extracts reveals a supershift (asterisk) in the static extracts but not the extracts from cells exposed to FSS (n = 3 for both S and FSS treatments). Runx2 binding was not altered by FSS (diamond). Abbreviations—Sp Comp: specific unlabeled competitor; Non-Sp Comp: non-specific unlabeled competitor.

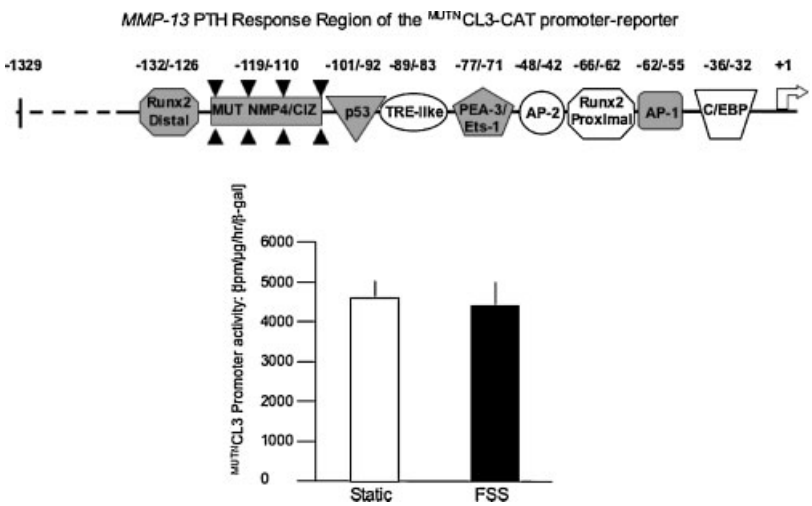

Fig. 4. Mutating the Nmp4/CIZ cis-element within the PTH Response Region abolishes FSS-induced changes in *MMP-13* promoter activity. The schematic diagram of the *MMP-13* rat PTH response region (−132/+1 nt) indicates a mutation in the Nmp4/CIZ site that abrogates binding [Shah et al., 2004]. MC3T3-E1 cells transiently transfected with the ^{MUTN}CL3-CAT promoter-reporter were exposed to static conditions or FSS (12 dynes/cm^2) for 5 h, harvested, and the promoter activity measured as described in Materials and Methods (static 4606.39 ± 715.02, n = 3; flow 4438.45 ± 643.69, n = 3, NS).

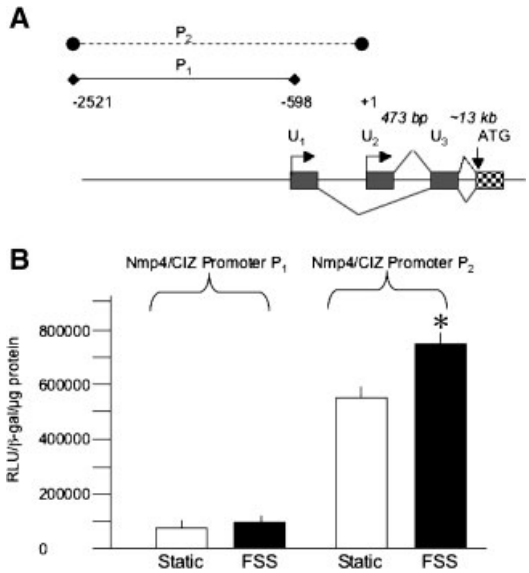

Fig. 5. FSS enhances Nmp4/CIZ P_2 promoter activity but not promoter P_1 activity. **A**: Schematic diagram of the Nmp4/CIZ P_1 and P_2 promoters: Exons 1–3 (U_1–U_3) are shown as gray boxes and the first coding exon is shown as a checkered box. The splice patterns up to the start of translation are indicated with straight lines. **B**: MC3T3-E1 cells transiently transfected with the P_1 or P_2 promoter-reporter were exposed to static or FSS (12 dynes/cm^2) for 5 h, harvested, and the promoter activity measured as described in Materials and Methods (average ± SE; P_1 static 90262.72 ± 2880.33, P_1 flow 93062.11 ± 8195.38, n = 3, NS; P_2 static 543946.47 ± 29644.69, P_2 flow 751440.19 ± 40003.43, n = 3, asterisk $P < 0.05$).

distribution in MC3T3-E1 cells since this mechano-responsive protein [Okuda et al., 1999; Zaidel-Bar et al., 2005; Geiger, 2006; Sawada et al., 2006] has been observed to associate with Nmp4/CIZ along the human *MMP-13* promoter in human chondrocytes and implicated as a potential transcription modulator [Fan et al., 2006]. Exposing osteoblasts to FSS for 5–20 min had no consistent effect on tyrosine phosphorylation of p130^{cas}, however we did observe that FSS induced a nuclear translocation of p130^{cas} in these cells. MC3T3-E1 osteoblast-like cells were subjected to 5 h of FSS or static conditions. Isolated nuclear and cytoplasmic subfractions of these cells were analyzed by Western blotting for p130^{cas} (Fig. 6A). In the cells maintained in static culture the p130^{cas} was primarily localized in cytoplasmic fraction, and the ratio of nuclear/cytoplasmic p130^{cas} was 0.64 (Fig. 6A). Interestingly, FSS induced a significant increase in the amount of p130^{cas} in the nuclear subfraction and the nuclear/cytoplasmic ratio increased significantly to 1.58 ($P < 0.03$, Fig. 6A). To confirm this observation we used a second approach, harvesting the cytoplasmic, membrane, and nuclear fractions via a differential detergent fractionation protocol [Ramsby et al.,

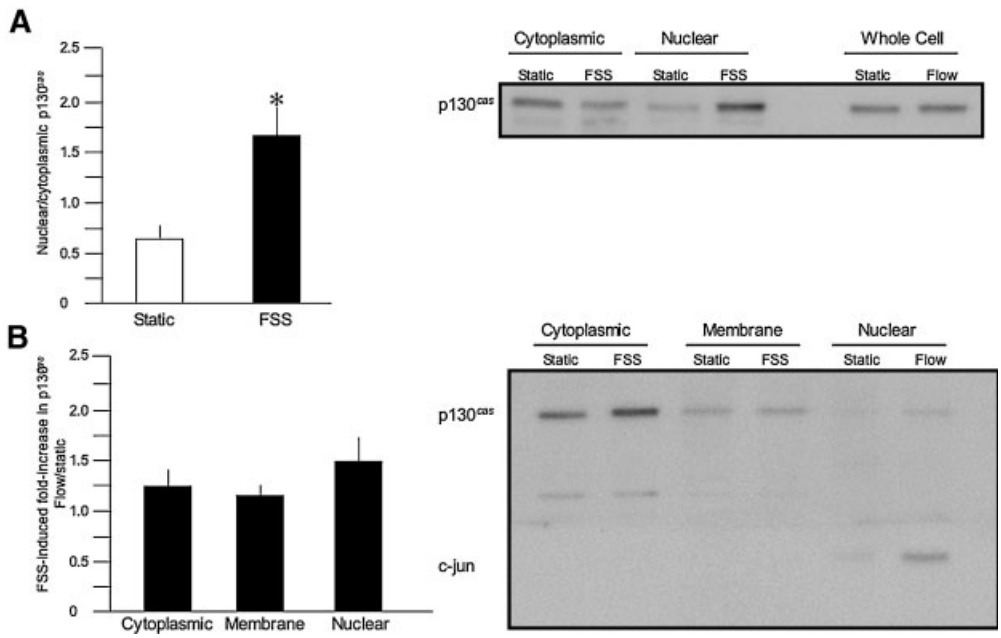

Fig. 6. FSS increases the presence of p130^{cas} in the nucleus. **A**: MC3T3-E1 cells exposed to static or FSS for 5 h show on average a shift in the nuclear/cytoplasmic ratio from ~0.6 to 1.6 (167%), $P < 0.03$ (average ± SE; static 0.64 ± 0.14, flow 1.58 ± 0.31, n = 5, asterisk $P < 0.05$). **B**: The observed increase in the presence of p130^{cas} in the nucleus persists using a differential detergent fractionation protocol comparing the fraction (cytoplasmic, membrane, nuclear) flow/static values (average ± SE; cytoplasmic 1.22 ± 0.20, membrane 1.16 ± 0.13, nuclear 1.49 ± 0.25, n = 4, NS). We also evaluated the presence of c-jun in each of these fractions as a means to assess cross-contamination by nuclear proteins.

1994]. The flow/static ratios of the three fractions did not differ statistically, however, the nuclear flow/static value was larger than the cytoplasmic and membrane fraction values consistent with an FSS-induced movement of $p130^{cas}$ into the nucleus (Fig. 6B). We also evaluated the presence of c-jun in each of these fractions as a means to assess cross-contamination by nuclear proteins. No cross-contamination was observed, however we did detect an increase in c-jun expression in the nuclear fraction with FSS (Fig. 6B). As expected this limited but significant FSS-induced $p130^{cas}$ nuclear translocation could not be consistently observed using immunocytochemical analysis.

DISCUSSION

The present data show that Nmp4/CIZ expression and DNA-binding activity are regulated by FSS and suggest that Nmp4/CIZ represses FSS induction of *MMP-13* transcription, consistent with the currently-held view that Nmp4/CIZ generally acts to suppress induction of osteoblast genes [Shen et al., 2002; Shah et al., 2004; Morinobu et al., 2005]. EMSA revealed that FSS attenuates Nmp4/CIZ DNA-binding along the *MMP-13* 5′ regulatory region (−119 nt/−110 nt) suggesting that *MMP-13* upregulation requires loss of Nmp4/CIZ association at this site. That Nmp4/CIZ binding to the *MMP-13* promoter may act as a suppressor of *MMP-13* expression is in accord with our previous study showing that Nmp4/CIZ represses *MMP-13* response to PTH [Shah et al., 2004]. Furthermore, Nmp4/CIZ suppresses BMP2-enhanced expression of *Runx2*, *alkaline phosphatase*, *osteocalcin*, *and type I collagen* genes in MC3T3-E1 cells [Shen et al., 2002]. Similarly, CIZ-knockout mice show enhanced BMP2-induced bone formation [Morinobu et al., 2005]. As to the manner of load application, the present data reveal that unidirectional FSS (12 dynes/cm^2) enhances *MMP-13* transcription and subsequent mRNA expression in MC3T3-E1 to the same degree as has been reported using a supraphysiological 8% biaxial stretch of these cells [Yang et al., 2004]. Although oscillatory shear stress might be a more accurate characterization of the canalicular fluid dynamics in vivo [Kim et al., 2006], we have previously reported no differences in gene expression profiles between the two fluid-shear models [Ponik et al., 2006].

Paradoxically, mutating the Nmp4/CIZ consensus sequence within the *MMP-13* gene, which abrogates Nmp4/CIZ binding, does not further enhance the FSS response. Rather, the mutation abolishes induction altogether, likely a reflection of distinct functional roles for trans-acting protein and cis-element and the functional interdependence between the multiple proximal cis-elements in the region of this gene. The Nmp4/CIZ element resides in a 110 bp region within the first 140 nucleotides of the 5′ regulatory region of the *MMP-13* gene. This region is comprised of multiple positive and negative regulatory elements that regulate PTH induction via combinatorial interactions [Selvamurugan et al., 1998; Hess et al., 2001; Shah et al., 2004, also see Fig. 3]. For example, an earlier study has determined that individually mutating either the AP-1 element (−62/−55 nt) or the distal Runx2 consensus element (−132/−126) (see Fig. 3) does not influence promoter activity in osteoblast-like cells. However, mutation of both elements abolishes both basal and PTH-induced transcription [Selvamurugan et al., 1998]. The helical arrangement of the multiple cis-elements within this region of the *MMP-13* gene is critical to the mechanism of PTH response [Selvamurugan et al., 1998; Hess et al., 2001; D'Alonzo et al., 2002]. Therefore, mutating the AT-rich Nmp4/CIZ element may negatively impact the helical character or some other structural feature of this region necessary for transcription regulation. As with previous investigations focused on interactions between these cis-elements and their cognate trans-acting proteins [Selvamurugan et al., 1998; Hess et al., 2001; D'Alonzo et al., 2002], similar experiments will be necessary to reveal the complete mechanism driving FSS induction of *MMP-13*.

Our data add to the growing body of evidence that mechanical force and PTH signal-to-gene response mechanisms overlap in the osteoblast [Ryder and Duncan, 2000; Li et al., 2003] but we also observed that the PTH- and FSS-induced nuclear events mediating osteoblast *MMP-13* induction are only partially coincident. PTH and FSS both target the 110 bp region (−132 nt/−32 nt) of the 5′ *MMP-13* promoter. However, PTH and FSS have opposing effects on Nmp4/CIZ DNA binding within −119 nt/−110 nt [Shah et al., 2004; this work]. We are currently addressing whether there are other differences between PTH-responsive and FSS-responsive

transcription mechanisms within this or other regions of the *MMP-13* gene.

Interestingly, of the two murine Nmp4/CIZ promoters (P_1 and P_2) that drive the expression of multiple Nmp4/CIZ isoforms [Alvarez et al., 2005], only one promoter is responsive to FSS (Fig. 5). These results suggest that a specific Nmp4/CIZ isoform or subset of isoforms is involved in mediating the osteoblast response to mechanical load. Our data show that promoter P_2 exhibits a modest but significant increase in activity upon challenge with FSS, whereas promoter P_1 is refractive to this agonist. This is strikingly different from our previous observations demonstrating that both promoters are positively autoregulated and both negatively regulated by PTH [Alvarez et al., 2005]. The two Nmp4/CIZ promoters comprise a genomic regulatory architecture that supports constitutive expression, as well as cell- and tissue-specific regulation. These observations are consistent with their generalized housekeeping function in addition to their tissue-specific roles in bone, blood, and testis [Martini et al., 2002; Nakamoto et al., 2004; Alvarez et al., 2005]. We are presently screening for this putative load-responsive Nmp4/CIZ isoform.

Our observation that FSS induces $p130^{cas}$ nuclear translocation is significant. Nmp4/CIZ physically associates with $p130^{cas}$ [Nakamoto et al., 2000] and this adaptor protein is mechano-sensitive [Okuda et al., 1999; Zaidel-Bar et al., 2005; Geiger, 2006; Sawada et al., 2006]. This provides a potential direct pathway for transferring mechanical information from the focal adhesions to the target genes by modulating Nmp4/CIZ transcription activity via $p130^{cas}$ nuclear translocation [Pavalko et al., 2003b]. This is consistent with a previous study showing an association between Nmp4/CIZ and $p130^{cas}$ along the *MMP-13* promoter in human chondrocytes [Fan et al., 2006]. EMSA of nuclear extracts from human chondrocytes showed the presence of Nmp4/CIZ and $p130^{cas}$ along the AG-rich element (AGRE) regulatory site (−128/−117 nt) of the human *MMP-13* gene [Fan et al., 2006]. Nmp4/CIZ and $p130^{cas}$ appear to regulate changes in *MMP-13* expression in human chondrocytes during the transition from a low osteoarthritic state to a high osteoarthritic state and $p130^{cas}$ may act as a transcription modulator [Fan et al., 2006]. In our study, however, EMSA revealed no association of this adaptor protein within the region of the Nmp4/CIZ cis-element (−137/−107 nt) of the *MMP-13* gene in cells that had been challenged with FSS and this is not surprising since Nmp4/CIZ binding was abolished with exposure to shear stress (Fig. 3). There may be an induced nuclear $p130^{cas}$–Nmp4/CIZ interaction in the early response to FSS resulting in the abrogation of Nmp4/CIZ DNA-binding and we are investigating this possibility. Consistent with this hypothesis is the observation that nuclear $p130^{cas}$ transiently concentrates in distinct speckles with two proteins, cell adhesion kinase β/proline-rich tyrosine kinase-2 (CAKβ/PYK2) and the transcriptional coactivator Hic-5 [Aoto et al., 2002].

The interaction of Nmp4/CIZ and its cis-element with the nuclear matrix [Alvarez et al., 1998] may play a role in *MMP-13* transcription response to FSS and PTH. Consistent with this hypothesis is our observation that the stably transfected *MMP-13* promoter-reporter constructs exhibited an attenuated response to FSS, perhaps the result of less than optimal chromatin integration. A similar phenomenon was observed in Sp1 regulation of the alpha-globin promoter [Pondel et al., 1995]. The association of transcriptional complexes to specific regions of the genome via interactions with the nuclear matrix is a critical aspect of gene expression [Stein et al., 2004; Tao et al., 2006 and references therein].

Mechanosomes are postulated multiprotein complexes comprised of focal adhesion, adherens junction, and nucleocytoplasmic shuttling DNA-binding proteins that transfer mechanical information from the osteoblast plasma membrane to target genes [Pavalko et al., 2003b]. The expanding list of proteins capable of translocating from the focal adhesions to the nucleus [Robinson et al., 2003; Shibanuma et al., 2003; Cattaruzza et al., 2004; Woods et al., 2005] strengthens the rationale for the authenticity of this aspect of mechanotransduction. Whether Nmp4/CIZ and $p130^{cas}$ are components of the same or distinct translocation complexes during FSS challenge is under study. Additionally, elucidating the relationship between PTH and mechanotransduction signaling pathways in the osteoblast has potential clinical significance for the treatment of bone disease; the overlap and divergence of these signaling mechanisms may direct therapeutic approaches for inducing anabolic bone growth [Sawakami et al., 2006].

ACKNOWLEDGMENTS

This study was supported by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases, NIH NIH-NIDDK 2 R01 DK053796-05A2 (JPB).

REFERENCES

Alvarez M, Shah R, Rhodes SJ, Bidwell JP. 2005. Two promoters control the mouse Nmp4/CIZ transcription factor gene. Gene 347:43–54.

Alvarez M, Thunyakitpisal P, Morrison P, Onyia J, Hock JM, Bidwell JP. 1998. A PTH-responsive osteoblast nuclear matrix architectural transcription factor binds to the rat type I collagen promoter. J Cell Biochem 69: 336–352.

Andrews NC, Faller DV. 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucl Acids Res 19:2499.

Aoto H, Sasaki H, Ishino M, Sasaki T. 2002. Nuclear translocation of cell adhesion kinase beta/proline-rich tyrosine kinase 2. Cell Struct Funct 27:47–61.

Basso N, Heersche JN. 2002. Characteristics of in vitro osteoblastic cell loading models. Bone 30:347–351.

Cattaruzza M, Lattrich C, Hecker M. 2004. Focal adhesion protein zyxin is a mechanosensitive modulator of gene expression in vascular smooth muscle cells. Hypertension 43:726–730.

Cowin SC. 2002. Mechanosensation and fluid transport in living bone. J Musculoskelet Neuronal Interact 2:256–260.

D'Alonzo RC, Selvamurugan N, Karsenty G, Partridge NC. 2002. Physical interaction of the activator protein-1 factors c-Fos and c-Jun with Cbfa1 for collagenase-3 promoter activation. J Biol Chem 277:816–822.

Ehrlich PJ, Lanyon LE. 2002. Mechanical strain and bone cell function: A review. Osteoporos Int 13:688–700.

Fan Z, Tardif G, Boileau C, Bidwell JP, Geng C, Hum D, Watson A, Pelletier JP, Lavigne M, Martel-Pelletier J. 2006. Identification in human osteoarthritic chondrocytes of proteins binding to the novel regulatory site AGRE in the human matrix metalloprotease 13 proximal promoter. Arthritis Rheum 54:2471–2480.

Frangos JA, McIntire LV, Eskin SG. 1988. Shear stress induced stimulation of mammalian cell metabolism. Biotechnol Bioeng 32:1053–1060.

Geiger B. 2006. A role for p130Cas in mechanotransduction. Cell 127:879–881.

Hess J, Porte D, Munz C, Angel P. 2001. AP-1 and Cbfa/runt physically interact and regulate parathyroid hormone-dependent MMP13 expression in osteoblasts through a new osteoblast-specific element 2/AP-1 composite element. J Biol Chem 276:20029–20038.

Iqbal J, Zaidi M. 2005. Molecular regulation of mechanotransduction. Biochem Biophys Res Commun 328:751–755.

Kim CH, You L, Yellowley CE, Jacobs CR. 2006. Oscillatory fluid flow-induced shear stress decreases osteoclastogenesis through RANKL and OPG signaling. Bone 39: 1043–1047.

Kurz B, Lemke AK, Fay J, Pufe T, Grodzinsky AJ, Schunke M. 2005. Pathomechanisms of cartilage destruction by mechanical injury. Ann Anat 187(5–6): 473–485.

Li J, Duncan RL, Burr DB, Gattone VH, Turner CH. 2003. Parathyroid hormone enhances mechanically induced bone formation, possibly involving L-type voltage-sensitive calcium channels. Endocrinology 144:1226–1233.

Li J, Liu D, Ke HZ, Duncan RL, Turner CH. 2005. The P2X7 nucleotide receptor mediates skeletal mechanotransduction. J Biol Chem 280:42952–42959.

MacLean JJ, Lee CR, Alini M, Iatridis JC. 2005. The effects of short-term load duration on anabolic and catabolic gene expression in the rat tail intervertebral disc. J Orthop Res 23:1120–1127.

Martini A, La Starza R, Janssen H, Bilhou-Nabera C, Corveleyn A, Somers R, Aventin A, Foa R, Hagemeijer A, Mecucci C, Marynen P. 2002. Recurrent rearrangement of the Ewing's sarcoma gene, EWSR1, or its homologue, TAF15, with the transcription factor CIZ/NMP4 in acute leukemia. Cancer Res 62:5408–5412.

Morinobu M, Nakamoto T, Hino K, Tsuji K, Shen ZJ, Nakashima K, Nifuji A, Yamamoto H, Hirai H, Noda M. 2005. The nucleocytoplasmic shuttling protein CIZ reduces adult bone mass by inhibiting bone morphogenetic protein-induced bone formation. J Exp Med 201: 961–970.

Mullender M, El Haj AJ, Yang Y, van Duin MA, Burger EH, Klein-Nulend J. 2004. Mechanotransduction of bone cells in vitro: Mechanobiology of bone tissue. Med Biol Eng Comput 42:14–21.

Nakamoto T, Shiratsuchi A, Oda H, Inoue K, Matsumura T, Ichikawa M, Saito T, Seo S, Maki K, Asai T, Suzuki T, Hangaishi A, Yamagata T, Aizawa S, Noda M, Nakanishi Y, Hirai H. 2004. Impaired spermatogenesis and male fertility defects in CIZ/Nmp4-disrupted mice. Genes Cells 9:575–589.

Nakamoto T, Yamagata T, Sakai R, Ogawa S, Honda H, Ueno H, Hirano N, Yazaki Y, Hirai H. 2000. CIZ, a zinc finger protein that interacts with p130(cas) and activates the expression of matrix metalloproteinases. Mol Cell Biol 20:1649–1658.

Okuda M, Takahashi M, Suero J, Murry CE, Traub O, Kawakatsu H, Berk BC. 1999. Shear stress stimulation of p130(cas) tyrosine phosphorylation requires calcium-dependent c-Src activation. J Biol Chem 274:26803–26809.

Owan I, Burr DB, Turner CH, Qiu J, Tu Y, Onyia JE, Duncan RL. 1997. Mechanotransduction in bone: Osteoblasts are more responsive to fluid forces than mechanical strain. Am J Physiol 273:C810–C815.

Pavalko FM, Gerard RL, Ponik SM, Gallagher PJ, Jin Y, Norvell SM. 2003a. Fluid shear stress inhibits TNF-alpha-induced apoptosis in osteoblasts: A role for fluid shear stress-induced activation of PI3-kinase and inhibition of caspase-3. J Cell Physiol 194(2):194–205.

Pavalko FM, Norvell SM, Burr DB, Turner CH, Duncan RL, Bidwell JP. 2003b. A model for mechanotransduction in bone cells: The load-bearing mechanosomes. J Cell Biochem 88:104–112.

Pondel MD, Murphy S, Pearson L, Craddock C, Proudfoot NJ. 1995. Sp1 functions in a chromatin-dependent manner to augment human alpha-globin promoter activity. Proc Natl Acad Sci USA 92:7237–7241.

Ponik SM, Triplett JW, Pavalko FM. 2006. Osteoblasts and osteocytes respond differently to oscillatory and unidirectional fluid flow profiles. J Cell Biochem [Epub ahead of print]

Quinn CO, Scott DK, Brinckerhoff CE, Matrisian LM, Jeffrey JJ, Partridge NC. 1990. Rat collagenase: Cloning, amino acid sequence comparison, and parathyroid hormone regulation in osteoblastic cells. J Biol Chem 265: 22342–22347.

Ramsby ML, Makowski GS, Khairallah EA. 1994. Differential detergent fractionation of isolated hepatocytes: Biochemical, immunochemical and two-dimensional gel electrophoresis characterization of cytoskeletal and noncytoskeletal compartments. Electrophoresis 15:265–277.

Rajakumar RA, Quinn CO. 1996. Parathyroid hormone induction of rat interstitial collagenase mRNA in osteosarcoma cells is mediated through an AP-1 binding site. Mol Endocrinol 10:867–878.

Robinson PA, Brown S, McGrath MJ, Coghill ID, Gurung R, Mitchell CA. 2003. Skeletal muscle LIM protein 1 regulates integrin-mediated myoblast adhesion, spreading, and migration. Am J Physiol Cell Physiol 284:C681–C695.

Ryder KD, Duncan RL. 2000. Parathyroid hormone modulates the response of osteoblast-like cells to mechanical stimulation. Calcif Tissue Int 67:241–246.

Sawada Y, Tamada M, Dubin-Thaler BJ, Cherniavskaya O, Sakai R, Tanaka S, Sheetz MP. 2006. Force sensing by mechanical extension of the Src family kinase substrate p130Cas. Cell 127:1015–1026.

Sawakami K, Robling AG, Ai M, Pitner ND, Liu D, Warden SJ, Li J, Maye P, Rowe DW, Duncan RL, Warman ML, Turner CH. 2006. The Wnt co-receptor LRP5 is essential for skeletal mechanotransduction but not for the anabolic bone response to parathyroid hormone treatment. J Biol Chem 281:23698–23711.

Schlaepfer DD, Broome MA, Hunter T. 1997. Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: Involvement of the Grb2, p130cas, and Nck adaptor proteins. Mol Cell Biol 17:1702–1713.

Selvamurugan N, Chou WY, Pearman AT, Pulumati MR, Partridge NC. 1998. Parathyroid hormone regulates the rat collagenase-3 promoter in osteoblastic cells through the cooperative interaction of the activator protein-1 site and the runt domain binding sequence. J Biol Chem 273:10647–10657.

Shah R, Alvarez M, Jones DR, Torrungruang K, Watt AJ, Selvamurugan N, Partridge NC, Quinn CO, Pavalko FM, Rhodes SJ, Bidwell JP. 2004. Nmp4/CIZ regulation of matrix metalloproteinase 13 (MMP-13) response to parathyroid hormone in osteoblasts. Am J Physiol Endocrinol Metab 287:E289–E296.

Shen ZJ, Nakamoto T, Tsuji K, Nifuji A, Miyazono K, Komori T, Hirai H, Noda M. 2002. Negative regulation of bone morphogenetic protein/Smad signaling by Cas-interacting zinc finger protein in osteoblasts. J Biol Chem 277:29840–29846.

Shibanuma M, Kim-Kaneyama JR, Ishino K, Sakamoto N, Hishiki T, Yamaguchi K, Mori K, Mashimo J, Nose K. 2003. Hic-5 communicates between focal adhesions and the nucleus through oxidant-sensitive nuclear export signal. Mol Biol Cell 14:1158–1171.

Stein GS, Lian JB, van Wijnen AJ, Stein JL, Javed A, Montecino M, Zaidi SK, Young D, Choi JY, Gutierrez S, Pockwinse S. 2004. Nuclear microenvironments support assembly and organization of the transcriptional regulatory machinery for cell proliferation and differentiation. J Cell Biochem 91:287–302.

Sun HB, Nalim R, Yokota H. 2003. Expression and activities of matrix metalloproteinases under oscillatory shear in IL-1-stimulated synovial cells. Connect Tissue Res 44:42–49.

Sun HB, Yokota H. 2002. Reduction of cytokine-induced expression and activity of MMP-1 and MMP-13 by mechanical strain in MH7A rheumatoid synovial cells. Matrix Biol 21:263–270.

Sun HB, Yokota H. 2001. Altered mRNA level of matrix metalloproteinase-13 in MH7A synovial cells under mechanical loading and unloading. Bone 28:399–403.

Tao Y, Kupfer R, Stewart BJ, Williams-Skipp C, Crowell CK, Patel DD, Sain S, Scheinman RI. 2006. AIRE recruits multiple transcriptional components to specific genomic regions through tethering to nuclear matrix. Mol Immunol 43:335–345.

Torrungruang K, Shah R, Alvarez M, Bowen DK, Gerard R, Pavalko FM, Elmendorf JS, Charoonpatrapong K, Hock J, Rhodes SJ, Bidwell JP. 2002. Osteoblast intracellular localization of Nmp4 proteins. Bone 30:931–936.

Turner CE. 2000. Paxillin interactions. J Cell Sci 113(Pt 23):4139–4140.

Turner CH, Robling AG. 2005. Mechanisms by which exercise improves bone strength. J Bone Miner Metab 23(Suppl):16–22.

Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, Horwitz AF. 2004. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol 6:154–161.

Weinbaum S, Cowin SC, Zeng Y. 1994. A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses. J Biomech 27:339–360.

Woods AJ, Kantidakis T, Sabe H, Critchley DR, Norman JC. 2005. Interaction of paxillin with poly(A)-binding protein 1 and its role in focal adhesion turnover and cell migration. Mol Cell Biol 25:3763–3773.

Wu W, Mwale F, Tchetina E, Kojima T, Yasuda T, Poole AR. 2001. Cartilage matrix resorption in skeletogenesis. Novartis Found Symp 232:158–170.

Yang CM, Chien CS, Yao CC, Hsiao LD, Huang YC, Wu CB. 2004. Mechanical strain induces collagenase-3 (MMP-13) expression in MC3T3-E1 osteoblastic cells. J Biol Chem 279:22158–22165.

Zaidel-Bar R, Kam Z, Geiger B. 2005. Polarized down-regulation of the paxillin-p130CAS-Rac1 pathway induced by shear flow. J Cell Sci 118(Pt 17):3997–4007.