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Nuclear factor of activated T cells mediates fluid shear stress-and tensile strain- induced Cox2 in human and murine bone cells

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Abstract

Mechanical loading such as interstitial fluid shear stress and tensile strain stimulates bone cells, which respond by changing bone mass and structure to maintain optimal skeletal architecture. Bone cells also adapt to bone implants and altered mechanical loading. Osseous integration between host bone and implants is a prerequisite for the stability of implants. Fluctuating fluid pressure and interfacial strains occur between bone cells and implants due to mechanical loading during walking and other daily activities. In this study, we examined the signaling mechanism by which mechanical stimulation activates a novel transcription factor in human and mouse bone cells. Nuclear factor of activated T cells (NFAT) is one of the transcription factors that act downstream of the Ca^{++} /Calcineurin (Ca^{++} /Cn) network: a well-known pathway of inflammation. In this study, we hypothesized that NFAT2 is activated in response to mechanical stimulation and mediates Cox2 expression. Fluid shear stress and tensile strain results in nuclear translocation of NFAT in cells of the osteoblastic lineage. A peptide inhibitor of the Cn/NFAT axis was found to block the mechanical stimulation-mediated Cox2 induction. Further, chromatin immunoprecipitation assay shows direct interaction between NFAT2 and the human Cox2 promoter region. Additionally, CnA β knockout calvarial bone cells were found to be less sensitive than control bone cells to mechanical stimulation. Our study provides new evidence for a novel role for NFAT in bone mechanotransduction in the context of cytokine gene induction in bone cells.

Keywords

NFAT; fluid shear stress; mechanical stimulation; Cox2; osteoblast

Introduction

Bone is a complex and dynamic tissue that is able to regulate its own mass and architecture. Its metabolic demands are managed primarily through hormones e.g. calcitonin, Vitamin D, and PTH. To maintain its structural and biomechanical integrity, bone adapts to its environment

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[1]. The bone remodeling process is a result of the coordinated activity of osteoblasts, which make new bone, and osteoclasts, which resorb bone. When bone cells sense alterations in mechanical load, bone mass and structure are modified to respond to this change and maintain optimal skeletal architecture [2]. Mechanical loading at the physiologic level can stimulate a net increase in bone mass *in vivo* [3,4], whereas prolonged absence of loading as in the case of extended bed rest or weightlessness can result in bone loss [5].

Mechanical loading is known to elicit a response from bone cells, where deformation of skeletal tissue generates substrate strains that drive the oscillatory movement of interstitial fluid. Bone cells respond to interstitial fluid flow inside the canalicular-lacunar networks, and trabecular spaces within bone tissue by increasing bone formation [6]. Several studies have been reported on the effects of oscillatory fluid flow on osteoblastic cells and osteocytic cells to include intracellular calcium transport, prostaglandin E2 release, increased osteopontin gene expression, increased MAPK activity, and inhibition of NFκB binding [7–9]. Studies have also shown that mechanical unloading leads to increased bone resorption, decreased bone mineral density, and decreased bone formation [8,10,11].

In addition to external loading, stimuli from the interfacial mechanical environment between host bone and implants also plays a crucial role in the long-term clinical success of joint replacement arthroplasties. Altered mechanical stimulation exerts immediate effects on periprosthetic bone mass. Fluid flow in the effective joint space disseminates wear particle debris, and fluctuating mechanical stimulation caused by hip joint motion and ambulation contribute to implant failure [12–14]. Clinical studies suggest that mechanical stimulation may initiate periprosthetic bone loss prior to wear particle generation, and the instability associated with migration may cause locally high fluid pressure surrounding the prosthesis [15–17]. In addition, rat and rabbit experimental models support a role for oscillatory fluid pressure in the initiation of periprosthetic bone loss [15,18].

In progression to implant failure and periprosthetic bone loss, host-inflammatory reaction to interfacial motion may be regarded as a host-immune response. Factors that mediate the host-inflammatory reaction may contribute to periprosthetic bone loss. Although mechanical stimuli are known to activate calcium signaling and cytokine secretion in osteoblasts, the mechanisms by which transcriptional factors transactivate target cytokine gene promoter regions have not been well elucidated. One such transcription factor is the nuclear factor of activated T cells (NFAT) in immune cells [19,20]. Initially identified in T cells, the role of NFAT in other cell types is emerging [21,22]. This family of transcription factors includes five members. NFAT1, NFAT2, NFAT3, and NFAT4 are under the regulation of the calcium/calcineurin (Ca^{++}/Cn) pathway, and are collectively called NFATc. NFAT5 responds to osmotic pressure changes [23]. NFAT proteins exist in a phosphorylated form in the cytoplasm, but translocate to the nucleus upon dephosphorylation by Cn and bind to their consensus sequence on the promoter of several cytokines. Deletion of NFAT2 results in embryonic death, while NFAT1/NFAT2 deletion results in anergy [24].

The functional role of NFAT2 in osteoblasts and osteoclasts is recently being explored. NFAT2 is expressed in osteoclast precursors and osteoblasts. NFAT2 is also essential in RANKL supported osteoclastogenesis and its overexpression can result in osteoclast formation in the absence of RANKL [25,26]. NFAT2 may also regulate osterix transcriptional activity [27]. In addition to its functional role in bone cells, NFAT has an important role in mechano-sensing of cardiac myocytes [28,29] as a downstream signaling component of the Ca^{++}/Cn axis. Mechanical stimulation elevates intracellular calcium in osteoblasts and the Cn signaling axis acts downstream, thus making the Cn/NFAT2 network a compelling candidate for mechanotransduction in osteoblasts. The role of the Cn/NFAT axis in bone cells is also clinically relevant because cyclosporine A, an inhibitor which blocks the binding of Cn to

NFAT, is commonly used for immunosuppression in patients with allogenic organ transplantation. Further, the Cox2 gene promoter is known to have the consensus sequence – CGAAA–, which is a binding site for NFAT. We hypothesize that the NFAT signaling mediates Cox2 in response to mechanical stimuli in osteoblasts.

The goal of this study was to show that NFAT2 is a mediator of mechanotransduction in human and mouse bone cells and that clinically relevant mechanical stimulation activates NFAT2, and induces Cox2 expression. Furthermore, we show that NFAT2 directly binds the Cox2 promoter and mechanically-induced Cox2 is inhibited upon blocking NFAT signaling with a synthetic peptide (VIVIT). These results suggest a novel role for NFAT in mechanobiology of bone cells.

Materials and Methods

Preparation of cells

Human mesenchymal stem cells (hMSC) (Cambrex Inc., Walkersville, MD) were maintained according to the manufacturer's recommendations. CnA β –/– mice [30] were kindly provided by Dr. Molkenkin, University of Cincinnati (Cincinnati, OH). Primary osteoblast cells were isolated as previously described [31]. Briefly, parietal bones of calvaria were isolated from 4-day-old CnA β +/+, +/- or –/– mice and then the parietal bones were cut into small pieces and treated with 2 mg/ml of collagenase (Sigma-Aldrich, St. Louis, MO) and cultured in MEM α supplemented with 10% FBS and 1% antibiotic/antimycotic solution.

Mechanical Loading

Two methods of clinically relevant mechanical loading were used in the studies: cyclical tensile strain and sinusoidal fluid shear stress (FSS). The cells were cultured on 6-well plates with a silicone elastomer membrane (Flexcell Inc., Hillsborough, NC) for tensile loading. Cells were subjected to physiologic and superphysiologic sinusoidal (0.5 and 5%, respectively) tensile stretching at 1Hz for 15 min (FX4000T, Flexcell Inc.). For physiologic and superphysiologic FSS, the cells were cultured on type-I collagen-coated glass slides (Flexcell Inc., Hillsborough, NC) and loaded at 16 dyn/cm² (1.6Pa) at 1Hz [32] and/or 1.03*10⁶ dyn/cm² (103 kPa) hydrostatic pressure for 15min in parallel plate fluid flow chamber. The sinusoidal flow profile was generated by a Masterflex L/S 7550-30 computerized pump (Cole-Parmer, Vernon Hills, IL) controlled by a custom-written LabVIEW program (National Instruments Corporation, Austin, TX). The sinusoidal fluid flow waveform had an accuracy of ± 0.4 dyn/cm² as determined by a TS410 Flowmeter with a ME4PXN201 flowsensor (Transonic Systems Inc., Ithaca, NY). For superphysiologic stress, the hydrostatic pressure was applied via a pressurized reservoir connected in parallel with the tubing from the peristaltic pump and flow chamber. The reservoir was kept approximately half-full with 200 ml of sterile media. Laboratory compressed air at approximately 50 psig was passed through a two-stage 50 SCFM extractor/dryer with a 5 micron filter to remove up to 99.9% of any in-line oil or water (Model 105, La-Man, Port Orange, FL), and then regulated by a precision pressure regulator (Type 700 Pressure Regulator, Control Air Inc, Amherst, NH, rated accuracy ± 0.03 psig). The hydrostatic pressure level was monitored with a 0 – 30 psig precision pressure gauge (WIKA Instrument Corporation, Lawrenceville, GA) with resolution of 0.5 psig and a manufacturer-rated accuracy of ± 0.3 psig. An inline HepaVent # L0650 HEPA filter (Whatman, GE Healthcare, Piscataway, NJ) was placed downstream of the pressure regulator to ensure a sterile airstream. The loading regimens used in this study represent normal physiologic and superphysiologic mechanical loading, which are observed in the periprosthetic interface between host bone and the implant [33–35]. Control sham cells were cultured under the same conditions with the omission of mechanical stimulation. For NFAT2 inhibition, cells were treated overnight with .5 μ M VIVIT peptide (Calbiochem, San Diego, CA) or control VEET peptide (Rockefeller Proteomics

Center, New York). VIVIT is a cell-permeable peptide inhibitor of NFAT (RRRRRRRRRRR-GGG-MAGPVIVITGPHEE), which does not inhibit Cn activity and VEET is the control peptide for VIVIT (RRRRRRRRRRR-GGG-MAGPPHIVEETGPHVI) [36,37].

Gene expression

RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and cDNA was synthesized with the Superscript III kit from Invitrogen (Carlsbad, CA). Real-time PCR (RT-PCR) reactions were conducted with gene specific primers using the Roche Lightcycler Fast Start DNA Master^{Plus} SYBR Green kit (Roche Applied Sciences, Indianapolis, IN). An Eppendorf Realplex⁴ system (Hamburg, Germany) was used for the RT-PCR reactions. The primers used were as follows: human Cox2 forward 5'-GAATGTTCCACCCGCAGTACA-3'; human Cox2 reverse 5'-GCATAAAGCGTTTGCAGTAC-3'; human GAPDH forward 5'-GAAGGTGAAGGTCGGAGTC-3'; human GAPDH reverse 5'-GAAGATGGTGATGGGATTTC-3'; human Alp forward 5'-CCGTGGCAACTCTATCTTTGG-3'; human Alp reverse 5'-GCCATACAGGATGGCAGTGA-3'; human Opn forward 5'-ACATGGAAAGCGAGGAGTTGA-3'; human Opn reverse 5'-CAATCAGAAGGCGCGTTCA-3'; mouse Cox2 forward 5'-ACATCGATGTGATGGAAGTCA-3'; mouse Cox2 reverse 5'-GGACACCCCTTCACATTATT-3'; mouse GAPDH forward 5'-AGAACATCATCCCTGCATCC-3'; mouse GAPDH reverse 5'-AGTTGCTGTTGACGTCGC-3'.

NFAT2 Immunocytochemistry

Immunocytochemistry can visualize NFAT2 in the cytoplasm and in the nucleus. Slides were fixed with 4% paraformaldehyde and ethanol, rinsed with PBS and air-dried overnight for immunocytochemistry. The slides were incubated in a blocking solution containing 5% mouse serum, washed and incubated with NFAT2 antibody (Abcam, Cambridge, MA) overnight at 4°C. The slides were then washed and incubated with a secondary antibody Alexa 488 (Invitrogen, Carlsbad, CA) for 1hr and visualized using the Axiovert imaging system (Zeiss, Thornwood, NY). Images were analyzed with NIH Image J program[38].

NFAT2 activity assay

For NFAT2 activation assays, nuclear extracts were collected with a Nuclear Extraction Kit (Active Motif, Carlsbad, CA), total protein concentration was determined (Prostain kit, Active Motif) and 2 µg of nuclear extract from control, and treatment groups were incubated on a 96-well plate containing the immobilized NFAT consensus sequence (Trans AM kit, Active Motif, Carlsbad, CA). The wells were washed and incubated with NFAT2 antibody, followed by HRP-conjugated anti-mouse antibody for colorimetric detection of the active NFAT2 (nuclear) bound to its consensus sequence. The assay included a positive control of nuclear extracts from Jurkat cells (PHA treated).

ALP stain

Slides were seeded with hMSC (200,000 cells/slide) and cultured for 6 days in osteogenic differentiation medium (Cambrex Inc., Walkersville, MD) prior to mechanical stimulation. The slides were prepared and stained with the Sigma alkaline phosphatase kit (Sigma, St. Louis, MO) according to the manufacturer's guidelines.

Western blot

Whole cell lysates were run on SDS-PAGE and incubated with the Cox2 primary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) followed by anti-goat HRP-conjugated

antibody. The membranes were stripped and reprobed with GAPDH antibody (Chemicon, Billerica, MA), followed by anti-mouse HRP conjugated antibody. Images were analyzed with NIH Image J program.

Chromatin Immunoprecipitation (ChIP) Assay

Soluble chromatin was extracted following the manufacturer's instructions (Upstate Biotechnologies, Billerica, MA). The cells were fixed with 1% formaldehyde, resuspended with 1% SDS lysis buffer including protease inhibitor and 1mM PMSF, and then sonicated 3 times each 10 seconds at 30% duty cycle on a Bransson Sonifier 250 (Bransson, Danbury, CT). The samples were centrifuged for 10 min at 13,000 rpm at 4°C. 200µl of the sonicated cell supernatant was transferred to a new 2mL tube. The supernatants were precleared with salmon sperm DNA/Protein A Agarose-50% slurry. The precleared 2mL of supernatant solution was incubated with anti-mouse NFAT2 antibody (Abcam, Cambridge, MA) overnight at 4°C with constant agitation. Immunoprecipitated complexes were treated with salmon sperm DNA/Protein A Agarose-50% slurry treatment for 1 h at 4°C with rotation. The histone-DNA complex was eluted with buffer containing 1% SDS and 0.1M NaHCO₃. Histone-DNA crosslinks were reversed and DNA was recovered by phenol/chloroform extraction, precipitated by ethanol, and resuspended with 30 µl Tris-EDTA. 2 µl of recovered DNA was used as a template for PCR with Cox2- promoter specific primers designed for the region (-904 to -641) of Cox2 promoter: sense: 5'-CCTGCAAATTCTGGCCATCG-3'; antisense: 5'-AAAACCAAGCCCATGTGACG-3'. Negative antibody control is incubation with nonspecific IgG.

Bioinformatics analysis

The genomic and mRNA sequences for human and mouse genes were obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov/>). The VISTA plot (<http://genome.lbl.gov/vista/mvista/submit.shtml>) of the promoter region was generated as described previously [39,40]. The putative NFAT binding sequences, whose consensus sequence is -A/TGGAAAA-, were predicted using MOTIF (<http://motif.genome.jp/>) and mapped on the VISTA plot. <http://motif.genome.jp/transfac-search.htm> was used for MOTIF score.

Luciferase assay

hMSC were transfected with pGL4-Cox2 construct and phRL using Lipofectamine LTX Plus reagent as described by manufacturer (Invitrogen, Carlsbad, CA). Cells were mechanically stimulated the next day and luciferase assay was conducted using the Dual-Glo Assay System (Promega, Madison, WI).

Statistical analysis

Treatment groups within experiments were performed in triplicate and reported as mean $\pm$ SEM. Statistical analysis was performed using Statview software (SAS Institute, Cary, NC) to determine significance among treatment groups. One-way analysis of variance tests followed by Tukey-Kramer *post hoc* tests were performed to test the effects of treatment for each experiment. Statistical significance was established at $p \leq 0.05$.

Results

NFAT2 is activated in response to mechanical stimulation

To determine the involvement of NFAT2 activation in hMSC in response to mechanical stimulation, we treated the cells with 16 dyn/cm² (1.6Pa) fluid shear stress (PS) in the presence or absence of VIVIT, a peptide inhibitor of NFAT. Upon activation by Cn, NFAT is activated

and translocates to the nucleus, but VIVIT blocks NFAT dephosphorylation by Cn. Using an NFAT2 monoclonal antibody for immunocytochemistry, we showed that NFAT2 translocates to the nucleus in response to FSS, but remains cytoplasmic if the cells were treated with VIVIT (Figure 1). We verified our results using an ELISA-format activation assay for NFAT2 (See Materials and Methods). We collected nuclear extracts after mechanical stimulation and assayed for NFAT2 amounts in the nuclear extracts. NFAT2 was activated in response to mechanical stimulation (Figure 2A, Figure 2B). Mechanical stimulation-induced NFAT2 activation was blocked by VIVIT.

Inhibition of the Cn/NFAT signaling diminishes fluid shear stress and tensile strain-induced Cox2 expression in osteoblasts

Cox2 is one of the first genes to be upregulated in response to mechanical stimulation. To determine the involvement of NFAT2 in mediating Cox2 expression in response to mechanical stimulation, we treated hMSC and collected total RNA for gene expression analysis. Cox2 was found to be upregulated in response to physiologic FSS (PS) 1hr post-stimulation (Figure 3B). NFAT inhibitor VIVIT blocks the mechanical stimulation-induced-effect. Cox2 protein levels were also increased in response to mechanical stimulation (Figure 3A).

Cox2 is frequently found in the interfacial membranes of failed arthroplasties. Superphysiologic stimulation was imposed on the cyclic fluid flow to mimic the loading environment seen by cells in the effective joint space after arthroplasty. There was no significant difference between the superphysiologic and physiologic levels of mechanical loading for Cox2 mRNA or protein expression (Figure 3A, 3C). This result could indicate a saturation effect due to mechanical overloading. Cox2 was induced by tensile strains at both the physiologic (0.5%) and superphysiologic (5%) levels (Figure 3D).

Genetic deletion of Cn inhibits Cox2 expression

NFAT acts downstream of the Ca^{++} /Cn axis. $\text{Cn}\beta^{-/-}$ cells are unresponsive to load-induced cardiac hypertrophy Bueno *et al.* [30]. To determine if primary bone cells from $\text{Cn}\beta^{-/-}$ mice respond to mechanical stimulation, we isolated calvarial osteoblasts from $\text{Cn}\beta$ knockout mice and their isogenic wild type mice and treated the cells with physiologic mechanical stimulation (PS). Our results show that the cells from knockout mice did not respond to mechanical stimulation with increased Cox2 expression, while the cells from isogenic wild type mice showed increased Cox2 (Figure 4).

NFAT2 directly binds to the Cox2 promoter in response to mechanical stimulation

We used a chromatin immunoprecipitation assay to confirm direct interaction between NFAT2 and the human Cox2 promoter. hMSC were treated with mechanical stimulation (PS) with/out VIVIT inhibitor and NFAT2 bound DNA was purified for PCR amplification with human Cox2 promoter specific primers. Our results show that there is enhanced NFAT2-Cox2 interaction in response to mechanical load (Figure 5B). Cox2-luciferase reporter assay confirms increased activity in response to mechanical loading and VIVIT inhibited Cox2 induction (Figure 5C).

A novel role for NFAT in mediating osteoblast differentiation induced by mechanical stimulation

We used a bioinformatics analysis to determine potential NFAT binding sites in the promoters of osteoblast differentiation genes. Cox2 is a key enzyme that is upregulated in response to mechanical stimulation [41,42]. NFAT2 is known to form a complex with osteoblast-specific transcription factor Osterix [41]. Our analysis showed that potential NFAT binding sites are present in the promoters of Alp, Ocn and Opn (Figure 6).

To determine if the fluid shear-induced-effect persists as hMSC differentiate to osteoblast lineage, hMSC were pretreated for 6 days in osteogenic media. The cells that were mechanically loaded showed increased NFAT2 activation (Figure 7A). Osteoblastic differentiation markers alkaline phosphatase (Alp) (Figure 7C), and osteopontin (Opn) were also increased (Figure 7B) in response to physiologic FSS, but superphysiologic levels did not affect these markers in comparison to control cells. Furthermore, the effect on Alp and Opn were blocked by NFAT inhibition. Cox2 expression increased in response to FSS in hMSC pretreated with osteogenic media, which was also blocked by NFAT inhibitor (unreported data, Aydemir 2008). NFAT2 continued to remain activated as the cells progressed into osteoblast lineage under mechanical stimulation (Figure 7A).

Discussion

The mechanosensitivity of bone is essential for its physiological functioning. Several studies have established a connection between mechanical stimulation and osteogenesis *in vitro* and *in vivo* [42–45]. It has been reported that in the initial phase of mechanotransduction, deformation of the cell membrane in response to stretch and canaliculi fluid flow-mediated shear stress is sensed by the osteoblasts and osteocytes [46,47]. The stretch activated cation channel mediates an increase in cytosolic Ca^{++} via release from the internal stores and entry through the L-type voltage-sensitive Ca^{++} channel. Ca^{++} signaling is known to mobilize downstream signaling events involving kinase cascades. Therefore, mechanical energy sensed at the cellular matrix is converted to chemical energy, which drives changes in protein conformation, phosphorylation and changes in DNA [47].

Some of the initial events that take place at the osteoblast cell adhesion complex in response to mechanical stimulation include the recruitment of integrins to focal adhesions and the reorganization of the actin filaments [48]. Fluid shear stress has been shown to increase integrin β 1 subunit expression and to activate $\alpha\text{v}\beta$ 3 integrin in osteoblasts, which may activate Ca^{++} /Cn/NFAT signaling [49]. Fluid shear stress induces Cox2, and c-fos, both of which are dependent on the mobilization of Ca^{++} [50]. It also mediates the release of prostaglandin (PGE2) via connexins, which can form regulatory channels between the cell and its environment (called hemi channels)[50]. The fluid shear induced rise in intracellular calcium stimulates ATP release, which may play a role in prostaglandin release [51]. In a recent study, ATP was implicated as a major player for fluid-flow induced increases in intracellular calcium concentration, activation of calcineurin, nuclear translocation of NFAT2, and proliferation of bone marrow stromal cells [52]. Therefore, purinergic receptor activation may have a role in the regulation of NFAT2 in response to mechanical stimulation. Further, activation of Erk1/2 by mechanical loading has been reported to be Ca^{++} and ATP-dependent, implicating a possible connection between NFAT2 and MAPK signaling specifically in the regulation of the Cox2 promoter [48,52–54].

We have shown that NFAT2 translocates to the nucleus in response to mechanical stimulation in hMSC (Figure 1). Donahue and colleagues [52] showed that ATP release mediates cell proliferation in the same type of cells. Another previous study concurs with this result where calcium oscillations induced by ATP have been associated with NFAT nuclear translocation in undifferentiated hMSC [55]. Conversely, we were unable to show NFAT2 nuclear localization in differentiated calvarial bone cells from CnA β knockout mice. Differentiated osteoblasts may lose inducibility of NFAT activity upon differentiation. This result is consistent with a study in which the calcium oscillations and NFAT nuclear localization cease after the differentiation of hMSC into adipocytes [55]. Unpublished data from our laboratory imply that macrophages require NFAT activity for proinflammatory cytokine expression, but NFAT activity itself does not stimulate the cells to differentiate (Unpublished data, Minematsu *et al.*, 2007) upon treatment with inflammatory reagents.

Our studies implicate NFAT signaling in Cox2 induction in response to mechanical stimulation using two different forms of stimulation: fluid flow-induced shear stress and cyclical tensile strain (Figure 3). Cox2 is upregulated in response to mechanical stimulation [56,57] and chromatin immunoprecipitation shows direct interaction between NFAT2 and Cox2 in response to mechanical stimulation (Figure 5). We used human mesenchymal stem cells (hMSC), which are mechanosensitive [8,58] and have the capability to contribute to the anabolic response of bone to mechanical load via differentiation/proliferation. Both FSS and tensile strain induced Cox2 expression, which was blocked by a peptide inhibitor of NFAT (Figure 3). We did not detect a significant increase due to superphysiologic mechanical stimulation, which may be attributable to desensitization of the cell receptors in response to mechanical overloading. Since NFAT acts downstream of Cn, we also used cells from CnA β mice. Cells from the wild type mice were responsive to FSS with increased Cox2 expression, whereas cells from the CnA β knockout mice were unresponsive (Figure 4).

The involvement of NFAT in bone formation has been established in several different studies. Winslow *et al.* reported that a constitutively active NFAT2 induces a high bone mass phenotype and an increase in osteoblast proliferation [41]. The authors also report an increase in Wnt4 signaling and hypothesize that NFAT2 may regulate Wnt signaling and cell cycle genes in osteoblasts. In a separate study, NFAT2 was found to be crucial for osteoclasts and bone formation, but did not affect osteoblast proliferation [27]. NFAT2 and Osterix (Osx) form a complex and the inhibition of Osx prevented NFAT2 recruitment to type I collagen promoter [27]. These studies implicate the importance of NFAT signaling in bone formation. In fact, NFAT remains activated during osteoblast differentiation [59]. Our analysis showed that potential NFAT binding sites are present in the promoters of Alp, Ocn and Opn (Figure 6). We report increased Alp expression and Alp stain in the presence of physiologic FSS (Figure 7). On the other hand, superphysiologic levels of mechanical stimulation did not increase Alp. It is possible that while physiologic levels (1Pa) of mechanical stimulation increase cell proliferation and early osteoblast differentiation, superphysiologic levels may suppress this phenotype. Osteogenic media alone increases Opn expression over 5-fold, whereas in the presence of physiologic FSS gene expression is increased 7.5-fold over the basal control. The use of VIVIT inhibitor decreases gene expression to 3-fold over the basal levels. NFAT2 is activated due to osteogenic differentiation media alone, and mechanical stimulation may not necessarily cause a synergistic increase in osteoblast marker gene expression, but it may maintain NFAT association with osteogenic factors. It would be interesting to monitor the mechanism and further delineate the role of NFAT at different stages of osteogenesis. Future studies should also involve the use of NFAT2 knockout mice.

NFAT could act as a fine-tuning agent in terms of its association with osteoblast specific promoters and the relay of the intracellular signaling. The NFAT complex with Osx could undergo additional modifications that favor osteoblast differentiation. One such modification may be through PIASx β , a SUMO E3 ligase, and an essential gene for bone mineralization [60]. Knockdown and overexpression experiments have shown that PIASx β is crucial for Alp, Osx and Ocn expression. When co-transfected with PIASx β and Osx, NFAT2 and NFAT4 further enhanced PIASx β /Osx transcriptional activity. On the other hand, NFAT2 or NFAT4 transfected with Osx alone did not affect its promoter activity. Kawashima and colleagues propose a mechanism whereby PIASx β sumoylates NFAT to cause a conformation change that enhances NFAT-Osx binding and transcriptional activation [60].

An interesting area that needs further exploration is the double-acting capability of NFAT2. Not only does NFAT2 seem to have an important role in bone formation, it is also important for cytokine production in osteoblasts. In addition, viable NFAT2-deficient mice fail to develop mature osteoclasts [61], and the retroviral expression of active NFAT2 is sufficient for the osteoclastic differentiation of a monocytic cell line [25]. Studies with cyclosporine A, an

inhibitor of calcineurin and NFAT, displays biphasic effects of NFAT on bone formation [62]. This study points out that a low dose is anabolic, whereas a high dose is catabolic for bone formation. NFAT2 may act autonomously for its roles in osteoblastogenesis and osteoclastogenesis. We showed that NFAT2 is a mediator of mechanotransduction in human and mouse bone cells and clinically relevant mechanical stimulation activates NFAT2. Furthermore, NFAT2 remains activated as hMSC differentiate to osteoblast lineage. Future studies need to focus on the elegant mechanism by which NFAT fine-tunes bone homeostasis. Further evidence may give us new guidelines for anabolic therapies for inflammatory bone loss.

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Figure 1.

NFAT2 translocates to the nucleus in mechanically stimulated hMSC. The cells were fixed on slides post stimulation, then treated with NFAT2 monoclonal antibody and 4'-6-Diamidino-2-phenylindole (DAPI nuclear stain) for immunofluorescence. A) NFAT2 translocates to the nucleus in response to physiologic fluid shear stress, (PS) which is inhibited by VIVIT (cytoplasmic NFAT), B) Image J quantitation of the cytoplasmic vs. nuclear NFAT2 in hMSC treated with physiologic fluid shear stress and/or VIVIT.

Figure 2.

Mechanical stimulation activates NFAT2. A) hMSC were treated with physiologic fluid shear stress (PS) or B) tensile strain physiologic (PS), superphysiologic (SPS) for 15min and nuclear extracts were collected, quantitated and NFAT2 activation assay (see Materials and Methods) was performed. For inhibition experiments, the cells were treated with VIVIT overnight. * indicates statistical significance over control, ** indicates statistical significance over corresponding treatment without VIVIT at $p < 0.05$, $n = 8$

Figure 3.

Inhibition of the Cn/NFAT signaling diminishes mechanical loading-induced Cox2 expression. A) Western blot analysis for Cox2 protein in response to physiologic (PS) or superphysiologic (PS) fluid shear stress with/out VIVIT. Densitometric analysis of the western blot image using Image J. B) The cells were treated with physiologic fluid shear stress (PS) with/out VIVIT (VIV) or control peptide VEET (VEE) and RNA was collected for Cox2 gene expression analysis C) Cox2 gene expression analysis, the cells were treated with physiologic (PS) or superphysiologic (PS) fluid shear stress D) Cox2 gene expression analysis, the cells were treated with physiologic (PS) or superphysiologic (PS) tensile strain for 15min and RNA was collected for gene expression analysis *indicates statistical significance over control at $p < 0.05$, ** indicates statistical significance over corresponding treatment without VIVIT $n = 3$.

Figure 4.

CnA β knockout cells are unresponsive to mechanical stimulation. Calvarial osteoblasts were treated with physiologic fluid shear stress (PS) for 15min and RNA was collected 1hr post stimulation for gene expression analysis. *indicates statistical significance over control at $p < 0.05$, $n = 5$.

A

Chromatin Immunoprecipitation (ChIP) assay NFAT2 binds to the promoter region of Cox2

B

C

Figure 5.

A working model of the involvement of NFAT2 in mediating Cox2 in response to mechanical load. A) $\text{Ca}^{++}/\text{Cn}/\text{NFAT}$ axis is activated in response to mechanical loading. NFAT2 translocates to the nucleus and binds to Cox2 promoter. Chromatin immunoprecipitation assay to show direct interaction between NFAT2 and the Cox2 promoter B) hMSC were treated with physiologic (PS) tensile strain for 30 min and chromatin immunoprecipitation assay was conducted (see Materials and Methods). Anti-mouse NFAT2 antibody was used for immunoprecipitation. Immunoprecipitated DNA was used as a template for PCR with primers which cover the region (−904 to −641) on the Cox2 promoter. Control (C), physiologic tensile strain (PS), physiologic tensile strain with VIVIT (PS+VIV), physiologic tensile strain with

VEET (PS+VEE), negative control (IgG), Input (total DNA after sonication) B) hMSC were transfected with Cox2-luciferase construct and luciferase assay conducted. *indicates statistical significance over control at $p < 0.05$, ** indicates statistical significance over corresponding treatment without VIVIT or VEET, $n=3$.

Figure 6.

NFAT consensus sequence exists in the promoters of *Alp*, *Cox2*, *Ocn* and *Opn*. VISTA plot (See Materials and Methods) comparing the promoter regions of osteoblast marker genes in human and mouse genomes. Numbers on X-axis indicate nucleotide positions in human. Transcription initiates at “1”. Plots show DNA sequence homology between humans and mice. The filled areas designate regions with high homology (>75%), blue in coding regions and red in non-coding regions. The top horizontal arrow indicates transcripts while the filled boxes just below indicate exons. Squares on the bottom refer to consensus sequences for NFAT binding found by MOTIF (See Materials and Methods). Colors of squares are based on a score, which indicates similarity to the consensus sequence.

Figure 7.

NFAT is activated during osteoblast differentiation. hMSC were cultured in osteogenic medium for 6 days prior to mechanical stimulation with physiologic (PS) or superphysiologic (SPS) fluid shear stress A) NFAT2 activation in response to osteogenic differentiation, using nuclear extracts from indicated treatment groups on Day7, RNA was collected and gene expression was analyzed for B) Opn, C) Alp, D) The cells were stained for Alp on the indicated timepoint. *indicates statistical significance over basal control, ** indicates statistical significance over corresponding treatment without VIVIT. Control2 stands for control cells maintained in osteogenic differentiation medium.