Published in final edited form as:

Cell Mol Bioeng. 2014 June 1; 7(2): 254–265. doi:10.1007/s12195-014-0321-3.

Enhancement of Flow-Induced AP-1 Gene Expression by Cyclosporin A Requires NFAT-Independent Signaling in Bone Cells

LEAH E. WORTON, RONALD Y. KWON, EDITH M. GARDINER, TED S. GROSS, and
SUNDAR SRINIVASAN

Department of Orthopaedics and Sports Medicine, University of Washington, Seattle, WA, USA

Abstract

Growing evidence suggests that aging compromises the ability of the skeleton to respond to anabolic mechanical stimuli. Recently, we reported that treating senescent mice with Cyclosporin A (CsA) rescued aging-related deficits in loading-induced bone formation. Given that the actions of CsA are often attributed to inhibition of the calcineurin/NFAT axis, we hypothesized that CsA enhances gene expression in bone cells exposed to fluid flow, by inhibiting nuclear NFATc1 accumulation. When exposed to flow, MC3T3-E1 osteoblastic cells exhibited rapid nuclear accumulation of NFATc1 that was abolished by CsA treatment. Under differentiation conditions, intermittent CsA treatment enhanced gene expression of late osteoblastic differentiation markers and activator protein 1 (AP-1) family members. Superimposing flow upon CsA further enhanced expression of the AP-1 members Fra-1 and c-Jun. To delineate the contribution of NFAT in this response, cells were treated with VIVIT, a specific inhibitor of the calcineurin/NFAT interaction. Treatment with VIVIT blocked flow-induced nuclear NFATc1 accumulation but did not recapitulate the CsA-mediated enhancement of flow-induced AP-1 component gene expression. Taken together, our study is the first to demonstrate that CsA enhances mechanically-induced gene expression of AP-1 components in bone cells, and suggests that this response requires calcineurin-dependent mechanisms that are independent of inhibiting NFATc1 nuclear accumulation.

Keywords

Mechanotransduction; CsA and VIVIT; Osteoblast; Fra-1; c-Jun; Fluid flow; Shear stress; Mechanical loading; MC3T3-E1

© 2014 Biomedical Engineering Society

Address correspondence to Leah E. Worton, Department of Orthopaedics and Sports Medicine, University of Washington, Seattle, WA, USA. lworton@u.washington.edu.

Leah E. Worton and Ronald Y. Kwon have contributed equally to this work.

CONFLICT OF INTEREST

Leah E. Worton, Ronald Y. Kwon, Edith M. Gardiner, Ted S. Gross, and Sundar Srinivasan declare that they have no conflicts of interest.

ETHICAL STANDARDS

No human studies were carried out by the authors for this article. No animal studies were carried out by the authors for this article.

INTRODUCTION

Given that mechanical loading is one of the primary determinants of bone strength, the mechanotransduction pathway is widely recognized as a promising target for non-pharmacological bone therapeutic strategies.⁴⁴ However, mechanically-based interventions for enhancing bone health such as physical exercise, resistance training, and low magnitude high frequency vibration have had limited efficacy in increasing bone mineral density in post-menopausal and age-related osteoporosis.¹¹ One potential reason for this lack of efficacy is that the osteogenic response to mechanical stimuli is markedly diminished in the aged.³⁸ A growing body of evidence suggests that this deficit in mechanotransduction is due to a compromised ability of cells from aged individuals to interpret and respond to anabolic signaling.^{14,58} Thus, developing interventions that overcome these deficits will be important in implementing exercise-based strategies for the treatment of bone loss with age.

A potential strategy for addressing age-related deficits in bone mechanotransduction is through supplementing physical exercise with low-dose pharmacological regimens.^{46–48} Recent evidence suggests that Cyclosporin A (CsA), a small peptide immunosuppressant that inhibits calcineurin and its substrate Nuclear factor of activated T cells (NFAT), may alter bone signaling pathways that affect bone mass accrual. For example, while one of the observed side effects of extended exposure to CsA and other calcineurin inhibitors has been severe bone loss in both animals and humans,⁵⁰ it has also been shown to inhibit osteoclast formation and function,³⁴ and enhance osteoblast differentiation.⁵⁴ In addition, it has been previously demonstrated that CsA may be both anabolic and catabolic in mice depending on the treatment dose.⁵³ In a mouse model of mechanical loading-induced bone formation, we have previously found that superimposing low dose CsA treatment (0.3 or 3.0 mg/kg) with direct mechanical loading of the tibia restored deficient bone formation in senescent mice (22 Mo) to the robust levels observed in young adult mice (4 Mo).⁴⁶ These data suggest that combining CsA at low doses (0.25–2.5% of that typically required for immunosuppression) together with mild physical activity could provide a readily implemented and cost effective treatment strategy to augment bone mass in the elderly. However, the mechanism by which CsA interacts with the mechanotransduction pathway to amplify mechanically-induced bone formation is unclear.

In other tissues, the actions of CsA have often been attributed to inhibition of the calcineurin/NFAT axis,¹⁶ suggesting that CsA may enhance bone mechanotransduction by inhibiting nuclear accumulation of the transcription factor NFAT. CsA acts through an association with cyclophilin to specifically inhibit the phosphatase activity of calcineurin, which in turn prevents the dephosphorylation and subsequent nuclear migration of NFAT proteins.^{16,29} In T-cells, the immunosuppressive properties of CsA are attributed to its inhibition of NFAT target genes (including cytokines, cell surface receptors and transcription factors) required to mount an immune response.⁴² In the nucleus, active NFATc1 binds to other protein partners, many of which have been implicated in the bone mechanotransduction pathway. These include the activator protein 1 (AP-1) group of dimeric transcription factors³⁰ which are composed of members of the Fos (c-Fos, FosB, Fra-1, Fra-2, FosB) and Jun (c-Jun, JunB, JunD) protein families. Inhibition of calcineurin

by CsA therefore, has the ability to inhibit NFATc1 transcriptional responses by preventing its localization in the nucleus in response to various stimuli.

An alternative mechanism by which CsA may impact mechanotransduction is through modulating calcineurin-dependent signaling pathways that function independently of nuclear NFAT translocation. Calcineurin is activated by Ca^{2+} bound calmodulin and is recognized as an important mediator of mechanotransduction in osteoblasts.⁸ In addition to promoting NFAT nuclear translocation, calcineurin also directly dephosphorylates the signaling molecule glycogen synthase kinase-3 β (GSK-3 β)²³ and transcription factor ETS-like transcription factor 1 (Elk-1),⁴⁹ and modulates the activity other transcription factors like nuclear factor κ B (NF- κ B), cyclic AMP response element-binding protein (CREB), and AP-1 by targeting upstream signaling molecules.^{7,17,39} These transcription factors are also activated in osteoblastic cells in response to shear stress.^{20,52,55} Thus inhibition of calcineurin *via* CsA has the ability to impact the expression of multiple downstream targets that are important in mechanotransduction through both nuclear NFAT-dependent and -independent mechanisms.

In this study, we hypothesized that CsA treatment enhances mechanically-induced gene expression in bone cells, and that this enhancement arises through its inhibition of nuclear NFATc1 accumulation. To assess this, we treated MC3T3-E1 osteoblastic cells with CsA and subjected them to dynamic fluid flow. Cells exposed to flow exhibited rapid nuclear accumulation of NFATc1 that was abolished by CsA treatment. Under differentiation conditions, intermittent CsA treatment enhanced gene expression of osteoblastic differentiation markers and of AP-1 family members. In addition, superimposing fluid flow upon CsA further enhanced expression of the AP-1 transcription factors Fra-1 and c-Jun. To assess the specific contribution of NFATc1 nuclear accumulation in this response, cells were treated with VIVIT, a specific inhibitor of the calcineurin/NFAT interaction that does not impair non-NFAT calcineurin substrates.⁴ VIVIT treatment blocked flow-induced nuclear NFATc1 accumulation but did not recapitulate the CsA-mediated enhancement of flow-induced AP-1 component gene expression. Taken together, our study is the first to demonstrate that CsA enhances mechanically-induced gene expression of AP-1 components in bone cells, and suggests that this response is mediated in part by calcineurin-dependent mechanisms that are independent of NFATc1 nuclear accumulation.

MATERIALS AND METHODS

Cell Culture and Fluid Flow

Tissue culture medium and supplements were purchased from Invitrogen (Life Technologies). Media was supplemented with heat inactivated FBS (HyClone, Thermo Scientific). MC3T3-E1 osteoblastic cells (clone 14, passage 9–12) were maintained in growth media (α -MEM with 10% FBS, 2 mM L-glutamine and 20 mM HEPES) at 37 °C and 5% CO₂. To mechanically stimulate cells, we subjected them to orbital shaking using a previously validated *in vitro* model of bone cell mechanotransduction.⁵¹ 72 h prior to experimentation, cells were seeded into 6-well plates (Corning) at a density of 2.5×10^4 cells per well in 2 mL of growth media. On the day of flow exposure, plates were treated with CsA (Calbiochem, EMD Millipore), VIVIT (Calbiochem) or DMSO at concentrations

indicated, and transferred to an orbital shaker (VWR, DS-500) placed in an incubator at 37 °C and 5% CO₂ 1 h prior to exposure to fluid flow. Cells were subjected to orbital shaking (2.2 Hz, 0.1–0.5 Pa) for up to 1 h as detailed previously.⁵¹ For differentiation experiments, cells were cultured in growth media supplemented with 10 mM β -glycerophosphate and 50 mg/L ascorbic acid. Cells were treated with CsA, VIVIT or DMSO as above. After 1 h of fluid flow, cells were incubated under stationary conditions for 24 h before culture media was replaced. Cells were subjected to a total of 4 treatments, on days 3, 5, 7 and 10 of culture, and harvested for RNA analysis 24 h after the final treatment.

Quantitative RT-PCR

For quantitative RT-PCR, total RNA was extracted using the RNeasy Mini Kit (Qiagen) or with TRIzol (Invitrogen, Life Technologies) according to the manufacturers' protocol. cDNA was synthesized using Super Script III reverse transcriptase (Invitrogen), and quantitative RT-PCR was performed using SYBR green and the Applied Biosystems 7900HT or ViiA7 sequence detection systems. Primer sequences are shown in Table 1. Relative gene expression levels were quantified using the $2^{(-CT)}$ method. To account for amplification differences between sample and housekeeping genes, amplification efficiencies were calculated using LinRegPCR,⁴¹ and amplification efficiency corrections were applied as in.³⁷ Similar to previous studies,⁴⁰ investigations of acute flow induced gene expression (after 1 h of flow stimulation $\pm$ 2 h of CsA treatment) were conducted using cyclophilin as a housekeeping gene. For longer term differentiation experiments, we used β -actin as the housekeeping gene due to concerns of a possible effect of CsA treatment on levels of cyclophilin expression over an extended period of time.⁵ Validation experiments comparing the levels of cyclophilin and β -actin expression in our cDNA samples showed little effect of CsA or flow treatment on expression of either housekeeping gene. PCR data were pooled from two to three independent experiments.

Antibodies, Fractionation and Immunoblotting

To detect changes in NFATc1 nuclear accumulation, cell fractionation was performed using NER-PER Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific). To assess ERK1/2 activity, total cellular protein was isolated in RIPA lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Nuclear and cytoplasmic fractions and total cell lysates were quantitated using BCA protein assay reagents (Thermo Scientific), then separated by electrophoresis in 4–12% NuPAGE Bis-Tris gels (Invitrogen) and transferred onto PVDF membranes (GE Healthcare). Membranes were probed with NFATc1 (Thermo Scientific, 1:1000), β -actin (Abcam, 1:5000) or p-ERK and ERK antibodies (Cell Signaling Technology, 1:1000). Bound primary antibodies were detected by chemiluminescent detection of HRP-conjugated secondary antibodies (GE Healthcare, 1:5000). Western blots results are representative of two independent experiments.

Data Analysis and Statistics

All data analyses were performed in the open source statistical environment R (<http://www.R-project.org/>). Two-way ANOVA and Fisher's PLSD *post hoc* tests were performed for gene expression studies. $p < 0.05$ was considered statistically significant.

RESULTS

Nuclear NFATc1 Accumulation is Rapidly Stimulated in MC3T3-E1 Cells by Fluid Flow, and Abolished by CsA Treatment

Stimulation of nuclear NFATc1 accumulation has been previously reported following 15 min of shear stress in human mesenchymal stem cells.⁹ Building on these findings, we assessed the capacity for fluid shear stress to activate NFATc1 nuclear accumulation in MC3T3-E1 bone cells, and characterized the temporal dynamics of this response. When exposed to flow, increased NFATc1 nuclear localization was detected within 5 min of the initiation of flow (Fig. 1a). Increased localization was also observed 10 min following initiation of flow, but not at 20 or 40 min. When treated with CsA, concentrations of 10 nM and 1 μ M were each capable of inhibiting NFATc1 nuclear accumulation in response to flow after 1 h of pre-treatment. To determine the degree to which this effect was attributable to a selective inhibition of NFATc1 nuclear accumulation by CsA or a decrease in total NFATc1 levels, we assessed cytoplasmic NFATc1 after 5 min of flow. NFATc1 levels in the cytoplasm were not changed by flow or CsA treatment at this time point (Fig. 1b). However, for more prolonged exposures, CsA appeared to decrease basal levels of nuclear NFATc1 when cells were incubated in no-flow conditions.

CsA Minimally Alters Acute Mechanotransduction Pathway Components That Have Been Implicated in the Calcineurin/NFAT Signaling Axis

We next sought to investigate the degree to which the calcineurin/NFAT axis cross-talks with known acute mechanotransduction pathways in bone cells, and whether these pathways are also inhibited by CsA. To investigate this, cells were exposed to fluid flow and assessed for a panel of known acute mechanotransduction pathway components that have previously implicated in the calcineurin/NFAT signaling axis. First, given that CsA has previously been found to alter MAPK signaling,⁴³ the effect of CsA on flow-induced ERK1/2 phosphorylation was investigated (Fig. 1c). In cells treated with DMSO, ERK1/2 phosphorylation was rapidly induced by 5 min of fluid flow and this response exceeded that induced by 10 min of flow. Treatment with CsA did not appear to alter the magnitude of the ERK1/2 response at either time point. In addition, no evidence of enhanced JNK or p38 MAPK phosphorylation was observed in CsA-treated cells exposed to fluid flow (data not shown). Finally, there were no effects of CsA in enhancing gene expression of c-Fos and Cox-2 (cyclooxygenase 2), two well-characterized mechanosensitive early response genes that have been implicated to be under regulatory influence of calcineurin/NFAT^{9,45} (Fig. 1d). CsA did not alter baseline or flow-induced c-Fos or Cox-2 mRNA expression. These studies suggest that CsA has little effect on early MAPK activation or c-Fos and Cox-2 gene expression in mechanically stimulated bone cells, despite the implication of these components in the calcineurin/NFAT signaling axis.

Repeated Treatment of Differentiating MC3T3-E1 Cells with CsA Leads to Increased Expression of Runx2 and Late Osteoblastic Markers

We next assessed the potential for CsA and mechanical loading to enhance osteoblastic gene expression. For these studies, we developed a multiday experimental regimen in which intermittent fluid flow and CsA were superimposed in cells undergoing osteoblastic differentiation. There was no effect of flow on the expression of genes for the early osteoblastic transcription factors Runx2 and Osx (Figs. 2a and 2b), but CsA treatment did have a significant effect on the expression of Runx2. CsA treatment also increased expression of genes associated with late osteoblastic differentiation including alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OCN), which was particularly evident at the highest concentration of CsA (Figs. 2c, 2d, and 2e). More specifically, in the no-flow samples, compared to the DMSO treated controls, there was a 1.8-fold elevation in both ALP and OPN expression and a 3-fold elevation in OCN expression in the cells treated with 1 μ M CsA. Expression of these genes was also elevated in cells exposed to 1 μ M CsA and flow; however, these changes did not differ from expression levels due to CsA alone. Together, these studies suggest the potential for CsA to enhance osteoblastic Runx2, ALP, OPN and OCN gene expression during MC3T3-E1 cell differentiation.

Treatment of Differentiating MC3T3-E1 Cells with CsA Leads to Increased Baseline and Flow-Induced Expression of AP-1 Genes

AP-1 signaling has long been understood to play a role in both osteoblast differentiation and the response of osteoblastic cells to mechanical stimulation.^{19,36} The capacity for CsA to enhance osteoblastic gene expression led us to investigate the potential role of AP-1 transcription factors, as CsA treatment has been found to increase expression of various AP-1 genes.^{39,53} Specifically, we investigated the expression of the members of the Fos and Jun protein families in MC3T3-E1 cells treated with the combination of CsA and fluid flow. After repeated CsA treatment, there were significant changes in the expression of c-Fos, Fra-1, c-Jun, JunB and JunD with CsA dose (Figs. 3a, 3c, 3f, 3g, and 3h), but no similar changes for the expression of other Fos family members, FosB, Fra-2 and FosB (Figs. 3b, 3d, and 3e). In the absence of flow, there was enhanced expression of the Fos family member Fra-1, with a 1.9-fold increase in cells treated with the highest dose of CsA compared to the DMSO control. Additionally, there was increased expression of all Jun protein family members under these treatment conditions, with 1.9-, 1.4- and 1.4-fold increases seen in c-Jun, JunB and JunD levels, respectively.

In cells exposed to fluid flow, there were significant flow-induced increases in expression of two AP-1 members, Fra-1 and c-Jun. In the absence of CsA, cells exposed to flow exhibited a 1.8-fold and 1.7-fold increase in mRNA expression relative to no-flow controls for Fra-1 and c-Jun respectively. For both Fra-1 and c-Jun, expression levels with the combination of high CsA and flow were elevated compared to the DMSO/Flow controls, suggesting that these interventions have an additive effect on the expression of these two genes. No other flow-induced AP-1 gene expression was observed. Collectively these data suggest that both the CsA treatment regimen alone and in combination with fluid flow enhances expression of the AP-1 components Fra-1 and c-Jun during MC3T3-E1 differentiation.

VIVIT Inhibits Flow-Induced Nuclear Accumulation of NFATc1 But Not ERK1/2 Activity or c-Fos/Cox-2 Gene Expression

We next sought to determine the role of nuclear NFATc1 accumulation in mediating the effects of CsA on enhancing flow-induced gene expression. Our strategy was to determine if the gene expression changes observed with the combination of CsA and fluid flow could be recapitulated by specifically inhibiting NFAT accumulation while preserving the non-NFAT related phosphatase activity of calcineurin. For this, cells were treated with the VIVIT peptide, a selective inhibitor of the interaction between calcineurin and NFAT that results in the inhibition of NFATc1 dephosphorylation but which does not affect non-NFAT substrates. We first sought to characterize the effects of VIVIT on flow-induced NFATc1 nuclear accumulation and acute mechanotransduction signaling events. In cells subjected to flow, treatment with VIVIT inhibited flow-induced NFATc1 nuclear accumulation at both concentrations tested (Fig. 4a). As both doses of VIVIT equivalently blocked NFAT accumulation, the lesser concentration (1 μ M) was used in subsequent VIVIT studies. We also assessed ERK1/2 signaling and c-Fos and Cox-2 gene expression (Figs. 4b and 4c); similar to cells treated with CsA (Figs. 1b and 1c), VIVIT treatment had little effect on these established mechanotransduction pathway components.

VIVIT Does Not Recapitulate the Enhancement of Flow-Induced Fra-1 and c-Jun Gene Expression Observed in CsA-Treated Cells

To assess the specific contribution of flow-induced NFATc1 nuclear accumulation on AP-1 gene expression, cells were subjected to differentiation with intermittent VIVIT treatment and fluid flow (using identical conditions as the CsA experiments) and then analyzed for gene expression. Interestingly, in contrast to the effects of CsA treatment (which enhanced flow-induced increases in Fra-1 and c-Jun; Figs. 3c and 3f), VIVIT treatment blocked flow-induced increases in Fra-1 and c-Jun gene expression (Figs. 5a and 5b). Specifically, while expression of both Fra-1 and c-Jun was enhanced in DMSO treated cells in response to repeated fluid flow bouts, inhibition of nuclear NFAT by VIVIT negated this flow response.

DISCUSSION

Previously, we found that superimposing mechanical stimulation on CsA treatment in senescent mice led to increased mineral apposition and bone formation rates,⁴⁶ suggesting that the combined therapy enhanced osteoblast function induced by mechanical loading. Here we used an established model of bone cell mechanotransduction to explore the signaling interactions that may underlie this response. We demonstrated that rapid accumulation of NFATc1 in response to fluid flow was inhibited by CsA treatment, and that CsA treatment, either alone or in combination with flow, has the potential to increase the expression of AP-1 genes important in the differentiation and activity of these osteoblastic cells. Additionally, we found that enhancement of mechanically-induced Fra-1 and c-Jun gene expression in cells exposed to CsA could not be recapitulated by treatment with VIVIT, a specific inhibitor of NFAT nuclear accumulation. Taken together, this study demonstrates the potential for CsA to enhance mechanically-induced gene expression of AP-1 components in bone cells, and suggests that this response is mediated in part by calcineurin-dependent mechanisms that are independent of NFATc1 nuclear accumulation.

We found that treatment of MC3T3-E1 cells with CsA enhanced expression of genes involved in osteoblastic differentiation and activity. Using an intermittent treatment schedule to mimic *in vivo* dosing of animals before a mechanical loading regimen, we detected amplified expression of three well characterized osteogenic markers, ALP, OPN and OCN. Interestingly expression of the early osteoblastic transcription factor Runx2 was also enhanced by CsA treatment. Additionally, transcripts of the AP-1 component c-Fos, together with components Fra-1, c-Jun, JunB and JunD were all increased with CsA. To our knowledge, this is the first report that CsA can enhance the gene expression of these latter four AP-1 members. While original characterization of the AP-1 transcription factors identified Fra-2 and JunD to predominate during differentiation of MC3T3-E1 cells,³² later studies have provided evidence for interactions of other Fos and Jun proteins on the promoters of genes like OCN, MGP and MMP-13,^{1,12,22} whose expression is important for osteoblast activity. Fitting with the findings of these previous studies, our results suggest the possibility that variation in the composition of AP-1 heterodimers may contribute to gene expression changes at progressive stages of osteoblast differentiation and in response to external stimuli.

When cells were exposed to flow, we found that CsA treatment additively enhanced mechanically-induced gene expression of AP-1 components. In particular, we observed an enhancement of flow-induced increases in expression of the AP-1 components Fra-1 and c-Jun. Both Fra-1 and c-Jun, as AP-1 transcription factor components, have been shown to positively regulate osteoblastic genes, including MGP, BSP, MMP-13 and OCN.^{1,12,22,28} Expression of Fos and Jun mRNA and proteins have been reported throughout *in vitro* osteoblast proliferation and differentiation.^{32,33} *In vivo*, c-Jun transgenic mice showed no bone pathology,¹⁸ but somatic inactivation of c-Jun was found to result in mice with malformed axial skeletons,⁶ while transgenic expression or conditional knockout of Fra-1 results in mice with increased or decreased bone formation rates respectively.^{15,21} Recently, Fra-1 has also been shown to be a transcriptional target of Runx2 during osteogenic differentiation of C2C12 cells.⁵⁷ Collectively, then, our results demonstrated that treatment of osteoblastic cells with CsA and fluid flow *in vitro* can additively increase gene expression of transcription factors that are directly involved in regulating osteoblast differentiation and function, and suggest a potential molecular basis for our previous observation of synergy between CsA and mechanical stimulation *in vivo*.⁴⁶

A surprising result of this study was the failure of VIVIT treatment to duplicate the positive effects of CsA on the mechanical induction of AP-1 genes, as the action of CsA is often attributed to inhibitory effects on NFAT signaling.¹⁶ In particular, unlike the enhancement of mechanically-induced gene expression observed in cells treated with CsA, cells treated with VIVIT exhibited abrogated flow-induced Fra-1 and c-Jun expression. The attenuation of flow-induced expression by VIVIT suggests that these genes may be under the influence of competing pathways that are differentially activated by CsA and fluid flow. In particular, the capacity for VIVIT to block flow-induced gene expression suggests that nuclear NFATc1 accumulation exerts a positive influence on the expression of these genes. However, the enhancement of flow-induced gene expression following CsA treatment (which blocks nuclear NFATc1 accumulation) suggests that the influence of inhibiting non-

NFAT targets of calcineurin may overcome the inhibition of flow-induced increased gene expression associated with loss of nuclear NFAT alone. Collectively, our results suggest that specific inhibition of NFATc1 dephosphorylation alone is not sufficient to capture the changes in AP-1 gene expression observed with the combination of CsA treatment and fluid flow, strongly suggesting the involvement of non-NFAT targets of calcineurin in this pathway.

While our results suggest that a non-NFAT dependent mechanism might underlie the CsA mediated increases in AP-1 gene expression observed, this mechanism remains to be determined. A survey of the literature suggests the potential for a number of different calcineurin substrates to mediate the effects of CsA in enhancing mechanically-induced expression of AP-1 genes. For example, in addition to NFAT, calcineurin also directly or indirectly affects the activity the NF- κ B, JNK, CREB, AP-1, GSK-3 β and ELK-1 signaling proteins.^{3,23} As CsA has effects on the activity of GSK-3 β ,²³ it would be interesting to investigate the contribution of downstream Wnt pathway involved signaling proteins on AP-1 gene expression in this system. Alternately, demonstrating a causal link between the inhibition of signaling upstream of NF- κ B by CsA^{17,35} with downstream increases in AP-1 gene expression and increased osteoblastic bone formation (as has been seen with NF- κ B inhibition *in vivo*),^{2,10} may illustrate a mechanism by which CsA may be enhancing gene expression in our system. Thus, future investigation into these non-NFAT targets of calcineurin may identify factors that regulate CsA-mediated enhancement of bone mechanotransduction.

Several important limitations should be considered when interpreting the results of this study. First, in order to broadly analyze possible mechanisms that may be affected in MC3T3-E1 cells treated with CsA and fluid flow, we used gene expression data as the primary outcome for this study. While such data are illustrative of broad signaling trends, it remains to be seen whether such gene expression changes result in differences at the protein level, especially considering that our changes were relatively mild. Post-transcriptional modifications are an important aspect of gene regulation, and initial analysis of Fos and Jun in osteoblastic cells found disparities between protein and mRNA levels during differentiation.³² Second, though the AP-1 genes identified in this study have been implicated in osteoblastic differentiation, we did not determine whether the CsA-mediated enhancement of AP-1 levels was responsible for the enhanced osteoblastic gene expression observed in our studies. Future studies will more comprehensively explore the interactions of these transcription factors on the specific promoter sequences of relevant osteoblastic function genes in response to fluid flow and CsA treatment in order to extend these initial findings. Third, we subjected cells to flow using orbital shaking, which results in a pulsatile waveform that differs from the oscillatory flow that is expected to occur *in vivo*. While recent studies suggest that orbital shaking and oscillatory flow in parallel plate chambers give rise to similar bone cell responses for a range of outcomes,⁵⁶ further studies are required to characterize the degree to which the findings from this study are robust to mechanical waveform. Finally, though our use of a well-characterized model of bone cell mechanotransduction (i.e. MC3T3-E1 osteoblastic cells exposed to fluid shear stress^{13,24,27,31};) greatly simplified our investigations, it does not recapitulate the complex,

multi-cellular interactions within intact loaded bone.²⁶ Particularly, bone adaptation to mechanical loading is mediated by osteocyte communication with osteoblasts,²⁵ and the degree to which the mechanisms explored in this study are conserved in osteoblasts and osteocytes *in vivo* remains to be investigated. Indeed, the mild effect of CsA in enhancing mechanically-induced expression of osteoblastic genes highlights the importance to confirm mechanisms elucidated here within intact loaded bone, an avenue of research we are actively exploring.

In summary, using an *in vitro* model of bone cell mechanotransduction we found that CsA treatment enhances mechanically-induced expression of AP-1 genes, and that this enhancement cannot be solely attributed to inhibition of nuclear NFAT accumulation. These data are consistent with the concept that CsA enhances bone mechanotransduction through inhibiting both NFAT and non-NFAT calcineurin targets in parallel, and suggest that future investigations should consider the interplay between NFAT and non-NFAT mediated signaling pathways during CsA-mediated enhancement of loading-induced bone formation.

Acknowledgments

The authors would like to acknowledge financial support from the University of Washington Department of Orthopaedics and Sports Medicine (RYK), the National Institutes of Health (SS: AR0562235, TSG: AR056652), and the Sigvard T. Hansen, Jr. Endowed Chair (TSG). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ABBREVIATIONS

AP-1	Activator protein-1
NFAT	Nuclear factor of activated T cells
CsA	Cyclosporin A
GSK-3β	Glycogen synthase kinase-3 β
Elk-1	ETS-like transcription factor 1
NF-κB	Nuclear factor κ B
CREB	Cyclic AMP response element-binding protein
ALP	Alkaline phosphatase
OPN	Osteopontin
OCN	Osteocalcin
Cox2	Cyclooxygenase 2

REFERENCES

1. Akhouayri O, St-Arnaud R. Differential mechanisms of transcriptional regulation of the mouse osteocalcin gene by Jun family members. *Calcif. Tissue Int.* 2007; 80:123–131. [PubMed: 17308994]
2. Alles N, Soysa NS, Hayashi J, Khan M, Shimoda A, Shimokawa H, Ritzeler O, Akiyoshi K, Aoki K, Ohya K. Suppression of NF-kappaB increases bone formation and ameliorates osteopenia in ovariectomized mice. *Endocrinology.* 2010; 151:4626–4634. [PubMed: 20810563]

3. Aramburu J, Rao A, Klee CB. Calcineurin: from structure to function. *Curr. Top. Cell Regul.* 2000; 36:237–295. [PubMed: 10842755]
4. Aramburu J, Yaffe MB, Lopez-Rodriguez C, Cantley LC, Hogan PG, Rao A. Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. *Science.* 1999; 285:2129–2133. [PubMed: 10497131]
5. Asanuma M, Ogawa N, Nishibayashi S, Kondo Y, Mori A. Effects of repeated injection of cyclosporin A on pentylentetrazol-induced convulsion and cyclophilin mRNA levels in rat brain. *Neurochem. Res.* 1995; 20:101–105. [PubMed: 7739751]
6. Behrens A, Haigh J, Mechta-Grigoriou F, Nagy A, Yaniv M, Wagner EF. Impaired intervertebral disc formation in the absence of Jun. *Development.* 2003; 130:103–109. [PubMed: 12441295]
7. Bito H, Deisseroth K, Tsien RW. CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. *Cell.* 1996; 87:1203–1214. [PubMed: 8980227]
8. Celil Aydemir AB, Lee S, Won Kim D, Gardner TR, Prince D, Mok Ahn J, Lee FY. Nuclear factor of activated T cell mediates proinflammatory gene expression in response to mechanotransduction. *Ann. N Y Acad. Sci.* 2007; 1117:138–142. [PubMed: 17584983]
9. Celil Aydemir AB, Minematsu H, Gardner TR, Kim KO, Ahn JM, Lee FY. Nuclear factor of activated T cells mediates fluid shear stress- and tensile strain-induced Cox2 in human and murine bone cells. *Bone.* 2010; 46:167–175. [PubMed: 19748606]
10. Chang J, Wang Z, Tang E, Fan Z, McCauley L, Franceschi R, Guan K, Krebsbach PH, Wang CY. Inhibition of osteoblastic bone formation by nuclear factor-kappaB. *Nat. Med.* 2009; 15:682–689. [PubMed: 19448637]
11. Cheung AM, Giangregorio L. Mechanical stimuli and bone health: what is the evidence? *Curr. Opin. Rheumatol.* 2012; 24:561–566. [PubMed: 22832826]
12. D'Alonzo RC, Selvamurugan N, Karsenty G, Partridge NC. Physical interaction of the activator protein-1 factors c-Fos and c-Jun with Cbfa1 for collagenase-3 promoter activation. *J. Biol. Chem.* 2002; 277:816–822. [PubMed: 11641401]
13. Donahue TL, Haut TR, Yellowley CE, Donahue HJ, Jacobs CR. Mechanosensitivity of bone cells to oscillating fluid flow induced shear stress may be modulated by chemotransport. *J. Biomech.* 2003; 36:1363–1371. [PubMed: 12893045]
14. Donahue SW, Jacobs CR, Donahue HJ. Flow-induced calcium oscillations in rat osteoblasts are age, loading frequency, and shear stress dependent. *Am. J. Physiol. Cell Physiol.* 2001; 281:C1635–C1641. [PubMed: 11600427]
15. Eferl R, Hoebertz A, Schilling AF, Rath M, Karreth F, Kenner L, Amling M, Wagner EF. The Fos-related antigen Fra-1 is an activator of bone matrix formation. *EMBO J.* 2004; 23:2789–2799. [PubMed: 15229648]
16. Flanagan WM, Cortes B, Bram RJ, Crabtree GR. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature.* 1991; 352:803–807. [PubMed: 1715516]
17. Frischbutter S, Gabriel C, Bendfeldt H, Radbruch A, Baumgrass R. Dephosphorylation of Bcl-10 by calcineurin is essential for canonical NF-kappaB activation in Th cells. *Eur. J. Immunol.* 2011; 41:2349–2357. [PubMed: 21674474]
18. Grigoriadis AE, Schellander K, Wang ZQ, Wagner EF. Osteoblasts are target cells for transformation in c-fos transgenic mice. *J. Cell Biol.* 1993; 122:685–701. [PubMed: 8335693]
19. Hipskind RA, Bilbe G. MAP kinase signaling cascades and gene expression in osteoblasts. *Front Biosci.* 1998; 3:d804–d816. [PubMed: 9682034]
20. Inoue D, Kido S, Matsumoto T. Transcriptional induction of FosB/DeltaFosB gene by mechanical stress in osteoblasts. *J. Biol. Chem.* 2004; 279:49795–49803. [PubMed: 15383527]
21. Jochum W, David JP, Elliott C, Wutz A, Plenk H Jr, Matsuo K, Wagner EF. Increased bone formation and osteosclerosis in mice overexpressing the transcription factor Fra-1. *Nat. Med.* 2000; 6:980–984. [PubMed: 10973316]
22. Julien M, Khoshniat S, Lacreusette A, Gatus M, Bozec A, Wagner EF, Wittrant Y, Masson M, Weiss P, Beck L, Magne D, Guicheux J. Phosphate-dependent regulation of MGP in osteoblasts: role of ERK1/2 and Fra-1. *J. Bone Miner. Res.* 2009; 24:1856–1868. [PubMed: 19419315]

23. Kim Y, Lee YI, Seo M, Kim SY, Lee JE, Youn HD, Kim YS, Juhnn YS. Calcineurin dephosphorylates glycogen synthase kinase-3 beta at serine-9 in neuroblast-derived cells. *J. Neurochem.* 2009; 111:344–354. [PubMed: 19659461]
24. Kwon RY, Jacobs CR. Time-dependent deformations in bone cells exposed to fluid flow in vitro: investigating the role of cellular deformation in fluid flow-induced signaling. *J. Biomech.* 2007; 40:3162–3168. [PubMed: 17559856]
25. Kwon RY, Meays DR, Meilan AS, Jones J, Miramontes R, Kardos N, Yeh JC, Frangos JA. Skeletal adaptation to intramedullary pressure-induced interstitial fluid flow is enhanced in mice subjected to targeted osteocyte ablation. *PLoS ONE.* 2012; 7:e33336. [PubMed: 22413015]
26. Kwon RY, Meays DR, Tang WJ, Frangos JA. Microfluidic enhancement of intramedullary pressure increases interstitial fluid flow and inhibits bone loss in hindlimb suspended mice. *J. Bone Miner. Res.* 2010; 25:1798–1807. [PubMed: 20200992]
27. Kwon RY, Temiyasathit S, Tummala P, Quah CC, Jacobs CR. Primary cilium-dependent mechanosensing is mediated by adenylyl cyclase 6 and cyclic AMP in bone cells. *FASEB J.* 2010; 24:2859–2868. [PubMed: 20371630]
28. Li Z, Wang Z, Yang L, Li X, Sasaki Y, Wang S, Araki S, Mezawa M, Takai H, Nakayama Y, Ogata Y. Fibroblast growth factor 2 regulates bone sialoprotein gene transcription in human breast cancer cells. *J. Oral Sci.* 2010; 52:125–132. [PubMed: 20339243]
29. Liu J, Albers MW, Wandless TJ, Luan S, Alberg DG, Belshaw PJ, Cohen P, MacKintosh C, Klee CB, Schreiber SL. Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. *Biochemistry.* 1992; 31:3896–3901. [PubMed: 1373650]
30. Macian F, Lopez-Rodriguez C, Rao A. Partners in transcription: NFAT and AP-1. *Oncogene.* 2001; 20:2476–2489. [PubMed: 11402342]
31. Malone AM, Batra NN, Shivaram G, Kwon RY, You L, Kim CH, Rodriguez J, Jair K, Jacobs CR. The role of actin cytoskeleton in oscillatory fluid flow-induced signaling in MC3T3-E1 osteoblasts. *Am. J. Physiol. Cell Physiol.* 2007; 292:C1830–C1836. [PubMed: 17251324]
32. McCabe LR, Banerjee C, Kundu R, Harrison RJ, Dobner PR, Stein JL, Lian JB, Stein GS. Developmental expression and activities of specific fos and jun proteins are functionally related to osteoblast maturation: role of Fra-2 and Jun D during differentiation. *Endocrinology.* 1996; 137:4398–4408. [PubMed: 8828501]
33. McCabe LR, Kockx M, Lian J, Stein J, Stein G. Selective expression of fos- and jun-related genes during osteoblast proliferation and differentiation. *Exp. Cell Res.* 1995; 218:255–262. [PubMed: 7737363]
34. Orcel P, Denne MA, de Vernejoul MC. Cyclosporin-A in vitro decreases bone resorption, osteoclast formation, and the fusion of cells of the monocyte-macrophage lineage. *Endocrinology.* 1991; 128:1638–1646. [PubMed: 1999178]
35. Palkowitsch L, Marienfeld U, Brunner C, Eitelhuber A, Krappmann D, Marienfeld RB. The Ca²⁺-dependent phosphatase calcineurin controls the formation of the Carma1-Bcl10-Malt1 complex during T cell receptor-induced NF-kappaB activation. *J. Biol. Chem.* 2011; 286:7522–7534. [PubMed: 21199863]
36. Peverali FA, Basdra EK, Papavassiliou AG. Stretch-mediated activation of selective MAPK subtypes and potentiation of AP-1 binding in human osteoblastic cells. *Mol. Med.* 2001; 7:68–78. [PubMed: 11474129]
37. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 2002; 30:e36. [PubMed: 11972351]
38. Pruitt LA, Taaffe DR, Marcus R. Effects of a one-year high-intensity versus low-intensity resistance training program on bone mineral density in older women. *J. Bone Miner. Res.* 1995; 10:1788–1795. [PubMed: 8592957]
39. Pyrzynska B, Mosieniak G, Kaminska B. Changes of the trans-activating potential of AP-1 transcription factor during cyclosporin A-induced apoptosis of glioma cells are mediated by phosphorylation and alterations of AP-1 composition. *J. Neurochem.* 2000; 74:42–51. [PubMed: 10617104]

40. Qiu N, Xiao Z, Cao L, Buechel MM, David V, Roan E, Quarles LD. Disruption of Kif3a in osteoblasts results in defective bone formation and osteopenia. *J. Cell Sci.* 2012; 125:1945–1957. [PubMed: 22357948]
41. Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* 2003; 339:62–66. [PubMed: 12618301]
42. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* 1997; 15:707–747. [PubMed: 9143705]
43. Rauch C, Loughna PT. Stretch-induced activation of ERK in myocytes is p38 and calcineurin-dependent. *Cell Biochem. Funct.* 2008; 26:866–869. [PubMed: 18956431]
44. Rubin C, Turner AS, Bain S, Mallinckrodt C, McLeod K. Anabolism. Low mechanical signals strengthen long bones. *Nature.* 2001; 412:603–604. [PubMed: 11493908]
45. Schaefer A, Magocsi M, Fandrich A, Marquardt H. Stimulation of the Ca²⁺-mediated egr-1 and c-fos expression in murine erythroleukemia cells by cyclosporin A. *Biochem. J.* 1998; 335:505–511. Pt 3. [PubMed: 9794788]
46. Srinivasan S, Ausk BJ, Prasad J, Threet D, Bain SD, Richardson TS, Gross TS. Rescuing loading induced bone formation at senescence. *PLoS Comput. Biol.* 2010; 6:e1000924. [PubMed: 20838577]
47. Srinivasan S, Gross TS, Bain SD. Bone mechanotransduction may require augmentation in order to strengthen the senescent skeleton. *Ageing Res. Rev.* 2012; 11:353–360. [PubMed: 22240208]
48. Srinivasan S, Threet D, Worton LE, Ausk BJ, Bain SD, Gardiner EM, Kwon RY, Gross TS. Distinct cyclosporin doses are required to enhance bone formation induced by cyclic and rest-inserted loading in the senescent skeleton. *PLoS ONE.* 2014; 9:e84868. [PubMed: 24404194]
49. Sugimoto T, Stewart S, Guan KL. The calcium/calmodulin-dependent protein phosphatase calcineurin is the major Elk-1 phosphatase. *J. Biol. Chem.* 1997; 272:29415–29418. [PubMed: 9367995]
50. Tamler R, Epstein S. Nonsteroid immune modulators and bone disease. *Ann. N Y Acad. Sci.* 2006; 1068:284–296. [PubMed: 16831929]
51. Worton LE, Ausk BJ, Downey LM, Bain SD, Gardiner EM, Srinivasan S, Gross TS, Kwon RY. Systems-based identification of temporal processing pathways during bone cell mechanotransduction. *PLoS ONE.* 2013; 8(9):e74205. [PubMed: 24040202]
52. Wu CC, Li YS, Haga JH, Wang N, Lian IY, Su FC, Usami S, Chien S. Roles of MAP kinases in the regulation of bone matrix gene expressions in human osteoblasts by oscillatory fluid flow. *J. Cell. Biochem.* 2006; 98:632–641. [PubMed: 16440309]
53. Yeo H, Beck LH, McDonald JM, Zayzafoon M. Cyclosporin A elicits dose-dependent biphasic effects on osteoblast differentiation and bone formation. *Bone.* 2007; 40:1502–1516. [PubMed: 17392048]
54. Yeo H, McDonald JM, Zayzafoon M. NFATc1: a novel anabolic therapeutic target for osteoporosis. *Ann. N Y Acad. Sci.* 2006; 1068:564–567. [PubMed: 16831953]
55. Young SR, Gerard-O’Riley R, Harrington M, Pavalko FM. Activation of NF-kappaB by fluid shear stress, but not TNF-alpha, requires focal adhesion kinase in osteoblasts. *Bone.* 2010; 47:74–82. [PubMed: 20353839]
56. Young SR, Hum JM, Rodenberg E, Turner CH, Pavalko FM. Non-overlapping functions for Pyk2 and FAK in osteoblasts during fluid shear stress-induced mechanotransduction. *PLoS ONE.* 2011; 6:e16026. [PubMed: 21283581]
57. Yu S, Geng Q, Sun F, Yu Y, Pan Q, Hong A. Osteogenic differentiation of C2C12 myogenic progenitor cells requires the Fos-related antigen Fra-1—a novel target of Runx2. *Biochem. Biophys. Res. Commun.* 2013; 430:173–178. [PubMed: 23159633]
58. Zhou S, Bueno EM, Kim SW, Amato I, Shen L, Hahne J, Bleiberg I, Morley P, Glowacki J. Effects of age on parathyroid hormone signaling in human marrow stromal cells. *Aging Cell.* 2011; 10:780–788. [PubMed: 21518242]

FIGURE 1.

Acute responses to fluid flow in CsA treated MC3T3-E1 cells. MC3T3-E1 cells pretreated with CsA or DMSO were subjected to fluid flow (FF). (a) To detect NFATc1 activation after dephosphorylation, cell fractions were collected at the time-points shown and analyzed by western blot for nuclear NFATc1 after treatment with doses of CsA. β -actin was used as a loading control. (b) Levels of NFATc1 in the cytoplasm were analyzed by western blot after CsA treatment and 5 min of flow. β -actin was used as a loading control. (c) After treatment with CsA doses cells were subjected to flow, and total cell lysates collected at the time-points shown. Lysates were analyzed for phosphorylated and total ERK1/2. (d) After 1 h incubation under stationary (grey bars) or flow (black bars) conditions, CsA or DMSO treated cells were analyzed by qRT-PCR for the expression of c-Fos and Cox2. Two-way ANOVA analyses were performed to detect significance of flow (denoted F), CsA dose (denoted D) and the combination of these factors (denoted F:D), for the expression of these genes. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. NF. Western blots results are representative of two independent experiments. qRT-PCR data are pooled from three replicate experiments and graphed as mean + SE (n = 9).

FIGURE 2.

Osteoblastic gene expression is stimulated by CsA. A panel of osteoblastic markers was analyzed by qRT-PCR for changes in mRNA levels with CsA and repeated fluid flow bouts. Expression under static conditions (grey bars) was compared to that seen after repeated flow bouts (black bars), for the early osteoblast markers (a) Runx2, (b) Osx, and (c) ALP, and later markers (d) OPN and (e) OCN for cells treated with CsA doses or with the DMSO control. Two-way ANOVA analyses were performed to detect significance of flow (denoted F), CsA dose (denoted D) and the combination of these factors (denoted F:D), for the expression of these genes. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. DMSO. Data are pooled from two independent experiments and graphed as mean + SE ($n = 4-7$).

FIGURE 3.

Expression of AP-1 genes is enhanced with CsA and fluid flow. After repeated treatment of MC3T3-E1 cells with CsA doses, expression of c-Fos, FosB, Fra-1, Fra-2, FosB, c-Jun, JunB and JunD (a-h) was analyzed in cells incubated under stationary conditions (grey bars) or with fluid flow (black bars). There was a flow-induced increase in expression of Fra-1 and c-Jun, after subjecting cells to repeated bouts of flow. Two-way ANOVA analyses were performed to detect significance of flow (denoted F), CsA dose (denoted D) and the combination of these factors (denoted F:D), for the expression of these genes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. NF; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. DMSO. Data are pooled from two independent experiments and graphed as mean + SE ($n = 7$).

FIGURE 4.

Acute responses to fluid flow in VIVIT treated MC3T3-E1 cells. MC3T3-E1 cells pretreated with VIVIT or DMSO were subjected to fluid flow (FF). (a) Cell fractions were collected at the time-points shown and analyzed by western blot for nuclear NFATc1 after VIVIT treatment. β -actin was used as a loading control. (b) After treatment with 1 μ M VIVIT cells were subjected to flow, and total cell lysates collected at the time-points shown. Lysates were analyzed for phosphorylated and total ERK1/2. (c) After 1 h incubation under stationary (grey bars) or flow (black bars) conditions, VIVIT or DMSO treated cells were analyzed by qRT-PCR for the expression of c-Fos and Cox2. Two-way ANOVA analyses were performed to detect significance of flow (denoted F), treatment (denoted T) and the combination of these factors (denoted F:T), for the expression of these genes. Western blots results are representative of two independent experiments. qRT-PCR data are pooled from 3 replicate experiments and graphed as mean + SE ($n = 10$).

FIGURE 5.

Enhanced gene expression with repeated flow bouts is not seen with VIVIT treatment. After repeated treatment with DMSO or 1 μ M VIVIT and fluid flow, cells were analyzed by qRT-PCR for (a) Fra-1 and (b) c-Jun mRNA levels. There was no increase in gene expression with VIVIT treatment in cells incubated under stationary conditions (grey bars). The flow-induced increase in Fra-1 and c-Jun expression observed in DMSO controls was not seen in VIVIT treated cells (black bars). Two-way ANOVA analyses were performed to detect significance of flow (denoted F), treatment (denoted T) and the combination of these factors (denoted F:T), for the expression of these genes. Data is pooled from 2 independent experiments and graphed as mean $\pm$ SE ($n = 3-7$).

TABLE 1

Primer sequences for qRT-PCR.

Target	F (5' → 3')	R (5' → 3')	Accession no.
cyc	GCCGATGACGAGCCCTTGGGCC	ACCAGTGCCATTATGGCGTGTG	NM_008907.1
β -actin	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG	NM_007393.3
c-Fos	CGCAGAGCATCGGCAGAAGG	TCTTGCAGGCAGGTCGGTGG	NM_010234
Cox-2	GCTGTACAAGCAGTGGCAAA	CCCCAAAGATAGCATCTGGA	NM_011198.3
Runx2	AAGTGCGGTGCAAACCTTTCT	TCTCGGTGGCTGGTAGTGA	NM_001146038.2
Osx	CCCTTCTCAAGCACCAATGG	AGGGTGGGTAGTCATTTCATAG	NM_130458.3
ALP	GCCCTCTCCAAGACATATA	CCATGATCACGTCGATATCC	NM_007431.2
OPN	TGCACCCAGATCCTATAGCC	CTCCATCGTCATCATCATCG	NM_009263
OCN	CTCTGTCTCTTGACCTCACAG	GGAGCTGCTGTGACATCCATAC	NM_007541.3
FosB	GTGTAGCTCATCACCTCCG	GTAGCTGGTTCCTGGCATGT	NM_008036.2
FosB	AGGCAGAGCTGGAGTCGGAGAT	GCCGAGGACTTGAACCTCACTCG	NM_008036.2
Fra-1	GCAAGTGTTTCAGCCCAAGAA	GTCCCAGGAAATGAGGCTGC	NM_010235.2
Fra-2	GTCTGCCTTGGTTAGGGGAC	GTGATGGCGTTGATTGTGGG	NM_008037.4
c-Jun	TGAGTGACCGCGACTTTTCA	GAGGGCATCGTCGTAGAAGG	NM_010591.2
JunB	AGGCAGCTACTTTTCGGGTC	GCGTCACGTGGTTCATCTTG	NM_008416.3
JunD	GACACGCAAGAACGCATCAA	TTGACGTGGCTGAGGACTTTC	NM_010592.4