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# Calcineurin/NFAT signaling pathway in osteoblast differentiation.

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# CALCINEURIN / NFAT SIGNALING PATHWAY IN OSTEOBLAST DIFFERENTIATION

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## ABSTRACT

Bone loss and osteoporosis result from an imbalance of bone formation and resorption. Cn/NFAT signaling is known to play a crucial role in the regulation of osteoclastogenesis, however, the mechanisms of the action of Cn/NFAT signaling in bone formation remain unclear. Here, we investigated the role of Cn/NFAT signaling in osteoblast differentiation and bone formation. We determined the role of Cn/NFAT signaling in osteoblast differentiation *in vitro* and bone mass *in vivo*. We observed these effects by the administration of CsA, a pharmacological inhibitor of Cn/NFAT signaling, and by a genetic osteoblast-specific deletion of Cnb1 in a mouse model.

Here, we show that the inhibition of Cn with low doses of CsA are anabolic and increase osteoblast differentiation *in vitro* and bone mass *in vivo*, whereas high doses exhibited a catabolic effect. In addition, osteoblast-specific deletion of Cnb1 in mice significantly increased bone formation and bone mass. This increase in bone mass is mediated by increasing osteoblast differentiation and by indirectly decreasing osteoclastogenesis. Mechanistically, the subsequent inhibition of Cn/NFAT signaling by CsA and the osteoblast-specific deletion of Cnb1 are accompanied by increased Fra-2 expression, thus promoting osteoblast differentiation. We also demonstrated enhanced expression of constitutively active NFATc1 leads to a decrease in osteoblast differentiation. Furthermore, NFATc1 forms a transcriptional repressor complex with histone deacetylase on the osteocalcin promoter, thus inhibiting osteoblast differentiation.

Taken together, these results demonstrated that Cn/NFAT signaling negatively regulates osteoblast differentiation and bone formation *in vitro* and *in vivo*. These findings will assist in developing a viable anabolic therapy to combat osteoporosis.

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INTRODUCTION



CYCLOSPORIN A ELICITS DOSE-DEPENDENT BIPHASIC EFFECTS ON OSTEOBLAST DIFFERENTIATION AND BONE FORMATION



CONDITIONAL DYSRUPTION OF CALCINEURIN B1 IN OSTEOBLASTS INCREASES BONE FORMATION AND REDUCES BONE RESORPTION

# INTRODUCTION

## Bone Turnover

Bone is a dynamic tissue that undergoes continuous turnover, the destruction of old bone and reformation of new bone, thereby maintaining bone mass and calcium homeostasis. Two primary cells, the osteoblasts and the osteoclasts, are responsible for bone formation and resorption, respectively. The presence of these two opposing cell types with contrasting activities requires tight regulation in order to maintain healthy and strong bones. Osteoblasts, which are derived from osteoprogenitor cells, become terminally differentiated to synthesize bone matrix proteins and regulate mineralization of the tissue. Multinucleated osteoclasts originate from hematopoietic cells and resorb bone. Therefore, bone loss may be caused by either a decrease in bone formation or an increase in bone resorption that results from an imbalance between the activity of osteoblasts and osteoclasts. The disruption of bone metabolism underlies a number of pathologies, including osteoporosis.

## Bone Formation and Remodeling

Bone is composed of two bone types, flat bones and long bones. These bones are derived from two different mechanisms, intramembraneous and endochondral ossification, respectively. Endochondral ossification occurs after patterning of skeletal elements when mesenchymal cells differentiate into chondrocytes, the cartilage-specific cell type. This cartilaginous skeleton is eventually replaced by mineralized bone containing osteoblasts and osteoclasts. During intramembraneous ossification, however, mesenchymal cells differentiate directly into osteoblasts [1]. Most bone turnover occurs

at the surface where calcified tissue interfaces with the bone marrow. Old bone is replaced by new bone by a remodeling process where bone growth and turnover occur through bone resorption by osteoclasts followed by bone formation by osteoblasts [2]. The interplay between osteoblasts and osteoclasts is crucial in bone remodeling. A key regulator of this process is the receptor activator of NF-κB (RANK)/receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) axis [3]. Osteoblasts express OPG (the osteoclast inhibitor factor) and RANKL (the major osteoclast differentiation factor) whereas osteoclasts express RANK, a cognate receptor of RANKL and OPG. RANKL, expressed on osteoblasts surface, interacts with RANK on osteoclasts, thus induce osteoclast differentiation, whereas OPG inhibits it. The alternative expression of OPG and RANKL from osteoblasts directly affects osteoclastogenesis. Therefore, the process of maintaining bone mass is tightly regulated by a balance in the level of OPG and RANKL between two opposing cells, osteoblasts and osteoclasts.

**Osteoclasts**

Osteoclasts are multinucleated giant cells, which are responsible for bone resorption. Osteoclasts are differentiated from monocyte/macrophage progenitor cells, which originate from hematopoietic stem cells. Osteoclasts attach to the bone surface, form a sealed bone-resorbing compartment and secrete enzymes, such as tartrate-resistant acid phosphatase (TRAP) and lysosomal enzymes, to dissolve the bone mineral and resorb the bone matrix. Osteoclast differentiation is regulated by interaction with cells of the early osteoblast lineage, which express two cytokines, RANK and macrophage-colony stimulating factor (M-CSF) [4].

**Osteoblast Differentiation**

Osteoblasts are derived from multipotent mesenchymal stem cells (MSC), which have the ability to differentiate into several different cell types, including chondrocytes, adipocytes, myoblasts and osteoblasts [5, 6]. Commitment of MSCs to tissue-specific cell types is regulated by specific transcription factors, which promote transcription of lineage-specific gene products. The osteoprogenitors are sequentially differentiated into pre-osteoblasts, mature, functional osteoblasts and osteocytes [6] (Figure 1). Osteoblast differentiation involves three stages of development: proliferation, extracellular matrix maturation and mineralization [7, 8]. During each stage of development, osteoblast-specific genes are sequentially expressed and repressed. Histone 4 (not shown in Figure1) and collagen type I are markers for the proliferation stage, alkaline phosphatase (ALP) for the extracellular matrix maturation stage and osteocalcin for mineralization *in vitro* and *in vivo* [8]. Osteoblasts are responsible for the production of collagenous, unmineralized bone matrix, known as osteoid. Osteoblast differentiation is known to be regulated at the transcriptional level [9, 10]. Several transcription factors and signaling pathway-associated molecules, such as activating protein-1 (AP-1) and Runx2, play essential roles in the regulation of osteoblast gene expression, phenotype and bone formation [11-13].

**Osteoporosis**

Osteoporosis is a metabolic disease characterized by a decrease in the quality and quantity of bone that results in skeletal fragility and increased susceptibility to fracture [14]. Although any bone can be affected, the most common fracture sites are the hip,

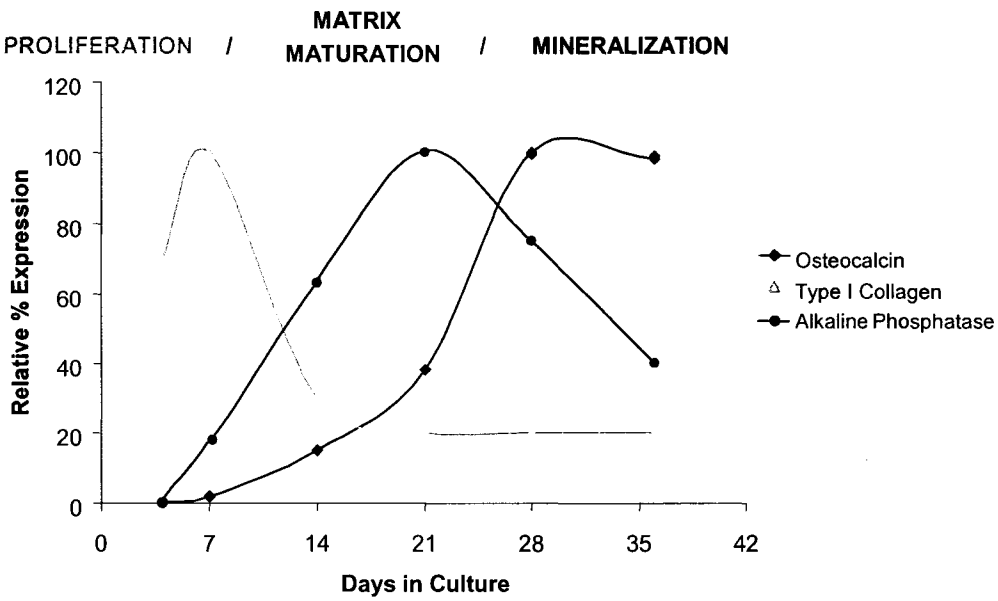


Figure 1. Development of osteoblast differentiation. Osteoblast differentiation involves three stages of development: proliferation, matrix maturation and mineralization. During development, osteoblast-specific genes are sequentially expressed and repressed. Type I collagen is predominantly expressed in the proliferation and early matrix maturation stages. The expression of alkaline phosphatase increases during matrix maturation, and beginning to decrease during the mineralization phase. Osteocalcin is predominantly expressed in the mineralization stage. Figure modified with permission from Owen *et al.*, *J. Cell. Physiol.*, Vol. 143, pp. 420-430. Copyright 1990, John Willey & Sons, Ltd.

vertebra (spine) and distal forearm (wrist). In 1994, the term osteoporosis was defined by the World Health Organization (WHO) as a condition of having a bone mineral density (BMD) value more than 2.5 standard deviations below the average for normal, young white women. Based on this criterion, 10 million Americans over age 50 are estimated to have osteoporosis. Furthermore, more than 33.6 million Americans have low bone mass, osteopenia, which places them at risk of developing osteoporosis [15-17]. One in two Americans over age 50 will have low bone mass and will suffer an osteoporosis-related fracture by 2020 [18]. Primary osteoporosis can occur in both sexes at all ages but often follows menopause in women and occurs later in life in men, referred to as age-related osteoporosis [19]. Secondary osteoporosis can occur as the result of other pathological conditions, like hypogonadism, or medications, such as glucocorticoids, which can contribute to the development of osteoporosis [20].

In order to prevent osteoporosis, several therapies are currently available: antiresorptive therapy, estrogen/hormone replacement and anabolic therapy. Antiresorptive drugs, including bisphosphonates (i.e. Alendronate), calcitonin, and estrogen analogs (Raloxifen), are currently approved for treatment of osteoporotic patients and function to reduce osteoclast-mediated bone resorption [21]. Although these antiresorptive agents reduce the bone remodeling rate, they do not restore or build new bone. Conversely, anabolic parathyroid hormone (PTH) therapy activates bone remodeling and leads to increased bone formation. Although intermittent PTH treatment dramatically increases bone mineral density and decreases fracture rates, the molecular mechanism for the anabolic actions of PTH remains to be elucidated [22, 23]. Therefore, finding new anabolic drugs whose molecular mechanisms are better understood is

necessary. Since osteoblast differentiation is essential for bone formation, further understanding of the molecular mechanisms that control osteoblast differentiation would potentially lead to an increase in development of new anabolic drugs to treat osteoporosis.

**Transplantation and Bone Disease**

Transplantation has been established as a common therapy for end-stage renal disease, liver disease, cardiac failure and pulmonary disease. The number of patients who have undergone organ transplantation has increased over the last few decades, and the number has already exceeded 300,000 in the United States [24, 25]. The survival rates of transplantation recipients have improved dramatically. However, bone disease, such as osteoporosis, has been identified as a common complication of organ transplantation that adversely affects the quality of life of patients. Mechanistically, post-transplantation bone loss is multi-factorial. The underlying disease by itself, such as cardiac, renal or lung failure, can indirectly affect bone disorders and mineral metabolism. Additional risk factors, such as, immobilization, nutritional deficiency, aging and hypogonadism, may also negatively impact the skeletal system in transplantation patients [26, 27]. After transplantation, patients are subjected to an immunosuppressive drug regimen that includes high doses of glucocorticoids, which have deleterious effects on bone turnover and are a major cause of secondary osteoporosis. During the first 3-6 months after grafting, BMD is decreased largely at the lumbar spine and femoral neck, which could be related to the high doses of glucocorticoids used for the first 6 months after transplantation [28-30]. Despite the fact that glucocorticoids are used to inhibit various inflammatory conditions, this drug causes profound impairment of bone formation by

reducing osteoblast numbers and differentiation and inducing excessive bone resorption [31]. However, after treatment with glucocorticoids for the first 3 months, steroid-free immunosuppressive agents such as Cyclosporin A are implemented following reduction of glucocorticoid doses. The BMD of post cardiac transplantation patients who received these regimens showed an increase BMD of femoral neck and lumbar spine at 3-6 months which was maintained at 12 months [32]. These data provide evidence that CsA could function as a positive effector on bone formation.

**The Effects of Immunosuppressive Agents on Bone**

Cyclosporin A (CsA), a fungal lipophilic cyclic undecapeptide, and FK506 (tacrolimus) have been widely used for treatment of graft-vs-host disease and allograft rejection following organ transplantation, because CsA alters the immune system by suppressing the activation of T lymphocytes [33]. Bone disease, such as osteopenia and osteoporosis, is a common and serious complication of organ transplantation. This incidence is not only due to underlying disease and the organ transplantation but also thought to be caused by the co-administration of CsA to the patients along with other immunosuppressive drugs, such as glucocorticoids. [34-37]. However, CsA monotherapy in renal transplantation patients has been shown to significantly increase lumbar bone mineral density while transplant patients who were administered both CsA and glucocorticoid exhibited a significant decrease [38]. These controversial issues are also contradictory when addressed in animal experimental studies. Most animal studies regarding bone loss have utilized rat models. In laboratory animals, the administration of CsA produces osteopenia, a condition known to be associated with either a high turnover

bone loss (increase in both osteoblasts and osteoclasts) or a low turnover bone loss [39, 40]. The administration of CsA to rats at a dose of 15mg/kg has shown significant bone resorption and trabecular bone loss as well as bone formation, resulting in severe high turnover osteopenia [39]. In contrast, substantial evidence indicating increased bone formation and decreased bone resorption has been reported after administration of CsA to rats at a dose of 7mg/kg [41]. These discrepancies could be due to different dosages, durations, genders and ages of the rats used in the experiments. *In vitro*, low doses of FK506 (10nM-1μM) have been shown to induce osteoblast differentiation [42]. Interestingly, it has recently been reported that a high concentration of FK506 (25μM) inhibits osteoblast differentiation by affecting the transcriptional activity of osteoblast marker proteins, such as osterix, and high doses of CsA decreases osteoblast differentiation by altering extracellular matrix synthesis and degradation [43, 44]. Based on these previous studies *in vivo* and *in vitro*, CsA and FK506 treatments have shown conflicting results ranging from increased bone formation to severe osteopenia. It is not understood why CsA treatment elicits these opposing results. However, these data suggest that CsA might have concentration-dependent biphasic effects. This has led us to focus on the biphasic effects of CsA on osteoblast differentiation.

**Calcineurin**

Calcium ($Ca^{2+}$) is one of the important intracellular messengers that controls many cellular responses [45]. Cellular $Ca^{2+}$ homeostasis is complex, being regulated by numerous processes including plasma membrane $Ca^{2+}$ channel, plasma membrane ($Ca^{2+}$+ $Mg^{2+}$)-ATPase, the endoplasmic reticulum $Ca^{2+}$ pump, inositol 1,4,5-triphosphate (IP3)

sensitive release and mitochondrial function [45, 46] 15101689. Once intracellular $Ca^{2+}$ levels increase, a small $Ca^{2+}$- binding protein, calmodulin (CaM), transduces many signals in response to an increased $Ca^{2+}$. CaM contains four EF-hand $Ca^{2+}$ binding motifs. When $Ca^{2+}$ binds CaM, it undergoes a conformational change, and the coupling of $Ca^{2+}$/CaM allows CaM to bind and activate several targets, including calcium/calmodulin-dependent protein kinase Π (CaMKΠ) and calcineurin (Cn) [47, 48]. Cn is a serine and threonine protein phosphatase, also called protein phosphatase 2B. Cn is a heterodimeric protein complex consisting of two subunits, the catalytic subunit A (CnA) and the regulatory subunit B (Cnb). There are three isoforms of the catalytic subunit A (CnAα, Aβ, and Aγ) and two isoforms of the regulatory subunit B (Cnb1 and b2) in mammals. CnAα and CnAβ are ubiquitously expressed while CnAγ expression is restricted to the testis and brain. Similarly, Cnb1 is expressed ubiquitously whereas Cnb2 is only found in the testis [49, 50]. CnA contains 4 domains: 1) the N-terminal catalytic domain, 2) the Cnb-binding domain, 3) the CaM-binding domain and 4) the autoinhibitory domain (Figure 2A). In addition, Cnb contains four EF-hand $Ca^{2+}$-binding motifs [49, 51]. In resting state, the autoinhibitory domain interacts with the active site of CnA, thus blocking the activation of Cn and maintaining it in an inactive state. When $Ca^{2+}$ levels are elevated, $Ca^{2+}$-bound CaM interacts with Cn and enhances the release of the autoinhibitory domain, leading to activation of phosphatase activity. Catalytic activity of Cn is inhibited by immunosuppressive drugs such as CsA and FK506, through binding to endogenous immunophilin proteins, known as cyclophilins, and to FK506 binding protein (FKBP12), respectively [51, 52]. Inhibition of Cn activity by these drugs results in many side effects due to the blocking of the dephosphorylation of the many Cn

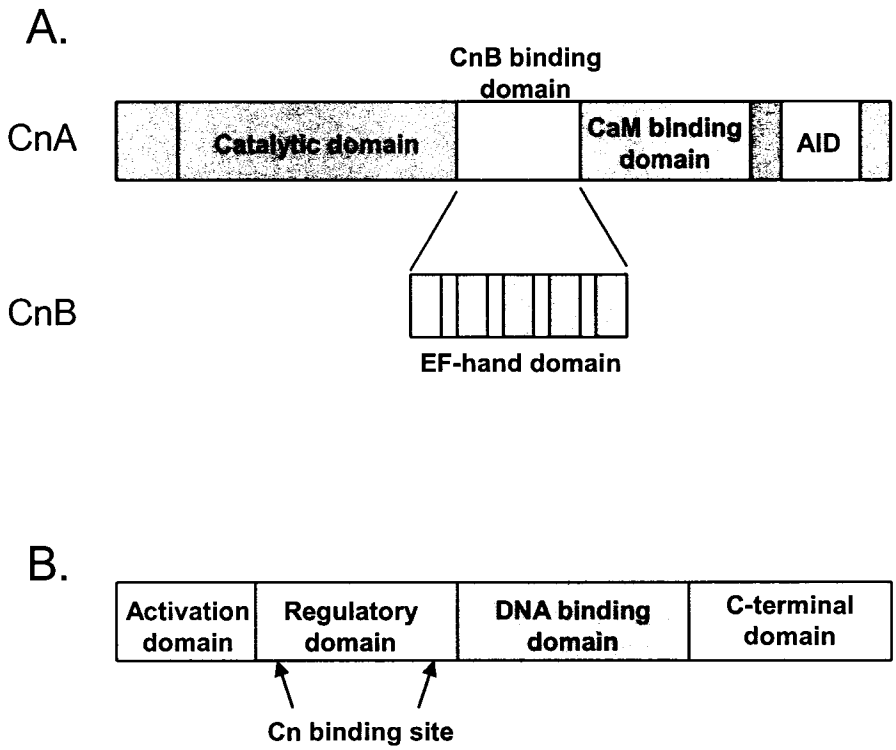


Figure 2. Structure of Cn and NFAT. (A). The catalytic calcineurin A subunit (CnA) has a catalytic domain, a calcineurin B (CnB)-binding domain, a calmodulin (CaM)-binding domain and an autoinhibitory domain (AID). The regulatory calcineurin B (CnB) subunit has 4 EF-hands, $Ca^{2+}$ binding sites. Figure modified with permission from Wilkins *et al.*, *Biochem Biophys Res Commun.*, Vol. 322, pp. 1178-1191. Copyright 2004, Elsevier. (B) This diagram is an overall structure of NFAT. NFAT has a transactivation domain in the N-terminal and the regulatory domain contains phosphorylation sites and two calcineurin binding sites. Also, NFAT has a DNA binding domain that is known as Rel homology regions (RHR) and a C-terminal domain Figure modified with permission from Hogan *et al.*, *Genes Devl.*, Vol. 17, pp. 2205-2231. Copyright 2003, Cold Spring Harvor Laboratory Press.

substrates, including nuclear factor-kappa B (NF-κB), cAMP response element-binding protein (CREB), myocyte enhancer factor 2 (MEF2) and a major target, nuclear factors of activated T cells (NFAT) [45, 53].

**NFAT**

The NFAT family of transcription factors, related to the Rel/NFκB family, is the best characterized of the calcineurin substrates [54]. The NFAT family of transcription factors is composed of five proteins: NFAT1 (NFATc2 or NFATp), NFAT2 (NFATc1 or NFATc), NFAT3 (NFATc4), NFAT4 (NFATc3 or NFATx) and NFAT5 (TonEBP) [54]. The NFAT 1-4 proteins have been shown to be regulated by Cn through $Ca^{2+}$/CaM-dependent signaling pathways while NFAT5 is not because NFAT5 lacks a regulatory domain that contains Cn docking sites [51, 54]. The NFAT 1-4 proteins consist of four major domains: 1) N-terminal transactivation domain, 2) regulatory domain, 3) DNA-binding domain and 4) C-terminal domain (Figure 2B). The regulatory domain contains two Cn binding motifs, an extended serine-rich region, three serine- and proline-rich regions and a nuclear localization signal [53-55]. In resting cells, the regulatory domain is highly phosphorylated on serine residues and resides in the cytoplasm. Upon activation of Cn through the coupling of $Ca^{2+}$/CaM, Cn binds to the regulatory domain of NFAT and dephosphorylates it. The dephosphorylated NFAT exposes the NFAT nuclear localization signal (NLS), causing it to translocate to the nucleus. Extensive NFAT dephosphorylation also induces NFAT DNA-binding activity to regulatory elements of NFAT target genes and activates transcription [54] (Figure 3). In the nucleus, NFAT forms as dimers to Rel/NF-κB-like elements of the DNA-binding domain and binds to the NFAT-binding

DNA site on the target gene promoter [56]. In addition, NFAT interacts with other transcription factors and coactivators, such as AP-1 and GATA-binding transcription factor (GATA) on composite NFAT/AP-1 sites and NFAT-GATA sites, leading to stimulation of gene expression [53, 57, 58]. When $Ca^{2+}$ levels returned to basal, NFAT is rephosphorylated by inducible or constitutive kinases, such as glycogen synthase kinase-3 (GSK-3) and casein kinase 1α (CK1α), which leads to exposure of the nuclear export signal, located at the C-terminus of the regulatory domain [53, 59, 60]. Cn and NFAT regulate expression of a number of immunological genes, including cytokine and cell-surface receptors which play a pivotal role in immune responses [55, 60].

**Calcineurin/NFAT Signaling Pathway and Bone**

The role of Cn and NFATs has been well defined in the immune system as well as in osteoclast differentiation and bone resorption [55, 60-64]. In contrast, the role of Cn and NFAT in osteoblast differentiation and bone formation is not well documented. Although the role of the Cn/NFAT signaling pathway in osteoblast differentiation and bone formation was recently reported in three different studies, these findings provoke several concerns [65-67]. For example, embryonic cells from the NFATc1$^{-/-}$ embryos exhibit a decrease in their osteoblastic potential, leading to the conclusion that NFATc1 is a positive regulator for osteoblast differentiation [65]. These embryonic stem cells are known to be pluripotent and capable of differentiating into several lineages such as chondrocytes, myocytes, adipocytes, as well as osteoblasts [5, 6]. Interestingly, NFAT has been shown to play a critical role in the lineage decision of stem cells to differentiate into chondrocytes, myocytes and adipocytes. Therefore, the decrease in osteoblast

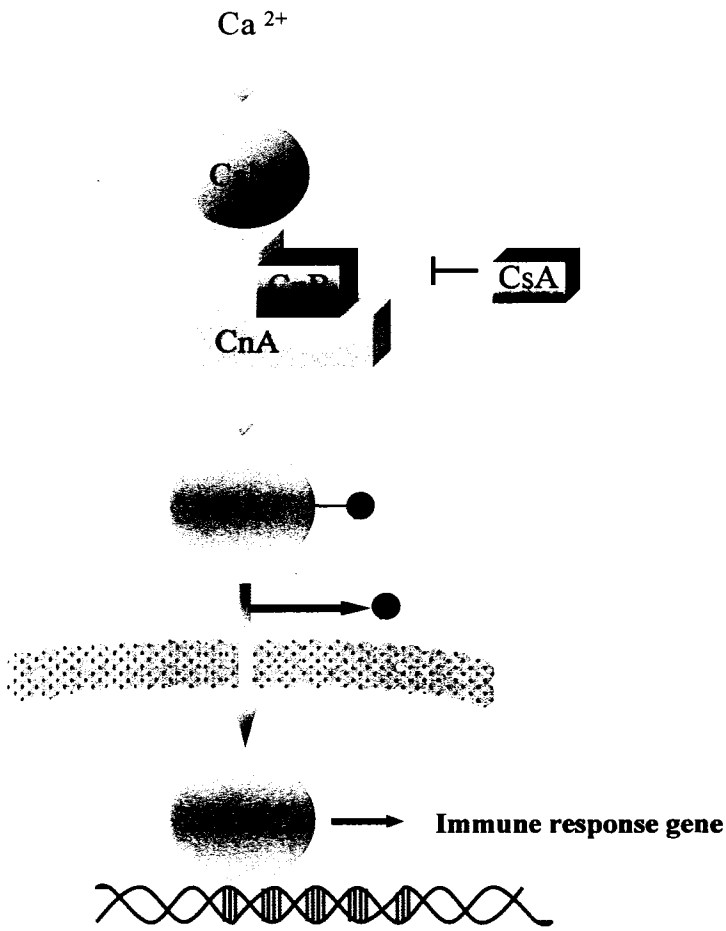


Figure 3. Regulation of the calcineurin-NFAT signaling pathway. After increased intracellular calcium, calcineurin (Cn) is activated by calmodulin (CaM) and binds to calcineurin A (CnA) catalytic subunit and calcineurin B (CnB) regulatory subunit, replacing an auto-inhibitory domain. Activated serine- and threonine- phosphatase calcineurin dephosphorylates NFAT, leading to translocation into the nucleus. CsA and cyclophilin complexes bind to calcineurin and inhibit its phosphatase activity. Dephosphorylated NFAT in the nucleus binds to its specific binding sites on target DNA and induces its target gene expression. Figure modified with permission from Parry *et al.*, *Nat Immunol.*, Vol. 4, pp. 874-881. Copyright 2003, Nature Publishing Group.

differentiation could possibly be due to a decrease in the lineage commitment of the NFATc1$^{-/-}$ embryonic cells, and not a direct osteoblastic response. In addition, it has been reported that the CnA Alpha deficient mice display severe osteoporosis and markedly reduced mineral apposition rates [66]. Furthermore, it was also reported that animals that globally overexpress a constitutively nuclear NFATc1 variant (NFATc1$^{nuc}$) develop high bone mass [67]. These reports utilized murine animal models where the Cn/NFAT signaling pathway was globally altered and/or in multiple tissues and were not reliable for examining the direct role of Cn and NFAT on osteoblast differentiation and bone mass. For instance, the global loss of CnA Alpha showed a decrease in bone mass that could be an indirect responses to an alteration in skeletal muscle phenotype or could be affected by altering T cell responses and activation [66, 68, 69]. Therefore, these reports did not consider that Cn and NFAT are also expressed in a variety of cells and tissues such as T cells, vasculature, skeletal muscle, chondrocytes and adipocytes that can indirectly alter the bone phenotype of these animals [60, 70]. To overcome these concerns and investigate the role of Cn and NFAT in osteoblast specifically, it is necessary to disrupt or overexpress Cn and NFAT signaling pathway molecules during osteoblastogenesis, using techniques such as osteoblast-specific conditional target gene deletion mice.

**AP-1 Transcription Factor**

The oncoproteins, c-Fos and c-Jun, are proto-typical members of the AP-1 family of transcription factors. This family also includes FosB, Fra-1, Fra-2, c-Jun, JunB, and JunD. AP-1 members are dimeric transcription factors composed of either homodimers of

the Jun family (c-Jun, JunB, and JunD) or heterodimers of the Jun and Fos family (c-Fos, Fra-1, Fra-2, and FosB) [71] or the Jun and ATF family (ATF-2, ATF-3, and ATF-4) [72]. AP-1 family members are well-known basic leucine-zipper (bZIP) proteins. Dimerization occurs through the leucine-zipper motif. These transcription factors also contain a basic region that recognizes the DNA element (TGACTCA), allowing binding to the DNA backbone. Dimerization of AP-1 family members is a prerequisite for DNA binding to their 12-*O*-tetradecanoylphorbol 13-acetate (TPA) response element (TRE) and cyclic adenosine monophosphate (cAMP) response element (CRE) consensus elements [72, 73]. AP-1 transcription factors are induced by several extracellular signals that increase mitogen-activated protein kinase (MAPK) activity. The transcriptional activity of AP-1 members is regulated by interactions with other transcription factors and is also controlled by upstream kinases, such as c-Jun $NH_2$- terminal kinase (JNK) and p38 kinase [74]. These kinases phosphorylate Fos and Jun proteins, thus regulating AP-1 transactivation and DNA binding activities. AP-1 transcription factors have been reported to regulate a variety of biological processes implicated in cell differentiation, proliferation, survival and transformation [75].

**AP-1 and Osteoblasts**

The role of AP-1 in skeletal development has been elucidated mainly by genetic studies in mice [13, 76, 77]. Expression of the AP-1 family members varies depending on the stage of osteoblast differentiation [78]. During osteoblast proliferation, all AP-1 members are known to be expressed, while Fra-2 and to a lesser extent, JunD are known to be the major AP-1 members expressed during mineralization, suggesting that Fra-2

expression is critical for osteoblast differentiation and mineralization [78]. This has been confirmed using Fra-2 antisense in osteoblasts, which results in inhibition of differentiation and mineralization [78]. Furthermore, it has been shown that hormones, such as PTH, increase osteoblast differentiation by increasing Fra-2 expression [79]. In many other systems, AP-1 proteins interact with other transcription factors, such as Runx2 and Smad, as well as NFAT, to regulate gene expression [80, 81]. Cooperative interaction between NFAT and AP-1 on specific DNA sites which contain adjacent NFAT and AP-1 binding sites is essential for regulating the expression of several genes, including cytokines, chemokines and cell surface receptors [55, 56]. Furthermore, a recent report states that inhibition of Cn/NFAT signaling increases AP-1 activity in human gingival fibroblasts [82], suggesting that NFAT does indeed play a role in regulating AP-1 member expression and activation.

**Osteocalcin**

One of the bone extracellular matrix (ECM) proteins, osteocalcin, was first discovered as a $Ca^{2+}$-binding protein in bone. Osteocalcin is the most abundant non-collagenous protein in bone and is only expressed in osteoblasts [83, 84]. During osteoblast development, type I collagen and ALP are highly expressed during matrix maturation stage, whereas, osteocalcin is highly expressed at the mineralization stage [8, 85]. The mouse osteocalcin protein is encoded by three genes, OG1 and OG2, which are only expressed in osteoblasts, and osteocalcin-related gene (ORG), which is not expressed in bone but in the kidney [86]. The osteocalcin promoter, both OG1 and OG2, contains two classical regions, the distal and proximal regions. The initiation of

osteocalcin gene expression starts at the proximal promoter region (-0.2 – 0 kbp), known to be responsible for basal, tissue-specific transcription. After initiation, transcriptional activity is enhanced by the distal region (-0.8 – 0.4kbp), which contains a 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) response element (VDRE) as well as Runx2 and AP-1 transcription factor binding sites [85]. These promoter regions serve to regulate osteocalcin gene expression. For example, the osteocalcin gene is suppressed in osteoprogenitor cells and then transcriptionally induced by Runx2 in mature osteoblasts [87]. Osteocalcin expression is also induced by the transcription factor CCAAA/enhancer binding protein beta and delta (C/EBP) and the hormone $1,25(OH)_2D_3$, which are maximized during the osteoblast mineralization stage [88]. The activation of osteocalcin gene expression is accompanied by the conversion of chromatin organization from inactive, closed chromatin to active, open chromatin, which allows transcription factors, including Vitamin D3, to access the osteocalcin gene promoter at the osteoblast differentiation stage [89, 90]. Many investigators have reported that the osteoblast-specific osteocalcin gene is controlled by modification in chromatin organization at the transcriptional level [89, 91].

**Chromatin Remodeling**

In eukaryotes, genomic DNA is compacted in the nucleus and packaged by the nucleoprotein complex as chromatin [92, 93]. The organization of DNA in chromatin contributes to its important roles, such as transcription, replication, recombination and DNA repair. Alteration of chromatin structure, termed chromatin remodeling, leads to changes in chromatin function and provides the primary mechanism for gene regulation

[94]. The nucleosome is the basic unit of chromatin, which contains DNA wrapped around a histone octamer that consists of two copies of histones (H2A, H2B, H3 and H4) [93]. Post-translational modifications of nucleosomal histones, such as acetylation, phosphorylation, ubiquitination and methylation, are known to regulate gene expression through transcriptionally active or inactive chromatin remodeling [95, 96]. One well-characterized modification of histone is lysine acetylation. Histone acetylation contributes to the active and open chromatin architecture, which leads to formation of the transcriptional environment and allows transcription factors to access target DNA templates [97-99]. In contrast, histone deacetylation is associated with the inactive, condensed and closed chromatin state that leads to inhibition of transcription machinery. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are the two essential enzymatic regulators of dynamic and reversible chromatin remodeling [100]. Active gene transcription is associated with acetylation of histone by HATs, whereas repressive chromatin structure correlates with deacetylation by HDACs.

**HDACs**

HDACs remove acetyl groups from histones and promote chromatin condensation, leading to transcriptional repression [101, 102]. HDACs are classified into three major groups according to their expression patterns, structures and catalytic mechanisms. Class I HDACs (HDAC 1, 2, 3, and 8) are expressed ubiquitously. They are mainly expressed in the nucleus of mammalian cells and inhibit specific target genes [100]. Class II HDACs (HDAC 4, 5, 6, 7, 9 and 10) are expressed in specific tissues, such as heart, brain and skeletal muscle [103]. These HDACs posses the ability to shuttle between nuclear

and cytoplasmic compartments in a specific cell types [104-106]. Finally, Class III HDACs are required for the deacetylase activity of the Nicotinamide adenine dinucleotide ($NAD^+$) [107].

In addition to deacetylating histones, HDACs are one of the multiprotein complexes that are recruited to target gene promoters as transcriptional corepressors. They interact with specific DNA-binding transcription factors in order to impede the transcription machinery [102]. Chemical inhibitors of HDACs (HDIs), such as trichostatin A (TSA), valporic acid (VPA), and sodium butyrate (NaB), mostly inhibit class I and II HDAC activity and promote histone acetylation and gene expression. In the presence of HDAC inhibitors, cells undergo cell cycle arrest, which may promote cell differentiation [108, 109].

**HDAC and NFAT**

The actions of the transcription factor, NFAT, are involved in numerous signaling functions from T lymphocyte activation to cardiac hypertrophy and musculoskeletal development [53, 60]. In general, NFATs are seen to be positive regulators of gene transcription by promoting the expression of cytokines and chemokines during T cell activation [55]. Also, NFATs form cooperative complexes with their binding partners, such as AP-1, myocyte enhancer factor 2 (MEF2) and GATA proteins, which lead to increased transcriptional activity. However, the negative role of NFAT has also been documented. Global NFATc2 deficient mice showed increased cartilage markers, including type II and type X collagen [110]. Baksh et al. reported that the Cn and NFAT signaling pathway suppresses cyclin-dependent kinase 4 (cdk4) gene expression through

a histone deacetylase-mediated mechanism involving the direct interaction between NFAT and HDAC in the CDK4 promoter [111]. In addition, Dai et al. demonstrated that the chaperone protein mammalian relative of DnaJ (Mrj) repressed NFAT transcriptional activity through direct recruitment of HDACs and suggested that HDACs suppressed gene expression in an NFAT-dependent manner [112]. It has also been shown that the association of NFAT1 and HDAC1 on intron 4 of *il10* gene silences IL-10 expression in T cells [113].

**HDAC and Osteoblasts**

To understand the molecular mechanisms of osteoblast differentiation, several reports have demonstrated that osteocalcin gene expression is transcriptionally regulated by modification in chromatin organization [89, 91]. Shen et al. demonstrated that the transcriptionally active osteocalcin promoter increased acetylated histones 3 and 4 to form the open chromatin conformation [114]. A recent study showed that HDAC3, a class I HDAC family member, associates with Runx2, an essential osteoblast transcription factor, and represses osteocalcin promoter and osteoblast differentiation [115]. Indeed, recent studies have shown that the HDAC inhibitors, TSA and VPA, induce osteoblast differentiation in calvarial-derived primary osteoblasts and human mesenchymal stem cells [116, 117]. TSA treatment in osteoblasts has been shown to accelerate osteoblast gene expression, including type I collagen, osteopontin and osteocalcin [116]. In addition, transforming growth factor-β (TGF-β) represses osteoblast differentiation by histone deacetylation through recruiting HDACs to the osteocalcin promoter [118]. These results suggest that transcription factors recruit HDACs, induce

histone deacetylation on target promoter sequences, and thus negatively regulate their gene expression.

**Animal Models**

For decades, genetically modified mouse models have been a powerful tool to advance studies in the field of bone development and metabolism. Various approaches are available to generate genetic modification of mice to analyze the role of target proteins *in vivo*, including overexpression of a target gene, global knockout and tissue-specific deletion of a target gene (e.g. *Cre-loxP* system). Transgenic animals, which overexpress target genes, were the first widely used tool to examine the function of a target gene *in vivo*. Transgenic overexpression models are moderately inexpensive and high levels of target gene expression often show an obvious phenotype. However, the integration of target DNA into the genome is randomly introduced, which results in different levels of transgene expression in different tissues and transgenic lines [119, 120]. Moreover, the transgene is expressed throughout the body and affects non-target tissues. Therefore, tissue-specific promoter animal models have been introduced to overcome these disadvantages and non-specific effects.

Global knockout mouse models that delete a target gene throughout the body are more reliable than the transgenic overexpression mouse model. However, global knockout models are also limited because there is no tissue specificity. As a result, it is often difficult to discern whether the outcome was the result of the direct cell- or tissue-specific effects or indirect secondary effects. Because of the uncertainties of the results

obtained from transgenic overexpression and global knockout mice models, techniques for generating tissue-specific mouse models were developed.

**Cre-loxP System**

This system is based on a *Cre-loxP* recombination method. Cre (Cyclization Recombination) protein is a 38kDa site-specific DNA recombinase from bacteriophage P1 that recognizes a specific site and catalyzes the recombination of DNA [121]. This specific site is known as the *loxP* sequence (locus of X-ing over P1). The *Cre-loxP* system utilizes two mouse lines: One is a transgenic mouse line that expresses Cre recombinase controlled by a tissue- or cell type-specific promoter [119, 120, 122]. The second is a floxed mouse line, a region of the target gene is flanked by two *loxP* sites. When these two mouse lines are mated, the *Cre* recombinase recognizes the *loxP* site and deletes the target gene between the two *loxP* sites (Figure 4). The *Cre-loxP* system is a powerful approach to address the function of a gene in a specific tissue. For example, the global knockout of the hypoxia-inducible factor 1α (HIF-1α) mice is lethal at E9, prior to the development of the skeleton. However, the chondrocyte-specific deletion of HIF-1α, using Col2a1-Cre mice, caused severe growth plate abnormality and chondrocyte cell death, resulting in skeletal deformity [123]. In addition, the global knockout of the type 1 insulin-like growth factor (IGF) receptor (IGF1r) caused death at birth [124]. However, osteoblast-specific IGF1r disrupted mice, using the osteocalcin promoter Cre mice, exhibited a decreased mineral apposition rate and reduced trabecular bone volume [125]. This result demonstrated that IGF signaling is required in the osteoblast mineralization process and these different kinds of animal models, global knockout and conditional

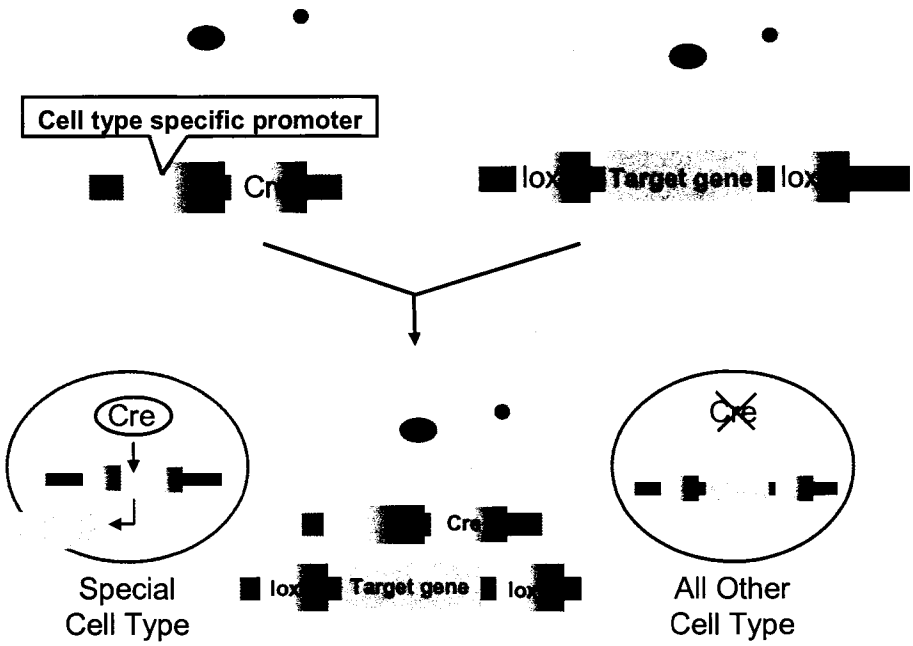


Figure 4. Cre and loxP system. Mice expressing Cre recombinase in a specific cell type promoter are mated with mice which carrying a target gene surrounded by loxP sites. The Cre recombinase recognizes the loxP sites and cleaves the loxP sites under the control of a target promoter in a cell-specific manner. This conditional knockout mouse displays the lack of target genes in specific cell types. Figure modified with permission from Pechisker, Issue two. Copyright 2004, The Science Creative Quarterly.

knockout, exhibited different results. Recently, osteoblast-specific conditional knockout mouse models have been accomplished using a 2.3kb fragment of the Col I-promoter and the human osteocalcin-promoter *Cre* mouse lines [125, 126]. The type I collagen promoter is expressed early in skeletal development, whereas osteocalcin is expressed in differentiated osteoblasts. It is reasonable to expect disruption of the Cn/NFAT signaling pathway during osteoblastogenesis in these mutant mice. Cnb1 and NFATc1 global knockout mice are embryonic lethal [127]. However, conditional deletion of Cnb1 in thymocytes led to inactivation and phosphorylation of NFATc1, c2 and c3 [128]. It has also been reported that the disruption of the regulatory domain of Cn, Cnb1, eliminated overall Cn phosphatase activity in somatic tissue [128]. Therefore, it is worthwhile to create a mouse model with osteoblast-specific Cnb1 disruption to investigate the role of Cn/NFAT signaling in bone formation and remodeling.

**Summary**

The role of the Cn and NFAT signaling pathway was first described in the T cells, and was soon followed by the discovery of its crucial functions in many other cells and tissues. Recent studies recognized that Cn and NFAT signaling is also associated with osteoblast differentiation and bone formation. Cn and NFAT are key promoters of osteoclast differentiation and bone resorption. In contrast, the role of Cn and NFAT in osteoblast differentiation and bone formation is not well defined. The goal of our research is to unequivocally determine the role of the Cn and NFAT signaling pathway in regulating bone mass. Here, we determine how the disruption of Cn and NFAT signaling in osteoblasts affects the bone phenotype and characterize the roles of Cn and NFAT in osteoblast growth and differentiation. Finally, we determine the molecular mechanisms by which Cn and NFAT regulates bone mass.

# CYCLOSPORIN A ELICITS DOSE-DEPENDENT BIPHASIC EFFECTS ON OSTEOBLAST DIFFERENTIATION AND BONE FORMATION

by

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## ABSTRACT

Cyclosporin A (CsA) is thought to prevent immune reactions after organ transplantation by inhibiting calcineurin (Cn) and its substrate, the Nuclear Factor of Activated T cells (NFAT). A dichotomy exists in describing the effects of CsA on bone formation. The concept that the suppression of Cn/NFAT signaling by CsA inhibits bone formation is not entirely supported by many clinical reports and laboratory animal studies. Gender, dosage and basal inflammatory activity have all been suggested as explanations for these seemingly contradictory reports. Here we examine the effects of varying concentrations of CsA on bone formation and osteoblast differentiation and elucidate the role of NFATc1 in this response. We show that low concentrations of CsA (<1μM *in vitro* and 35.5 nM *in vivo*) are anabolic as they increase bone formation, osteoblast differentiation, and bone mass, while high concentrations (>1μM *in vitro* and *in vivo*) elicit an opposite and catabolic response. The overexpression of constitutively-active NFATc1 inhibits osteoblast differentiation, and treatment with low concentrations of CsA does not ameliorate this inhibition. Treating osteoblasts with low concentrations of CsA (<1μM) increases fra-2 gene expression and protein levels in a dose-dependent manner as well as AP-1 DNA binding activity. Finally, NFATc1 silencing with siRNA increases Fra-2 expression, whereas NFATc1 overexpression inhibits Fra-2 expression. Therefore, NFATc1 negatively regulates osteoblast differentiation, and its specific inhibition may represent a viable anabolic therapy for osteoporosis.

# INTRODUCTION

Bone disease, in the form of osteopenia and osteoporosis, is a serious complication of organ transplantation in humans [1-3]. This phenomenon is not only due to the underlying disease and the organ transplantation but also is thought to be a side effect of the many drugs that are administered pre- and post- transplantation, including glucocorticoids and CsA [3-6]. However, controversial evidence exists that CsA might have a bone protective effect both in human and laboratory animals [7]. Based on the literature, both *in vivo* and *in vitro* data suggest that CsA has potential biphasic effects on bone formation and osteoblast differentiation, and the seemingly contradictory reports and paradoxical results stem, in part, from the wide variation in the conditions used for these studies and in the concentrations of the immunosuppressive drugs used, ranging between 1 nM-25 μM. It has been shown that post-transplantation patients that are treated with immunosuppressive drugs including CsA develop osteoporosis [8]. Interestingly, CSA monotherapy in renal transplantation patients has been shown to significantly increase lumbar bone mineral density while transplant recipients who were treated with both CsA and glucocorticoids exhibited a significant decrease [9]. In laboratory animals, the administration of CsA to rats at high doses (15 mg/kg) shows significant bone resorption and trabecular bone loss, resulting in severe high turnover osteopenia [10]. However, administration of a lower dose of CsA (7 mg/kg) to rats results in a decrease in bone resorption and an increase in bone formation *in vivo* [11]. *In vitro*, using low concentrations of another immunosuppressive drug, FK506 (10 nM-1 μM), has been shown to induce osteoblastic differentiation [12]. In contrast, a high concentration of FK506 (25 μM) inhibits osteoblast differentiation [13].

The NFAT (Nuclear Factor of Activated T Cells) family of transcription factors, best known for its role in T lymphocyte activation, is composed of five proteins related to the Rel/NFκB family (NFATc1-c4 and NFAT5) [14]. In resting cells, NFAT proteins are highly phosphorylated and reside in the cytoplasm. Upon activation by increases in intracellular $Ca^{2+}$, they are dephosphorylated by the $Ca^{2+}$/calmodulin-dependent serine-threonine phosphatase, calcineurin, (Cn); then translocate to the nucleus to become transcriptionally active [14,15]. The immunosuppressive agents CsA or FK506 are thought to elicit their response by inhibiting the Cn/NFAT signaling pathway in T cells [16]. These agents bind to cyclophilin or to FK506 binding protein, respectively. The binding proteins then bind Cn, thus inhibiting the dephosphorylation and nuclear translocation of NFAT and thus, T-cell activation [17].

The role of Cn/NFAT signaling in osteoblast differentiation and bone formation was recently reported in three different studies [13,18,19]. The findings from these reports were recently described as thought provoking for several reasons [20]. Although these studies identify osteoblast factors that could positively be regulated by Cn/NFAT signaling pathway and lead to osteoblastic anabolic response, they are inconsistent with the clinical observations that bone loss-related to CsA and Cn/NFAT inhibition is associated with high bone turnover [20,21]. It is possible that these reports are misinterpreted due to the nature of the animal models which were used in these studies [13,18,19]. These reports used global CnA alpha and NFATc1 overexpression and knockout animal models to examine the function of Cn or NFAT specifically in osteoblast differentiation and bone formation. However, these reports do not consider that Cn and NFAT are also globally expressed in different tissues and have been shown

to be critical for the differentiation of many cells and organs including heart, vasculature, skeletal muscle, chondrocytes and adipocytes [22,23]. Therefore, their results may be secondary and not direct osteoblast-specific effects.

Osteoblasts are the bone forming cells. They originate from mesenchymal progenitors that, with appropriate stimulation, undergo proliferation and differentiate into preosteoblasts and then into mature, functional osteoblasts [24]. In culture, as *in vivo*, osteoblasts form bone-like mineralized nodules by undergoing three stages of development: proliferation, extracellular matrix maturation, and mineralization [25,26]. During each stage of development, specific subsets of genes are sequentially expressed or repressed. For example, histone 4 is a marker for proliferation, alkaline phosphatase for extracellular matrix maturation, and osteocalcin for mineralization [26]. Several transcription factors and signaling pathways, such as AP-1 and Runx2, have been shown to play major roles in regulating osteoblast gene expression, phenotype, and ultimately bone formation [27,28].

Activator Protein-1 (AP-1) is a dimeric transcription factor family composed of either homodimers of the Jun family (c-Jun, JunB, and JunD) or heterodimers of the Jun and Fos family (c-Fos, Fra-1, Fra-2, and FosB) [29]. Expression of the AP-1 family members varies depending on the stage of osteoblast differentiation [30]. During osteoblast proliferation, all AP-1 members are known to be expressed, while during mineralization, Fra-2 and to a lesser extent, JunD are the major AP-1 members expressed, suggesting that Fra-2 expression is critical for osteoblast differentiation and mineralization [30]. AP-1 proteins interact with other transcription factors, such as Runx2 and Smad, as well as NFAT, to regulate gene expression [31-33]. Cooperative interaction

between NFAT and AP-1 on specific DNA composite sites which contain adjacent NFAT and AP-1 binding sites is essential for regulating the expression of several genes including cytokines, chemokines and cell surface receptors [34,35].

The aims of this study were to examine the potential biphasic effects of CsA on osteoblast differentiation and bone formation and to determine the role of NFATc1 in this response. Here we show paradoxical effects of CsA on bone formation and bone mass *in vivo* and osteoblast differentiation *in vitro*, resulting from different CsA concentrations. Furthermore, we demonstrate that the osteoblast specific overexpression of constitutively-active NFATc1 inhibits osteoblast differentiation. Finally, we determine the role of NFATc1 in regulating Fra-2 expression and AP-1 activation. Taken together, our work provides a critical explanation to seemingly controversial reports describing the effects of immunosuppressive agents on bone and the role of Cn/NFAT signaling on osteoblast differentiation.

## MATERIAL AND METHODS

### Cell Culture and Differentiation.

MC3T3-E1 pre-osteoblast cells [36] were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Primary calvarial osteoblasts were isolated from 1 day-old Balb/C mice. Calvariae were subjected to three sequential digestions with collagenase (Worthington) at 37 °C. Osteoblasts were collected from the $2^{nd}$ and $3^{rd}$ digestions. Cells were maintained as previously described [36]. Osteoblastic induction was performed by supplementing medium with 8 mM β-glycerophosphate and 250 μM ascorbic acid-2-phosphate [36,37].

**Alkaline Phosphatase and Mineral Deposition Assays.**

Cultured MC3T3-E1 and primary cells were fixed in 2% v/v paraformaldehyde for 10 minutes and then incubated at 37°C with alkaline phosphatase substrate solution [36]. Mineralization was assessed by either von Kossa staining of the cultures (3 minutes in 3% w/v AgNO3) [36] or by measurement of calcium deposition as previously described [38].

**Transient Transfections and Luciferase Reporter Assays.**

MC3T3-E1 osteoblasts were plated at a density of 2 x $10^4$ cells/$cm^2$ in 6-well plates. Twenty-four hours after plating, cells were transfected with 1 μg of NFAT luciferase plasmid (Clontech) and 0.2 μg CMV-β-galactosidase reporter construct using LipofectAMINE (Invitrogen). 24 hours post-transfection, cells were treated with CsA, as indicated. Reporter activity was measured using a luciferase (Promega) or β-galactosidase (Clontech) assay system [36,37].

**Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay.**

MC3T3-E1 Nuclear protein was extracted and the AP-1 oligonucleotide was labeled as previously described [36,38]. The oligonucleotide sequences for wild-type and mutant AP-1 were previously described [39]. Competition binding experiments were performed in the presence of a 50-fold molar excess of unlabeled wild type or mutant AP-1 oligonucleotides. For the supershift experiments, eight μg of nuclear extracts were

incubated with the $^{32}$P-labeled AP-1 oligonucleotide and antibodies against Fra-2 and junD (Santa Cruz Biotechnology) overnight at 4°C.

**Gene Silencing by siRNA.**

MC3T3-E1 Osteoblasts were plated at a density of 2 x $10^4$ cells/cm$^2$ in 6-well plates. NFATc1 or control non-functional siRNA (Santa Cruz Biotechnology) were transfected into the cells using TransIT-TKO transfection reagent as recommended by the manufacturer, (Mirus, Madison, WI). The medium was changed 24 hours post-transfection and cells were cultured in fresh medium. Cells were harvested 72 hours later [36].

**Retrovirus production and infection.**

We used retroviral expression vectors pMSCV-GFP and pMSCV-NFATc1 [40]. Retroviruses were produced by cotransfecting pMSCV vectors with pVSV-G into BOSC23 cells using Lipofectamine (Invitrogen). Twenty four hours after transfection, the media was replaced, and retroviral supernatant was collected after 48 hours. For infection, 2 x $10^4$ cells/cm$^2$ MC3T3 cells were plated onto 6-well plates. The culture media was replaced with 500μl of retroviral supernatant with 8μg/ml polybrene (Sigma) and plates were incubated for 2 hours at 37 °C with 5% $CO_2$. Retroviral supernatant was then removed and cells were cultured in regular growth medium.

**Cyclosporin in vivo Treatment.**

Male, six-week old, BALB/c mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana). CsA was purchased from Calbiochem/EMD Biosciences

(San Diego, CA) and dissolved in DMSO. CsA solution was appropriately diluted in vehicle (2% v/v DMSO, 5% v/v ethanol, 60% v/v polyethylene glycol and 33% v/v PBS) to obtain concentrations of 1, 5, 10 and 30 mg/kg body weight of the mice. Vehicle or CsA was administered daily by subcutaneous injection for 28 days. All mice were sacrificed 24 hours after the last administration of CsA. Blood samples were collected by cardiac puncture and placed into tubes containing an anticoagulant (EDTA). CsA blood levels were measured using the Abbott TdxFLx Cyclosporine Monoclonal Whole Blood assay according to manufacturer's protocol (Abbott Laboratories, Abbott Park, IL). All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

**Dual energy X-ray absorptiometry (DXA) and μCT analysis of bone.**

Non-cephalic bone mineral density (BMD; $g/cm^2$) was determined by DXA, which was measured by a Lunar PIXImus densitometer (GE Lunar Corp, Madison, WI). After euthanizing the animals, tibiae were removed and dissected free of soft tissue. The bones were then fixed in 10% v/v formaldehyde and analyzed by a $\mu$CT system and the manufacturer's included 3-D analysis software ($\mu$CT 40, Scanco Medical, Basserdorf, Switzerland). The region of interest analyzed was the metaphysis of the proximal tibia, for a total of 25 specimens scanned. The trabecular bone in the tibia was scanned using a 12- $\mu$m slice increment on 100 slices, starting 360 $\mu$m distal to the growth plate and extending for 120 $\mu$m.

**Bone histomorphometric analysis.**

Mouse tibiae and femora were fixed in 10% v/v buffered formalin, decalcified in EDTA, embedded in paraffin, sectioned and stained for TRAP. Also, tissues were fixed, embedded in methyl methacrylate, sectioned and stained for von Kossa. Bone histomorphometry parameters were measured using an area at least 0.5mm below growth plate, excluding the primary spongiosa and trabecular connected cortical bone [41].

**Statistical analysis.**

All statistical analyses were performed using the Microsoft Excel data analysis program for Student's t-test analysis. Experiments were repeated at least three times unless otherwise stated. Values are expressed as the mean ±SE.

## RESULTS

To resolve the contradiction regarding the effects of CsA on bone *in vivo*, we administered different concentrations of CsA (1, 5, 10 and 30 mg/kg) to mice daily by subcutaneous injections for 28 days. At the end of the study, we examined the steady-state CsA concentrations in the blood (CsA levels 24 hours after the last administration) and their effects on bone. The daily subcutaneous administration of a low dose of CsA (1 mg/kg) to mice results in a steady-state concentration of the drug of 35.5 ± 5.9 nM. The steady-state CsA concentrations averaged 1234.2 and 2230.4 nM after treatment with 10 and 30 mg/kg, respectively (Fig. 1A). Low dose CsA (1 mg/kg) dramatically increases tibial trabecular bone volume while high doses (10 and 30 mg/kg) decrease trabecular bone volume as demonstrated by representative μCT images (Fig. 1B). Furthermore, non-

cephalic whole body bone mineral density measured by DXA (Dual energy X-ray absorptiometry) is significantly increased after 28 days of treatment with low dose CsA (1 mg/kg) and decreased after treatment with high doses (10 and 30 mg/kg, respectively) (Fig. 1C). Histological analyses of femora from these animals demonstrate that treatment with low dose CsA (1 mg/kg) increases mineralization (black, von Kossa) as well as the mineral apposition rate (MAR), and decreases TRAP (Tartrate Resistant Acid Phosphatase) -positive osteoclasts (red, TRAP) compared to untreated mice (Fig. 2). In contrast, high doses of CsA (10 and 30 mg/kg) decrease trabecular bone, mineralization and MAR and increase TRAP -positive osteoclasts compared to untreated mice (Fig 2). These parameters were quantitated using BioQuant Image Analysis System® (R&M Biometrics, Inc, Nashville, TN) and demonstrate that the *in vivo* treatment with CsA (1 mg/kg) results in 37% increase in Bone Volume/Tissue Volume (BV/TV), a 27% increase in trabecular number and 26% decrease in trabecular separation, as compared to untreated mice. These changes in bone volume in response to a low dose of CsA (1 mg/kg) were accompanied by a significant increase in the number of osteoblasts (50%) and a 42% decrease in the number of osteoclasts. In contrast, high doses of CsA (10 and 30 mg/kg) resulted in a 20% and 36% decrease in BV/TV, 26% and 22% increase in trabecular separation, respectively. Interestingly, the decrease in bone volume is associated with a significant increase in the number of the osteoclasts (56% and 60%, respectively) without significant change in the osteoblast number (Table 1). Taken together, these results demonstrate that the *in vivo* treatment with CsA differentially affects both bone formation and resorption; ultimately leading to an increase in bone

mass by low CsA concentrations and a decrease in bone mass by high CsA concentrations.

In order to directly demonstrate the effects of CsA on osteoblast differentiation, MC3T3-E1 pre-osteoblasts were used. Cells were cultured for four days in proliferation medium and osteoblastic differentiation was induced by supplementing the culture medium with β-glycerophosphate and ascorbic acid. Differentiated osteoblasts were cultured for 14 and 21 days and treated with different concentrations of CsA. At the end of the study, cells were fixed and stained for alkaline phosphatase (ALP) activity and for mineralization. Figure 3A demonstrates that low doses of CsA dose-dependently increase both ALP activity (red, upper panel) and mineralization (black, lower panel). Interestingly, although ALP and mineralization are increased in response to 1μM CsA compared to untreated cells, they are decreased compared to treatment with 500 nM CsA. Furthermore, calcium deposition was quantitated and shows a significant increase after treatment with 1 μM CsA (Fig. 3B). To examine the effect of CsA on osteoblast proliferation, we performed [$^3$H]-thymidine incorporation. Fig. 3C demonstrates that CsA treatment significantly decreases osteoblast proliferation, as measured by [$^3$H]-thymidine incorporation, with a 25% decrease at 500 nM CsA and a 35% decrease at 1000 nM, while 100 nM CsA had no effect.

The increase in the osteoblastic phenotype in response to low dose CsA was confirmed by examining the expression of the early and late osteoblastic gene markers, ALP and osteocalcin, respectively. Treating osteoblasts with low concentrations of CsA (<1μM) significantly increased *ALP* and *osteocalcin* gene expression, in a dose-dependent manner, as shown by real-time RT-PCR (Figs. 3D and E).

Consistent with numerous reports, we also demonstrate that osteoblast differentiation is inhibited when CsA is used at high concentrations (10 and 25 μM) (Fig. 4A). The decrease in differentiation was not due to an increase in apoptosis, as we show that CsA does not affect osteoblast apoptosis while treatment with 10 μM Actinomycin D for 6 hours, as a positive control [42] , results a 270% increase in the number of apoptotic cells (Fig. 4B). Furthermore, we examined the dose-dependent effects of CsA on the differentiation of primary mouse calvarial osteoblasts to confirm that this response is not only limited to the MC3T3-E1 osteoblastic cell line. As expected, primary calvarial osteoblasts responded similarly to MC3T3-E1 cells after treatment with CsA (100 nM- 25 μM) for 14 and 21 days (Fig. 4C). Our results confirm that CsA elicits biphasic dose-dependent response on primary osteoblasts depending on its concentration.

In order to characterize the role of NFAT in the paradoxical effect of CsA on osteoblast differentiation we first characterized the expression of NFAT isoforms in MC3T3-E1 osteoblasts. Cells were cultured for 11 days in the presence of β-glycerophosphate and ascorbic acid. RNA, cytoplasmic (Cyto) and nuclear (Nuc) proteins were extracted and the expression of NFAT isoforms examined by RT-PCR and Western blotting. Here we show that osteoblasts express NFATc1, c2 and c3 genes (Fig. 5A). Furthermore, NFATc1 and c3 proteins were detected in osteoblasts in both the cytoplasm and in the nucleus. In contrast, NFATc2 was only detectable in the cytoplasm (Fig. 5B). Treatment with 1 μM CsA dramatically decreased the nuclear translocation of both NFATc1 and c3 (Fig. 5B). Positive controls, proteins and RNAs extracted from Jurkat cells, were examined and show expression of NFATc1, c2 and c3 (data not shown).

In order to confirm the effect of CsA on NFAT transactivation, we transfected MC3T3-E1 cells with pNFAT-TA-luciferase for 24 hours, and cells were treated with different concentrations of CsA for another 24 hours. Cells were then lysed and luciferase activity measured. Here we show that CsA significantly decreases the transactivation of NFAT at concentrations between 10 nM and 25 μM (Fig. 5C), thus demonstrating the effectiveness of CsA in inhibiting NFAT activity. Although, CsA at 1μM concentration decreases NFAT transactivation compared to untreated osteoblasts, it slightly increases the NFAT transactivation when compared to cells treated with 500 nM CsA. The inhibition of NFATc1 nuclear translocation in osteoblasts by CsA is rapid. Three hours after treating MC3T3-E1 osteoblasts with 500 nM CsA, NFATc1 protein was mainly detected in the cytoplasm. The inhibition of NFATc1 nuclear translocation persisted even after 24 hours from the start of CsA treatment (Fig. 5D). Next, we examined NFATc1 nuclear translocation in response to different concentrations of CsA ranging between 100 nM to 25 μM. CsA at concentrations ranging from 100 nM to 25 μM inhibit the nuclear localization of active NFATc1. Interestingly, CsA when used at high concentrations (>1μM) not only decreases nuclear NFATc1, but also the cytoplasmic inactive form of this protein (Fig. 5E). Taken together, these data suggest that the inhibition of NFAT (by low concentrations of CsA, <1 μM) induces osteoblast differentiation while high concentrations (>1μM) inhibit it.

In order to examine the role of NFATc1 specifically in osteoblasts, we overexpressed constitutively-active NFATc1 in MC3T3-E1 cells using a retroviral system. These constructs were a generous gift from Dr. Neil Clipstone, Northwestern University, Chicago, IL [40]. Transduction efficiency was approximately 95% (data not

shown). Here we demonstrate that the overexpression of constitutively-active NFATc1 in MC3T3-E1 osteoblasts inhibits osteoblast differentiation as demonstrated by a decrease in ALP activity (red, upper panel) and mineralization (black, lower panel) (Fig. 6A). The inhibition in osteoblast differentiation was not due to a decrease in osteoblast cell number in response to an increase in NFATc1 expression. In fact, despite the decrease in differentiation, the number of the osteoblast cells that express constitutively-active NFATc1, 48 hours after culturing in serum free medium, was 25% more than that of the GFP expressing cells, as determined by MTT assay (data not shown). With the purpose of determining the role of NFATc1 in the biphasic effects of CsA on osteoblast differentiation, we treated MC3T3-E1 osteoblasts that express constitutively-active NFATc1 with different concentrations of CsA (100 nM-25 μM) for 14 and 21 days. As expected, CsA increased the differentiation of MSCV-GFP (GFP empty vector) osteoblasts when used at low concentrations (100 and 500 nM) which is demonstrated by an increase in ALP activation and mineralization, while high CsA concentrations (10 and 25 μM) produced the opposite results (Figs. 6B and 6C, upper panels). Interestingly, CsA at low concentrations does not rescue the inhibition of osteoblast differentiation in response to the overexpression of NFATc1 while high CsA concentration completely inhibits the basal ALP activation and mineralization (Figs. 6B and 6C, lower panels). Taken together, these results confirm that the anabolic effect of low CsA concentrations is NFATc1-dependent while the effects of the high concentrations are NFATc1-independent.

In order to elucidate the mechanism by which NFAT inhibition induces osteoblast differentiation, we examined the effects of CsA on the expression of Fra-2, an AP-1

family member. It has been shown that during the mineralization stage of differentiation, Fra-2, and to a lesser extent, JunD are the major AP-1 members in osteoblasts [30]. MC3T3-E1 cells were cultured for 11 days with different concentrations of CsA. Cells were harvested and RNA extracted. Real time RT-PCR was performed for *fra-2*. Here we show that CsA at concentrations lower than 1 μM, dose-dependently increases the expression of *fra-2* in osteoblasts (Fig. 7A). Furthermore, CsA concentrations higher than 1 μM failed to increase *fra-2* despite the inhibition of NFAT transactivation, which confirms the involvement of an NFAT-independent response (Fig. 7A). In addition, we examined the effects of NFAT inhibition by CsA on nuclear Fra-2 protein levels. MC3T3-E1 cells were cultured for 11 days and nuclear proteins extracted. Not surprisingly, the inhibition of NFAT by low concentrations of CsA dose-dependently increases Fra-2 nuclear protein levels while high concentrations have no effect (Fig. 7B). We also examined the nuclear protein levels of JunD, which is known to be the AP-1 partner of Fra-2 during osteoblast differentiation. Here we show that CsA does not affect JunD proteins at either low or high concentrations (Fig. 7B).

In order to demonstrate the specific role of NFATc1 in osteoblast differentiation and Fra-2 expression, we silenced NFATc1 expression using siRNA, and overexpressed constitutively-active NFATc1 in MC3T3-E1 cells. The effectiveness of the NFATc1 silencing or overexpression of NFATc1 was first confirmed by demonstrating the corresponding changes in NFATc1 protein expression, compared to osteoblasts transfected with a non-functional scrambled siRNA or transduced with GFP empty vector (Figs. 7C and 7D). NFATc1 siRNA dramatically induces Fra-2 expression compared to control siRNA without affecting JunD expression (Fig. 7C). In contrast, overexpression

of a constitutively-active NFATc1 dramatically decreases Fra-2 expression compared with control MSCV-GFP infected osteoblasts without affecting JunD (Fig. 7D).

Fra-2 is a member of the Fos family of AP-1 transcription factors that form heterodimers with AP-1 Jun family members and functions as a transcriptional regulator [43]. We examined the effects of CsA at concentrations lower than 1 μM on AP-1 DNA binding activity by performing an electrophoretic mobility shift assay (EMSA). MC3T3-E1 osteoblasts were cultured for 11 days and treated with different concentrations of CsA. Nuclear proteins were extracted and incubated with radiolabeled oligonucleotide containing an AP-1 consensus sequence, and an EMSA was performed. Inhibition of NFAT by CsA increases AP-1 DNA binding activity dose-dependently in osteoblasts (Fig. 8A). Competition assays using excess wild-type AP-1 oligos abolish the AP-1 response while mutant oligos have no response (Fig. 8B). Furthermore, super shift analyses using antibodies to AP-1 family members confirm that Fra-2 and JunD are increased in the AP-1 DNA-protein complex (Fig. 8C). These findings suggest that inhibition of NFAT increases osteoblast differentiation which is accompanied by increased Fra-2 expression, leading to an increase in Fra-2/JunD binding to the AP-1 consensus sequence. We also examined the effects of CsA treatment on NFAT/AP-1 DNA binding activity. Consistent with literature [44,45], treatment with different concentrations of CsA effectively inhibited DNA binding activities of NFAT/AP-1 consensus sequence (data not shown). These results suggest that the activation of NFAT is critical for the activation of NFAT/AP-1 DNA binding, and AP-1 alone is not capable of substituting for NFAT on these consensus sequences.

## DISCUSION

Early reports concerning the role of Cn in regulating bone density arose from clinical observations noting that treatment with immunosuppressive drugs, CsA and FK506, is associated with increased incidence of bone fractures [46]. However, CsA monotherapy in renal transplantation patients has been reported to significantly increase lumbar bone mineral density [9]. Administration of CsA to laboratory animals also produces conflicting results ranging from increased bone formation to severe osteopenia [10,11]. It is not completely understood why CsA treatment would elicit opposing effects, but it is possible that these differences in results are due to the use of different species, genders, ages, and most importantly, different drug concentrations and duration of administration in these studies [10,11,47-49].

Reports examining the specific roles of Cn and NFAT in osteoblast differentiation are not consistent. Some studies have reported that CsA and FK506 enhance osteoblastic differentiation and bone formation both *in vivo* and *in vitro* [11-13,49], while other studies report that they decrease osteoblast differentiation by a variety of mechanisms. Consistent with the work of others, we demonstrate that osteoblast differentiation is indeed inhibited by CsA but only when used at high concentrations (10 μM and 25 μM). This inhibition was not due to an increase in apoptosis. Furthermore, we show that the administration of high doses of CsA (above 5 mg/kg) to mice significantly decreases bone mineral density and trabecular bone volume. In contrast, low concentrations of CsA (10 nM-1 μM) dose-dependently increase ALP staining and mineralization. These results are consistent with studies reporting that both CsA and FK506 increase the mineralization of osteoblast-like cells[12,50]. Similarly, we observe an increase in

trabecular bone volume and BMD *in vivo* after treating mice with a low dose of CsA (1 mg/kg). Our results are consistent with many reports that describe an increase in osteoblast differentiation in response to treatment with a low doses of CsA as well as those which report a decrease in bone mass in response to treatment with high doses of CsA [10,11]. We demonstrate that these seemingly contradictory reports are potentially due to differences in the dosage of CsA. Furthermore, CsA at low concentrations increases bone mass not only by increasing bone formation but also by decreasing bone resorption as shown by the decrease in TRAP activity. The precise mechanism of this is currently not known. It is possible that treatment with a low dose of CsA acts on osteoclasts directly by inhibiting differentiation and/or activation, or indirectly by inhibiting the expression of RANKL and increasing osteoprotegerin expression by osteoblasts.

Nevertheless, dissimilarities between the *in vivo* and the *in vitro* systems exist. We found that CsA 10-500 nM is optimal for increasing ALP and mineralization of osteoblasts *in vitro*. In contrast, the steady-state concentration of CsA in the blood that was effective in increasing bone formation and osteoblast number was 35 nM. This difference is most likely due to the *in vivo* generation of at least 6 known highly lipophilic CsA metabolites that are also effective in inhibiting the Cn/NFAT signaling pathway, which are not detected when CsA levels are measured in the blood [51]. Furthermore, high concentrations of CsA significantly decrease osteoblast proliferation *in vitro* while low concentrations have no effect. In contrast, our *in vivo* data show that low doses of CsA increase osteoblast number. This disparity is likely due to the involvement of other factors that are present *in vivo* which could act indirectly on osteoblasts, resulting

in an increase in their proliferation. For example, it was shown that *in vivo* CsA treatment increases the expression of TGF-beta, which is known to induce osteoblast proliferation [52,53].

Here we demonstrate that in osteoblasts both NFATc1 and c3 proteins are cytoplasmic and nuclear while NFATc2 is only detectable in the cytoplasm. Basal Cn activity would naturally result in some nuclear localization of NFAT proteins. It is not yet known why NFATc2 is only present in the cytoplasm of osteoblasts, but it is likely due to special characteristics of this isoform [54]. Treatment with CsA significantly decreases NFAT nuclear localization and transactivation, but does not cause a complete inhibition. This could be due to the presence of several phosphorylation sites in the serine-rich gatekeeper region-2 of NFAT that are not susceptible to Cn dephosphorylation [14]. Furthermore, the inhibition of NFAT transactivation in osteoblasts by different concentrations of CsA does not appear to be linear. CsA begins to inhibit NFAT transactivation and NFAT protein nuclear localization at 10 nM and plateaus around 500 nM. Interestingly, at 1 μM concentration, CsA decreases NFAT transactivation compared to untreated osteoblasts but increases it compared to the 500 nM-treated cells. This suggests that one of the mechanisms responsible for the paradoxical effect of CsA on osteoblasts could be a Cn and/or NFAT-independent response. It was reported that CsA decreases the activation of p38 MAP kinase [55]. Interestingly, p38 is known to phosphorylate NFAT, which leads to an increase in its nuclear export and ultimately decreases its activity [56]. Taken together, CsA in the 1 μM range could be inhibiting both the Cn pathway leading to a decrease in NFAT transactivation as well as inhibiting p38 activation leading to a decrease in NFAT nuclear export and causing a counteracting

increase in NFAT transactivation.

We examined the specific role of NFATc1 on osteoblast differentiation by overexpressing a constitutively-active NFATc1 in MC3T3-E1 cells. We show that the increase in NFATc1 expression and activation inhibits the ability of these fibroblastic pre-osteoblasts to differentiate into mature osteoblasts. Similar to our results, it was reported that the overexpression of NFATc1 in pre-adipocytes inhibits their differentiation into mature adipocytes [57]. In addition, it was shown that the increase in NFATc1 in these fibroblastic cells causes them to adopt a transformed cell type. The same phenomena could also be present in pre-osteoblasts. More work needs to be performed to confirm this.

Transcription factors, namely Fra-2, which is an AP-1 family member, are known to play a critical role in the differentiation of a variety of cells, including osteoblasts [30,58]. Our results demonstrate that treatment with CsA and the specific inhibition of NFATc1 by siRNA increase the expression of Fra-2. Analysis of the *fra-2* promoter [59] by a Transcription Element Search System (TESS) program [60] identified three potential NFAT consensus sequences. NFAT binding to these sites could be responsible for the negative regulation of *fra-2* expression. Nevertheless, it is possible that the increase in *fra-2* expression is indirect and results from the increase in osteoblast differentiation, after the inhibition of Cn/NFAT by CsA. Furthermore, we cannot rule out the involvement of other AP-1 family members in osteoblast differentiation in response to NFAT inhibition.

The increase in Fra-2 expression leads to a dose-dependent increase in AP-1 DNA binding activity and an increase in the formation of Fra-2/JunD heterodimers. NFAT

inhibition and treatment with CsA did not change the expression of JunD, suggesting that the increase in Fra-2 is sufficient to recruit JunD and increase AP-1 DNA binding activity. It is also possible that other Jun family proteins could be interacting with Fra-2, causing the increase in AP-1 DNA binding activity. Interestingly, it has been shown previously that CsA inhibits the NFAT/AP-1 DNA binding activity [44,45]. Our data suggest that the cooperation between NFAT and AP-1 in binding to NFAT/AP-1 binding sites is not only regulated by AP-1 family members but also by NFAT. Inhibition of NFAT in osteoblasts increases AP-1 DNA binding activity. But due to the unavailability of NFAT proteins, AP-1 by itself is not able to be recruited to bind to the NFAT/AP-1 binding sites.

Finally, it was recently reported that embryonic cells from the NFATc1$^{-/-}$ embryos exhibit a decrease in their osteoblastic potential, leading the authors to conclude that NFATc1 is critical for osteoblast differentiation [13]. This was explained by the ability of NFATc1 to enhance the transcriptional activation of osterix. These embryonic stem cells are known to be pluripotent and capable of differentiating into several lineages such as chondrocytes, myocytes, adipocytes, as well as osteoblasts [61]. Interestingly, NFAT has been shown to play a critical role in the lineage decision of stem cells to differentiate into chondrocytes, myocytes and adipocytes. Furthermore, it has been reported that the lineage decision of mesenchymal stem cells is dependent on their redox state and oxidative stress has been reported to play a role in the activation of the Cn/NFAT signaling pathway [62,63]. Therefore, the decrease in osteoblast differentiation that was observed by Koga et al [13] could possibly be due to a decrease in the lineage commitment of the NFATc1$^{-/-}$ embryonic cells, and not a direct osteoblastic response. Similarly, it has recently been reported that the CnA Alpha$^{(-/-)}$ mice display severe

osteoporosis and markedly reduced mineral apposition rates [18]. Furthermore, it was also reported that animals that globally overexpress a constitutively nuclear NFATc1 variant ($NFATc1^{nuc}$) develop high bone mass [19]. These mice were generated by either disrupting the CnA Alpha gene, or overexpressing NFATc1 variant in embryonic cells, which were later injected into mice blastocytes [64]. These mice not only lack CnA Alpha or overexpressed NFATc1 from early stage of osteoblast differentiation before mesenchymal lineage commitment, but also had the Cn/NFAT signaling disrupted in all NFAT-dependent biological systems in the body, which could play a critical role in their skeletal phenotype. This is supported by other skeletal abnormalities that these mice exhibit, such as altered skeletal muscle phenotypes [65] and several other abnormalities that are related to the global loss of CnA Alpha or overexpression of NFATc1. This can indirectly affect bone phenotype, such as altered T-cell response or changes in muscle mass, which can ultimately and indirectly determine bone phenotype [64]. To support this argument we have generated a CnB1-osteoblast specific knockout mouse model using the Cre/lox system. Consistent with our results, we found that mice lacking the CnB1 gene in osteoblasts exhibit a significant increase in osteoblast differentiation and bone formation [66].

Our results provide new insight of a novel mechanism for osteoblast differentiation. Our work suggests that the Cn/NFAT signaling pathway plays a critical role in the negative regulation of osteoblast differentiation. NFATc1 acts, directly or indirectly, by negatively regulating *fra-2* expression. Clinically, the toxicity of CsA as a monotherapy at very low concentrations for a short period of time is not known. If CsA at the 1 mg/kg dose or other novel and non-toxic Cn inhibitors are found to be safe and

without significant side effects *in vivo*, they may provide novel anabolic therapies for osteopenia and osteoporosis.

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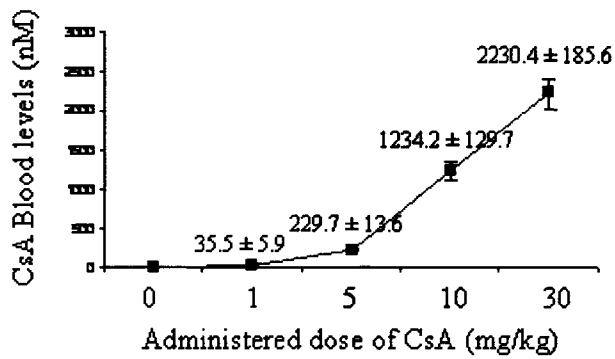
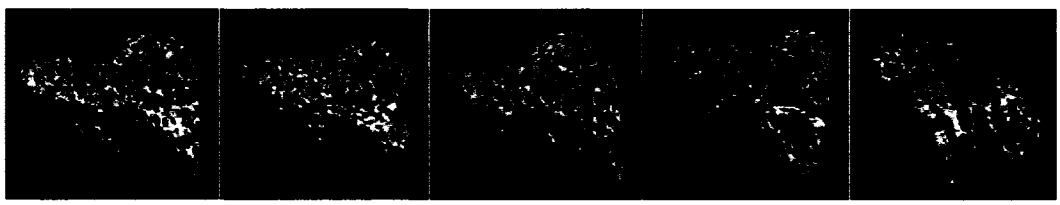
Figure 1. CsA exhibits differential effects on bone formation *in vivo*. CsA at concentrations ranging between 1 and 30 mg/kg was subcutaneously administered to mice every day for 28 days. **(A)** Steady state levels of CsA in whole blood were measured 24 hours after the last CsA injection by fluorescence polarization immunoassay using CsA monoclonal antibody. Values were obtained from 5 mice in each group and represent the mean ±SE. **(B)** μCT images of proximal metaphysis of tibia of CsA treated mice. The photographs shown are representative of 5 different mice in each group. **(C)** Bone mineral density was determined by using Dual-Energy X-ray absorptiometry (DXA). Values were obtained from 5 mice in each group and represent the mean ±SE of BMD of treated mice; *, $p \leq 0.03$, #, $p \leq 0.05$, **, $p \leq 0.004$ compared to those that were untreated.

A.




B.




C.

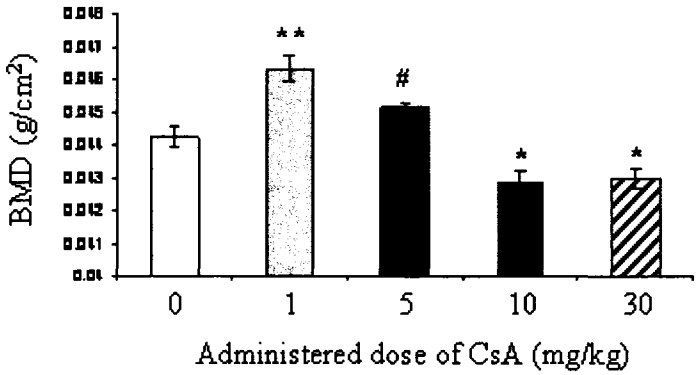


Figure 2. Histological analysis of proximal femur of CsA treated animals. All mice were injected with calcein 8 days and 1 day before euthanasia. After treatment of CsA for 28 days, femora were dissected. Tissues were fixed, decalcified with EDTA, embedded in paraffin, sectioned and stained for hematoxylin and eosin staining (left column, pink) as well as tartrate-resistant acid phosphatase (TRAP) (right column, red). Also, tissues were fixed, embedded in methyl methacrylate, sectioned and stained for von Kossa (middle column, black) or examined unstained under a florescence microscope to visualize and measure the distance between the 2 calcein layers (white arrow) that reflects the mineral apposition rate (MAR). Representative pictures are shown from 5 different mice in each group.

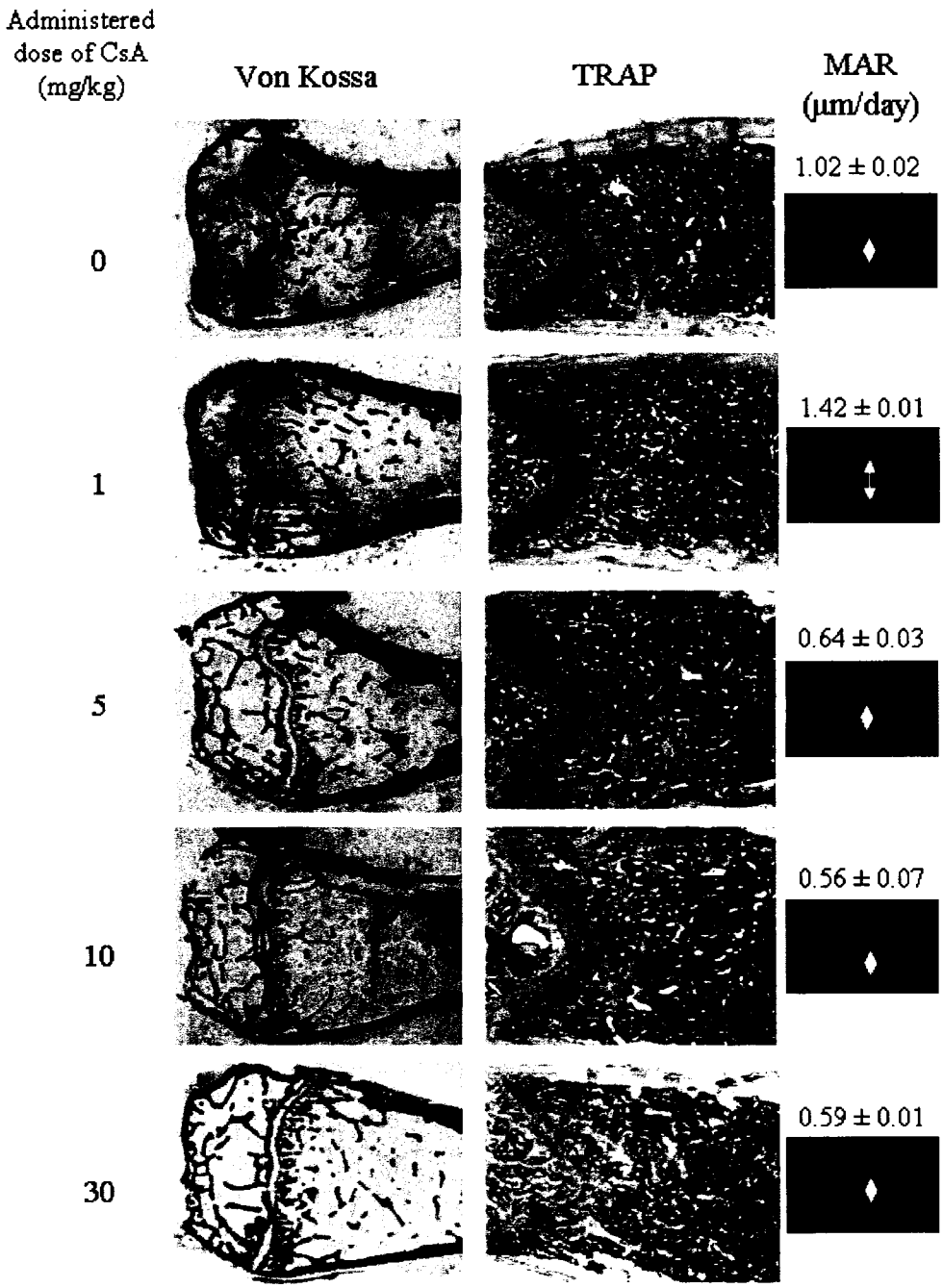


Figure 3. CsA increases osteoblast differentiation *in vitro*. MC3T3-E1 cells were cultured for 14 days or 21 days and treated with different concentrations of CsA. **(A)** Cells were fixed and stained for alkaline phosphatase activity (ALP) (upper panel) or for mineralization by von Kossa (lower panel). **(B)** Then, cells were lysed and calcium content was measured. Represent the mean ±SE of the calcium content relative to protein; *, $p \leq 0.02$. **(C)** MC3T3-E1 cells were cultured for 7 days and treated with CsA. Then, cells were incubated with [$^3$H]-thymidine and incorporation was measured. Represent the percent mean ±SE of the thymidine incorporation relative to total DNA; *, $p \leq 0.04$. After cultured 14 days, RNA was extracted and Real time RT-PCR was performed for **(D)** alkaline phosphatase, **(E)** osteocalcin and actin. Represent the mean ±SE of ALP and osteocalcin mRNA expression relative to actin expression; *, $p \leq 0.05$ compared to control.

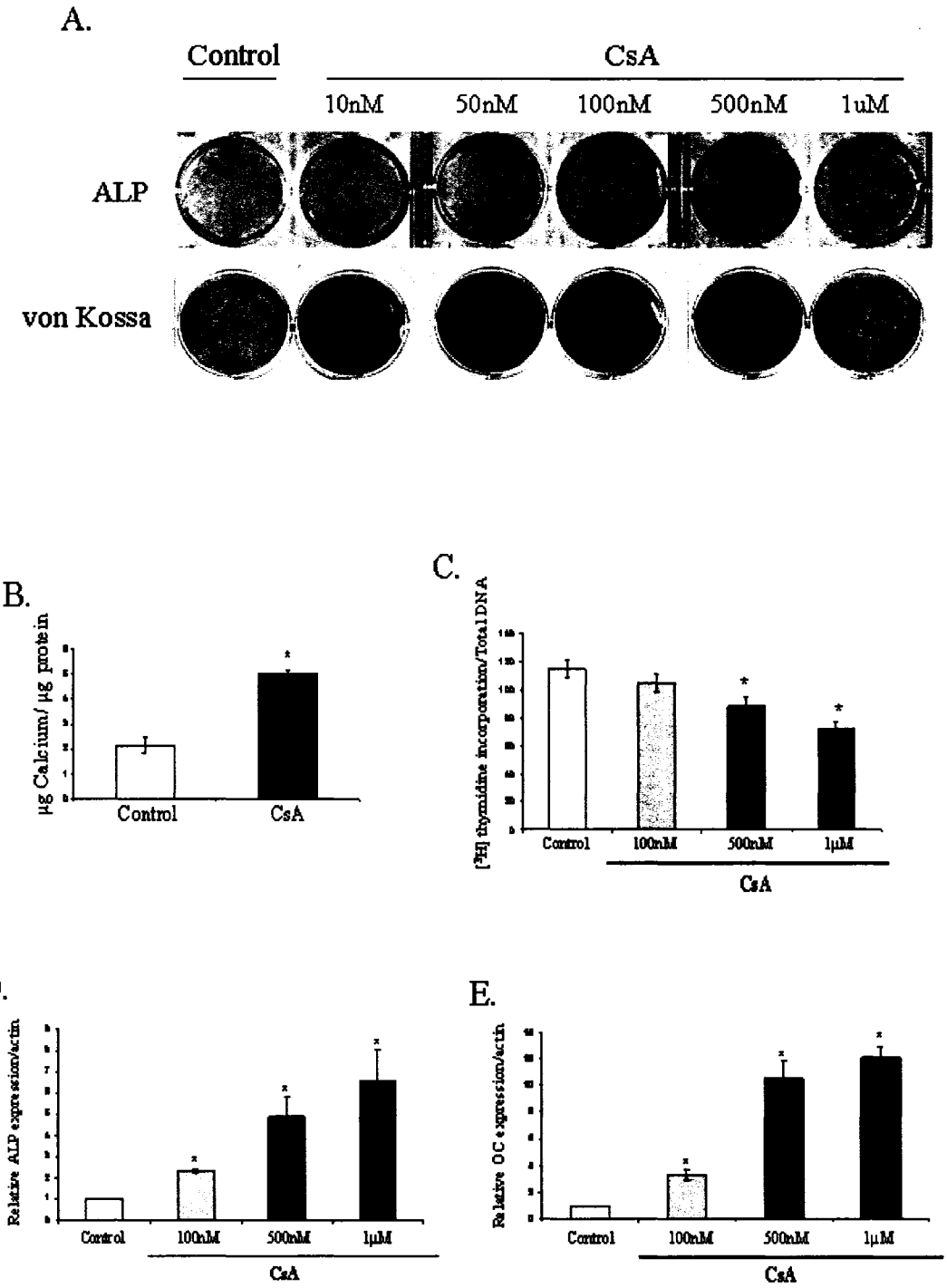
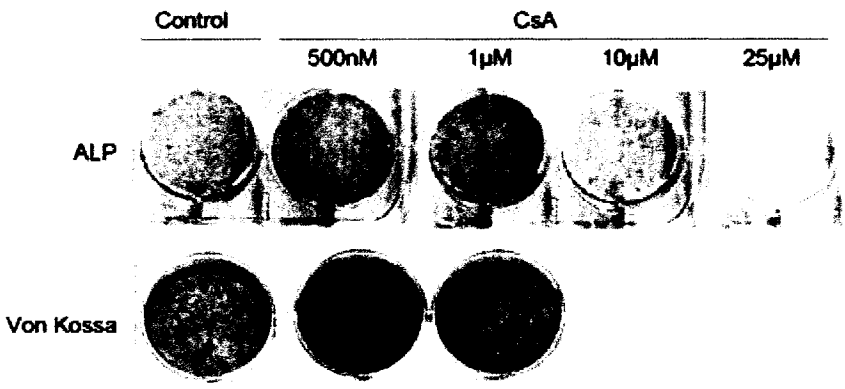


Figure 4. CsA exhibits differential effects on osteoblast differentiation *in vitro*. MC3T3-E1 **(A)** and primary mouse calvarial osteoblasts **(C)** were cultured and treated with either DMSO or different concentrations of CsA for 14 days (upper panel) or 21 days (lower panel). Cells were fixed and stained for ALP activity (upper panel) or for mineralization by von Kossa (lower panel). Similar results were seen in three other experiments. **(B)** MC3T3-E1 cells were cultured for 4 days and treated with CsA for 24 hours or Actinomycin D (ACT D, 10μM) for 6 hours. Cells were then harvested and stained with annexin V-FITC and PI. Apoptotic cell death was analyzed by flow cytometry. Values represent the mean ± SE of 4 separate experiments each performed in triplicate; *, $p \leq 0.05$ compared to control.

A.

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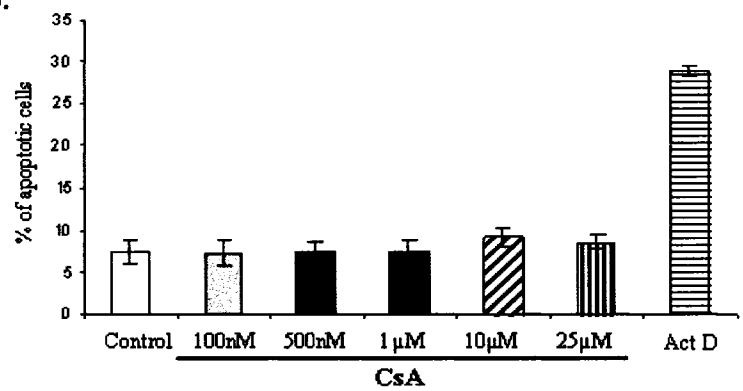


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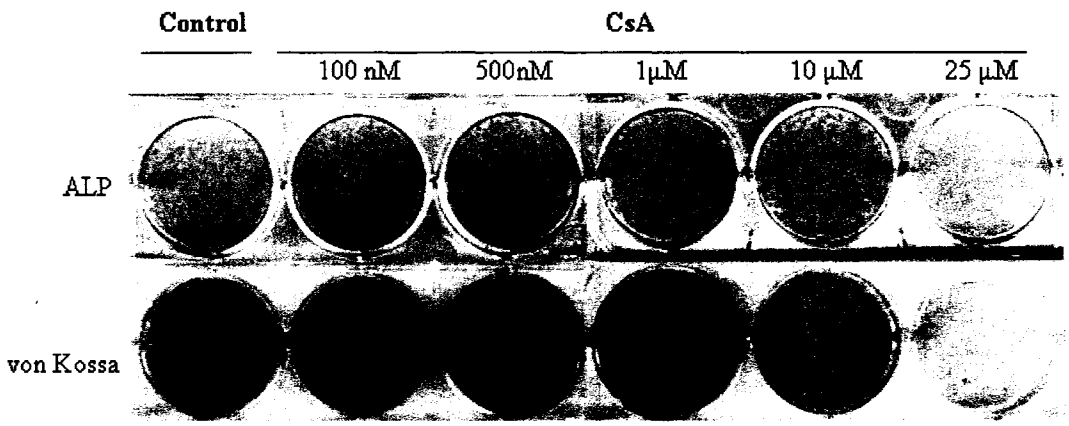


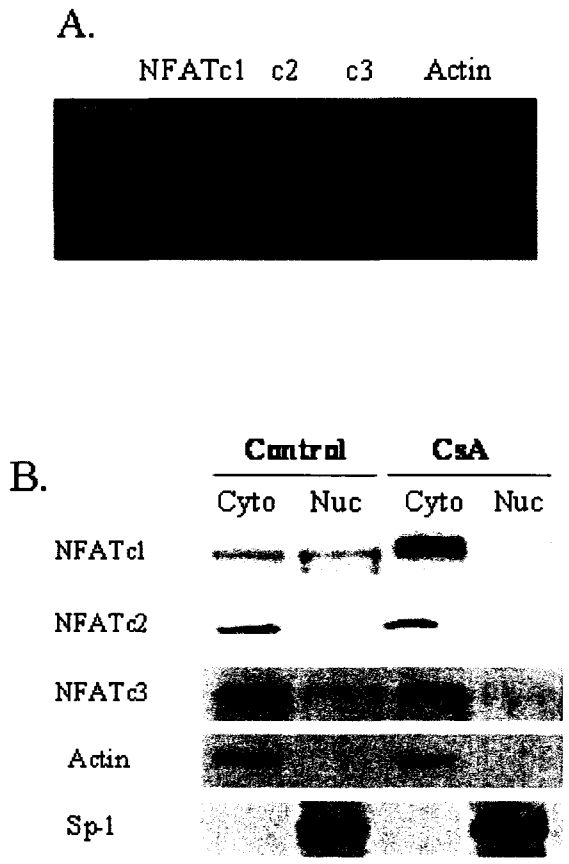
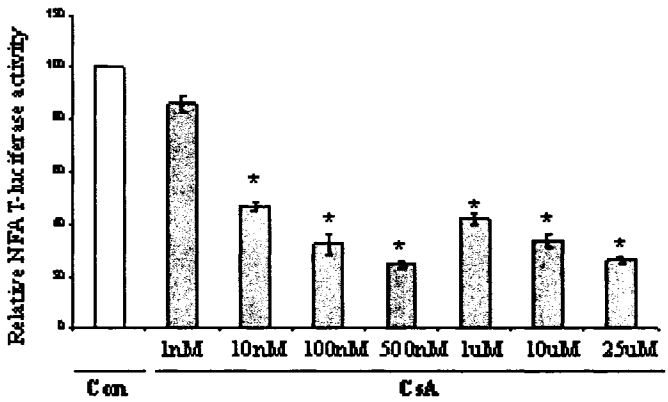



Figure 5. Osteoblasts express functional NFAT isoforms. MC3T3-E1 cells were cultured and treated with CsA (1μM) for 10 days. **(A)** Representative pictures of regular RT-PCR for NFATc1, c2 and c3 are shown. **(B)** Cytoplasmic and nuclear protein extracts (20μg/lane) were separated by SDS-PAGE. Immunoblots were developed using antibodies against NFATc1, c2, c3, actin and SP-1. **(C)** MC3T3-E1 cells were cultured and transfected with a pNFAT-TA-luciferase construct. Cells were treated with CsA and luciferase activity measured. Data are expressed relative to CMV-β-galactosidase and values represents the mean ± SE of 5 separate experiments; *, $p \leq 0.05$. **(D)** MC3T3-E1 osteoblasts were cultured for 4 days and then with 500nM of CsA for different time points as indicated. **(E)** MC3T3-E1 cells were cultured for 14 days and treated with CsA and DMSO. Cytoplasmic and nuclear protein extracts were separated by SDS-PAGE. Immunoblots were developed using antibodies against NFATc1, actin and SP-1.

C.

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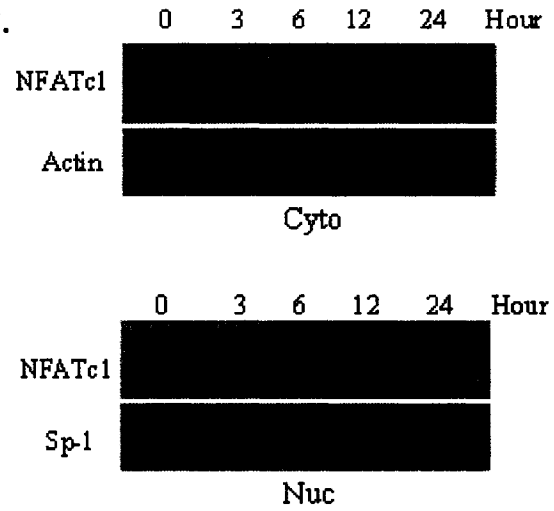


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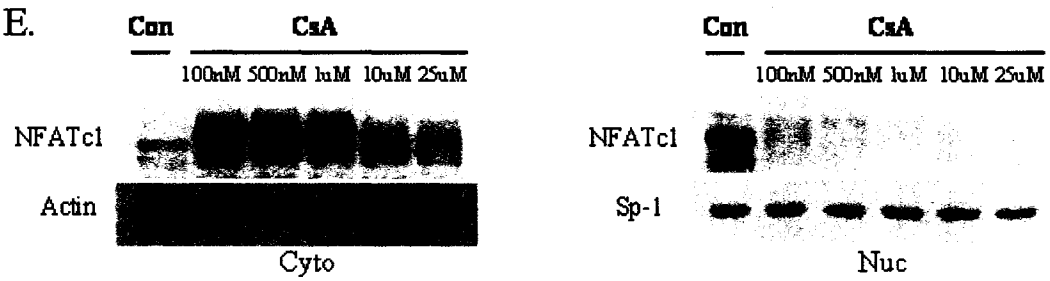


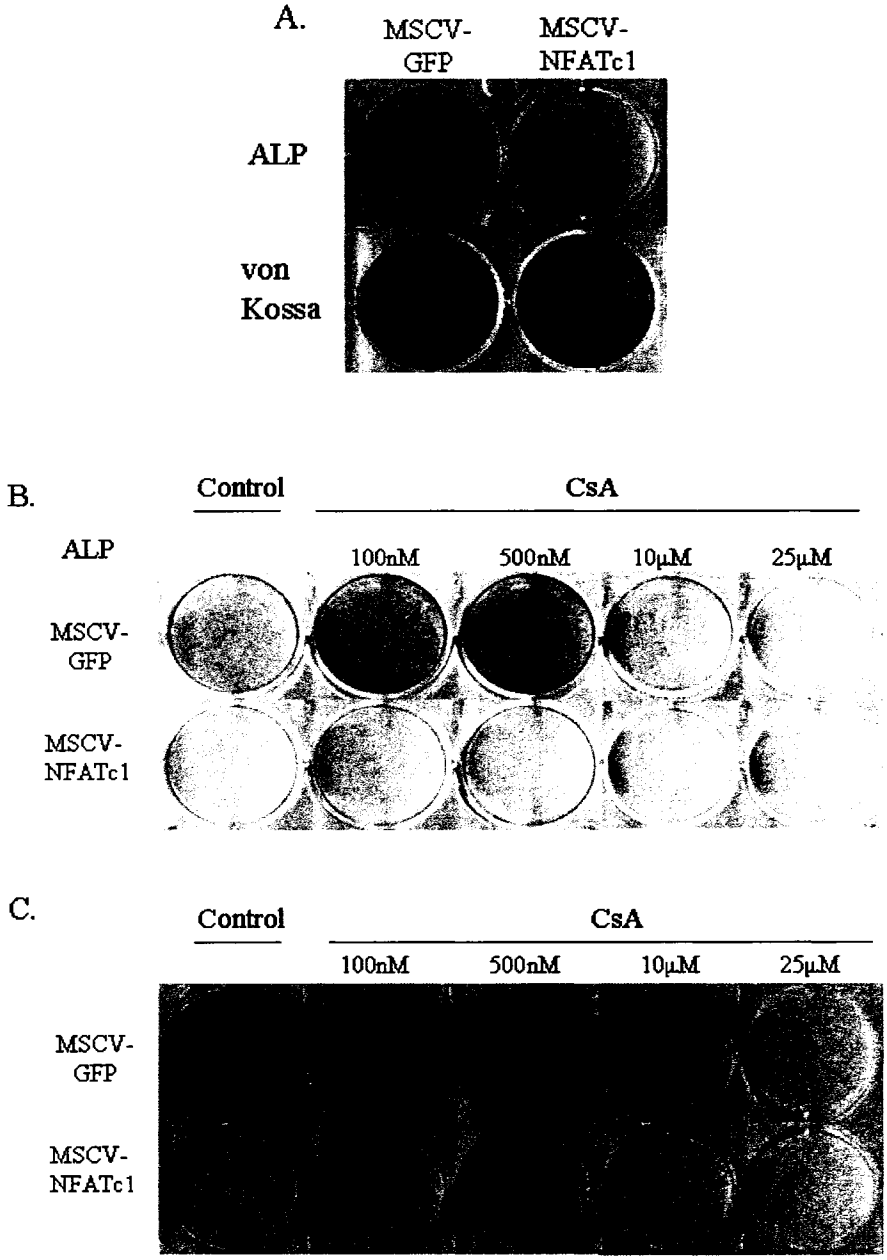
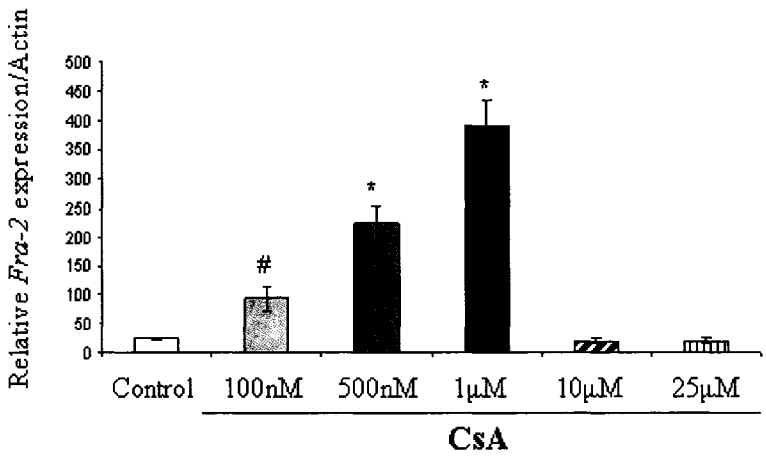



Figure 6. NFATc1 negatively regulates osteoblast differentiation. MC3T3-E1 cells were transduced with GFP-empty vector (MSCV-GFP) or the constitutively-active NFATc1 (MSCV-NFATc1-GFP) retrovirus and then cultured either without **(A)** or with CsA, as indicated, for **(B)** 14 or **(C)** 21 days. Cells were fixed and stained for ALP activity (red) or for mineralization by von Kossa (black/brown). Similar results were seen in three other experiments.

Figure 7. NFATc1 negatively regulates Fra-2 expression. MC3T3-E1 cells were cultured for 14 days and treated with different concentrations of CsA and DMSO as a control. Total RNA was extracted and real time RT-PCR was performed using primers for *fra-2*. Values were obtained from three separate experiments and represent the mean ±SE of *fra-2* mRNA expression relative to actin expression; *, $p \leq 0.01$ and # $p \leq 0.05$. **(B)** Nuclear proteins were extracted and separated by SDS-PAGE. Immunoblots were developed using antibodies directed against Fra-2 and total SP-1. **(C)** MC3T3-E1 cells were transfected with either control siRNA or NFATc1 siRNA. Cells were then harvested and lysed for protein extraction. **(D)** MC3T3-E1 cells were transduced with GFP-empty vector (MSCV-GFP) or the constitutively-active NFATc1 (MSCV-NFATc1) retrovirus and then cultured for 10 days. Immunoblots were developed using antibodies against NFATc1, Fra-2, JunD and SP-1. Shown is a representative of three separate experiments.

A.

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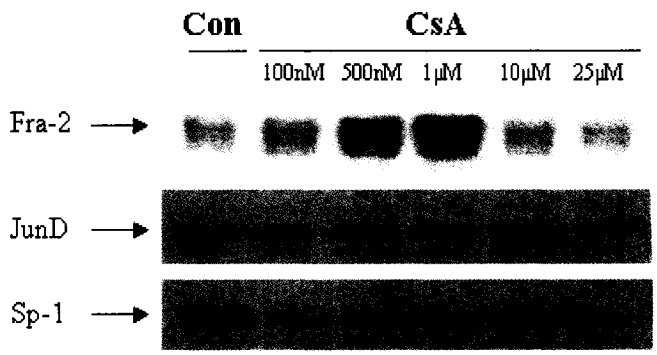


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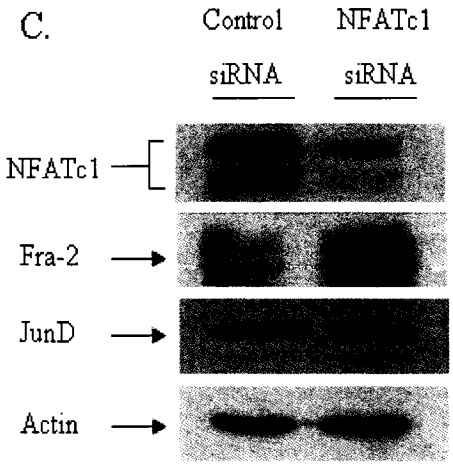


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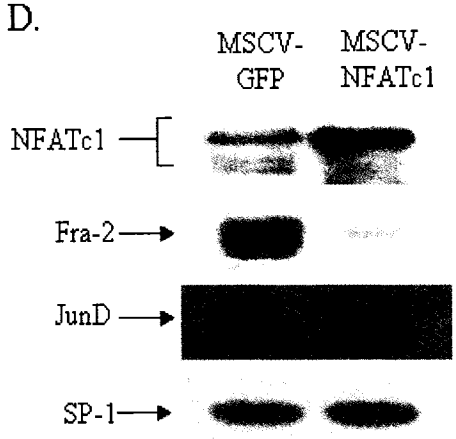
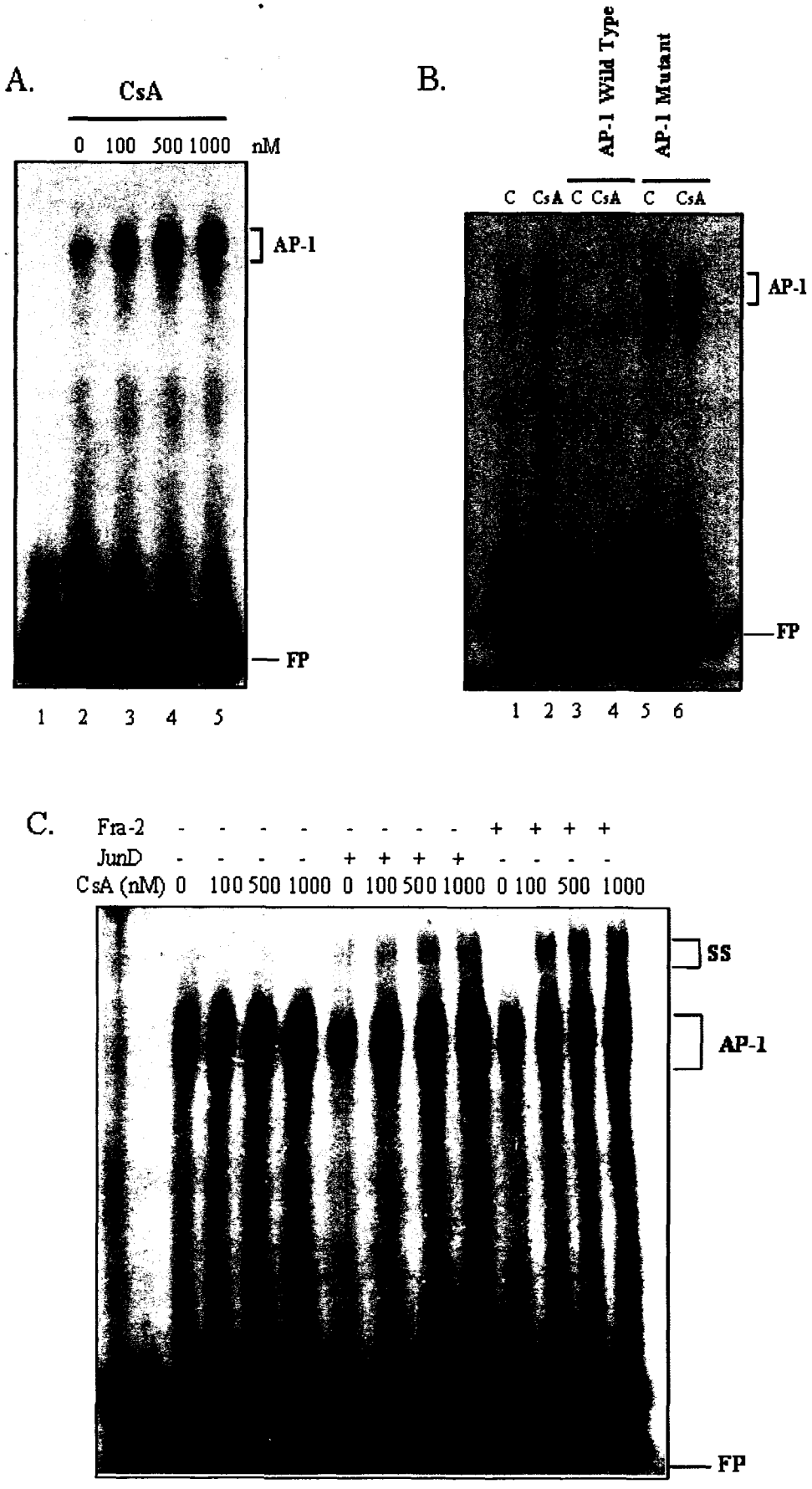


Figure 8. CsA increases AP-1 DNA binding activity in osteoblasts. MC3T3-E1 cells were cultured for 14 days and treated with CsA. **(A)** Nuclear protein extracts (4μg) were incubated with a $^{32}$P-labeled oligonucleotide containing an AP-1 consensus sequence (5'-GCGTTGA**TGAGTCA**GCCGGAA-3') and an electrophoretic mobility shift assay was performed. Lane 1, DNA probe alone and FP is the free unbound probe. **(B)** Four micrograms of nuclear extracts from osteoblasts treated with either DMSO (C) or 1μM CsA were incubated with 50-fold molar excess of unlabeled AP-1 wild type (lane 3 and 4) as well as 50-fold molar excess of mutant AP-1 oligonucleotides (lane 5 and 6). The autoradiograph is representative of 2 separate experiments. **(C)** Nuclear extracts (8μg) were incubated with Fra-2 and JunD antibodies followed by incubation with AP-1 $^{32}$P-labeled oligonucleotide sequence before performing an electrophoretic mobility shift assay. The autoradiograph is representative of 3 separate experiments.



# Table 1

**Table 1.** Histomorphometric quantification of femora from CsA-treated mice

| CsA | Control | 1mg/kg | 5mg/kg | 10mg/kg | 30mg/kg |
|---|---|---|---|---|---|
| BV/TV | 15.0 ± 0.5 | 20.5 ± 0.7 [a] | 9.5 ± 0.3 [b] | 12.0 ± 0.1 | 9.5 ± 0.2 [a] |
| Trabecular number ($mm^{-1}$) | 3.7 ± 0.1 | 4.7 ± 0.2 [a] | 3.1 ± 0.3 | 3.1 ± 0.1 [a] | 3.2 ± 0.2 |
| Trabecular separation (μm) | 230.6 ± 6.8 | 170.7 ± 7.5 [a] | 231.8 ± 17.5 | 290.9 ± 14.1 [b] | 283.2 ± 17.0 [b] |
| N.Ob/BS | 5.5 ± 0.7 | 8.2 ± 0.3 [b] | 5.4 ± 0.6 | 4.6 ± 1.0 | 4.2 ± 0.3 |
| N.Oc/BS | 1.0 ± 0.0 | 0.6 ± 0.1 [a] | 1.4 ± 0.1 [a] | 1.6 ± 0.0 [a] | 1.7± 0.1 [a] |

All values are the mean ± SEM. n=5

BV/TV : Bone Volume/Total Volume.
N.Ob/BS : Bone Number of Osteoblast/Bone Surface
N.Oc/BS : Bone Number of Osteoclast/Bone Surface

[a] $P<0.01$ *vs.* control
[b] $P<0.05$ *vs.* control

**Table 1.** Histomorphometric quantification of femora from CsA -treated mice

# CONDITIONAL DYSRUPTION OF CALCINEURIN B1 IN OSTEOBLATS INCREASES BONE FORMATION AND REDUCES BONE RESORPTION

by

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## ABSTRACT

We recently reported that the pharmacological inhibition of calcineurin (Cn) by low concentrations of Cyclosporin A increases osteoblast differentiation *in vitro* and bone mass *in vivo*. To determine whether Cn exerts direct actions in osteoblasts, we generated mice lacking Cnb1 (Cn regulatory subunit) in osteoblasts ($\Delta Cnb1^{OB}$) using Cre-mediated recombination methods. Transgenic mice expressing *Cre*-recombinase, driven by the human osteocalcin promoter, were crossed with homozygous mice that express *loxP*-flanked-Cnb1 ($Cnb1^{f/f}$). Micro-CT analysis of tibiae at three months showed that $\Delta Cnb1^{OB}$ mice had dramatic increases in bone mass compared to controls. Histomorphometric analyses showed significant increases in mineral apposition rate (67%), bone volume (32%), trabecular thickness (29%) and osteoblast numbers (68%), as well as a 40% decrease in osteoclast numbers as compared to the values from control mice. To delete Cnb1 *in vitro*, primary calvarial osteoblasts, harvested from $Cnb1^{f/f}$ mice, were infected with adenovirus expressing the *Cre*-recombinase. Cre-expressing osteoblasts had a complete inhibition of Cnb1 protein levels, but differentiated and mineralized more rapidly than control, GFP-expressing cells. Deletion of Cnb1 increased expression of osteoprotegerin and decreased expression of RANKL. Co-culturing Cnb1-deficient osteoblasts with wild type osteoclasts demonstrated that osteoblasts lacking Cnb1 failed to support osteoclast differentiation *in vitro*. Taken together, our findings demonstrate that the inhibition of Cnb1 in osteoblasts increases bone mass by directly increasing osteoblast differentiation and indirectly decreasing osteoclastogenesis.

## INTRODUCTION

Bone is a highly dynamic structure that is constantly renewing through a process called remodeling [1]. This process is critical for maintaining healthy bones and is mainly controlled by the activities of bone-forming osteoblasts and bone-resorbing osteoclasts. The presence of these two opposing cell types, with contrasting activities in close proximity, requires tight regulation in order to maintain healthy and strong bones. Bone resorption is attained by the action of osteoclasts, which are specialized macrophages whose differentiation is primarily regulated by Receptor Activator of NF-kappa-B Ligand (RANKL) and osteoprotegerin (OPG) [2]. Osteoblasts originate from multipotent mesenchymal progenitors that replicate as undifferentiated cells, but have the potential to differentiate into different lineages of mesenchymal tissues including: bone, cartilage, fat, muscle, and marrow stroma [3, 4]. Osteoblasts control bone formation not only by synthesizing bone matrix proteins and regulating mineralization but also by orchestrating the process of bone resorption through the modulation of RANKL and OPG expression [2, 4].

We have recently shown that the pharmacologic inhibition of Calcineurin (Cn) by low concentrations of Cyclosporin A (CsA) increases osteoblast differentiation and bone formation [5]. Cn is a $Ca^{2+}$/Calmodulin-dependent serine/threonine -protein phosphatase that regulates several physiological processes [6]. Cn exists as a heterodimeric protein complex consisting of two subunits: the 61-kDa calmodulin-binding, catalytic subunit A (CnA) and the 19-kDa $Ca^{2+}$-binding, regulatory subunit B (CnB) [7]. Three mammalian isoforms of CnA (α, β and γ) and two B isoforms (1 and 2) have been identified. The isoforms CnAα , β and B1 are ubiquitously expressed, while CnAγ and B2 are

specifically expressed in the testis [8]. The CnA subunit contains the phosphatase domain, a CnB binding domain, a calmodulin binding domain, and an autoinhibitory loop. In resting cells, the autoinhibitory domain obscures the phosphatase domain and is displaced upon the binding of CnB and $Ca^{2+}$/CaM to CnA, resulting in the full activation of Cn [8]. Nuclear Factors of Activated T cells (NFAT) are members of a family of transcription factors that are known to be dependent on Cn activation. In resting cells, NFAT proteins are highly phosphorylated and reside in the cytoplasm. Upon Cn activation, NFAT proteins are dephosphorylated and translocate to the nucleus where they regulate the transcription of NFAT-dependent genes [6].

Cn and NFAT are key promoters of osteoclast differentiation and bone resorption [9-12]. In contrast, the role of Cn and NFAT in osteoblast differentiation and bone formation is not well-defined. It has been reported that the Cn/NFAT signaling pathway is also a positive regulator of osteoblast differentiation and bone formation [13-15]. The conclusions of these reports were based on data from murine animal models where the Cn/NFAT signaling pathway was disrupted either globally and/or in multiple tissues and was not limited to differentiated osteoblasts. These reports did not consider that Cn and NFAT are also expressed in a variety of tissues such as vasculature, T-cells, skeletal muscles, chondrocytes and adipocytes which can indirectly alter the bone phenotype of these animals [16, 17].

In contrast to the notion that Cn/NFAT is a positive regulator of bone formation, we discovered that the pharmacologic inhibition of Cn by low concentrations of Cyclosporin A (CsA) increased osteoblast differentiation *in vitro* and bone mass *in vivo* [5]. The increase in bone mass was associated with enhanced bone formation and a

decrease in bone resorption [5]. To determine whether Cn directly impacts osteoblast differentiation, we utilized Cre-mediated recombinase technology to create mice lacking the calcineurin regulatory subunit (Cnb1) specifically in osteoblasts. Our results demonstrate that a block in the Cn/NFAT signaling pathway, when restricted to osteoblasts, leads to an increase in bone mass by directly increasing osteoblast differentiation and indirectly decreasing osteoclastogenesis.

## MATERIAL AND METHODS

### Generation of ΔCnb1$^{OB}$ mice.

Mice expressing the *Cre* recombinase under the control of an osteoblast-specific human osteocalcin promoter (OC-*Cre*) were provided by Dr. Thomas L. Clemens (University of Alabama at Birmingham) [18]. Mice expressing *loxP*-flanked calcineurin b1 (Cnb1$^{f/f}$) were generously provided by Dr. Gerard Crabtree (Stanford University) [19]. Cnb1$^{f/f}$ mice were crossed with OC-*Cre* mice to generate OC-*Cre*/ΔCnb1$^{OB}$ progeny, which were used in subsequent mating. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. For mice genotyping, DNA was prepared from tail biopsies. PCR-based strategies were then used to genotype these mice according to the manufacturer's recommendations (Sigma, St. Louis, MO). The sequences for the primers that were used for genotyping were previously published [18, 19]. The animals that had a genotype of (Cre$^{-/-}$/Cnb1$^{f/f}$) were considered control, (Cre$^{+/-}$/Cnb1$^{f/-}$) were considered heterozygote and (Cre$^{+/-}$/Cnb1$^{f/f}$) were considered ΔCnb1$^{OB}$.

**Histology and Histomorphometry.**

Tibiae and femora were harvested from three-month old male mice (Control and $\Delta Cnb1^{OB}$). Bones were fixed in 10% (*v/v*) buffered formalin, decalcified in EDTA, embedded in paraffin, sectioned and then stained for tartrate resistant acid phosphatase (TRAP). Also, bones were fixed, embedded in methyl methacrylate, sectioned and stained with Goldner's Trichrome. A region of interest, an area at least 0.5mm below the growth plate (excluding the primary spongiosa and trabecular-connected cortical bone) was selected and remained constant for all animals regardless of the shape of the section [5]. Standard bone histomorphometry as described by Parfitt et al. [20] was performed using the BioQuant image analysis software (R & M Biometrics, Nashville, TN) in the Histomorphometry and Molecular Analysis Core Laboratory of the University of Alabama at Birmingham Bone Center [5, 21].

**Microcomputed Tomography (μCT).**

After euthanizing the animals, tibiae were removed and dissected free of soft tissue. The bones were then fixed in 10% (*v/v*) buffered formalin for 24 hours and analyzed by a μCT system using the manufacturer's included 3-D analysis software (μCT 40, Scanco Medical, Basserdorf, Switzerland). The region of interest analyzed from each mouse was the metaphysis of the proximal tibia. The trabecular bone in the tibia was scanned using a 12- μm slice increment on 100 slices, starting 360 μm distal to the growth plate and extending for 120 μm [5].

**Adenovirus-GFP and -Cre Infection.**

Primary calvarial osteoblasts were isolated from 1-day-old Cnb1$^{f/f}$ mice as previously described [5]. Briefly, mice calvariae were subjected to three sequential digestions in an enzymatic solution containing 0.1% (*w/v*) collagenase (Worthington, Lakewood, NJ) and 0.05% (*v/v*) trypsin containing 0.53 mM ethylene-diamine-tetra-acetic acid (EDTA) at 37°C. Osteoblasts were collected from the second and third digestions. Cells were maintained in Minimum Essential Medium Eagle, Alpha Modification (α-MEM) (Sigma) containing 10% FBS (Atlanta Biologicals), 100 units/ml penicillin G and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C with 5% $CO_2$. Adenovirus-GFP and -*Cre* were purchased from Vector Biolabs (Philadelphia, PA). At confluency, cells were infected for 48 hours with adenovirus-GFP and -*Cre* at an moi (Multiplicity Of Infection) of 100 [22]. Osteoblastic induction was performed by supplementing the culture medium with 5 mM β-glycerophosphate and 250 μM ascorbic acid-2-phosphate [5, 21].

**Alkaline Phosphatase (ALP) and Mineral Deposition Assays.**

Cultured Cnb1$^{f/f}$ primary osteoblasts that were infected with adenovirus-GFP or -*Cre* were fixed in 2% paraformaldehyde/PBS (*v/v*) for 10 minutes and then incubated at 37°C with freshly-prepared alkaline phosphatase substrate solution (100mM Tris-Maleate buffer (pH 8.4), 2.8% N,N-dimethyl formamide (*v/v*), 1 mg/ml Fast Red TR and 0.5 mg/ml naphthol AS-MX phosphate) [5]. Mineralization was assessed by von Kossa staining (3 minutes in 3% (*w/v*) $AgNO_3$) as previously described [5].

**Osteoblasts/Osteoclasts Co-Culture.**

Primary osteoclast precursor cells were isolated by flushing bone marrow cells from the long bones of two-month-old control and $\Delta Cnb1^{OB}$ mice. Cells were plated and cultured overnight in α-MEM containing heat inactivated 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 100 units/ml penicillin G, and 100 μg/ml streptomycin (Invitrogen). Suspended cells were collected and layered on a Ficoll (Amersham, Pittsburgh, PA), then centrifuged at 340 x *g* for 15 min at room temperature. Bone marrow macrophage precursor cells were then collected from the interface. Primary Adenovirus-GFP and -*Cre* infected $Cnb1^{f/f}$ osteoblasts ($10^5$ per well) were co-cultured with bone marrow cells ($10^6$ per well) in α-MEM containing 10% FBS, $10^{-7}$ M Dexamethasone and $10^{-8}$ M $1\alpha,25(OH)_2D_3$-dihydroxy-vitamin $D_3$ in 24 well plates. Culture media were changed every 3 days for 10 days. At the end of the study, cultures were stained for TRAP using a TRAP staining kit (Sigma) [23].

**Real-time PCR Analysis.**

Total RNA was extracted by the Trizol method, as recommended by the manufacturer (Invitrogen) [5]. One μg of RNA was reverse-transcribed and the equivalent of 10 ng was used for real-time PCR, as previously described [24]. Expression of actin was used for the normalization of gene expression values. The sequences for the specific primers used in this study were previously described: ALP, osteocalcin, osteoprotegerin, RANKL, and actin [21, 22, 25].

**Enzyme Serum Measurements.**

Serum was collected from the blood of control and $\Delta Cnb1^{OB}$ animals immediately after euthanasia. OPG and RANKL serum levels were measured using the mouse OPG/TNFSRSF11B and mouse TRANCE immunoassay according to manufacturer's protocol (R&D System, Minneapolis, MN).

**Statistical analysis.**

All statistical analyses were performed using the Microsoft Excel data analysis program for student's t-test analysis. Experiments were repeated at least three times unless otherwise stated. Values are expressed as the mean ±SE.

## RESULTS

To determine whether Cnb1 exerts direct actions in osteoblasts, we generated mice lacking Cnb1 in osteoblasts ($\Delta Cnb1^{OB}$) using *Cre*-mediated recombination. Transgenic mice expressing the *Cre* recombinase driven by the human osteocalcin promoter were crossed with homozygous mice that express *loxP*-flanked-Cnb1 ($Cnb1^{f/f}$). In order to confirm that Cnb1 deletion in $\Delta Cnb1^{OB}$ mice is indeed specific to osteoblasts, we performed PCR analysis using a combination of primers that specifically detect floxed Cnb1 alleles ($Cnb1^{f}$) and null alleles ($Cnb1^{\Delta}$). Genomic DNA was extracted from several non-skeletal tissues such as: brain, kidney, lung, liver, heart and muscle, as well as skeletal tissues, including: calvarium, femur and spine from the $\Delta Cnb1^{OB}$ offspring. Figure 1A demonstrates that *Cre*-mediated recombination ($Cnb1^{\Delta}$) occurred exclusively in tissues that contain osteoblastic cells, whereas non-skeletal tissues retained the Cnb1

floxed alleles ($Cnb1^{f}$). Non-osteoblastic cells in skeletal tissues such as chondrocytes, adipocytes, and myocytes also retained their $Cnb1^{f}$ alleles (Figure 1A).

We then assessed the specific deletion of Cnb1 in osteoblasts by examining the levels of Cnb1 protein in primary calvarial osteoblasts that were harvested from 1-day-old control, $f/\Delta Cnb1^{OB}$ and $\Delta Cnb1^{OB}$ pups. Primary calvarial osteoblasts were cultured for 30 days in the presence of β-glycerophosphate and ascorbic acid in order to induce osteoblast differentiation and ensure the activation of the osteocalcin promoter *in vitro*, and thus the expression of *Cre*-recombinase. $\Delta Cnb1^{OB}$ osteoblasts completely lacked Cnb1 as compared to controls and heterozygotes ($f/\Delta Cnb1^{OB}$) (Figure 1B, left panel). The deletion of Cnb1 isoform (regulatory subunit) in osteoblasts did not affect the levels of the CnAα (catalytic subunit), but it was sufficient to inhibit the dephosphorylation of NFATc1. Loss of one allele of Cn ($f/\Delta$ $Cnb1^{OB}$) in osteoblasts resulted in a slight decrease in the dephosphorylation of NFATc1 (Figure 1B, left panel). Furthermore, protein levels of Cnb1, CnAα and NFATc1 were not affected in non-osteoblastic tissues such as the liver (Figure 1B, right panel). Taken together, these data confirm that the $\Delta Cnb1^{OB}$ mice represent a suitable model for determining the direct role of Cnb1 in osteoblasts on bone formation and bone mass.

There were no gross abnormalities in the skeletal development of the $f/\Delta Cnb1^{OB}$ or $\Delta Cnb1^{OB}$ mice as compared to controls. To examine the effects of Cnb1 ablation in osteoblasts on bone architecture, tibiae from 3-month-old male $\Delta Cnb1^{OB}$ and control mice were compared by microcomputed tomography (μCT) analysis. Three-dimensional images revealed a pronounced increase in the trabecular bone volume in the $\Delta Cnb1^{OB}$ mice as compared to control mice, using sagittal and transverse scanning of the

metaphysis of the proximal tibia (Figure 2A). There was no difference between heterozygote and control animals, even though Cnb1 was somewhat deleted in these animals (data not shown). This microarchitectural increase in trabecular bone volume was further substantiated by a decrease in the structure model index (SMI) (Figure 2B). This indicates the conversion of trabecular bone to plate-like rather than rod-like structures because of an increase in bone connectivity and a decrease in perforation. Static histomorphometric analyses of distal femora of 3-month-old male mice confirmed the μ-CT findings. Mutant mice that have had Cnb1 specifically deleted in osteoblasts demonstrated a 32% increase in trabecular bone volume/tissue volume, a 29% increase in trabecular thickness, a 10% increase in trabecular number, and a 17% decrease in trabecular separation, when compared to controls (Figures 2C-2F). The combination of these microarchitectural and histomorphometrical results indicates that Cnb1 in osteoblasts negatively regulates bone mass.

The increased bone volume seen in the $\Delta Cnb1^{OB}$ mice was accompanied by an increase in bone formation measured histomorphometrically. We show dynamic changes in bone remodeling by injecting calcein twice at 7-day intervals before euthanizing the animals. The distance between two consecutive labels in the trabecular bone of the femur was dramatically greater in the $\Delta Cnb1^{OB}$ mice than in the controls (Figure 3A. upper panel). Goldner's Trichrome staining showed that $\Delta Cnb1^{OB}$ mice had an increase in bone volume (green/blue) and newly-formed bone (red) (Figure 3A, lower panel). These parameters were quantitated using BioQuant image analysis system and demonstrated that $\Delta Cnb1^{OB}$ mice had an increase in the osteoid perimeter (131%), mineral apposition rate (MAR) (12%) , osteoblast surface (70%) and osteoblast number (68%) when

compared to the values from control mice (Figures 3B-3E). Furthermore, all of the bone mass and bone formation indices were similar between heterozygote and control animals (Data not shown). Taken together, these results demonstrate that the increase in bone mass in the $\Delta Cnb1^{OB}$ mice is associated with an increase in bone formation.

Bone mass is known to be controlled by the activities of bone-forming osteoblasts and bone-resorbing osteoclasts. Therefore, we next examined bone resorption in the $\Delta Cnb1^{OB}$ mice. TRAP staining demonstrated that the activity and the number of bone-resorbing osteoclasts are dramatically decreased in the $\Delta Cnb1^{OB}$ as compared to control mice (Figure 4A). This is confirmed by a quantitative static histomorphometrical analysis showing that $\Delta Cnb1^{OB}$ mice have a decreased osteoclast surface (50%) and osteoclast number (42%) when compared to the values from control mice (Figures 4B and 4C). These results indicate that the deletion of Cnb1 in osteoblasts not only induces bone formation, but also decreases bone resorption.

In order to determine the role of Cnb1 in osteoblast differentiation, we examined the effects of the *in vitro* deletion of Cnb1 in primary calvarial osteoblasts. Primary osteoblasts were harvested from mice carrying Cnb1 floxed alleles ($Cnb1^{f/f}$) and then infected with either adenovirus expressing *Cre* recombinase ($Cre^{+}$) or a control adenovirus directing the expression of green fluorescent protein (GFP) ($Cre^{-}$). Cells were cultured in osteogenic differentiation media (in the presence of ascorbic acid and β-glycerophosphate) for 14 days. To confirm the efficiency of the adenovirus-*cre* in deleting Cnb1, proteins were extracted and the levels of Cnb1 protein were examined by western blotting. Here we show that infecting primary osteoblasts harvested from $Cnb1^{f/f}$ mice with adenovirus-*Cre* ($Cre^{+}$) completely abolished Cnb1 protein, as compared to

adenovirus-GFP infected cells ($Cre^-$). The deletion of Cnb1 in these cells also inhibited the activation of Cn-downstream targets, as we show that $Cre^+$ cells have a dramatic increase in the levels of the phosphorylated and inactive form of NFATc1 protein (Figure 5A). $Cre^+$ and $Cre^-$ primary osteoblasts were then cultured for 14 and 21 days under osteogenic conditions to examine their osteogenic capabilities. Consistent with the phenotype of the $\Delta Cnb1^{OB}$ mice, osteoblasts deficient in Cnb1 ($Cre^+$) showed an enhanced osteoblastic potential demonstrated by a marked increase in alkaline phosphatase activation (red) and calcified nodule formation (von Kossa staining, black nodules) (Figure 5B). This was accompanied by an increase in the expression of osteoblast-specific genes, ALP (150%) and osteocalcin (134%), in differentiated osteoblasts that are deficient in Cnb1 ($Cre^+$) as compared to control cells ($Cre^-$) (Figure 5C and 5D).

Our initial observation that $\Delta Cnb1^{OB}$ mice exhibit a decrease in bone resorption parameters could be due to a disruption in the OPG-RANKL axis. To further explore this possibility, we examined the gene expression of RANKL (the major osteoclast differentiation factor) and OPG (the osteoclast inhibitory factor) in primary osteoblasts that were Cnb1 deficient. Our results show that differentiated osteoblasts which lack Cnb1 exhibit a 155% increase in OPG expression, accompanied by a 76% decrease in the expression of RANKL when compared to control cells (Figures 6A and 6B). To confirm this *in vivo*, we measured serum levels of OPG and RANKL using mouse OPG and RANKL immunoassays. Our results demonstrate that the levels of OPG protein in the serum of $\Delta Cnb1^{OB}$ mice were 193% increased when compared to control mice, while RANKL was 78% decreased (Figure 6C and 6D). Finally, in order to determine whether

Cnb1-deficient osteoblasts are capable of supporting osteoclast differentiation, bone marrow macrophages (BMMs) from control and $\Delta Cnb1^{OB}$ mice were co-cultured with $Cnb1^{f/f}$ primary calvarial osteoblasts that had been infected with either $Cre^-$ or $Cre^+$ adenovirus in the presence of Dexamethasone and 1,25 vitamin $D_3$ for 10 days. Cultures were then stained for TRAP activity to identify TRAP-positive, multinucleated osteoclasts. Control primary calvarial osteoblasts that lack *Cre* recombinase were able to support osteoclasts generated from BMMs collected from either control or $\Delta Cnb1^{OB}$ mice, whereas $Cre^+$ osteoblasts which lack Cnb1 failed to do so (Figure 6E). Taken together, these results demonstrate that the inhibition of Cnb1 in osteoblasts increases bone mass by directly increasing the activation of bone-forming osteoblasts and indirectly decreasing the formation of bone-resorbing osteoclasts (Figure 7).

## DISCUSSION

We have recently reported that the pharmacologic inhibition of Cn by low concentrations of Cyclosporin A (CsA) increased osteoblast differentiation *in vitro* and bone mass *in vivo* [5]. However, these findings alone do not confirm that this bone-anabolic response is due to the specific inhibition of Cn in osteoblasts. This is because Cn is ubiquitously expressed and CsA in the blood could also be acting elsewhere. To determine whether Cn directly impacts osteoblast differentiation, we utilized Cre-mediated recombinase technology to create mice lacking the calcineurin regulatory subunit (Cnb1) specifically in osteoblasts. We discovered that in response to Cnb1 deletion, when limited to osteoblasts, bone formation is enhanced and bone resorption is diminished, which ultimately lead to an increase in bone mass.

We generated mutant mice that have Cnb1 gene deleted only in osteoblasts ($\Delta Cnb1^{OB}$). Mice lacking Cnb1 in osteoblasts developed normally. Mice were viable and showed no significant differences in size, weight and survival rate (data not shown). However, mice that lacked Cnb1 in osteoblasts developed an increase in bone volume and bone mass. This is consistent with our previous findings where we reported that low concentrations of the Cn antagonist cyclosporin A increase osteoblast differentiation and bone mass *in vitro* and *in vivo* [5]. The $\Delta Cnb1^{OB}$ mice revealed a significant increase in bone formation, as demonstrated by an increase in calcein double labeling, implying accelerated osteogenesis. The osteoid perimeter and mineral apposition rate, which are markers of osteoblastic bone formation, were significantly increased in $\Delta Cnb1^{OB}$ mice as compared to the control mice. Finally, osteoblast surface/bone surface as well as number of osteoblasts were significantly higher in $\Delta Cnb1^{OB}$ mice, confirming that bone formation is greatly increased in response to Cnb1 deletion in osteoblasts. However, the increase in osteoblast number in the $\Delta Cnb1^{OB}$ mice could not be explained by an increase in osteoblast proliferation. In this mouse model we deleted Cnb1 in mature osteoblasts by expressing Cre-recombinase that is driven by the osteocalcin promoter. It is known that osteocalcin promoter is activated at a late stage of osteoblast differentiation, long after osteoblasts exit the cell cycle. Therefore, it is unlikely that osteoblast proliferation was increased in response to Cnb1 deletion during the late stage of osteoblast differentiation. Thus, the increase in the number of osteoblasts as a result of deleting Cnb1 in osteoblasts could be due to an increase in the recruitment of new osteoblasts to the bone surface by an unknown mechanism. This notion was supported by examining the proliferation of primary cells that lack Cnb1 *in vitro*. Cre-mediated deletion of Cnb1

in primary calvarial osteoblasts showed no significant changes in osteoblast proliferation, as compared to control (data not shown). Together, these results suggest that Cnb1 in osteoblasts may regulate an autocrine or paracrine growth factor in the bone microenvironment that increases osteoblast numbers *in vivo*.

The increase in bone mass may result from enhancing bone formation or/and decreasing bone resorption. Mice lacking Cnb1 in osteoblasts develop a decrease in bone resorption demonstrated by a decrease in TRAP activation and TRAP-positive osteoclasts. This decrease in osteoclast number appears to be secondary to the deletion of Cnb1 in osteoblasts for several reasons. First, monocytes or macrophages from either $\Delta Cnb1^{OB}$ mice or control mice differentiated into multinucleated osteoclasts when cultured with control osteoblasts indicating that osteoclasts harvested from $\Delta Cnb1^{OB}$ mice are normal. Second, deletion of Cnb1 in osteoblasts rendered these cells incapable of inducing osteoclastogenesis. Finally, $\Delta Cnb1^{OB}$ mice have reduced osteoclast surface and numbers. These results suggest that the deletion of Cnb1 in osteoblasts not only increases osteoblastic bone formation but also reduces bone resorption.

It is has been shown that decreases in bone resorption could result from dysregulation in the OPG-RANKL axis. In the process of examining this possibility, we discovered that OPG protein levels were indeed increased in $\Delta Cnb1^{OB}$ mice as compared to control mice, while RANKL levels were dramatically decreased. This was also confirmed by demonstrating that the deletion of Cnb1 in osteoblasts increased the expression of OPG and decreased that of RANKL. It is not completely clear how Cn regulates the expression of RANKL and OPG in osteoblasts. This could be through a direct binding of different transcription factors that are regulated by Cnb1 that leads to an

activation of the OPG promoter and inhibition of RANKL. Alternatively, Cnb1 could be controlling the expression and/or activation of some unidentified factor(s) that indirectly regulate the expression of OPG and RANKL. Nevertheless, our data demonstrate that the absence of Cnb1 in osteoblasts indirectly regulates osteoclastogenesis in $\Delta Cnb1^{OB}$ mice *in vivo* and *in vitro* (Figure 7).

Our results are in contrast to three recent reports describing the Cn/NFAT signaling pathway as a positive regulator of osteoblast differentiation and bone formation [13-15]. In these studies, this conclusion was based on data from murine animal models where the Cn/NFAT signaling pathway was globally altered and/or expressed in multiple tissues. Together, this makes the global genetic alteration of Cn/NFAT signaling unreliable for examining the direct roles of Cn and NFAT in osteoblast differentiation and bone mass. For example, the reported decrease in bone mass in response to the global loss of CnAα could be an indirect response to an alteration in skeletal muscle phenotype [14, 26]. Also, the global loss of CnAα is known to alter T cell responses and activation that may indirectly affect the bone phenotype [27]. Furthermore, bone-forming osteoblasts are known to originate from the osteogenic differentiation of mesenchymal stem cells. These bone marrow stem cells are pluripotent and capable of differentiating into several lineages such as chondrocytes, myocytes, and adipocytes, in addition to osteoblasts [3]. Interestingly, Cn/NFAT signaling has been shown to play a critical role in the lineage decision of stem cells to differentiate into chondrocytes, myocytes, and adipocytes [26, 28, 29]. Indeed, the global deletion of NFATc2 in mice has been shown to increase the expression of chondrocytic specific genes and chondrogenesis, suggesting that NFATc2 is a negative regulator of chondrocyte growth and differentiation [29].

Global NFATc2/c4 double knockout mice exhibit defects in fat accumulation and reduced adiposity, suggesting that NFAT signaling is positive regulator of adipogenesis [30]. Finally, it has been shown that the number of primary myofibers and muscle size are decreased in the global NFATc3 deficient mice [31]. Taken together, it is likely that the global modulation of Cn or NFAT expression in murine animal models does not provide a reliable tool for examining osteoblast differentiation and bone mass, as Cn/NFAT signaling is globally expressed and is involved in the lineage decision of mesenchymal stem cells.

The work described here is consistent with our previous reports that describe the effects of the pharmacologic inhibition of Cn by low concentrations of CsA on bone formation [5]. Indeed, the skeletal phenotypes of mice that were treated with a low dose of CsA and osteoblast-specific Cnb1 deleted are similar. Both had increased bone mass and osteoblastic bone formation, and showed a decrease in osteoclastogenesis and bone resorption. Clinically, this work provides a better understanding of the mechanisms by which Cn/NFAT regulates osteoblastic bone formation, and may have important implications in the development of new anabolic drugs which target the Cn/NFAT pathway in osteoblasts to treat osteoporosis and bone loss.

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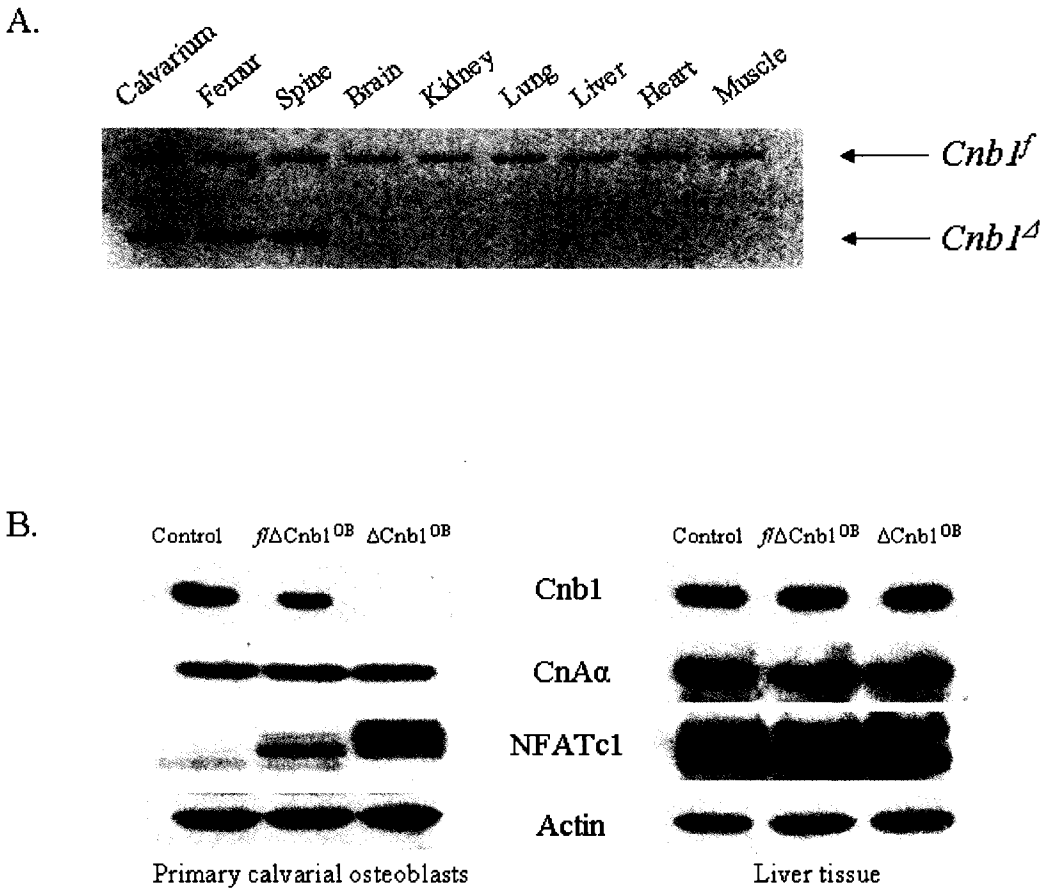


FIGURE 1. Generation of $\Delta Cnb1^{OB}$ mice. (**A**) RT-PCR analysis was performed using different tissues that were harvested from $\Delta Cnb1^{OB}$ mice to examine specific deletion of the Cnb1 gene. As indicated, bands represent Cnb1 gene alleles that are either flanked with a loxP site ($Cnb1^{f}$) or deleted ($\Delta$). (**B**) Cnb1 and NFATc1 protein levels were determined by western blot analysis using protein extracts from primary calvarial osteoblasts and liver tissues that were harvested from control, heterozygote ($fl/\Delta Cnb1^{OB}$) and homozygote Cnb1 deleted mice ($\Delta Cnb1^{OB}$). CnA$\alpha$ and actin were used as controls. Photographs are representative of 3 different experiments using cells and tissues from 3 different animals.

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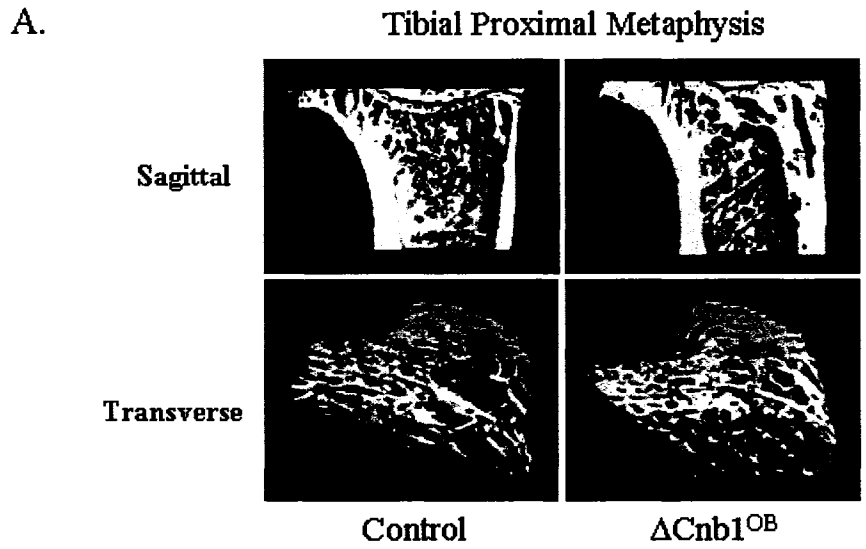
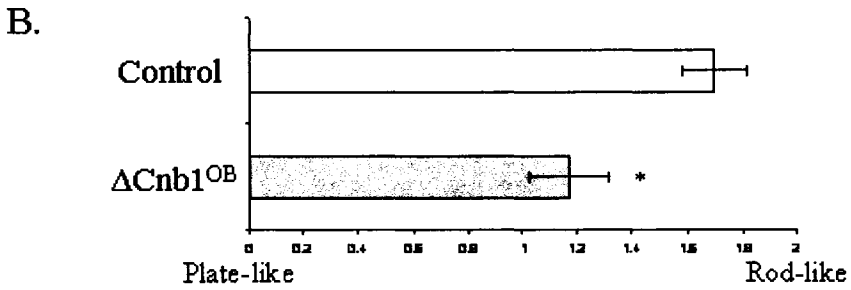
FIGURE 2. Bone volume is increased in osteoblast-specific $\Delta Cnb1^{OB}$ mice. (**A**) Microcomputed tomography (μCT) 3D reconstruction images of proximal metaphysis of tibia from control and $\Delta Cnb1^{OB}$ mice are shown. Sagittal and transverse views were reconstructed. Images were obtained from control mice (n=4) and $\Delta Cnb1^{OB}$ mice (n=6). (**B**) Mean values of structure model index, calculated by μCT of tibiae, demonstrating a conversion of trabecular bone in the $\Delta Cnb1^{OB}$ mice to plate-like rather than rod-like structures as shown in control mice. (**C-F**) Quantitative analysis of histomorphometrical indices was performed (**C**) trabecular bone volume/tissue volume (BV/TV), (**D**) trabecular thickness (Tb.Th), (**E**) trabecular number (Tb.N), and (**F**) trabecular separation (Tb.Sp). Values represent the mean ± SE of $\Delta Cnb1^{OB}$ mice (n=6) compared to control (n=4); *, $p \leq 0.05$.



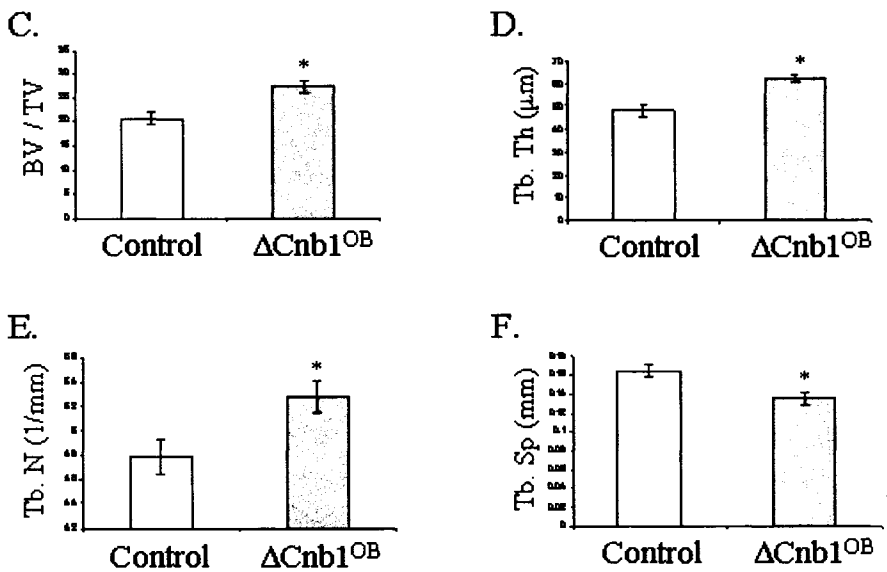
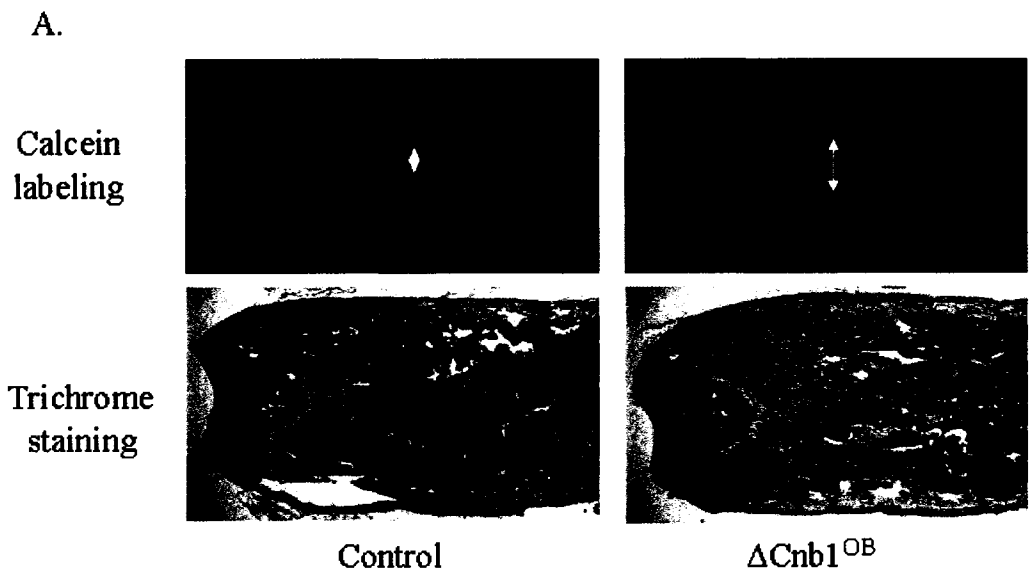
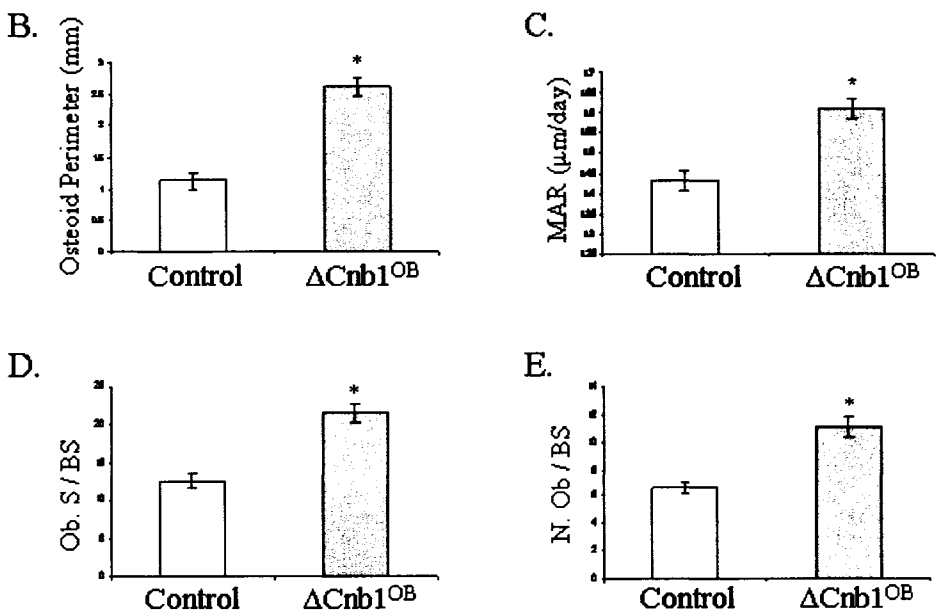



FIGURE 3. Conditional deletion of Cnb1 in osteoblasts increases bone formation *in vivo*. Distal femora from 3-month-old male control and $\Delta Cnb1^{OB}$ mice were harvested; tissues were fixed, embedded in methyl methacrylate, sectioned and examined. **(A, upper panel)** Unstained sections were examined under a florescence microscope to visualize and measure the distance between the 2 calcein layers (white arrow) which reflects the mineral apposition rate (MAR). **(A, lower panel)** Tissues were stained with Trichrome to distinguish the mineralized bone (green/blue), and osteoid that lines bone (red). Representative pictures are shown from 4 control and 6 $\Delta Cnb1^{OB}$ mice. **(B-E)** Quantitative analysis of histomorphometrical indices was performed and shows **(B)** osteoid perimeter (mm), **(C)** MAR (μm/day), **(D)** osteoblast surface/bone surface (Ob.S/BS) and **(E)** number of osteoblasts/bone surface (N.Ob/BS). Values represent the mean ± SE of $\Delta Cnb1^{OB}$ mice (n=6) compared to control (n=4); *, $p \leq 0.05$.



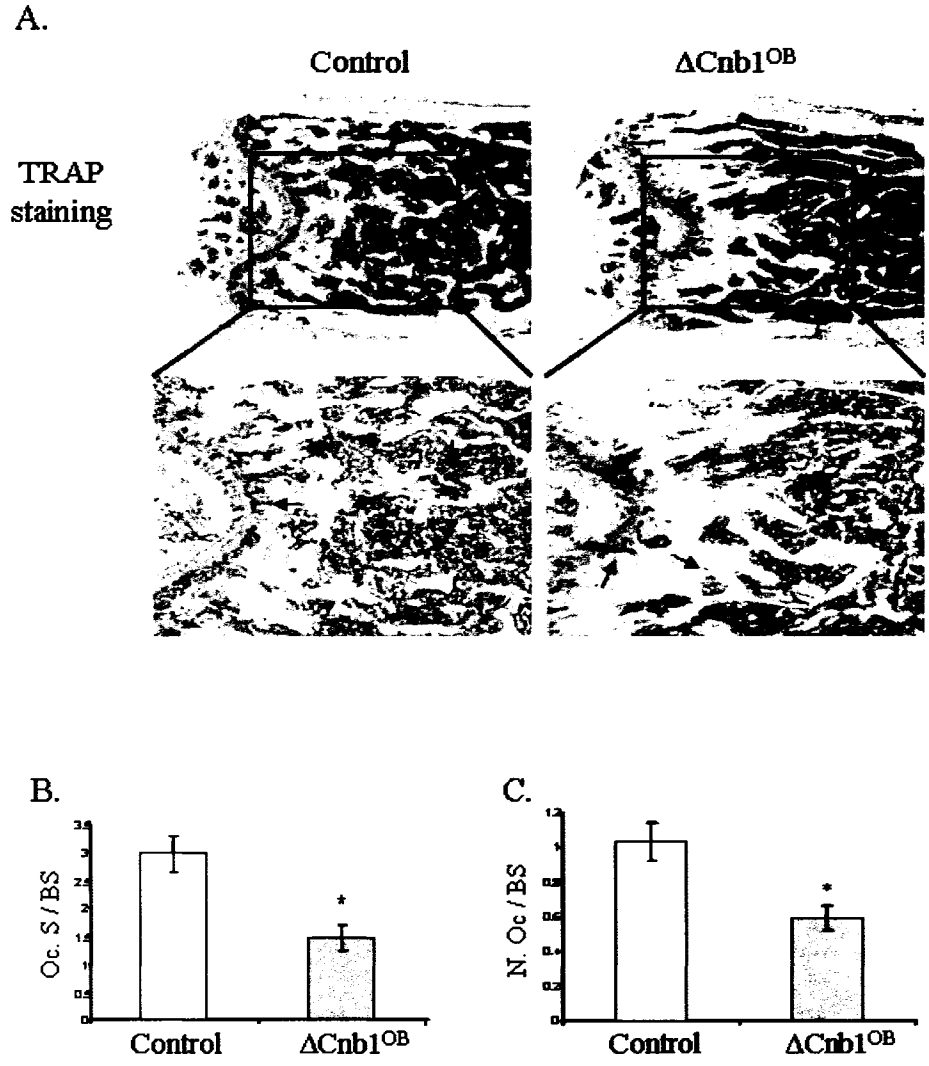



FIGURE 4. Conditional deletion of Cnb1 in osteoblasts decreases bone resorption *in vivo*. (**A**) Femora were harvested from control and $\Delta Cnb1^{OB}$ mice at 3 months old. Tissues were fixed, decalcified with EDTA, embedded in paraffin, sectioned and stained for TRAP (red). Arrows indicate TRAP-positive, multinucleated osteoclasts. Representative pictures are shown from 4 control and 6 $\Delta Cnb1^{OB}$ mice. (**B-C**) Quantitative analysis of histomorphometrical indices was performed and shows (**B**) osteoclast surface/bone surface (Oc.S/BS), and (**C**) number of osteoclasts/bone surface (N.Oc/BS). Values represent the mean ± SE of $\Delta Cnb1^{OB}$ mice (n=6) compared to control (n=4); *, $p \leq 0.05$.

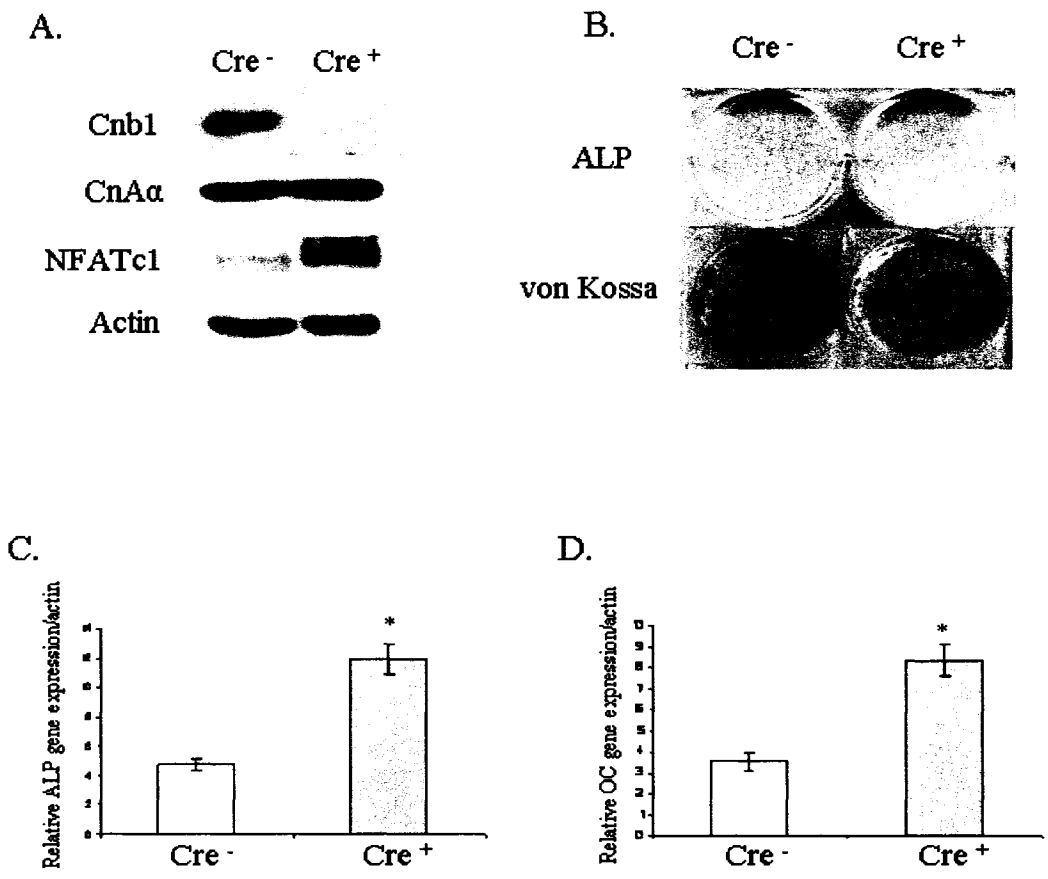
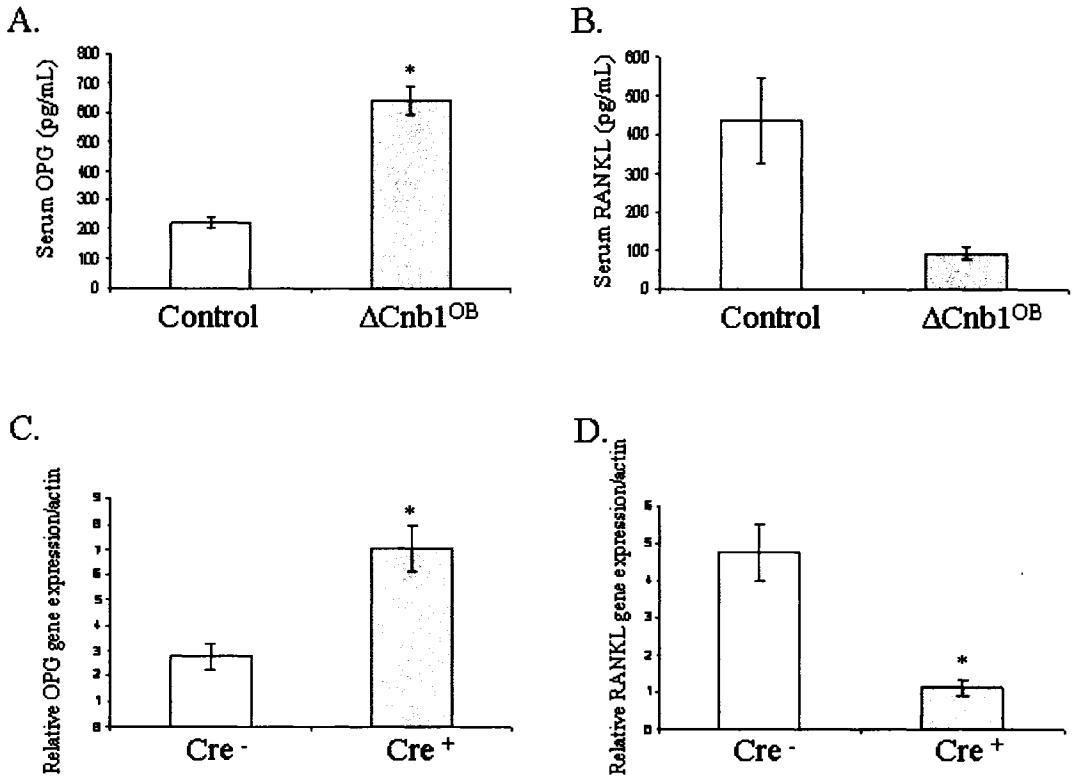
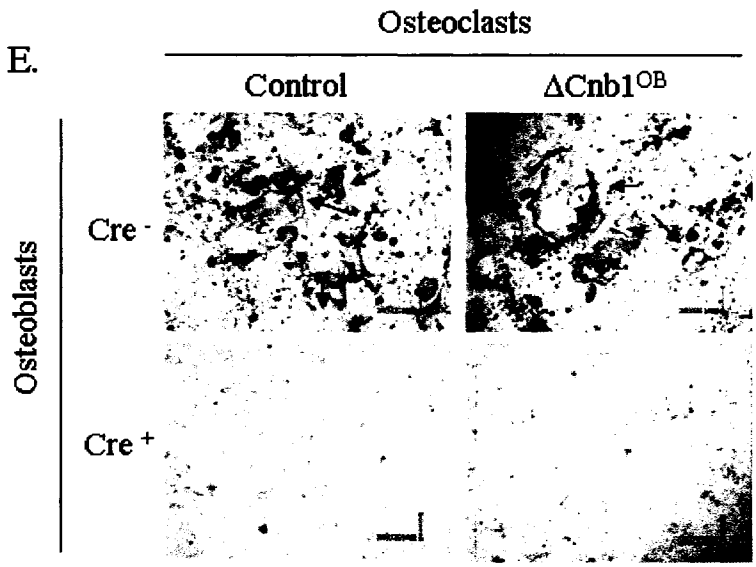



FIGURE 5. The deletion of Cnb1 in osteoblasts increases osteoblast differentiation *in vitro*. Primary calvarial osteoblasts from Cnb1$^{f/f}$ mice were transduced with adenovirus-GFP (Cre$^-$) or adenovirus-Cre (Cre$^+$). (**A**) Proteins were extracted and separated by SDS-PAGE. Immunoblots were developed using antibodies against Cnb1, CnAα, NFATc1 and Actin. (**B**) Cre$^-$ and Cre$^+$ primary calvarial osteoblasts from Cnb1$^{f/f}$ mice were cultured in osteogenic differentiation media (in the presence of ascorbic acid and β-glycerophosphate) for 14 or 28 days and then stained for alkaline phosphatase (ALP; upper panel) and von Kossa (lower panel). (**C-D**) RNA was extracted and Real-time RT-PCR was performed for (**C**) alkaline phosphatase, (**D**) osteocalcin and actin. Values represent the mean ± SE of ALP and osteocalcin mRNA expression relative to actin expression; *, $p \leq 0.05$ compared to control. All studies were repeated 3 times using cells harvested from 3 different animals.

FIGURE 6. Osteoblasts lacking Cnb1 cannot maintain osteoclastogenesis. (**A-B**) Serum was collected from the blood of control and $\Delta Cnb1^{OB}$ 3-month-old mice immediately after euthanasia. Protein levels of (**A**) OPG and (**B**) RANKL in serum were measured using OPG and RANKL serum Enzyme-linked immunoassay. Values were obtained from 4 mice in each control and $\Delta Cnb1^{OB}$ group and represent the mean ± SE; *, $p \leq 0.05$. (**C-D**) Primary calvarial osteoblasts were harvested from $Cnb1^{f/f}$ mice and transduced with adenovirus-GFP ($Cre^-$) or adenovirus-Cre ($Cre^+$). Cells were cultured in osteogenic differentiation media (in the presence of ascorbic acid and β-glycerophosphate) for 7 days. RNA was extracted and Real-time RT-PCR was performed for **(C)** OPG and **(D)** RANKL. Values represent the mean ± SE of OPG and RANKL mRNA expression relative to actin expression; *, $p \leq 0.05$ compared to control. All studies were repeated 3 times, each repeated in triplicate. (**E**) $Cre^-$ and $Cre^+$ primary calvarial osteoblasts from $Cnb1^{f/f}$ mice were co-cultured with bone marrow macrophages from control and $\Delta Cnb1^{OB}$ mice in the presence of $10^{-8}$ M 1,25 vitamin $D_3$ and $10^{-7}$ M Dexamethasone. After 10 days, cells were fixed and stained for TRAP to detect multinucleated osteoclasts (indicated by black arrows). Representative pictures from 2 separate experiments are shown.



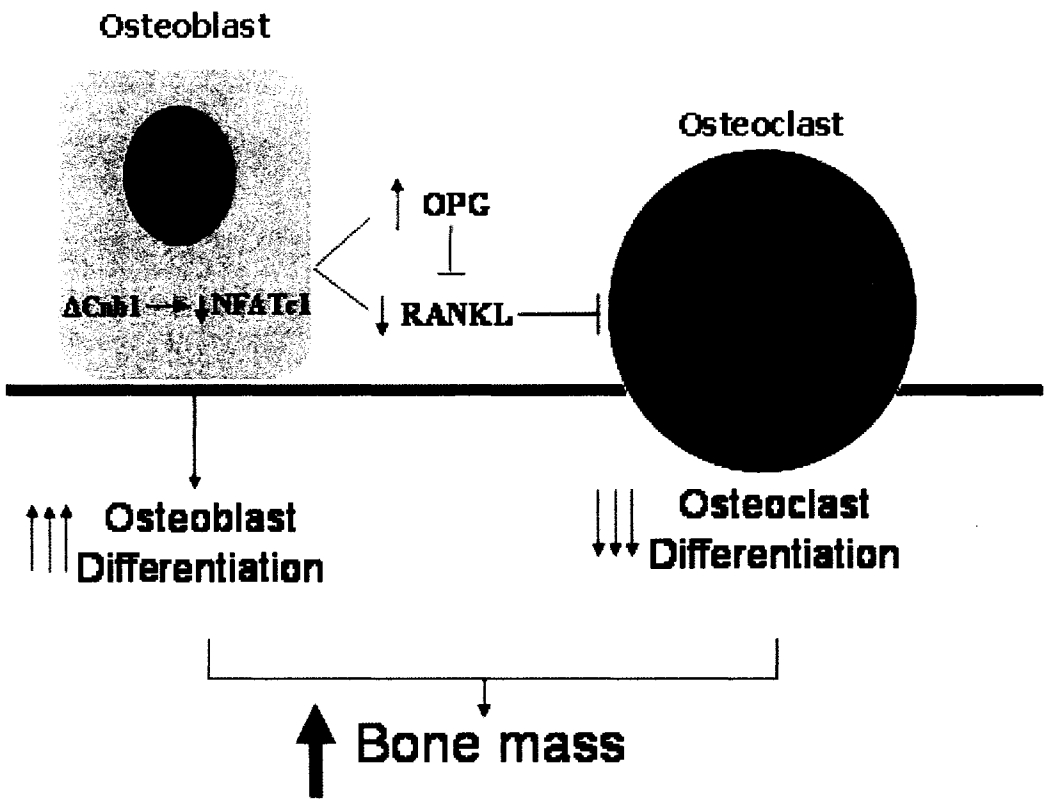



FIGURE 7. A proposed mechanism depicting the role of Cnb1 in regulating bone mass. Cnb1 in osteoblasts is a negative regulator of osteoblast differentiation. The *in vivo* deletion of Cnb1 in osteoblasts directly increases osteoblast differentiation and bone formation. The deletion of Cnb1 also alters the OPG/RANKL axis by increasing the expression of OPG and decreasing that of RANKL, which ultimately leads to a decrease in osteoclastic bone resorption. Together, this leads to an increase in bone mass.

# NFATC1 REPRESSES OSTEOCALCIN GENE EXPRESSION THROUGH THE RECRUITMENT OF HDAC3

by

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## ABSTRACT

We recently reported that pharmacologic inhibition of NFAT by Cyclosporine A and specific inhibition of NFATc1 expression increases osteoblast differentiation in MC3T3-E1 osteoblasts. To determine the role of NFATc1 in the regulation of osteoblast differentiation, we infected retrovirus-expressing constitutively active NFATc1 (ca-NFATc1) and control GFP into MC3T3-E1 osteoblasts. Overexpression of ca-NFATc1 in MC3T3-E1 osteoblasts confirmed that NFATc1 protein level and NFAT transactivation increased compared to control GFP MC3T3-E1 osteoblasts. The overexpression of ca-NFATc1 decreased osteoblast differentiation, demonstrated by ALP activity and calcium deposition. Moreover, ca-NFATc1 also decreased the gene expression of osteocalcin, an osteoblast specific marker of the mineralization stage as well as osteocalcin promoter activity. Here we show that ca-NFATc1 has the ability to inhibit osteocalcin gene expression and promoter activity through TCF/LEF recognition site on the osteocalcin promoter. This repression occurs through the action of histone deacetylase 3 (HDAC3), which interacts with NFATc1 in MC3T3-E1 osteoblasts. Accordingly, ca-NFATc1 recruits HDAC3 on the TCF recognition sequence of the osteocalcin promoter, thus inhibiting its gene expression and osteoblast differentiation. Our results indicate that ca-NFATc1 acts as a transcriptional corepressor interacting with HDAC3 in differentiating osteoblasts.

## INTRODUCTION

Post-translational modifications of nucleosomal histones, such as acetylation, phosphorylation, ubiquitination and methylation, are known to regulate gene expression through the transcriptionally active or inactive chromatin remodeling in eukaryotes [1, 2]. Histone acetylation is a reversible modification and has been shown to stabilize nucleosomes to activate gene expression. Two enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC) are responsible for transferring or removing acetyl groups to lysine residue on histones. Activation of transcription is associated with HAT, which catalyzes the acetylation of histones that contributes to relaxing the chromatin complex, thus allowing transcriptional activators access to the DNA complex to induce target gene expression. In contrast, HDAC removes acetyl groups from histones and promotes chromatin condensation, which leads to transcriptional repression [3, 4]. HDACs are classified into three major groups, Class I (HDAC 1, 2, 3, and 8) which are expressed ubiquitously, and Class Π (HDAC 4, 5, 6, 7, 9 and 10) which are expressed in a tissue-specific manner, such as heart, brain and skeletal muscle [5]. Class Ш HDACs are specifically required by $NAD^+$ for maintaining its deacetylase activity [6]. HDACs are one of the multiprotein complexes, which work as transcriptional repressors. Also, they are recruited to target gene promoters by interacting with specific DNA binding transcription factors [4]. It has been reported that Class I HDACs are ubiquitously expressed in mammalian cells in the nucleus, and thus induce the inhibition of the specific target genes and the entire chromosome [7]. In addition, it has been documented that Class Π HDACs are able to shuttle between nuclear and cytoplasmic compartments in specific cell types, including myoblasts and T cells [8-10]. Some HDACs have been

known to be expressed in osteoblasts, such as HDAC4 and HDAC5 of Class Π in primary calvarial osteoblasts, and HDAC1 and HDAC3 of Class I in MC3T3-E1 osteoblasts [11-13].

Bone is a dynamic tissue that is continuously remodeled by destruction and reformation, thereby maintaining bone mass and calcium homeostasis [14]. Two primary cells, osteoblasts and osteoclasts, are responsible for bone formation and resorption, respectively. Osteoblasts are derived from multipotent mesenchymal stem cells and osteoprogenitors are sequentially differentiated into preosteoblasts, mature, functional osteoblasts and osteocytes [15]. The major functions of ostoblasts are the production of collagenous unmineralized bone matrix and the expression of genes which are necessary for the mineralization of the bone extracellular matrix (ECM). The well known osteoblast-specific molecules are type I collagen and alkaline phosphatase, which are expressed during osteoblast proliferation and matrix maturation, as well as osteocalcin, which is highly expressed at the osteoblast mineralization stage *in vitro* and *in vivo* [16]. In proliferating osteoprogenitor cells, the osteocalcin gene is suppressed and then transcriptionally induced by Runx2 in mature osteoblasts [17]. Thus, osteocalcin expression is induced by the transcription factor CEBP, and hormone $1,25(OH)_2D_3$, and thus maximized during the osteoblast mineralization stage [18]. To understand the molecular mechanism of osteoblast differentiation, several reports have focused on the osteoblast specific osteocalcin gene that is regulated by transcriptional control with chromatin remodeling modification [19, 20].

It has been demonstrated that the transcriptionally active osteocalcin promoter increases the level of acetylated histone 3 and 4 to form the open and relaxed chromatin

conformation in differentiating osteoblasts [21]. In addition, another group showed that HDAC3, a Class I HDAC member associated with Runx2, repressed osteocalcin promoter activity and osteoblast differentiation [13]. In addition, it has been demonstrated that transforming growth factor (TGF-β) repressed osteoblast differentiation by histone deacetylation through the recruitment of HDAC to the osteocalcin promoter [11]. These results suggest that transcription factors recruit HDACs to target promoter sequences to form transcriptional repressor complexes, leading to the induction of histone deacetylation, and thus negatively regulating target gene expression.

The NFAT (Nuclear Factors of Activated T cells) family of transcription factors plays a pivotal role in the immune response by regulating cytokine and cell surface receptors [22]. NFAT proteins have been shown to be regulated by calcineurin, a serine- and threonine- phosphatase through the $Ca^{2+}$/Calmodulin-dependent signaling pathway [23, 24]. In resting cells, the regulatory domain of NFAT is highly phosphorylated on serine and threonine residues and resides in the cytoplasm. Upon activation, calcineurin binds and dephosphorylates NFAT transcription factors, leading to translocation into the nucleus and induction of target gene expression. It has been reported that NFATc1 is an important transcription factor in the regulation of osteoclast differentiation and the inhibition of calcineurin/NFAT signaling by either Cyclosporin A (CsA) or FK506 inhibits osteoclastogenesis [25]. We have previously demonstrated that inhibition of NFAT by low doses of CsA increases osteoblast differentiation *in vitro* and bone mass *in vivo*. In addition, NFATc1 negatively regulates osteoblast differentiation and overexpression of constitutively active NFATc1 decreases osteoblast differentiation. It has been reported that NFAT has the ability to function as a mediator of gene repression.

Ranger et al. demonstrated that NFATc2 plays a role as a repressor of chondrogenesis and NFATc2 deficient mice exhibited an increase in the expression of cartilage markers, collagen type Π and type X [26]. It has also been reported that the direct association between NFATc1 and HDAC1 binds to the cyclin-dependent kinase 4 (cdk4) promoter and decreases osteocalcin gene expression and its promoter activity [27]. It has also been shown that the association of NFAT1 and HDAC1 on intron 4 of *il10* gene silences IL-10 expression in T cells [28]. Therefore, NFAT could function to repress target gene expression in an HDAC-dependent manner.

Here we demonstrate that ectopic expression of ca-NFATc1 inhibited osteoblast differentiation in MC3T3-E1 osteoblasts, and overexpression of ca-NFATc1 reduced both osteocalcin gene expression and promoter activity. Furthermore, we show that ca-NFATc1 forms a repressor complex on the osteocalcin promoter by recruiting HDAC3 and thus inhibiting its gene expression. Our findings indicate that NFATc1 negatively regulates osteocalcin gene expression by recruiting HDAC3, leading to decreased osteoblast differentiation.

## MATERIALS AND METHODS

### Cell Culture and Differentiation

MC3T3-E1 cells, a murine osteoblastic cell line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA) [29]. Cells were maintained in Minimum Essential Medium Eagle, Alpha Modification (α-MEM) (Sigma) containing 10% fetal bovine serum (Atlanta Biologicals), 100 units/ml penicillin G and 100 μg/ml streptomycin (Invitrogen) at 37°C with 5% $CO_2$. Osteoblastic induction was performed

by supplementing the maintenance medium with 8 mM β-glycerophosphate and 250 μM ascorbic acid-2-phosphate [30, 31].

**Retrovirus production and infection.**

We used retroviral expression vectors pMSCV-GFP and pMSCV-ca-NFATc1 [32]. Retroviruses were produced by cotransfecting pMSCV vectors with pVSV-G into BOSC23 cells using Lipofectamine (Invitrogen). Twenty four hours after transfection, the media was replaced, and retroviral supernatant was collected after 48 hours. For infection, $2 \times 10^4$ cells/cm$^2$ MC3T3-E1 cells were plated onto 6-well plates. The culture media was replaced with 500 μl of retroviral supernatant with 8 μg/ml polybrene (Sigma) and plates were incubated for 2 hours at 37 °C with 5% $CO_2$. Retroviral supernatant was then removed and cells were cultured in regular growth medium.

**Alkaline Phosphatase and Calcium Measurement**

Cultured GFP MC3T3-E1 and ca-NFATc1 MC3T3-E1 cells were fixed in 2% paraformaldehyde/PBS for 10 minutes and then incubated at 37 °C with freshly-prepared alkaline phosphatase substrate solution (100mM Tris-Maleate buffer (pH 8.4), 2.8% N,N-dimethyl formamide (v/v), 1 mg/ml Fast Red TR and 0.5 mg/ml naphthol AS-MX phosphate) [29]. Cells were lysed with double distilled $H_2O$, accompanied by three freeze/thaw cycles. Calcium was measured using a calcium detection kit (Arsenazo III; Sigma).

**RNA Extraction and real time RT-PCR**

Total RNA was extracted by the TRIzol method, as recommended by the manufacturer (Invitrogen). One μg of RNA was reverse-transcribed and the equivalent of 10 ng was used for quantitative RT-PCR, as previously described [33]. Expression of actin was used for normalization of gene expression values. The sequences and conditions for the specific primers of *osteocalcin* used in this study were as previously described [33].

**Transient Transfections and Luciferase Reporter Assays**

GFP MC3T3 and ca-NFATc1 MC3T3 osteoblasts were plated at a density of 2 x $10^4$ cells/$cm^2$ in 6-well plates. Twenty-four hours after plating, cells were transfected with 1 μg of luciferase reporter plasmids driven by the TOP flash (Clontech), pOSE6-luciferase plasmid and rat osteocalcin promoter-luciferase plasmid (pOC-285 and pOC-1050) and 0.2 μg CMV-β-galactosidase reporter construct (as a control) using LipofectAMINE (Invitrogen), according to the manufacturer's instructions. Fourty-eight hours post-transfection, cells were lysed and reporter activity was measured using a luciferase (Promega) or β-galactosidase (Clontech) assay system and a luminometer. Transfection efficiency was evaluated by transfecting CMV-GFP. An efficiency of 70-80% was achieved.

**Nuclear Protein Extraction and Western Blot Analysis.**

GFP MC3T3-E1 and ca-NFATc1 MC3T3-E1 cells were cultured and washed with chilled PBS. The nuclei were isolated in NP-40 lysis buffer (10 mM Tris, 10 mM

NaCl, 3 mM $MgCl_2$, 0.5 % NP-40 and 0.56 M sucrose). The extracts were further treated with hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$ and 10 mM KCl) and finally isolated with extraction buffer (20 mM HEPES, 20 % glycerol, 600 mM KCl, 1.5 mM $MgCl_2$ and 0.2 mM EDTA) followed by 30 minutes incubation in ice and centrifuged at 14000Xg for 30 minutes at 4°C. The protein concentration was measured by the Bio-Rad DC protein assay. Nuclear protein extracts (20 μg/lane) were subjected to SDS-PAGE, and immunoblotting analysis was performed using antibodies directed against NFATc1 and SP-1 (Santa Cruz Biotechnology) [29, 33].

**HDAC activity assay**

GFP MC3T3-E1 and ca-NFATc1 MC3T3-E1 cells were cultured as indicated. Nuclear fractionation was performed to reduce the loss of activity of HDAC as recommended (Abcam, Cambridge, MA). Cells were treated with buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.05 % NP-40) and incubated on ice for 10 minutes followed by centrifugation for 10 minutes at 3000 rpm at 4°C. The pellets were resuspended with buffer B (5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 26 % glycerol) and 300 mM NaCl and homogenized with 20 strokes in a Dounce homogenizer. After incubating for 30 minutes on ice, extracts were centrifuged at 24000 Xg for 20 minutes at 4 °C. HDAC activity was measured using an HDAC activity assay kit, as recommended by the manufacturer (Abcam, Cambridge, MA).

**Immunoprecipitation**

GFP MC3T3-E1 and ca-NFATc1 MC3T3-E1 cells were harvested and lysed with the HDAC activity assay buffer as described above. Equal amounts of lysates were precleared with protein G-agarose beads (Upstate Biotechnology) for 1 hour and precipitated overnight with 4 μg of NFATc1 and normal mouse IgG antibodies as a control (Santa Cruz Biotechnology), followed by the addition of protein G-agarose beads. Immunoprecipitated proteins were washed 4 times with lysis buffer and eluted with protein sample buffer and boiling. Immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotting analysis using antibodies directed against NFATc1 and HDAC3 (Santa Cruz Biotechnology).

**CHIP assay**

GFP MC3T3-E1 and ca-NFATc1 MC3T3-E1 cells were grown as indicated. To cross-link DNA-protein complex, cells were washed with PBS and treated with 1% formaldehyde at room temperature for 10 minutes. Cells were then collected into 100 mM Tris-HCl (pH 9.4), 10 mM DTT and incubated on ice 5 minutes and centrifuged at 2000 Xg for 5 minutes. Cross-linked cells were resuspended in Tris-EDTA buffer and sequentially sonicated (Fisher Sonic dismembrator, Model 300). The soluble chromatin was diluted in RIPA buffer (0.1 % sodium dodecyl sulfate, 1 % Triton X-100, 0.1 % sodium deoxycholate, 140 mM NaCl) and immunocleared with 2 μg of salmon sperm DNA/Protein A agarose beads (Upstate Biotechnology) for 1 hour at 4°C. Immunoprecipitation was performed with appropriate antibodies for NFATc1, HDAC3 and Acetyl-Histone 4 or normal rabbit or mouse IgG (Santa Cruz Biotechnology)

overnight at 4°C, followed by adding salmon sperm DNA/Protein A agarose for 1 hour. Immunoprecipitants were sequentially washed with the following buffers : once with low salt buffer (0.1 % SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Hcl (pH 8.1) and 150 mM NaCl), once with high salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1 % NP-40, 1 % Na-Deoxycholate, 1 mM EDTA and 10 mM Tris-HCl (pH 8.1)) and twice with Tris-EDTA buffer. Cross-linking was reversed by heating at 65 °C for 24 hours with 0.2 M NaCl. DNA was precipitated with Phenol/Choloroform and the DNA template was amplified of the specific binding site of TCF/LEF in the osteocalcin promoter: 5'-gcccctcagggaagaggtctt-3' (-236 bp to -216 bp) and 5'-ctgcaccctccagcgtccag-3' (-20 bp to -1 bp from the transcription start site). The 210 bp product was separated by agarose gel electrophoresis.

**Statistical analysis**

All statistical analyses were performed using the Microsoft Excel data analysis program for Student's t-test analysis. Experiments were repeated at least three times unless otherwise stated. Values are expressed as the mean ±SE.

## RESULTS

We have previously observed that pharmacological inhibition of NFAT by low concentrations of Cyclosporin A increases osteoblast differentiation *in vitro* and bone mass *in vivo*. Also, specific inhibition of NFATc1 using siRNA has been shown to increase the expression of Fra-2 which plays a crucial role in osteoblast differentiation.

To assess the potential contribution of the NFATc1 to osteoblast differentiation, we overexpressed constitutively active NFATc1 (ca-NFATc1) in MC3T3-E1 osteoblasts. Ca-NFATc1 was already known to localize in the nucleus, and to have a high affinity DNA binding activity, as well as being able to activate endogenous gene expression [34]. MC3T3-E1 osteoblasts were infected with either retrovirus encoding GFP (MSCV-GFP) or ca-NFATc1 (MSCV-ca-NFATc1), called GFP MC3T3-E1 and ca-NFATc1 MC3T3-E1 cells. To confirm the efficacy of retroviral infection of ca-NFATc1, protein levels of NFATc1 were examined. As shown in Fig 1A, enforced expression of ca-NFATc1 in MC3T3-E1 osteoblasts increased its protein level and showed as active and dephosphorylated form, compared to GFP expressing cells. In order to confirm the effect of overexpression of ca-NFATc1, we transfected GFP and ca-NFATc1 MC3T3-E1 cells with pNFAT-TA-luciferase construct for 24 hours. Cells were then lysed and luciferase activity measured. Here we show that enhanced expression of ca-NFATc1 significantly increases the NFAT transactivation (Fig. 1B). To determine the effects of ca-NFATc1 on osteoblast differentiation, GFP and ca-NFATc1 MC3T3-E1 cells were induced to differentiate for 14 and 21 days in the presence of ascorbic acid and β-glycerophosphate. At the end of the study, cells were fixed and stained for ALP activity and measured intracellular calcium deposition. As shown in Figure 1C and 1D, overexpression of ca-NFATc1 in MC3T3-E1 inhibited differentiation into mature osteoblasts, as demonstrated by ALP activity and calcium deposition, compared to control GFP expressing MC3T3-E1 osteoblasts. Taken together, these data indicate that ectopic expression of ca-NFATc1 inhibits osteoblast differentiation and function.

In order to further characterize the mechanism by which ca-NFATc1 decreased osteoblast differentiation, we analyzed the expression of the osteoblast-specific marker gene, osteocalcin. It has been shown that osteocalcin is highly expressed during the matrix maturation and mineralization stages in osteoblasts. Thus, MC3T3-E1, GFP-MC3T3-E1 and ca-NFATc1 MC3T3-E1 cells were induced to differentiate for 11days in the presence of ascorbic acid and β-glycerophosphate. Cells were harvested, RNA extracted and real time RT-PCR was performed using specific primers for osteocalcin. Differentiated control osteoblast cells, both parent MC3T3-E1 and GFP expressing MC3T3-E1 cells, induce the expression of osteocalcin, as expected. However, the osteocalcin gene expression is significantly decreased in ca-NFATc1 overexpressing MC3T3-E1 cells (Fig. 2A). These data suggest that constitutively active NFATc1 signaling may prevent the differentiation of MC3T3-E1 osteoblasts by inhibiting the expression of the critical osteoblast differentiation marker, osteocalcin.

The expression of the osteocalcin gene is known to be controlled at the transcriptional level by the coordinated activation of basal tissue specific or enhancer molecules that regulate the osteocalcin promoter [35]. Therefore, we examined whether or not ca-NFATc1 plays a role in regulating osteocalcin promoter activity. We transfected GFP and ca-NFATc1 MC3T3-E1 cells with pOC-285-luciferase and pOC-1050-luciferase constructs for 24 hours. These constructs were a generous gift from Dr. Amjad Javed, University of Alabama at Birmingham. The pOC-285-luciferase plasmid was constructed using the initial 285 base pair (-285 to +1) of the osteocalcin promoter region, and the pOC-1050-luciferase plasmid was constructed using the 1050 base pair (full length) (Fig. 3A). Cells were then lysed and luciferase activity measured. Enforced

expression of ca-NFATc1 significantly decreased osteocalcin promoter activity of the full length osteocalcin promoter fragments (Fig. 2B). Moreover, ca-NFATc1 also significantly decreased the proximal 285 base pair fragment of osteocalcin promoter activity (Fig. 2C). These results indicate that ca-NFATc1 could repress the proximal region of osteocalcin promoter (-285 to +1 bp). Taken together, these data indicate that ca-NFATc1 represses the osteocalcin gene expression and promoter activity in osteoblasts, thereby defining a novel molecular mechanism, the negative regulation of osteocalcin activity during osteoblast differentiation by ca-NFATc1.

Since cells expressing ca-NFATc1 appeared to decrease osteoblast differentiation by repressing osteocalcin promoter activity, we were interested in knowing whether or not ca-NFATc1 regulates the binding elements of specific transcription factors on the proximal region of osteocalcin promoter. It has been reported that a Runx2 binding site and a consensus TCF/LEF binding site are adjacent on the proximal osteocalcin promoter region (Fig. 3A) [36]. These binding sites were reported to be conserved in mouse, rat and human osteocalcin promoters [36]. To determine whether Runx2 or TCF/LEF binding sites are affected by ca-NFATc1, we examined the effects of ca-NFATc1 on their transactivation. We transfected GFP and ca-NFATc1 MC3T3-E1 cells with a pOSE6-luciferase construct for Runx2 transactivation and a TOP flash construct for TCF transactivation. Cells were then lysed and luciferase activity measured. Ectopic expression of ca-NFATc1 does not affect Runx2 transactivation, whereas TOP flash activity was significantly decreased in cells expressing ca-NFATc1 compared to GFP expressing MC3T3-E1 osteoblasts (Fig. 3B and 3C). Therefore, these data indicate that

ca-NFATc1 might regulate the osteocalcin gene expression and promoter activity mainly through the activity of TCF transcription factors.

TCF/LEF transcriptional activity has been reported to be modulated by transcriptional regulation, to form complexes with co-repressors or co-activators on the target gene promoter. Without Wnt signaling, TCF/LEF forms complexes with co-repressors, such as TLE (Gro/TLE family of corepressor) and HDACs, and inhibits target gene expression, such as c-myc [37]. Differentiated primary bone marrow cells and ROS17/2.8 cells have been shown to have decreased total HDAC activity [12]. In addition, HDAC inhibitors, especially Trichostatin A (TSA), has been reported to promote osteoblast differentiation in MC3T3-E1 cells [38]. Therefore, we were interested in determining whether or not HDAC could be involved in ca-NFAT mediated osteocalcin repression mechanism as a transcriptional repressor complex. To determine the effects of ca-NFATc1 on HDAC activity in osteoblast differentiation, the total HDAC activity was measured in ca-NFATc1 and GFP expressing MC3T3-E1 cells. Consistent with a previous report, differentiated control GFP expressing MC3T3-E1 osteoblasts showed significantly decreased HDAC activity, whereas differentiated ca-NFATc1 expressing MC3T3-E1 osteoblasts retained total HDAC activity (Fig. 4A). These results suggest that HDAC could be associated directly or indirectly with the mechanism by which ca-NFATc1 decreased osteoblast differentiation.

It has also been reported that HDAC3 is expressed in osteoblasts and functions to inhibit osteocalcin gene expression and promoter activity through interacting with a Runx2 transcription factor [11, 13]. The direct association between NFATc1 and HDAC1 has been shown to bind to a cdk4 promoter and decrease cdk4 gene expression and its

promoter activity [27]. To explore the possibility that ca-NFATc1 recruits HDAC3 to the osteocalcin promoter to repress gene expression, we first examined the direct interaction between NFATc1 and HDAC3, in ca-NFATc1 and GFP expressing MC3T3-E1 cells. GFP-MC3T3-E1 and ca-NFATc1 MC3T3-E1 cells were induced to differentiate for 11 days in the presence of ascorbic acid and β-glycerophosphate. Cells were harvested, nuclear proteins extracted and immunoprecipitation performed. As shown in Figure 4B, HDAC3 directly interacts with NFATc1 in GFP MC3T3-E1 cells and ca-NFATc1 overexpressing MC3T3-E1 osteoblasts showed an increase in their interaction. These results indicate that ca-NFATc1 interacts with HDAC3 and sustains its activity in differentiated osteoblasts. Therefore, this interaction could act as transcriptional repressor on the osteocalcin promoter.

We used the chromatin immunoprecipitation assay (CHIP) to assess whether the interaction between ca-NFATc1 and HDAC3 could repress osteocalcin transcription at the TCF/LEF element of the osteocalcin promoter to inhibit osteoblast differentaition. GFP-MC3T3-E1 and ca-NFATc1 MC3T3-E1 cells were induced to differentiate for 11 days in the presence of ascorbic acid and β-glycerophosphate. Cells were harvested, fixed and nuclear fraction extracted by sonication. DNA-protein complexes were immunoprecipitated using the specific antibodies for NFATc1, HDAC3 and acetyl-Histone 4, and then RT-PCR was performed using specific primer sets to show specific protein interaction in the TCF/LEF sequence on osteocalcin promoter. Control GFP MC3T3-E1 cells showed an increased acetylation of histone 4 on the TCF/LEF binding site of the osteocalcin promoter, whereas it was decreased in ca-NFATc1 overexpression cells. The decreased histone deacetylation at the TCF/LEF element of the osteocalcin

promoter in ca-NFATc1 cells indicated that HDAC medicated repression. Surprisingly, HDAC3 recruitment at the TCF/LEF element of the osteocalcin promoter was decreased in GFP MC3T3-E1 cells whereas their binding was sustained in ca-NFATc1 MC3T3-E1 osteoblasts. In addition, HDAC3 is specific for this complex on the TCF/LEF element in the osteocalcin promoter, since HDAC1 was not detected by RT-PCR (data not shown). As we expected, NFATc1 was also involved in the osteocalcin repressor complexes with HDAC3. We previously reported that expression of NFATc1 gradually decreased during osteoblast differentiation, therefore, differentiated control GFP MC3T3-E1 cells showed a decrease binding of NFATc1 whereas ca-NFATc1 overexpressiong cells maintain its binding. Ultimately, the function of NFATc1 in osteoblasts is to negatively regulate osteoblast differentiation through forming the transcriptional repressor complexes with HDAC3 on the osteocalcin promoter.

## DISCUSSION

We have previously observed that pharmacological inhibition of calcineurin and NFAT by low concentrations of Cyclosporin A increased osteoblast differentiation *in vitro* and bone mass *in vivo* in mice. Also, overexpression of constitutively active NFATc1 was shown to inhibit osteoblast differentiation, as well as the expression of fra-2 which plays a crucial role in osteoblast differentiation. Consistent with this previous data, we observed a decrease of the osteoblast differentiation in ca-NFATc1 overexpressed MC3T3-E1 osteoblasts, demonstrated by decreased ALP activity and calcium deposition (Fig. 1C and 1D). The mechanism by which NFATc1 regulates osteoblast differentiation has not been elucidated. NFAT is a well characterized transcription factor that induces

transcription genes of the immune system, such as IL-2, IL-3 and CD-40L [22]. However, it has been shown by others that NFAT also functions as a gene repressor. For instance, NFAT repressed the transcription of cdk4 by inhibiting promoter activity [27]. Therefore, the negative regulation of ca-NFATc1 in MC3T3-E1 osteoblasts may be due to repression of target gene expression of osteoblast development molecules.

Osteocalcin plays a crucial role in osteoblast development and is well established as a bone-specific marker. Its synthesis, secretion and deposition occur at the late stages of the maturation and mineralization of osteoblasts [39]. Due to its tissue specific expression, many studies have been performed on the regulation of osteocalcin expression in bone cells. Since osteocalcin is expressed in differentiated osteoblasts, not during the proliferating stage, it suggests that osteocalcin gene expression may be controlled or repressed during osteoblast differentiation at the transcriptional level. Here we showed that ca-NFATc1 decreased osteoblast differentiation, significantly inhibiting osteocalcin gene expression (Fig. 2A).

The osteocalcin promoter consists of two regions, a proximal promoter region (-200bp to -70bp) and a distal promoter region (-600bp to -400bp) [40, 41]. Functional and physical interactions between transcription factors and the two regions of the osteocalcin promoter could be responsible for the regulation of osteocalcin expression in osteoblasts. Many regulatory elements have been identified in the distal promoter region of the osteocalcin promoter, including OSE1, OSE2 and AP-1/VDRE (vitamin D response element) as well as in the proximal promoter region, including OSE, AP-1 and TCF/LEF [36, 42, 43]. To determine whether or not ca-NFATc1 plays a role in the regulation of the osteocalcin gene transcription, we analyzed the effects of ca-NFATc1 on the activity of

the full length (1050 bp) osteocalcin promoter luciferase reporter construct in MC3T3-E1 osteoblasts. Enhanced expression of ca-NFATc1 decreased full length osteocalcin promoter activity, inhibiting the promoter activity of the proximal region (285 bp) (Fig. 2B and C). These results indicate that ca-NFATc1 significantly inhibited osteocalcin promoter activity in the proximal region which is composed of OSE, AP-1 and TCF/LEF binding sites.

We previously reported that inhibition of NFAT by both CsA treatment and NFATc1 siRNA increased Fra-2 gene expression and protein level, and ectopic expression of ca-NFATc1 decreased them. It has been shown that the expression of AP-1 members changes during osteoblast development and that Fra-2 is the major AP-1 member found in differentiated and mineralized osteoblasts [44]. Therefore, it is possible that decreased Fra-2 expression by ca-NFATc1 could indirectly inhibit osteocalcin gene expression in the AP-1 binding element on the proximal osteocalcin promoter. However, the inhibition of NFAT by CsA treatment did not alter Runx2 gene expression, protein level or transactivation (data not shown). The lack of change in Runx2 transactivation described here is therefore consistent with this observation. These data indicate that the effects of ca-NFATc1 on osteoblast differentiation could be Runx2-independent.

TCF/LEF are nuclear effectors of the canonical Wnt signaling pathway. Under the activation of Wnt, β-catenin is dephosphorylated, leading to the interaction with TCF/LEF to convert them into transcriptional activators [37, 45]. Rachel et al has reported that the functional TCF/LEF binding element is located near the Runx2 binding site in the proximal osteocalcin promoter region [36]. Also, this group has reported that the Wnt signaling activated LEF1, leads to the repression of the Runx2-dependent

osteocalcin promoter activity. It is becoming increasing evident that the repression mechanism of TCF/LEF factors could be conserved. We demonstrated that the enhanced expression of ca-NFATc1 decreased TCF transactivation in MC3T3-E1 osteoblasts (Fig. 3B). It is plausible that NFATc1 may repress osteocalcin gene expression not through Runx2 but through the TCF/LEF binding element on proximal osteocalcin promoter.

Vietor et al has reported that overexpression of TIS7 inhibited osteopontin (OPN) gene expression by forming a transcriptional repressor complex with β-catenin/TCF-4 and HDAC on osteopontin (OPN) promoter [46]. There are some reports that HDACs function as a negative regulator of osteoblast differentiation. Kang et al has reported that HDAC4 and HDAC5 acted as corepressors in TGF-β/Smad3 mediated repression of Runx2 function during osteoblast differentiation and mineralization [11]. Also, the specific inhibition of HDAC3 has been shown to accelerate MC3T3-E1 osteoblast differentiation [13]. It also has been reported that differentiated osteoblasts showed decreased total HDAC activity [12]. Consistent with these reports, we demonstrated that differentiated control GFP MC3T3-E1 osteoblasts had significantly decreased total HDAC activity whereas differentiated ca-NFATc1 expressing MC3T3-E1 osteoblasts retained its activity. It is therefore likely that HDAC could be involved in ca-NFATc1 mediated osteocalcin gene repression in MC3T3-E1 osteoblasts.

We further demonstrated that NFATc1 binds to HDAC3, thus overexpression of ca-NFATc1 increased their binding in differentiated MC3T3-E1 osteoblasts (Fig 4A). In addition, ca-NFATc1 recruited HDAC3 to the TCF/LEF binding element on the osteocalcin promoter in differentiated ca-NFATc1 overexpressing osteoblasts (Fig. 5). These results demonstrated that ca-NFATc1 interacted with HDAC3 to form the

transcriptional repressor complex in TCF/LEF binding element on osteocalcin promoter, thus inhibiting osteocalcin gene expression and finally decreasing osteoblast differentaition. Hence, osteoblast specific inhibition of NFATc1 could be the potential mechanism whereby osteoblast differentiation is increased, leading to an increase in bone mass and bone formation.

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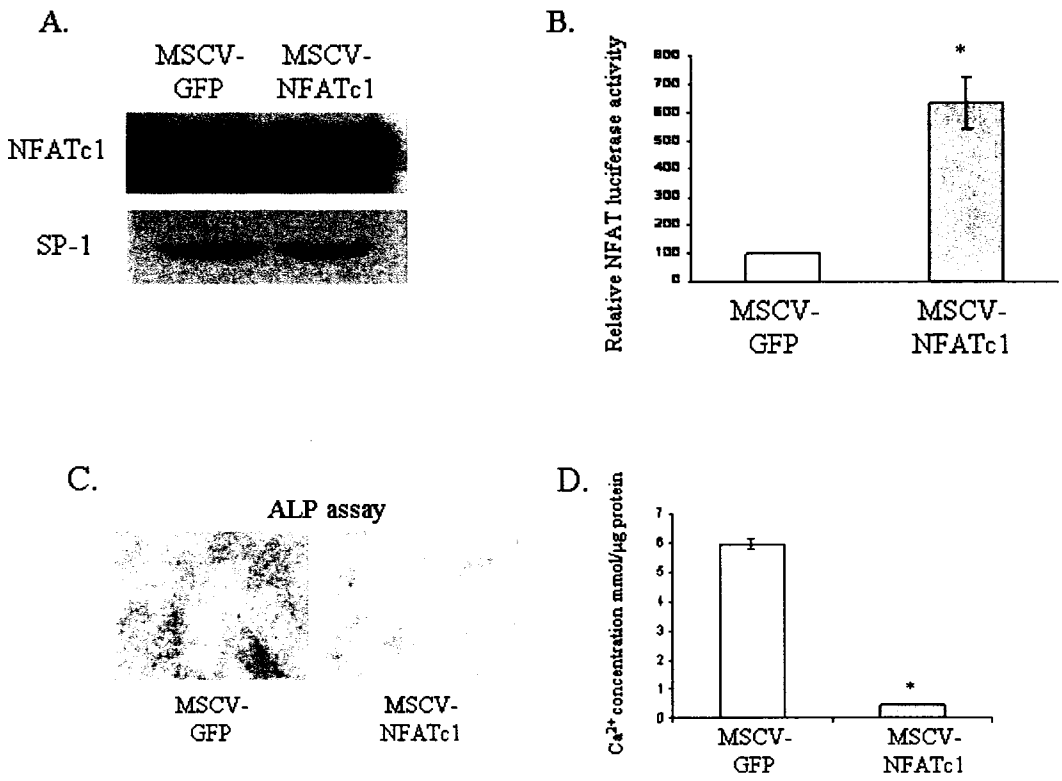


Figure 1. Overespression of ca-NFATc1 inhibits osteoblast differentiation. (A) GFP-MC3T3-E1 and caNFATc1-MC3T3-E1 cells were cultured for 4 days and nuclear proteins were extracted. Nuclear protein extracts were separated by SDS-PAGE and immunoblots were developed using antibodies directed against total NFATc1 and SP-1. Photographs are representative of three separate experiments. (B) GFP-MC3T3-E1 and caNFATc1-MC3T3-E1 cells were cultured for 24 hours and then transfected with pTA-NFAT luciferase construct. Forty eight hours after transfection, cells were harvested and luciferase activity was measured. Data are expressed relative to an internal control (CMV-β-galactosidase) and values represents the mean ±SE of 3 separate experiments each performed in triplicated; *, $p<0.001$. GFP-MC3T3-E1 and caNFATc1-MC3T3-E1 cells were cultured for 14 days (C) and 21 days (D) in the presence of β-glycerophosphate and ascorbic acid-2-phosphate to induce differentiation. Cells were fixed and alkaline phosphatase activity staining was performed (C). Photographs are representative of three separate experiments. Cells were lysed and calcium content was measured (D). Values were obtained from three separate experiments, each performed in triplicate and represent the mean ±SE of calcium content; *, $p< 0.001$, compared to control.

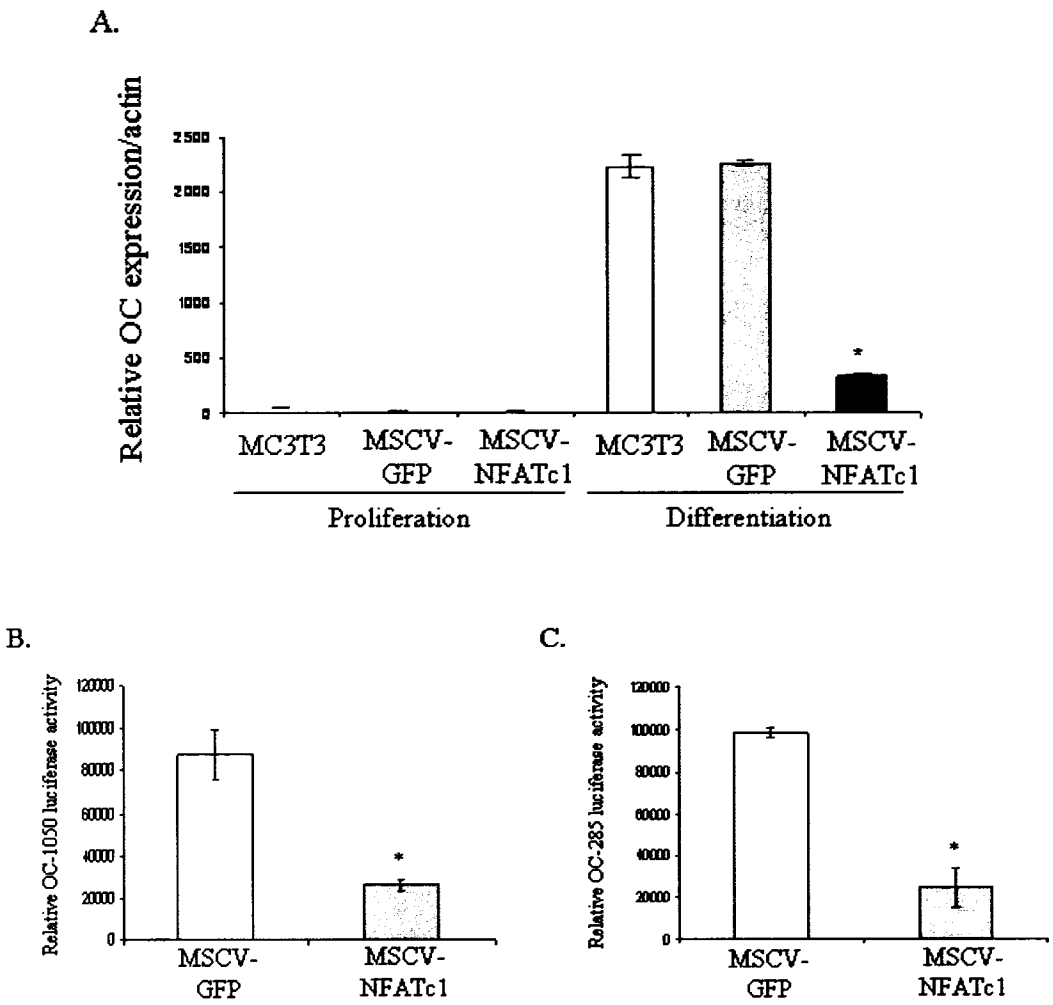


Figure 2. Overexpression of ca-NFATc1 decreased osteocalcin gene expression and promoter activity. (A) MC3T3-E1, GFP-MC3T3-E1 and caNFATc1-MC3T3-E1 cells were cultured for 0 and 11 days in the presence of β-glycerophosphate and ascorbic acid-2-phosphate. Total RNA was extracted and quantitative RT-PCR was performed using a specific primer for osteocalcin. The values were obtained from three separate experiments and represent the mean ±SE of osteocalcin mRNA expression relative to actin expression; *, $p<0.01$ (B) GFP-MC3T3-E1 and caNFATc1-MC3T3-E1 cells were cultured for 24 hours and then transfected with rat OC-luciferase constructs, including pOC-285 and pOC-1050. Forty eight hours after transfection, cells were harvested and luciferase activity measured. Data are expressed relative to an internal control (CMV-β-galactosidase) and values represents the mean ±SE of 3 separate experiments each performed in triplicate; *, $p<0.01$.

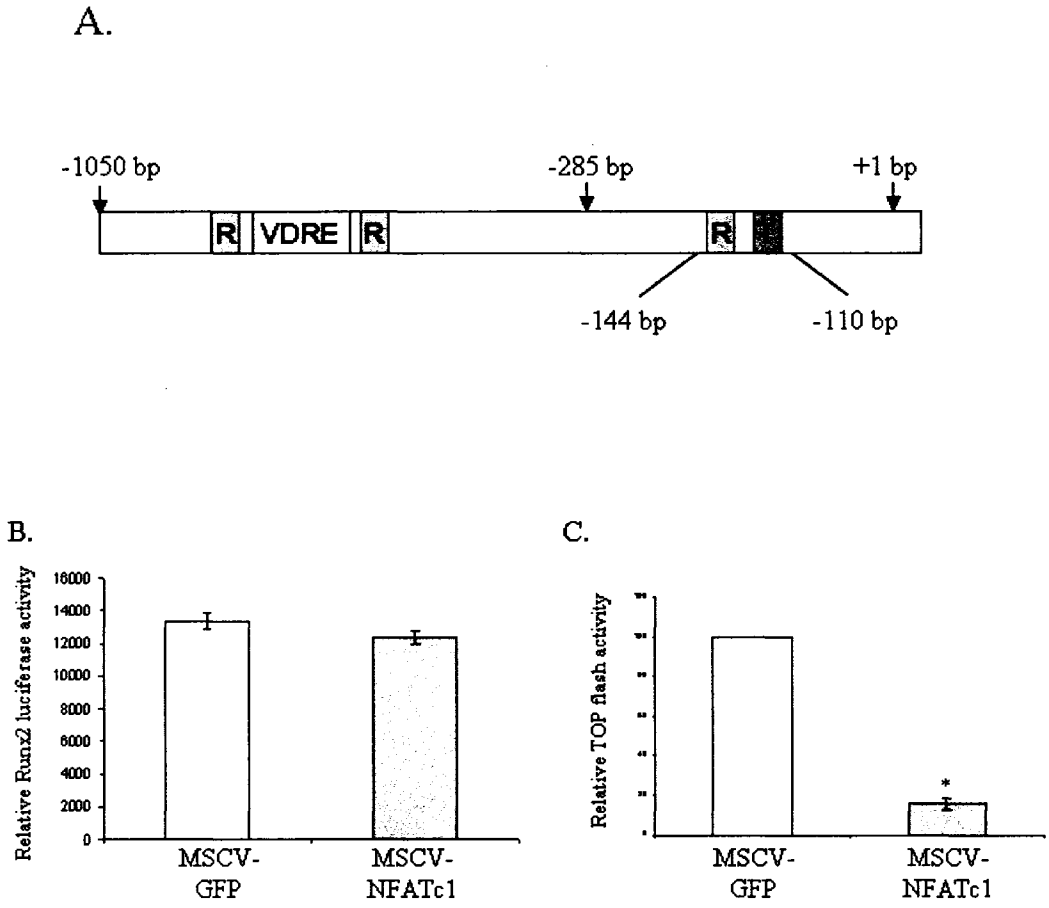


Figure 3. ca-NFATc1 decreased TOP flash activity. (A). The representative positions of the distal promoter region (-600bp to -400bp) of osteocalcin consist of vitamin D response element (VDRE) and two Runx2 (R) binding elements. Also, the proximal region of osteocalcin promoter has Runx2 (R) and TCF/LEF (T) binding elements. GFP MC3T3-E1 and caNFATc1 MC3T3-E1 cells were cultured for 24 hours and then transiently transfected with Runx2 luciferase contruct, pOSE6 (B) and TOP flash-luciferase construct (C). Forty eight hours after transfection, cells were harvested and luciferase activity measured. Data are expressed relative to an internal control (CMV-β-galactosidase) and values represents the mean ±SE of 3 separate experiments each performed in triplicate; *, $p<0.02$

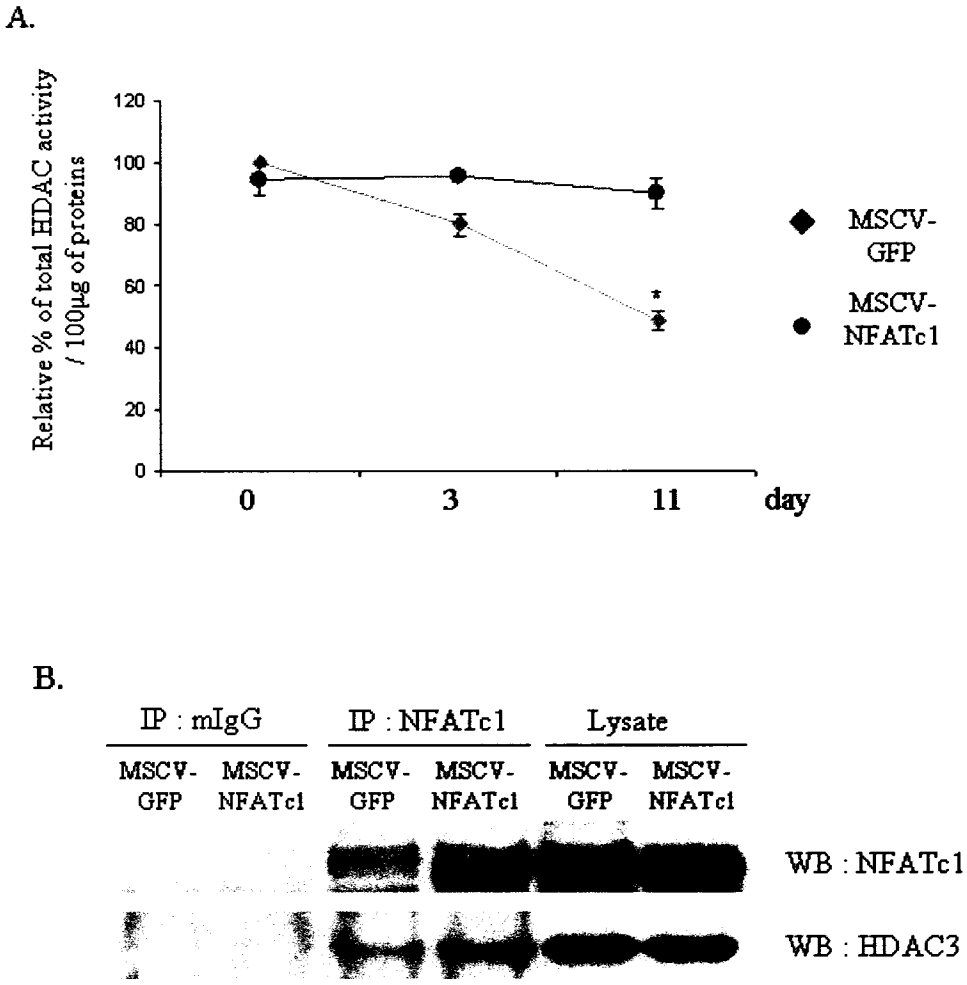


Figure 4. ca-NFATc1 sustains total HDAC activity and interacts with HDAC3. (A) GFP MC3T3-E1 and caNFATc1 MC3T3-E1 cells were cultured for 11 days in the presence of β-glycerophosphate and ascorbic acid-2-phosphate and nuclear proteins were extracted. Total histone deacetylase activity was assayed. Values were obtained from three separate experiments, each performed in triplicate and represent the mean ±SE of histone deacetylase activity per 100 μg concentration of protein; *, $p<0.05$ compared to control. (B) Protein lysates were collected from GFP MC3T3-E1 and caNFATc1 MC3T3-E1 cells cultured for 11 days in the presence of β-glycerophosphate and ascorbic acid-2-phosphate and co-immunoprecipitation was performed using antibodies control mouse IgG, and NFATc1. Immunoprecipitated proteins were detected by western blotting using antibodies directed against NFATc1 and HDAC3. Lysates (20 μg) were used as a control. Photographs are representative of three separate experiments.

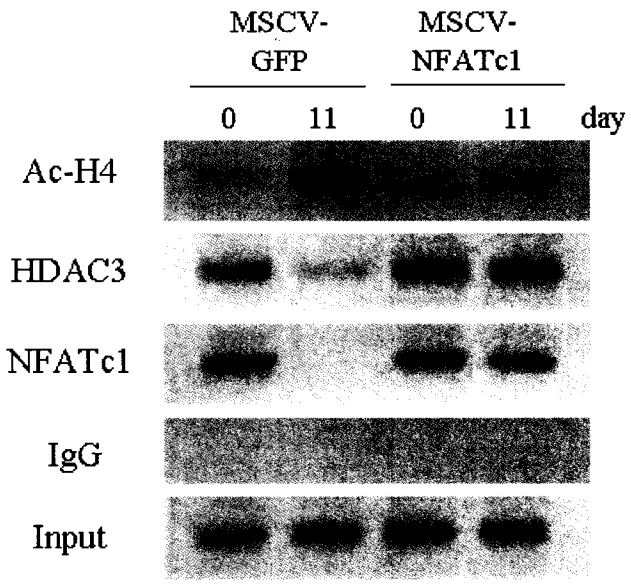


Figure 5. ca-NFATc1 forms a transcriptional repressor complex through the interaction with HDAC3 on the TCF binding element of the osteocalcin promoter. GFP MC3T3-E1 and caNFATc1 MC3T3-E1 cells were cultured for 11 days in the presence of β-glycerophosphate and ascorbic acid-2-phosphate. Then, cells were cross-linked with formaldehyde and the soluble chromatin fragments were immunoprecipitated with antibodies against acetylated histone 4, NFATc1, HDAC3 and normal mouse IgG as a control. The representative figures were generated by PCR using primers that span 210 base pair sequences that include the TCF/LEF consensus sequence. The pictures are representative of 3 separate experiments.

## SUMMARY AND DISCUSSION

The major objective my research is to determine a novel therapeutic approach for building and retaining bone mass in humans. The specific work that was described in this thesis has unequivocally determined the role of the Cn/NFAT signaling pathway in regulating bone mass. Osteoporosis is the major metabolic bone disease characterized by low bone mass and skeletal fragility. Osteoblasts play a central role in bone formation. Therefore a better understanding of osteoblast differentiation and the manipulation of this process could potentially be used to develop anabolic therapeutic targets to combat osteoporosis. Bone loss after organ transplantation is a serious complication in humans that occurs secondary to treatment with immunosuppressive drugs such as CsA resulting in osteopenia or osteoporosis [32, 34, 129]. Similarly, administration of CsA to laboratory animals produces osteopenia that is known to be associated with either high turnover bone loss or low turnover bone loss [39, 40]. In contrast to the notion that the Cn/NFAT signaling pathway positively regulates bone formation, here, we discovered that the pharmacological inhibition by low dose of CsA increased osteoblast differentiation *in vitro* and bone mass *in vivo*. In addition, our results demonstrated that blocking Cn/NFAT signaling when restricted to osteoblasts increased bone mass by directly increasing osteoblast differentiation and indirectly decreasing osteoclastogenesis. Therefore, the impact of our work is far reaching because it provides a better understanding by which the Cn/NFAT signaling pathway regulates osteoblast differentiation and it has important implications in developing new anabolic drugs to treat osteoporosis and bone loss.

Prior to our research, *in vivo* studies of CsA administration to examine bone remodeling have been performed in rats [39, 41, 130]. In laboratory animals, the administration of high doses of CsA (15 mg/kg) to rats demonstrated severe high turnover osteopenia leading to an increase in bone resorption and trabecular bone loss [39]. In contrast, administration of CsA at low doses (7 mg/kg) to rats showed an increase in bone formation and a decrease in bone resorption [41]. Moreover, *in vitro*, high concentrations of FK506 (25μM) have been shown to inhibit osteoblast differentiation, whereas low concentrations of FK506 (<1μM) induce osteoblast differentiation [42, 43]. Therefore, in order to more accurately examine the effects of diverse concentrations of CsA *in vivo*, we hypothesized that there would be a biphasic effect of CsA in osteoblast differentiation and bone formation depending upon its concentration.

Here, in Chapter 1, we subcutaneously injected CsA using different concentrations, 0, 1, 5, 10 and 30 mg/kg, for 30 days in 6-week old male mice. At the end of the study, animals were sacrificed and the bone phenotype of these mice was examined using μCT and histology analyses *in vivo* (Figures 1 and 2 in Chapter 1). Results of this study demonstrated that the administration of the lowest dose of CsA (1mg/kg) significantly increased tibial trabecular bone volume *in vivo*, whereas high doses (10 and 30 mg/kg) dramatically decreased tibial trabecular bone volume. Histomorphometry analysis further supports that the lowest dose of CsA (1mg/kg) increased the following parameters: bone volume/total volume ratio, trabecular numbers and number of osteoblasts, whereas high doses of CsA (10-30 mg/kg) significantly decreased these endpoints (Table 1 in Chapter 1). Therefore, our results indicate that administration of

low concentrations of CsA could be used as a potential anabolic therapy for treatment of osteoporosis.

Based on previous *in vivo* investigations, it is possible that CsA could cause indirect effects on bone formation. Therefore, we confirmed the effects of different concentrations of CsA *in vitro*, using MC3T3-E1 osteoblasts and calvarial primary osteoblasts. Consistent with our animal experiments, treatment of osteoblasts with low doses of CsA (<1uM) enhanced osteoblast differentiation, demonstrated by increased ALP activity and osteoblast mineralization as well as enhanced expression of osteoblastic gene markers, including ALP and osteocalcin (Figure 3 in Chapter 1). Consistent with our data that CsA induces differentiation in osteoblasts, similar functions have been reported using human cementoblastoma-derived cells [131]. In addition, high doses of CsA (10 and 25 μM) decreased osteoblast differentiation, as we expected. However, the mechanism by which high doses of CsA inhibit osteoblast differentiation still remains unclear. Extracellular signaling pathways may explain the effect of high doses of CsA on osteoblasts. It has been reported that high doses of CsA stimulate the production of transforming growth factor β (TGF-β), a multifunctional cytokine [132-134]. TGF- β has been shown to inhibit osteoblast differentiation in cell culture systems via decreased expression of osteoblast gene markers, such as Runx2 and osteocalcin [135]. In addition, it has been demonstrated that renal transplantation patients who receive CsA have an increase in active TGF- β expression in renal biopsy specimens [136]. Hence, to confirm the effects of high doses of CsA on bone formation, it would be necessary to examine the expression level of TGF- β after treatment of high doses of CsA *in vivo* and *in vitro*.

We have chosen to focus on the potential anabolic effects of low doses of CsA. To fully understand the low dose anabolic effects of CsA, we first needed to confirm its function in bone formation. Although we found that low dose administration of CsA increased bone formation in male mice, it will be necessary at some point to examine these effects in post-menopausal females. Estrogen deficiency is one of the major causes of bone loss and osteoporosis. Estrogen deficiency has been shown to increase the production of Tumor Necrosis Factor (TNF) by activated T cells, thus increasing immune function and inducing osteoclastogenesis by enhancing the expression of RANKL, ultimately resulting in bone loss [137]. Importantly, our data demonstrated that the administration of low doses of CsA decreased the numbers of osteoclasts *in vivo* (Table 1 in Chapter 1). It has also been reported that estrogen significantly inhibits Cn immunoreactivity in the hippocampus [138]. In addition, estradiol benzoate (EB) injections into ovariectomized female rats have been shown to decrease cytosolic Cn [139]. These reports suggest that estrogen-deficient bone loss may occur through activation of Cn, the major target of CsA. Therefore, to determine whether or not low dose CsA (1mg/kg) can overcome osteoporosis after menopause, CsA must be administered to normal and ovariectomized female mice and the bone phenotype evaluated.

It is unclear why CsA inhibits osteoclast formation *in vitro* but increases the differentiation of osteoclasts *in vivo* [39, 140]. However, as shown in Chapter 1, the administration of low doses of CsA decreased osteoclast numbers while high doses increased them (Table 1 in Chapter 1). Because intact bones depend on the interactions between osteoblasts and osteoclasts to maintain bone homeostasis, it will be necessary to

confirm the efficacy of potential therapies for osteoporosis that utilize a combination of CsA as an anabolic treatment of bone loss and bisphosphonate, an antiresorptive drug which inhibits osteoclastogenesis.

Third, unloading (disuse) conditions lead to the development of osteoporosis and increased fracture risk in elderly, bedridden patients [141, 142]. Skeletal unloading has been shown to reduce bone mass and bone formation, thus uncoupling bone formation from bone resorption [143]. The mechanism of skeletal unloading bone loss remains unclear. However, it is possible that low doses of CsA may prevent the bone loss induced in unloading animal models and that CsA therapy may provide an effective treatment for skeletal unloading osteoporosis. Therefore, it will be necessary to conduct more animal experiments to confirm the effect of low doses of CsA as an anabolic therapy for osteoporosis.

The Cn and NFAT signaling pathway is well known to be inhibited by CsA and FK506, both of which block the transcription of cytokine genes in activated T cells [144]. NFAT transcription factors are known to regulate several genes which are important for the development and function of many cells and organs, including T cells, osteoclasts and skeletal muscle fibers [54, 60, 145]. Additionally, inhibition of NFAT by FK506 treatment in mesenchymal cells has been shown to induce osteoblast differentiation *in vitro* [42]. Therefore, we examined the effects of CsA and its downstream targets Cn and NFAT *in vitro* to determine whether or not low doses of CsA contributed to increase osteoblast differentiation in a Cn and NFAT-dependent manner. Here, we demonstrated that CsA treatment in MC3T3-E1 osteoblastic cell lines inhibited NFAT transactivation and translocation into the nucleus (Figure 5 in Chapter 1). The inhibition of NFAT is

accompanied by increased osteoblastic gene expression, including ALP and osteocalcin, as well as enhanced osteoblast differentiation, demonstrated by quantitative real-time PCR and von Kossa staining (Figure 3 in Chapter 1). In addition, overexpression of constitutively-active NFATc1 decreased osteoblast differentiation. Thus, we concluded that NFATc1 negatively regulates osteoblast differentiation (Figure 6 in Chapter 1). Interestingly, low doses of CsA could not rescue the inhibition of osteoblast differentiation in response to the overexpression of constitutively-active NFATc1. In contrast, high doses of CsA completely inhibited osteoblast differentiation even in the absence of constitutively-active NFATc1 (Figure 6 in Chapter 1). Therefore, we demonstrated that low doses of CsA (<1μM) increased osteoblast differentiation in a Cn and NFAT-dependent manner while high doses of CsA (>1μM) decreased osteoblast differentiation in an NFAT-independent manner.

AP-1 transcription factors are well characterized for their regulatory role in osteoblastic growth and development [78]. Dimerization of AP-1 family members is a prerequisite for DNA binding and for regulation of gene expression [75]. It has been reported that CsA has the ability to increase AP-1 DNA binding activity in other cell types, including renal cortex and smooth muscle cells [146, 147]. Here, we demonstrate that inhibition of NFAT by CsA treatment coincides with increased AP-1 DNA binding activity in MC3T3-E1 osteoblasts (Figure 8 in Chapter 1). The expression of AP-1 family members changes during osteoblast development, and Fra-2 is the major AP-1 member found in differentiated and mineralized osteoblasts [78]. Consistent with our results, CsA enhanced AP-1 DNA binding activity, as dimerization of Fra-2 and JunD, in memory T helper cells [148]. Indeed, our results show that CsA treatment leads to an increase in the

formation of Fra-2 and JunD heterodimers and ultimately activates AP-1 DNA binding activity in osteoblasts (Figure 8 in Chapter 1). These findings suggest that NFAT plays a negative role in regulating osteoblast differentiation by mediating AP-1 activity.

NFAT was previously thought to be associated only with activation of gene transcription in the immune system, such as interleukin-2 (IL-2), IL-3, IL-4, CD-40L and Fas L [55]. Nevertheless, it is becoming increasingly evident that NFAT also mediates gene repression in other systems. Similar to our results that NFATc1 negatively regulates osteoblast differentiation, it has been shown that activation of Cn and NFAT signaling represses the transcription of cdk4 through negative regulation of cdk-4 promoter activity [111]. In addition, overexpression of NFATc2 in cartilage cells eliminates the cartilage phenotype, demonstrating that NFATc2 functions as a repressor of chondrogenesis [110]. Indeed, inhibition of NFAT by CsA treatment, increased Fra-2 gene expression and protein levels, whereas overexpression of constitutively-active NFATc1 decreased Fra-2 protein levels in osteoblasts (Figure 7 in chapter 1). It is therefore likely that NFAT negatively regulates differentiation, not only in osteoblasts, but in other systems as well. The Transcription Element Search System (TESS) program identified three potential NFAT consensus sequences on the Fra-2 promoter [149, 150]. NFAT binding to these sites could be responsible for the negative regulation of Fra-2 expression. However, it is possible that the increase in Fra-2 expression is either an indirect effect or occurs in an NFATc1-independent manner. Therefore, experiments are needed to examine whether or not NFAT directly inhibits Fra-2 expression on the Fra-2 promoter.

Recently, three studies have reported on the role of Cn and NFAT in bone formation and osteoblast differentiation in several animal models. First, Koga et al.

demonstrated that NFATc1 null (-/-) and NFATc2 (-/-) osteoblasts suppressed bone formation and provided evidence that cooperation between NFAT and osterix induces type I collagen promoter activation [43]. Second, Sun et al. reported that overexpression of CnAα in MC3T3-E1 osteoblasts induced differentiated osteoblastic gene expression, and CnAα deficient mice displayed osteoporosis and decreased mineral apposition [66]. Third, Winslow et al. reported that transgenic animals overexpressing constitutively active NFATc1 displayed increased bone mass and corresponding increases in osteoblast numbers and osteoblast activation [67].

Although these animal models indicate potential roles for Cn and NFAT signaling in regulating bone formation and osteoblast differentiation, they do not provide a specific mechanism. Embryonic stem cells are well characterized to have a pluripotent capability and thus, may differentiate into several cell lineages, such as chondrocytes, myocytes, and adipocytes, as well as osteoblasts [5]. Interestingly, it has been shown that NFAT functions in the lineage commitment of chondrocytes, myocytes and adipocytes. Therefore, the changes in osteoblast differentiation and bone formation that were described by Koga et al. and Winslow et al. might in fact be caused by a decrease in the lineage commitment decision of NFATc1 (-/-) embryonic cells, NFATc1 nuc mice cells, as well as CnAα (-/-) mice cells. In addition, these reports do not indicate direct roles of Cn and NFAT in osteoblast differentiation specifically, because CnAα (-/-) mice lacked CnAα before mesenchymal lineage commitment. The mice also lack CnAα throughout their entire body, which could play a critical role in their skeletal phenotype as well. For instance, the global loss of CnAα or global overexpression of NFATc1 in animal models resulted in an altered skeletal muscle phenotype that could indirectly affect the bone

phenotype [68]. Also, global loss of CnAα altered T cell responses that may indirectly affect the bone phenotype [69].

Therefore, to resolve these debates, we generated a conditional deletion mutant mouse model specific to osteoblasts using the *Cre-loxP* technique. The *Cre-loxP* system is a powerful approach to characterize the tissue-specific function of a target gene. Here, we generated osteoblast-specific deletion of Cnb1 in mice ($\Delta Cnb1^{OB}$) using *Cre-loxP* recombination. Transgenic mice engineered to express *Cre*-recombinase under the control of the human osteocalcin promoter (OC-*Cre*) were crossed with mice in which the E3-5 exon of Cnb1 was flanked by *loxP* sequences ($Cnb1^{f/f}$). Notably, consistent with our previous work in Chapter 1 that inhibition of Cn by CsA treatment increased bone formation, the disruption of Cnb1 in osteoblasts ($\Delta Cnb1^{OB}$) caused a significant increase in bone mass and bone formation, as observed by μCT, histology and histomorphometry analyses (Figures 2 and 3 in Chapter 2). The increasing bone formation could result from increased osteoblastogenesis, decreased osteoclastogenesis or a combination of the two. Indeed, $\Delta Cnb1^{OB}$ mice revealed an increase in osteoblast numbers and differentiation as well as decreased osteoclast numbers and TRAP positive cells in the femora (Figures 3 and 4 in Chapter 2).

It is known that osteoclast differentiation is induced by bone marrow stromal cells and osteoblasts that express the RANKL and inhibited by osteoprotegerin (OPG), which is a decoy receptor of RANKL [4]. Here, we demonstrated that the deletion of Cnb1 in osteoblasts inhibited osteoclastogenesis most likely by increasing OPG expression, thereby inhibiting RANKL *in vitro* (Figure 6 in Chapter 2). Finally, our finding in Chapter 2 is consistent with previous data in Chapter 1 that Cnb1 is a negative regulator

of osteoblast differentiation and bone formation, and that the Cn and NFAT signaling pathway negatively regulates osteoblast differentiation and bone formation.

Despite our demonstration of increased bone mass and bone formation in the $\Delta Cnb1^{OB}$ mouse model, we have one concern regarding the timing of expression of osteocalcin induced *Cre* recombinase. Osteocalcin is an osteoblast-specific protein that is expressed at the differentiation stage. Therefore, the *Cre* recombinase may not be expressed early enough to cause Cn and NFAT inhibition at other stages of observed. To address this possibility, an alternative approach would be to use type I collagen 2.3-*Cre* mice, which also express *Cre* recombinase specifically in osteoblasts but at an early stage of osteoblast development [126].

In addition to the osteoblast-specific effects of Cn, it is necessary to confirm the role of NFAT in an animal model with osteoblast specificity to support our previous work. Although we showed that NFATc1 negatively regulates osteoblast differentiation *in vitro*, 4 isoforms of NFAT are expressed in osteoblasts. Each of these isoforms could have either redundant or opposite functions in osteoblasts. The phenotype of NFATc1-deficient mice is embryonic lethality. However, an osteoblast-specific NFAT overexpression mouse model can be generated using the osteoblast-specific promoter regions of type I collagen 2.3 [126]. Also, there are currently two available NFAT constructs. One is constitutively-active NFATc1, and the other is dominant negative NFAT, which has already been shown to inhibit NFAT 1-4 isoforms *in vitro* [151, 152]. It is likely that osteoblast-specific overexpression of constitutively active NFATc1 mice will show a decreased bone phenotype and will confirm our previous data. In addition, transgenic animal that overexpresses dominant negative NFAT in osteoblasts will likely

show increased bone formation. Taken together, these animal models would provide further support for our hypothesis that the Cn and NFAT signaling pathway negatively regulates osteoblast differentiation and bone formation *in vivo*.

Another potential mechanism by which NFAT negatively regulates osteoblast differentiation could be through regulation of the osteocalcin promoter. Osteocalcin is a well-known protein, which is expressed specifically in osteoblasts during the osteoblast mineralization stage [83, 85]. We previously described that overexpression of constitutively active-NFATc1 (ca-NFATc1) in MC3T3-E1 osteoblast cells inhibited osteoblast differentiation and mineralization *in vitro* (Figure 4 in Chapter 1). Therefore, we proposed that NFATc1 negatively regulates osteoblast differentiation by inhibiting osteocalcin gene expression and promoter activity. Here, we confirmed that overexpression of ca-NFATc1 in MC3T3-E1 osteoblasts increased NFAT transactivation and decreased calcium deposition and alkaline phosphatase activity, thus inhibiting osteoblast differentiation (Figure 1 in Chapter 3). Furthermore, ca-NFATc1 also showed decreased osteocalcin gene expression and promoter activity in MC3T3-E1 osteoblasts *in vitro* (Figure 2 in Chapter 3).

Recently, it has also been reported that osteocalcin gene expression is regulated by transcriptional control exerted by chromatin organization [89, 91]. Transcriptionally active and inactive chromatin remodeling is regulated by two enzymes, HDACs and HATs. HDACs remove acetyl groups from histones, enhance chromatin condensation and result in transcriptional repression [101, 102]. The HDAC inhibitor, TSA, has been shown to induce osteoblast matrix maturation in calvarial primary osteoblasts and human mesenchymal stem cells, leading to an increased expression of the osteoblastic gene

markers, osteocalcin, type I collagen and osteopontin [116]. Here, we demonstrated that MC3T3-E1 osteoblasts, which overexpressed ca-NFATc1, retained HDAC activity, whereas its activity decreased in control GFP-expressing MC3T3-E1 osteoblasts (Figure 4 in Chapter 3). This suggests that HDACs might function as mediators between NFAT and osteoblast differentiation. Therefore, we hypothesized that ca-NFATc1 inhibits osteocalcin gene expression through an HDAC associated mechanism.

It has been shown that NFAT negatively regulates cdk4 gene expression due to the direct interaction between NFAT and HDAC1 on the cdk4 promoter, thus decreasing promoter activity of cdk4 and its gene expression [111]. In addition, there is evidence that NFAT and HDAC1 association silences IL-10 expression in T cells [113]. It has been documented that HDACs 1, 3 (Class I) and 6 (Class II) are expressed in osteoblasts [116, 153]. Inhibition of HDAC3 by shRNA increased osteoblast differentiation in MC3T3-E1 osteoblasts [116]. It is possible that NFATc1 and HDAC3 form a transcriptional repressor complex that enhances the inhibition of osteocalcin gene expression and promoter activity. Both HDAC3 and NFAT have a Rel homology region (Rel A), which functions to bind to DNA and to form protein-protein interactions [54, 154]. Here, we demonstrated that overexpression of ca-NFATc1 in MC3T3-E1 osteoblasts increased the interaction between NFATc1 and HDAC3. This association may enhance the inhibition of osteocalcin gene expression in ca-NFATc1 overexpressed osteoblasts (Figure 4 in Chapter 3).

The association between NFATc1 and HDAC3 may occur through the T-cell factor 1 (TCF1) binding site. TCF1 is a transcription factor that is a downstream target of the Wnt and beta-catenin signaling pathway. The TESS program indicates one TCF

binding site on the osteocalcin promoter. It has been reported that 12-O-Tetradecanoylphorbol-13-acetate-induced sequence 7 (TIS7) downregulates beta-catenin/TCF4 transcriptional activity and that the interaction between beta-catenin and HDAC inhibited the target gene expression of osteopontin (OPN) in myocytes [155]. Here, we demonstrated that NFAT decreased TCF transactivation in osteoblasts overexpressing ca-NFATc1. Ca-NFATc1 and HDAC3 form a complex on the TCF1 binding site of the osteocalcin promoter, decreasing acetylation of histone 4 (Figure 6 in Chapter 3).

Taken together, we propose the following model (Figure 1). The Cn and NFAT signaling pathway negatively regulates osteoblast differentiation *in vitro* and bone mass *in vivo*. We demonstrated that the pharmacological inhibition of Cn and NFAT signaling by CsA and the genetical deletion of Cnb1 in osteoblasts increases osteoblast differentiation *in vitro* and bone mass *in vivo*. The proposed mechanism for this action is that inhibition of Cn/NFAT signaling pathway increases Fra-2 expression and inhibits the activity of HDAC, leading to an increase in osteoblast differentiation. Furthermore, the inhibition of Cn/NFAT signaling in osteoblasts indirectly decreased osteoclast differentiation and bone resorption by inducing OPG and decreasing RANKL expression. In conclusion, we have demonstrated the negative role of Cn and NFAT signaling in osteoblast differentiation and bone formation. Our results confirm that this signaling pathway may provide a target for anabolic therapy to combat osteoporosis.

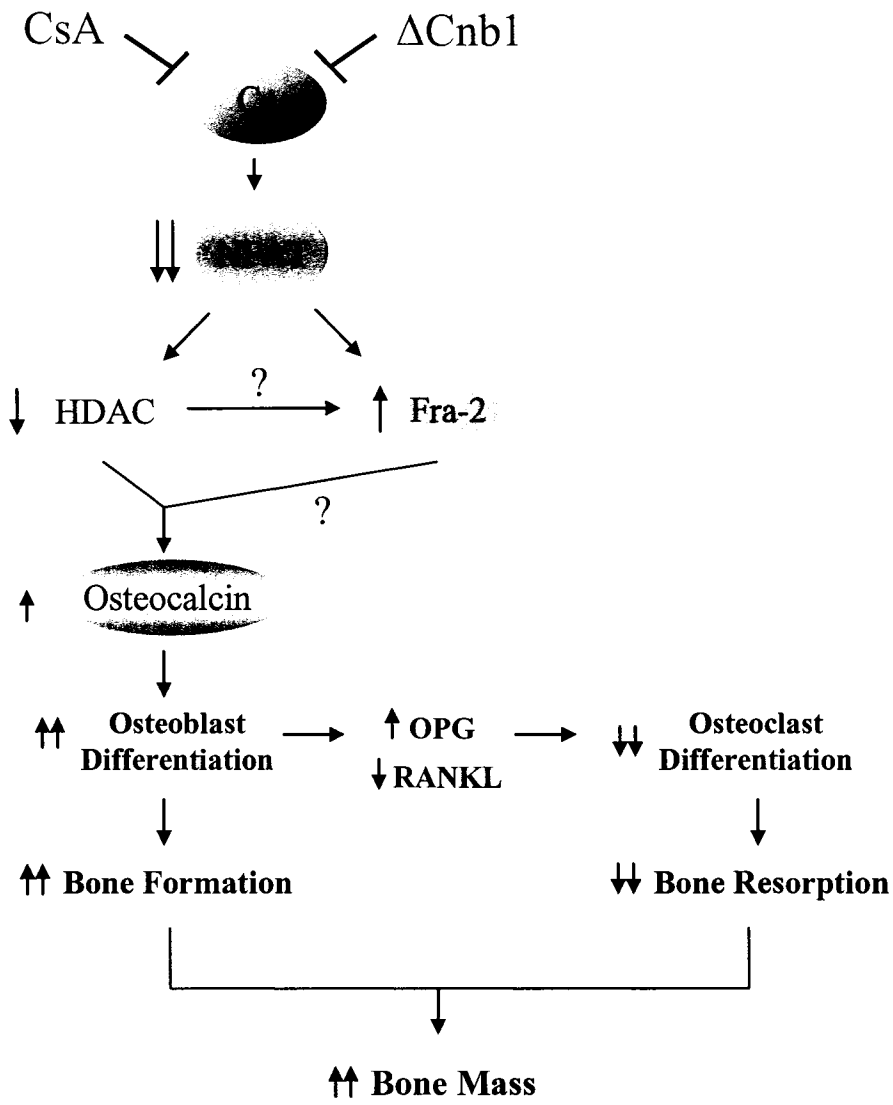


Figure 1. The regulation of bone mass by the Cn and NFAT signaling pathway. The inhibition of Cn and NFAT signaling in osteoblasts by CsA or deletion of Cnb1 decreases HDAC activity and increases Fra-2 expression, leading to an increase in osteoblast differentiation. This is accompanied by a decrease in osteoclastogenesis by the induction of OPG and reduction in RANKL expression. Therefore, the inhibition of Cn/NFAT signaling in osteoblasts induces uncoupling between osteoblasts and osteoclasts, thus finally increasing bone mass.

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# APPENDIX A

## INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

**MEMORANDUM**

**DATE:** August 7, 2006

**TO:** Majd Zayzafoon, M.D., Ph.D.
LHRB-533 0007
FAX: 975-9927

**FROM:** Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

**SUBJECT:** **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

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The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on July 26, 2006.

Title of Application: CORE: UAB Core Center for Basic Skeletal Research (Dr. McDonald); P&F #1: NFAT Negatively Regulates Osteoblast Differentiation and Bone Formation

Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW) (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

**Institutional Animal Care and Use Committee**
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