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Calcineurin/NFAT Signaling in Osteoblasts Regulates Bone Mass

Monte M. Winslow,[1] Minggui Pan,[2] Michael Starbuck,[3] Elena M. Gallo,[1] Lei Deng,[3] Gerard Karsenty,[3] and Gerald R. Crabtree[1,4,*]
[1] Program in Immunology
[2] Division of Oncology
Stanford University
Stanford, California 94305
[3] Department of Molecular and Human Genetics and the Bone Disease Program of Texas
Baylor College of Medicine
Houston, Texas 77030
[4] Howard Hughes Medical Institute and Departments of Pathology and Developmental Biology
Stanford University
Stanford, California 94305

Summary

Development and repair of the vertebrate skeleton requires the precise coordination of bone-forming osteoblasts and bone-resorbing osteoclasts. In diseases such as osteoporosis, bone resorption dominates over bone formation, suggesting a failure to harmonize osteoclast and osteoblast function. Here, we show that mice expressing a constitutively nuclear NFATc1 variant (NFATc1^{nuc}) in osteoblasts develop high bone mass. NFATc1^{nuc} mice have massive osteoblast overgrowth, enhanced osteoblast proliferation, and coordinated changes in the expression of Wnt signaling components. In contrast, viable NFATc1-deficient mice have defects in skull bone formation in addition to impaired osteoclast development. NFATc1^{nuc} mice have increased osteoclastogenesis despite normal levels of RANKL and OPG, indicating that an additional NFAT-regulated mechanism influences osteoclastogenesis in vivo. Calcineurin/NFATc signaling in osteoblasts controls the expression of chemoattractants that attract monocytic osteoclast precursors, thereby coupling bone formation and bone resorption. Our results indicate that NFATc1 regulates bone mass by functioning in both osteoblasts and osteoclasts.

Introduction

Bone is a dynamic organ, undergoing remodeling throughout life. The elaborate processes of bone formation and resorption result from the intricately coupled actions of bone-forming osteoblasts (Harada and Rodan, 2003; Karsenty and Wagner, 2002) and bone-resorbing osteoclasts (Boyle et al., 2003). When these two processes are not balanced and bone resorption dominates over bone formation, osteoporosis results (Zelzer and Olsen, 2003).

Osteoblasts and osteoclasts are derived from distinct stem cell pools and serve opposite but coordinated roles during bone remodeling. Osteoblasts are derived from multipotent mesenchymal progenitors and form the proteinacious bone matrix and orchestrate the mineralization of this extracellular matrix (Zelzer and Olsen, 2003). Bone mass is regulated both by the number and bone-forming activity of mature osteoblasts. Osteoblast number is regulated by differentiation and proliferation, and recent data have highlighted several signaling pathways and transcription factors that regulate bone mass (Harada and Rodan, 2003; Karsenty and Wagner, 2002; Zelzer and Olsen, 2003). The Wnt/wingless pathway is an established regulator of morphogenesis, and human and mouse studies have implicated this pathway as a regulator of bone mass (Glass et al., 2005; Gong et al., 2001; Kato et al., 2002).

Bone formation by osteoblasts is opposed by bone resorption by osteoclasts, which develop from hematopoietically derived monocytic cells (Ash et al., 1980). These osteoclast progenitors are recruited to the bone, where they interact with osteoblasts. Chemokines play a crucial role in recruitment and positioning of immune cells in tissue microenvironments (Butcher, 1991). Numerous monocyte chemoattractants have been identified, several of which have been implicated in osteoclast progenitor recruitment (Graves et al., 1999; Posner et al., 1997; Votta et al., 2000; Yu et al., 2003, 2004). By controlling cell positioning, chemokines may regulate the interactions between osteoclast progenitors and osteoblasts that are critical for osteoclast development. Osteoblasts regulate osteoclastogenesis by providing differentiation and survival signals through receptor activator of NFκB (RANK) and monocyte colony stimulating factor receptor (M-CSF receptor) (Lacey et al., 1998; Lagasse and Weissman, 1997). Multiple levels of positive and negative feedback balance the activities of osteoblasts and osteoclasts and preserve the steady state of mineralized bone (Boyle et al., 2003; Harada and Rodan, 2003; Karsenty and Wagner, 2002; Zelzer and Olsen, 2003).

NFAT transcriptional complexes were initially identified in T cells in an effort to define the biochemical pathway that leads from the antigen receptor to the genes that regulate T cell development and differentiation (Shaw et al., 1988). In unstimulated cells, NFATc transcription factors are highly phosphorylated and cytoplasmic. When a stimulus results in an increase in intracellular calcium ($[Ca^{2+}]_i$), the heterodimeric serine/threonine phosphatase calcineurin dephosphorylates NFATc, resulting in an allosteric switch that exposes a nuclear localization sequence and conceals a nuclear export sequence (Beals et al., 1997; Crabtree and Olson, 2002). NFATc then translocates to the nucleus and binds to specific regions in the promoters of target genes. DNA binding requires a nuclear partner protein. The nuclear import and activation of NFATc is opposed by rephosphorylation of NFATc by GSK3 and other NFAT kinases and subsequent nuclear export (Crabtree and Olson, 2002). The regulation of NFATc by nuclear import, nuclear export, and the assembly with nuclear partners allows many different pathways to influence NFAT activity.

In vivo studies have shown that calcineurin/NFAT signaling is an important regulator of immune development

*Correspondence: crabtree@stanford.edu

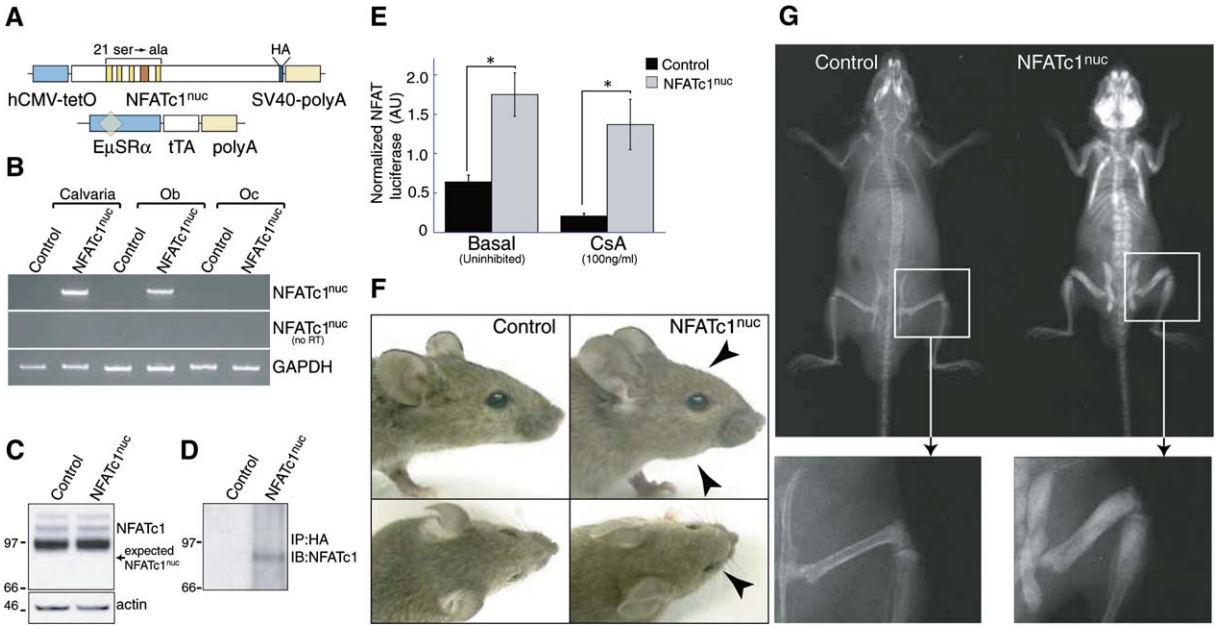

Figure 1. A Constitutively Active Cyclosporine-Resistant NFATc1 Is Expressed in Osteoblasts from NFATc1^{nuc} Mice

(A) Design of the doxycycline-regulatable NFATc1^{nuc} transgene.

(B) RT-PCR analysis of P4 calvaria, passage 2 in vitro-cultured osteoblasts, and in vitro-differentiated (5 days with RANKL and M-CSF) osteoclasts. GAPDH is a control for RT, and no-RT is a control for genomic DNA contamination.

(C) NFATc1 Western blot on protein from control and NFATc1^{nuc} osteoblasts. NFATc1^{nuc} (79 kDa) cannot be detected, indicating that its expression is much lower than that of endogenous NFATc1.

(D) IP Western shows expression of NFATc1^{nuc} protein (HA-tagged) in calvaria extract.

(E) Increased NFAT activity in NFATc1^{nuc} osteoblasts. NFATc1^{nuc} is constitutively active and CsA insensitive (n = 3, mean ± SD, *p < 0.01).

(F) Lateral and dorsal photos of 12-week-old control and NFATc1^{nuc} mice. Arrowheads indicate visually apparent changes in facial structure in NFATc1^{nuc} mice.

(G) Representative X-ray of 11-week-old control and NFATc1^{nuc} mice (n > 20).

and function (Neilson et al., 2004; Peng et al., 2001; Ranger et al., 1998; Winslow et al., 2006), cardiac development (Chang et al., 2004), angiogenesis (Graef et al., 2001), neural development and function (Graef et al., 1999, 2003), muscle cell fusion (Horsley et al., 2003), and chondrogenesis (Ranger et al., 2000). Patients treated with the calcineurin inhibitors cyclosporine (CsA) and FK506 develop osteopenia, and CsA-treated patients have an increased incidence of fractures (Katz and Epstein, 1992; Rodino and Shane, 1998; Sprague, 2000; Sprague and Josephson, 2004). These in vivo effects may be due to inhibition of calcineurin in osteoblasts and osteoclasts, or they may be indirect, due to calcineurin inhibition in other cell types. We generated mouse lines that allowed us to assess the role of NFATc1 in osteoblasts and osteoclasts in vivo. Our results indicate that calcineurin/NFAT signaling regulates osteoblast proliferation, osteoclast differentiation, and the coordination of bone formation and resorption.

Results

To determine whether the NFAT pathway has the potential to function in osteoblasts, we examined the expression of various components of the NFAT pathway in osteoblasts. Primary mouse osteoblasts express *NFATc1*, *NFATc3*, and *NFATc4*, but not *NFATc2* (Figure S1; see the Supplemental Data available with this article online). The components of the heterodimeric calcineurin complex, calcineurin A and calcineurin b1, are also expressed in primary osteoblasts (unpublished data). Pharmacological stimulation of osteoblasts or MC3T3-E1 osteoblast-like cells leads to the calcineurin-dependent dephosphorylation of NFATc1, NFATc1 nuclear translocation, and increased NFAT transcriptional activity (Figure S1 and unpublished data). These results prompted us to closely examine the role of NFAT signaling in osteoblasts in vivo.

Generation of Mice Expressing Active NFATc1 in Osteoblasts

To characterize the function of NFATc1 in osteoblasts in vivo, we generated mice expressing a constitutively nuclear NFATc1 variant (NFATc1^{nuc}) under the control of the tetracycline-responsive operator (tetO) and bred them to Eμ-tTA mice (Figure 1A) (Felsher and Bishop, 1999; Jain et al., 2002). In NFATc1^{nuc}, the serines that are dephosphorylated by calcineurin are mutated to alanines, rendering it constitutively nuclear, constitutively active, and insensitive to nuclear kinases (Figure S2) (Beals et al., 1997). In NFATc1^{nuc};Eμ-tTA mice (referred to as NFATc1^{nuc} mice), the tetracycline transactivator (tTA) drives expression of NFATc1^{nuc} in the thymus, splenic lymphocytes, and bone (Figures 1B–1D and unpublished data).

The expression of *NFATc1^{nuc}* in bone could indicate expression in osteoblasts, osteoclasts, or other cell lineages. *NFATc1^{nuc}* is expressed in osteoblasts, but not in

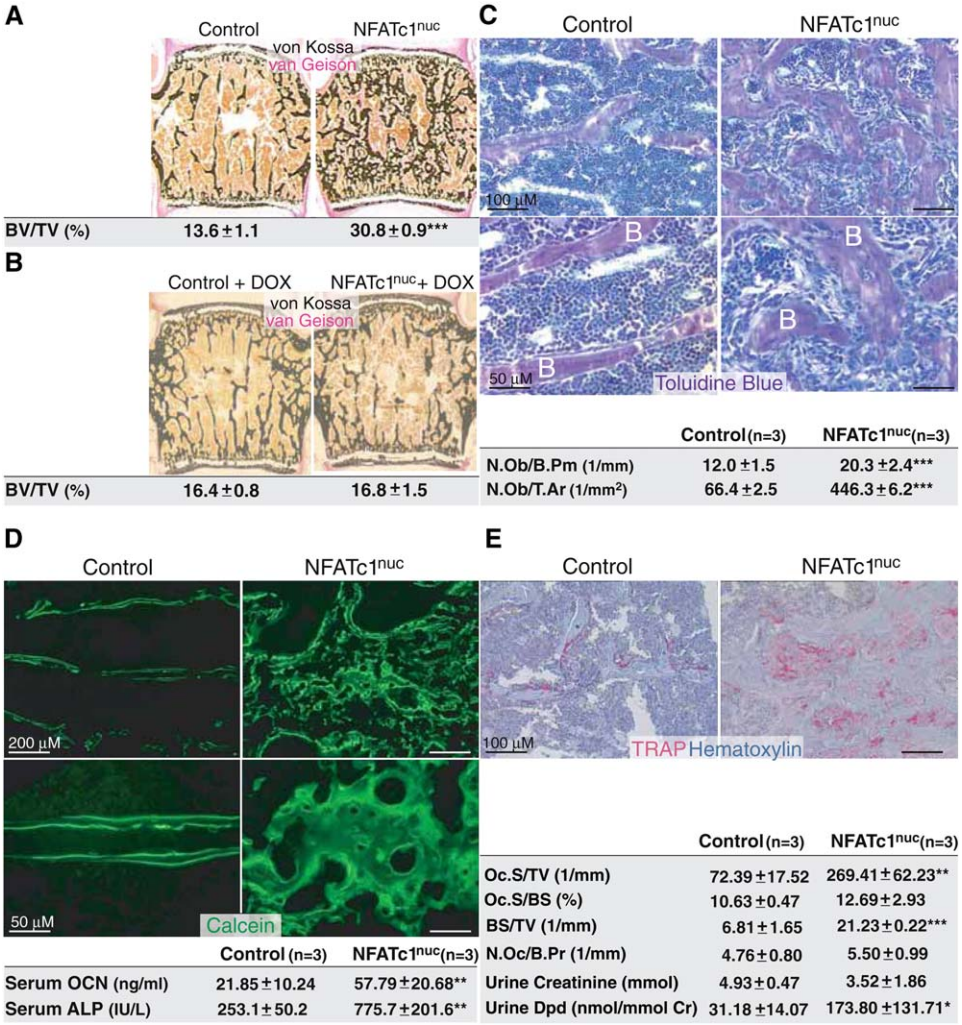

Figure 2. NFATc1^{nuc} Mice Have Increased Bone Volume and Increased Osteoblast Number

(A) von Kossa/van Gieson-stained vertebrae from 4-week-old mice (bone is black). Bone volume/tissue volume (BV/TV) is shown (mean ± SD, ***p < 0.0001).

(B) Vertebrae from 4-week-old mice treated with doxycycline throughout gestation and life (NFATc1^{nuc} off; n = 4) have normal bone mass. Bone volume/tissue volume (BV/TV) is shown (mean ± SD).

(C) Increased osteoblast number in NFATc1^{nuc} vertebrae from 4-week-old mice (n = 4). B, bone. Number of osteoblasts/bone perimeter (N.Ob/B.Pm) and number of osteoblasts/tissue area (N.Ob/T.Ar) are shown (mean ± SD, ***p < 0.0001).

(D) Disorganized calcein double labeling in NFATc1^{nuc} vertebrae from 4-week-old mice (n = 4). Serum osteocalcin and alkaline phosphatase is shown (mean ± SD, **p < 0.001).

(E) Increased osteoclasts (TRAP stained) in vertebrae from 4-week-old NFATc1^{nuc} mice (n = 4). Osteoclast surface/tissue volume (Oc.S/TV), osteoclast surface/bone surface (Oc.S/BS), bone surface/tissue volume (BS/TV), and number of osteoclasts/bone perimeter (N.Ob/B/Pr) are shown. Serum creatinine (Cr) and deoxypyridonoline (Dpd) is also shown (mean ± SD, *p < 0.01, **p < 0.001, ***p < 0.0001).

in vitro-differentiated osteoclasts or monocytes from NFATc1^{nuc} mice (Figure 1B; Figure S2). Expression of the 79 kDa NFATc1^{nuc} protein in bone or primary osteoblasts from NFATc1^{nuc} mice is below the level of detection by conventional NFATc1 or HA Western blotting. NFATc1^{nuc} was detectable by IP Western, indicating that the transgene is expressed, but at a level well below that of endogenous NFATc1 (Figures 1C and 1D). This low level expression was functional, as NFATc1^{nuc} osteoblasts have higher basal NFAT activity than control osteoblasts, and this NFAT activity was CsA resistant (Figure 1E). Collectively, these data indicate that osteoblasts from NFATc1^{nuc} mice express a constitutively active NFATc1.

NFATc1^{nuc} Mice Have Increased Bone Mass and Osteoblast Number

Visual, radiographic, and histological analyses indicated that NFATc1^{nuc} mice have increased bone (Figures 1F, 1G, and 2A). This high-bone mass phenotype was observed in all NFATc1^{nuc};Eμ-tTA mice regardless of sex or strain and in NFATc1^{nuc};Eμ-tTA from two independent NFATc1^{nuc} transgenic founder lines (Figures 1E, 1F, and 2A). High bone mass was not observed in NFATc1^{nuc} or Eμ-tTA single transgenic mice. Addition of doxycycline to the animals' drinking water (which turns off NFATc1^{nuc} expression by inhibiting the tTA; Furth et al., 1994) prevented or reversed the high-bone mass phenotype (Figure 2B; Figure S5). Additionally, a control

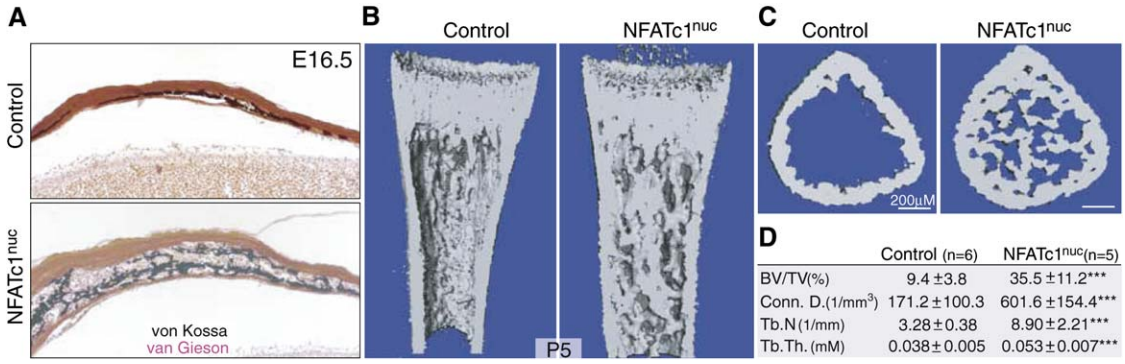

Figure 3. Increased Bone in NFATc1^{nuc} Embryos and Increased Trabecular Number and Trabecular Thickness in P5 Mice

(A) von Kossa/van Giesen-stained E16.5 calvaria show increased mineralized bone (bone is black; n = 4).

(B) Increased bone in tibiae of P5 NFATc1^{nuc} mice.

(C) Cross-section of the diaphysis of P5 tibia.

(D) Bone volume/tissue volume (BV/TV), connectivity (Conn. D.), trabecular number (Tb. N), and trabecular thickness (Tb. Th.) are shown (mean ± SD, ***p < 0.0001).

transgenic mouse strain expressing wild-type NFATc1 (tetO-NFATc1^{wt}) does not develop high bone mass (Figures S2 and S3). Therefore, the phenotype observed in the NFATc1^{nuc} mice is due to an increase in NFAT nuclear occupancy and NFAT activity.

Histological analysis of the vertebrae and tibia revealed that 4-week-old NFATc1^{nuc} mice have a 2-fold increase in bone mass and an increase in osteoblast number (Figure 2C and unpublished data). Osteoblasts in the NFATc1^{nuc} mice are in multiple layers in bone that lacks normal trabecular order, while chondrocytes within the growth plate appear normal (Figure 2C; Figures S4 and S5). Despite the remarkable increase in osteoblast number, we have never detected osteosarcomas in >200 NFATc1^{nuc} mice.

Bone formation was assessed by in vivo calcein labeling and by measuring serum markers of bone formation. NFATc1^{nuc} mice have increased calcein incorporation without consistent double labeling, reflecting the increased number and disorganization of the osteoblasts as well as the rapid osteogenesis that likely results in woven bone (Figure 2D). Serum osteocalcin and alkaline phosphatase are also significantly higher in NFATc1^{nuc} mice, confirming that bone formation is increased (Figure 2D) (Kruse and Kracht, 1986).

Increased Osteoclast Number and Bone Resorption in NFATc1^{nuc} Mice

A second cellular phenotype was observed in the NFATc1^{nuc} mice. Although neither osteoclasts nor their progenitors express *NFATc1^{nuc}*, osteoclast number is increased 4-fold in 4-week-old NFATc1^{nuc} mice (Figures 1B and 2E; Figure S2). NFATc1^{nuc} mice also have a 5-fold increase in urine deoxypyridinoline crosslinks, a biochemical marker of osteoclast function (Robins et al., 1994), indicating that there is increased osteoclast function (Figure 2E). Thus, NFATc1^{nuc} mice have both increased bone formation and increased bone resorption.

Increased Bone Mass Early during Development of NFATc1^{nuc} Mice

To determine whether active NFATc1 alters the initiation or extent of bone formation during embryogenesis, we analyzed the skeletal elements of control and NFATc1^{nuc} embryos. Ossification of skeletal elements initiates at the same developmental stage in control and NFATc1^{nuc} embryos. However, increased bone mass is apparent in NFATc1^{nuc} embryos at E16.5 by von Kossa staining and at E17.5 by alizarin red staining. This increase in bone mass includes skeletal elements formed by intramembranous and endochondral ossification (Figure 3A; Figure S6). Five-day-old NFATc1^{nuc} mice have greatly increased trabecular bone volume and trabecular number in the diaphysal region (Figures 3B–3D). Importantly, the trabecular thickness and connectivity are also greater in tibiae from NFATc1^{nuc} mice.

Active NFAT in Osteoblasts Is Sufficient to Produce High Bone Mass

To determine whether the expression of active NFATc1 in osteoblasts is sufficient to drive the high-bone mass phenotype, we transplanted E13.5 embryonic tibial and femoral anlage (containing the cartilage scaffold and osteoblast progenitors) under the kidney capsule of adult recipients. In this system, the wild-type host provides the osteoclasts, the systemic environment, and the bone marrow (Jochum et al., 2000). The NFATc1^{nuc} transplants had higher bone volume and connectivity and more osteoblasts than control transplants (Figures 4A and 4B and unpublished data). These studies strongly suggest that the high bone mass is due to NFATc1^{nuc} expression in osteoblasts. Additionally, lethally irradiated wild-type mice reconstituted with NFATc1^{nuc} bone marrow do not develop high bone mass, indicating that hematopoetically derived cells (including osteoclasts) are not responsible for the high bone mass. These results indicate that although the expression of NFATc1^{nuc} in osteoblasts is extremely low, it is sufficient to drive the dramatic phenotype observed in the NFATc1^{nuc} mice.

To determine the mechanism that leads to increased osteoblast number, we assessed osteoblast proliferation and cell death. NFATc1^{nuc} embryos have increased osteoblast proliferation in the calvaria and mandibles at E16.5 and E17.5. Osteoblast cell death in vitro and in vivo was comparable (Figures 4C–4E; Figure S7). The clear increase in osteoblast proliferation and bone

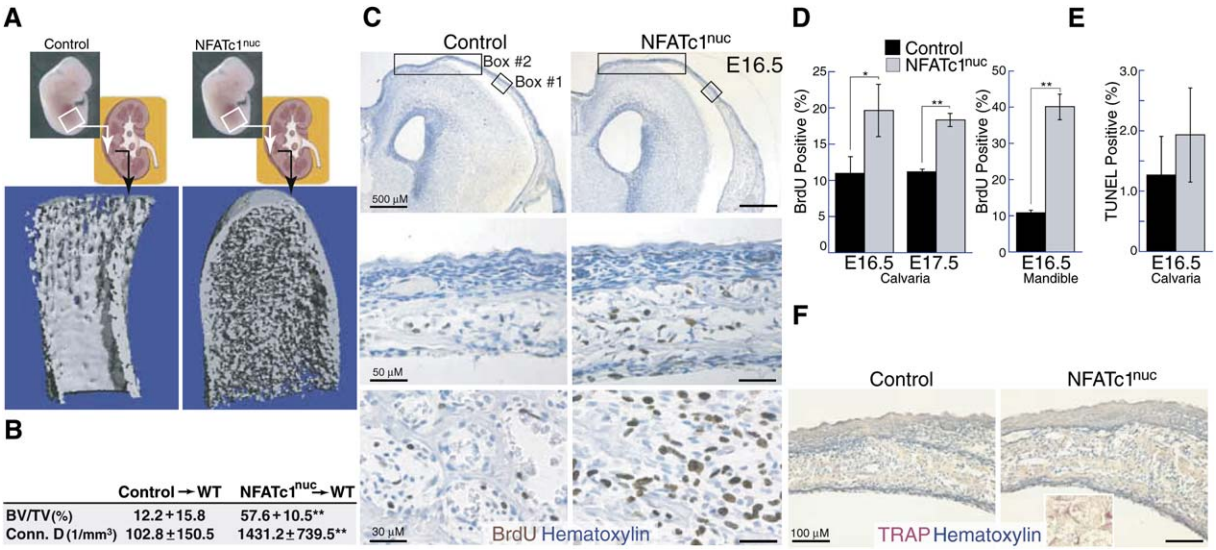

Figure 4. NFATc1^{nuc} Mice Have Increased Osteoblast Proliferation In Vivo

(A) μCT of the transplanted fetal limb.

(B) Quantification of BV/TV and connectivity (Conn. D.) of transplanted femoral anlage (n = 4, mean ± SD, **p < 0.01).

(C) Coronal sections of in vivo BrdU-labeled E16.5 calvaria. (Middle) Higher magnification of Box #1 in top panels. (Lower) Higher magnification of the mandible.

(D) Quantification of percent BrdU^{+} osteoblasts in the area defined by Box #2 and within the mandible (n = 4 for each age, mean ± SD, *p < 0.01, **p < 0.001).

(E) Quantification of osteoblast cell death in E16.5 calvaria (n = 4, mean ± SD).

(F) E16.5 control and NFATc1^{nuc} calvaria were TRAP stained for osteoclasts. Neither control nor NFATc1^{nuc} calvaria have TRAP^{+} cells at this age. The inlay shows P0 NFATc1^{nuc} calvaria staining as a positive control. A scale bar is included in each panel.

mass at E16.5 in NFATc1^{nuc} mice precedes the increase in osteoclast number, as TRAP^{+} cells were not present in either control or NFATc1^{nuc} skull bones at this age (Figure 3F). This indicates that increased osteoclast number and enhanced bone resorption do not drive the increased osteoblast proliferation.

NFATc1^{nuc} osteoblasts continue to express the NFATc1^{nuc} transgene throughout in vitro mineralization, therefore, NFATc1^{nuc} may function in mature bone-forming osteoblasts (Figure S8). Control and NFATc1^{nuc} osteoblasts produce mineralized bone and induce alkaline phosphatase activity comparably during in vitro mineralization (Figure S8). Our results cannot rule out the possibility that NFATc1^{nuc} subtly enhances osteoblast function in vivo, but they indicate that active NFATc1 does not alter their ability to differentiate and form mineralized bone in vitro.

NFATc1 Is Required In Vivo for Osteoclastogenesis

To examine the function of endogenous NFATc1, we generated and analyzed viable NFATc1$^{-/-}$;Tie2-NFATc1^{+} mice. Expression of an NFATc1 transgene under the control of the endothelial-specific Tie-2 promoter rescues the lethal heart valve development defects in NFATc1$^{-/-}$ embryos (Figure 5A) (Chang et al., 2004). Neither osteoblasts nor osteoclast progenitors from Tie2-NFATc1^{+} mice express the NFATc1 transgene (Figure S9). NFATc1$^{-/-}$;Tie2-NFATc1^{+} mice appear normal at birth but gain weight slower than their littermates and die before adulthood. These mice have greatly reduced osteoclastogenesis, as revealed by the absence of tooth eruption, osteopetrosis, and very few small TRAP^{+} cells (Figures 5B–5D). These results are consistent with previous in vitro and vivo results and indicate that NFATc1 is required for osteoclast development (Hirotani et al., 2004; Humphrey et al., 2004; Ishida et al., 2002; Takayanagi et al., 2002). The osteoclast defect and the early lethality of NFATc1$^{-/-}$;Tie2-NFATc1^{+} mice compromised our ability to closely analyze bone formation in adult mice. However, examination of skeletal elements during development revealed delayed frontal, parietal, and occipital bone formation in NFATc1$^{-/-}$;Tie2-NFATc1^{+} mice, and these bones are thinner even at 2 weeks of age (Figures 5E and 5F and unpublished data). These results suggest a bone formation defect in viable NFATc1-deficient mice.

Active NFATc1 Coordinately Regulates Genes Critical for Wnt Signaling

To understand the mechanism by which active NFATc1 enhances osteoblast proliferation and leads to increased osteoclastogenesis, we performed global gene expression profiling on control and NFATc1^{nuc} postnatal day 4 (P4) calvaria. We chose to analyze whole P4 skull bone in order to get a snapshot of the total bone genes early in the development of this phenotype. Several known osteoblast functional genes, growth factors, and growth factor receptors are changed (Figure 6; Table S1), reflecting a global change in the genetic program that drives increased immature osteoblast proliferation. Two established NFAT target genes, DSCR1 and Egr2, are increased in NFATc1^{nuc} bone (Figure 6A). Interestingly, there is a coordinated change in the expression of genes within the Wnt/wingless signaling pathway: Wnt4 and Frizzled 9 are increased, while the Wnt inhibitors Dickkopf 2 (DKK2) and secreted

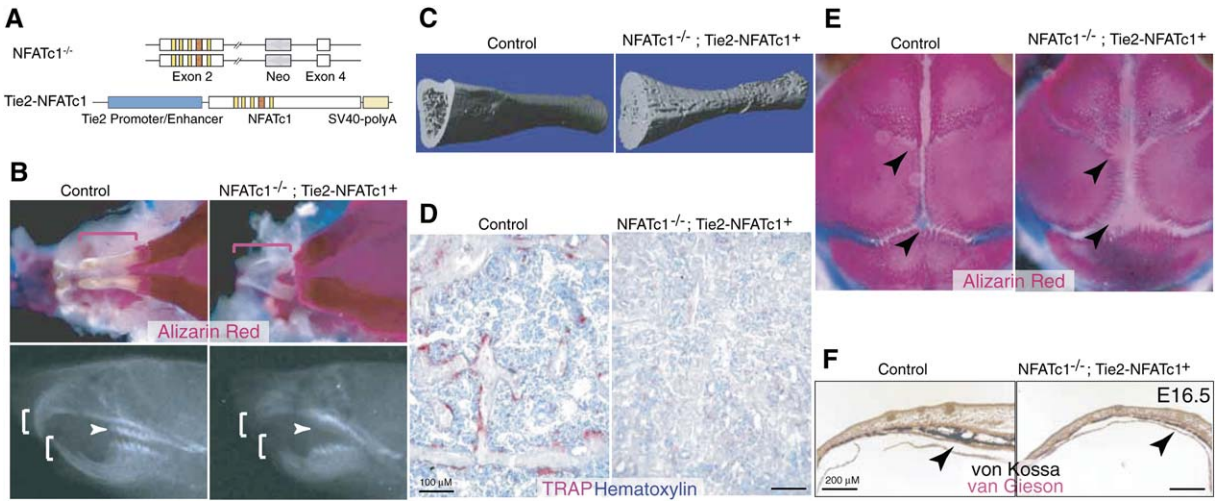

Figure 5. Viable NFATc1$^{-/-}$ Mice Lack Osteoclasts and Have Delayed Skull Bone Formation

(A) Design of the NFATc1$^{-/-}$;Tie2-NFATc1^{+} transgene to rescue heart valve development in NFATc1-deficient mice.

(B) Ventral view of 3-week-old skulls. The pink bracket highlights the lack of incisors in the NFATc1$^{-/-}$;Tie2-NFATc1^{+} mouse (n = 3). (Lower) The lateral X-ray of the skull shows the lack of both upper and lower incisors (white brackets) and the absence of molars (white arrowhead) in NFATc1$^{-/-}$;Tie2-NFATc1^{+} mice.

(C) µCT analysis of tibia shows osteopetrosis in NFATc1$^{-/-}$;Tie2-NFATc1^{+} mice.

(D) NFATc1$^{-/-}$;Tie2-NFATc1^{+} mice lack TRAP^{+} osteoclasts (n = 3).

(E) NFATc1$^{-/-}$;Tie2-NFATc1^{+} mice have delayed calvaria formation. Dorsal view of P4 whole calvaria (n = 3).

(F) Decreased bone in NFATc1$^{-/-}$;Tie2-NFATc1^{+} skulls. Comparable coronal sections of E16.5 frontal bones are shown (n = 3).

frizzled-related protein 2 (sfrp2) are decreased (Figures 6A and 6C). Studies in both humans and mice have implicated Wnt signaling in the regulation of bone mass (Gong et al., 2001; Kato et al., 2002). These Wnt components could function in either an autocrine or a paracrine fashion to regulate osteoblast proliferation.

Expression of many other genes previously implicated in osteoblast proliferation, differentiation, and function is not changed (Table S2). Importantly, expression of the key osteoblast transcription factor, Runx2, is similar at the RNA and protein levels (Figure 6D; Table S2). Consistent with the increased in vivo proliferation, many cell cycle regulators are increased in NFATc1^{nuc} calvaria (Figures 4C, 4D, and 6B).

Increased Monocyte Chemoattractant Expression in NFATc1^{nuc} Bone

Several monocyte chemoattractants (CCL8/monocyte chemoattractant protein-2 [MCP-2], CCL6/C10, and CCL12/MCP-5) are increased in the NFATc1^{nuc} calvaria (Figure 6E). These chemokines may recruit monocytes to bone, where interactions with osteoblasts allow these precursors to differentiate into mature osteoclasts, thereby balancing osteoblast number with osteoclast differentiation. In support of this hypothesis, many monocyte/osteoclast genes are increased in the NFATc1^{nuc} calvaria and these calvaria have increased TRAP staining, consistent with increased osteoclastogenesis (Figures 6F and 7A).

While chemokines can control the recruitment and localization of hematopoietic cells, the enhanced osteoclastogenesis in NFATc1^{nuc} mice could also be driven by other changes in osteoblast gene expression. Osteoblasts control the maturation of osteoclasts in vivo, and coculture of osteoblasts with monocytes leads to osteoclast development in vitro (Takahashi et al., 1988). We determined whether NFATc1^{nuc} osteoblasts have an enhanced ability to direct osteoclast development in vitro. Cultured osteoblasts from control and NFATc1^{nuc} calvaria direct osteoclast differentiation comparably in vitro (Figure 7B). Additionally, control and NFATc1^{nuc} calvaria express similar levels of the key osteoclast differentiation and survival factors *RANKL*, *OPG*, and *M-CSF* (Figure 7C). Control and NFATc1^{nuc} mice also have similar serum levels of RANKL and OPG (Figure 7D). These results indicate that the increase in osteoclastogenesis in NFATc1^{nuc} mice is not driven by changes in RANKL or OPG expression by osteoblasts.

Active NFATc1 has been shown to be sufficient to promote osteoclastogenesis of a monocyte cell line, suggesting that the increased osteoclastogenesis in NFATc1^{nuc} mice could be due to expression of the transgene in monocytes or osteoclasts (Hirotani et al., 2004). Despite our inability to detect *NFATc1^{nuc}* expression in monocytes or osteoclasts, an extremely low level that affects osteoclastogenesis in these mice could be present. To address this, splenocytes from young control and NFATc1^{nuc} mice were cultured in vitro with RANKL and M-CSF, and the number of multinucleated TRAP^{+} cells was determined after 5 days. Splenocytes from 4-day-old mice were used because adult NFATc1^{nuc} mice have an altered bone marrow environment and extramedullary hematopoiesis in the spleen. Control and NFATc1^{nuc} splenocytes gave rise to comparable numbers of TRAP^{+} multinucleated cells in vitro, indicating that these cells have an equivalent intrinsic potential to become osteoclasts (Figure 7E). The differentiation of these cells into osteoclasts was sensitive to the calcineurin inhibitor CsA, consistent with the absence of NFATc1^{nuc} expression (Figure 7E).

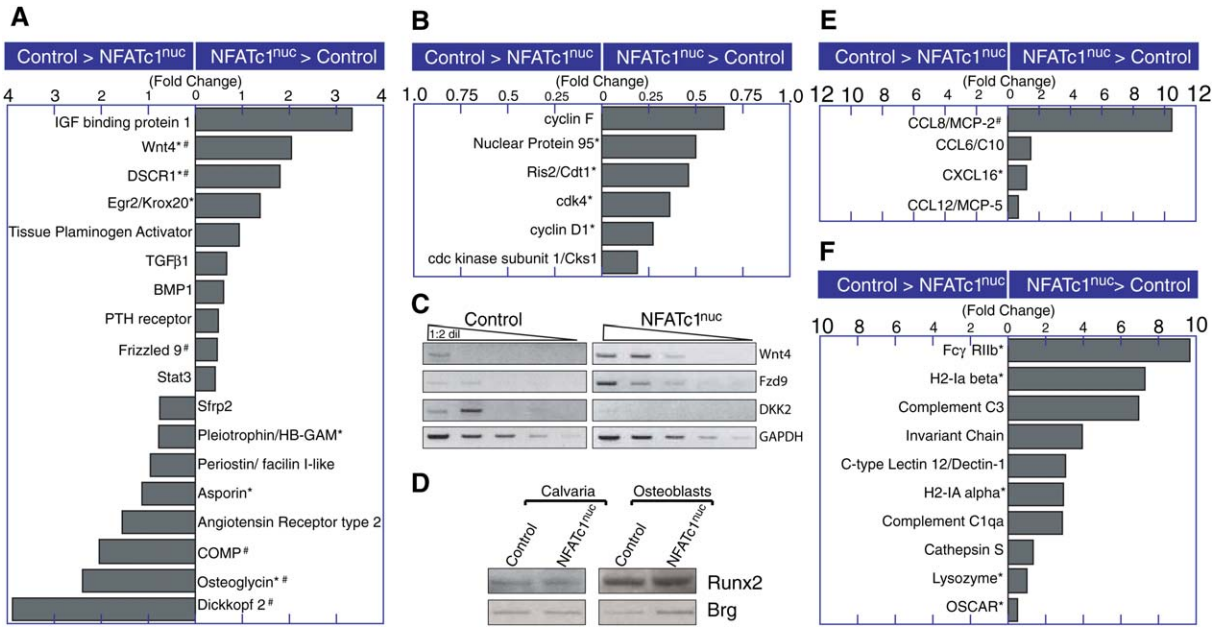

Figure 6. NFATc1^{nuc} Mice Have Changes in Gene Expression Consistent with Increased Proliferation and Monocyte Recruitment

(A–F) (A, B, E, and F) Gene expression changes between control and NFATc1^{nuc} calvaria expressed as fold change. (A) Osteoblast genes, (B) cell cycle genes, (E) monocyte chemoattractants, and (F) monocyte genes. *Fold change is an average of two or more features for that gene. #Change validated by RT-PCR on independent calvaria RNA. (C) RT-PCR verification of *Wnt4, Frizzled9* (*Fzd9*), and *DKK2* expression in NFATc1^{nuc} and control calvaria. A 2-fold dilution series is shown with *GAPDH* as a control. (D) Runx2 protein expression is unaltered in NFATc1^{nuc} calvaria and in vitro-cultured osteoblasts. Brg shows equal nuclear loading.

CCL8 Is a Direct Target of Calcineurin/NFAT Signaling

NFATc1^{nuc} osteoblasts have an equivalent ability to direct osteoclast development in vitro and express normal levels of RANKL and OPG. NFATc1^{nuc} mice have greatly increased osteoclast numbers in vivo, suggesting that active NFATc in osteoblasts may control an additional regulator of osteoclastogenesis. Based on our gene expression data and the significant increase in osteoclasts in calvaria from 4-day-old NFATc1^{nuc} mice, we further investigated the expression and regulation of the monocyte chemoattractants. CCL8 was originally cloned from an osteogenic sarcoma line, and both CCL8 and CCL12 have been shown to specifically attract monocytes in vitro and in vivo (Sarafi et al., 1997; Van Coillie et al., 1997; Van Damme et al., 1992). We first verified the increased expression of CCL8 in calvaria from NFATc1^{nuc} mice by RT-PCR (Figure 7F), and then determined whether NFAT could regulate CCL8 expression. The 5′ flanking regions and introns of *CCL8* are conserved between multiple species and have many potential NFAT binding sites. To create a reporter with all the control regions of endogenous CCL8, we inserted the luciferase gene in-frame into the first exon. This reporter is induced in osteoblasts by PMA/Ionomycin stimulation, blocked by CsA, and upregulated by NFATc1^{nuc} expression, suggesting that NFAT signaling directly regulates CCL8 expression (Figures 7G and 7H).

Discussion

Bone is constantly resorbed and rebuilt; thus, the proper regulation of bone mass requires the precise coordination of osteoblasts and osteoclasts. In osteoporosis, an imbalance between bone formation and bone resorption over many years leads to crippling consequences. Our data indicate that calcineurin/NFAT signaling functions in osteoblasts and osteoclasts to regulate bone mass (Figure 7I). NFATc1 is essential for osteoclast lineage specification, while active NFAT drives osteoblast proliferation in vivo. Additionally, calcineurin/NFAT signaling in osteoblasts enhances chemokine expression, which may recruit osteoclast precursors to bone and influence osteoclastogenesis.

NFATc1 Regulates Osteoblast Proliferation In Vivo

Our results indicate that mice expressing a low level of active NFATc1 develop severe osteosclerosis caused by a dramatic increase in osteoblast number. Several lines of evidence indicate that active NFATc1 in osteoblasts is sufficient to direct osteoblast proliferation in vivo. Embryonic tibial and femoral NFATc1^{nuc} anlage transplanted under the kidney capsule of wild-type mice develop the high-bone mass phenotype. NFATc1^{nuc} was also expressed in thymic and splenic lymphocytes, but T and B cells are not required for the high-bone mass phenotype, as NFATc1^{nuc} mice on *TCR*$\alpha^{-/-}$ (which lack $\alpha\beta$T cells) or *Rag2*$^{-/-}$(which lack all T and B cells) backgrounds still develop high bone mass (unpublished data). Involvement of a gene at the transgene integration site is excluded by the suppression of the phenotype by doxycycline administration (transgene off), the lack of high bone mass in Eμ-tTA and NFATc1^{nuc} single transgenic mice, and the presence of high bone mass in two independent NFATc1^{nuc} mouse lines. Collectively, our results indicate that NFATc1^{nuc} directs osteoblast proliferation (perhaps more exuberant osteoblast overgrowth than any other genetically modified mouse) by slightly

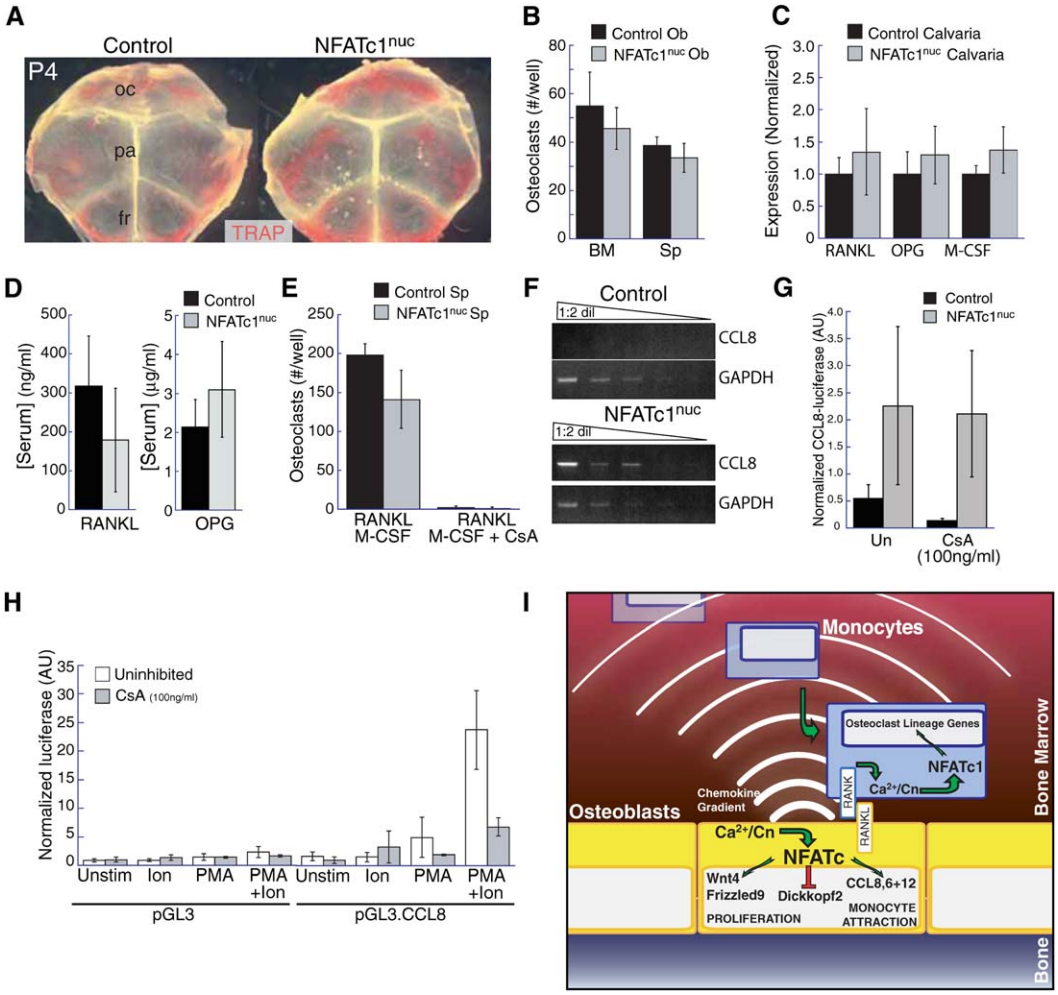

Figure 7. Calcineurin/NFAT Signaling Regulates Chemokine Expression

(A) Dorsal view of TRAP-stained whole P4 calvaria (n = 4). Major skull bones are indicated. fr, frontal; pa, parietal; oc, occipital.

(B) Coculture of control or NFATc1^{nuc} osteoblasts with control bone marrow (BM) or splenocytes (Sp) leads to equivalent TRAP^{+} multinucleated osteoclast development in vitro.

(C) Control and NFATc1^{nuc} calvaria express similar levels of *RANKL*, *OPG*, and *M-CSF* as assessed by microarray. Data are the average of three repeats and of multiple features for each gene.

(D) Similar serum levels of RANKL and OPG in control and NFATc1^{nuc} mice.

(E) RANKL and M-CSF induce equivalent osteoclast development from control and NFATc1^{nuc} splenocytes (Sp) in vitro. Osteoclast development is sensitive to CsA (100 ng/ml).

(F) RT-PCR for *CCL8* on control and NFATc1^{nuc} calvaria. A 2-fold dilution series is shown with *GAPDH* as a control.

(G) MC3T3-E1 cells were transfected with pGL3.CCL8 with or without an NFATc1^{nuc} expression plasmid. Expression of NFATc1^{nuc} increased basal CCL8 luciferase expression, which was not blocked by the calcineurin inhibitor CsA (100 ng/ml) (luciferase activity is normalized to renilla luciferase, n = 3, mean ± SD).

(H) CCL8 expression is activated by PMA/Ionomycin and is calcineurin dependant (n = 3, mean ± SD).

(I) NFAT functions in both osteoblasts and osteoclasts to regulate bone mass. Small changes in the nuclear occupancy of NFATc1 lead to a dramatic increase in osteoblast progenitor proliferation. Active NFATc1 also directs a genetic program that leads to monocyte recruitment. Chemokines attract monocytes from the blood and surrounding tissue and position monocytes in close proximity to osteoblasts, where they receive RANK and M-CSF receptor signaling. NFATc1 functions downstream of RANK in osteoclast progenitors and is indispensable for the development of mature osteoclasts in vivo.

enhancing the nuclear occupancy of NFATc1. This observation indicates that subtle yet stable changes in transcription factor activity can produce dramatic effects.

Our gene expression profiling indicates that components of the Wnt signaling pathway are coordinately changed in NFATc1^{nuc} calvaria. While the Wnt signaling pathway may regulate osteoblast differentiation, proliferation, and function, the signaling pathways that regulate the expression of Wnt signaling components in osteoblasts are poorly defined. Our studies suggest that NFAT signaling may coordinately alter *Wnt4*, *Frizzled9*, and *DKK2* expression, providing a potential mechanism by which NFAT signaling regulates osteoblast proliferation. Additional studies will be required to determine the extent to which enhanced Wnt signaling in vivo contributes to the increased osteoblast number and high bone mass in NFATc1^{nuc} mice.

Osteopetrosis in Viable NFATc1$^{-/-}$ Mice

Viable NFATc1-deficient mice fail to develop mature osteoclasts. Gene-targeted mice that lack RANK or FcγRIII and DAP12 fail to activate and induce NFATc1 and do not develop mature osteoclasts, suggesting that NFATc1 activation downstream of RANK may be critical for osteoclastogenesis in vivo (Humphrey et al., 2004; Ishida et al., 2002; Koga et al., 2004; Takayanagi et al., 2002). NFATc1 autoregulation (Northrop et al., 1994) appears to be a necessary positively reinforcing feedback loop, which likely explains the specific requirement for NFATc1 during osteoclastogenesis (Asagiri et al., 2005). The retroviral expression of active NFATc1 is sufficient to drive the differentiation of a monocytic cell line to osteoclasts in vitro (Hirotani et al., 2004). These data indicate that NFATc1 plays a second role in regulating bone mass by acting cell autonomously to regulate osteoclastogenesis.

Active NFAT in Osteoblasts Increases Osteoclastogenesis

NFATc1^{nuc} mice have increased osteoclast number and increased bone resorption. Several observations indicate that this increase in osteoclast number is secondary to the expression of active NFATc1 in osteoblasts. First, *NFATc1^{nuc}* expression is undetectable in monocytes or osteoclasts, and NFATc1^{nuc} splenocytes do not have enhanced osteoclastogenesis in vitro (Figures 1B and 7E; Figure S2). Second, the increased osteoblast proliferation in NFATc1^{nuc} mice precedes the increased osteoclast number during embryogenesis (Figure 4F). Third, transplanted NFATc1^{nuc} fetal limbs have osteoclasts similar to untransplanted NFATc1^{nuc} vertebrae and thin trabeculae, indicating that there is increased osteoclastogenesis in NFATc1^{nuc} bone even in a situation where the osteoclast progenitors are wild-type (Oc.S/BS is 6.11 ± 2.02 and Oc.S/TV is 202.3 ± 61.8 [n = 5]; see Figure 2I).

How does enhanced NFAT activity in osteoblasts enhance osteoclastogenesis? The expression of *RANKL* and the soluble decoy receptor *OPG* are unchanged in NFATc1^{nuc} bones, and the ability of NFATc1^{nuc} osteoblasts to direct osteoclast differentiation in vitro is comparable to that of control osteoblasts. These results indicate that an additional mechanism regulates osteoclastogenesis in NFATc1^{nuc} mice in vivo. Several monocyte chemoattractants (CCL8, CCL6, and CCL12) are upregulated in NFATc1^{nuc} calvaria and could function to control the recruitment and/or differentiation of these osteoclast progenitors within bone. The addition of CCL8 to in vitro osteoclastogenesis cultures did not alter the development of osteoclasts, ruling out a direct morphogenic function of CCL8 (data not shown). Chemokines and integrins coordinate the spatially and temporally discrete patterns of cell migration in vivo, and they would not be expected to influence in vitro coculture of monocytes on a confluent monolayer of osteoblasts (Butcher, 1991; Parfitt, 1998). Consistent with the established ability of CCL8 and CCL12 to attract monocytes in vivo, there was increased expression of many monocyte genes in NFATc1^{nuc} calvaria, indicating that there was an increased number of monocytes within this tissue. The dramatic increase in the expression of several monocyte chemoattractants in NFATc1^{nuc} calvaria led us to test whether the calcineurin/NFAT pathway regulates these chemokines. We found that calcineurin/NFAT signaling controls the expression of CCL8 in osteoblasts. Bone formation and bone resorption are integrated processes, and the production of monocyte chemoattractant proteins by osteoblasts may provide an additional level of coordination.

Regulation of Bone Mass by NFAT

Previous studies have attempted to determine the role of the calcineurin/NFAT pathway in osteoblasts (Buchinsky et al., 1996; Cvetkovic et al., 1994; Fornoni et al., 2001; Goodman et al., 2001; Klein et al., 1997; Koga et al., 2005; McCauley et al., 1992; Movsowitz et al., 1988; Tang et al., 2002). These studies have been contradictory, potentially due to the systemic effects of the injected calcineurin inhibitors, the systemic nature of NFATc2 deficiency, and the excessively high concentrations of calcineurin inhibitors used in vitro. A recent report describes a potential role for NFAT in regulating osteoblast function (Koga et al., 2005). Their results complement our findings and indicate that NFAT may function at different stages to regulate osteoblast proliferation and function, consistent with the sequential roles of calcineurin/NFAT signaling in other cell types (Crabtree and Olson, 2002).

The role of calcineurin/NFAT signaling in osteoblasts and osteoclasts is interesting since these two cell types oppose each other's actions. We find that one function of calcineurin/NFAT signaling in osteoblasts is to coordinately regulate the expression of Wnt pathway and cell cycle genes. Our studies also indicate that NFAT signaling in osteoblasts directly activates genes encoding chemokines. Thus, calcineurin/NFAT plays multiple roles in osteoblasts and osteoclasts to regulate bone mass.

Experimental Procedures

Generation of TetO-NFATc1^{nuc} Mice

NFATc1^{nuc} (mSRR+SP123) (Beals et al., 1997) was cloned into pUD10-3 downstream of the hCMVTetO promoter. The transgene was restriction digested to remove the plasmid backbone and was purified, and pronuclear injection was performed with B6BAF1/J-derived oocytes (Jackson). TetO-NFATc1^{wt} mice were generated in a similar manner. Genotype was determined by using the NFATc1^{nuc} specific primers 5′-AAGAAGATGGTCCTGTCTGG-3′ and 5′-GTAG TCTGGTACGTCGTAC-3′. Eμ-tTA mice (FVB/N) (Felsher and Bishop, 1999), NFATc1$^{-/-}$;Tie2-NFATc1^{+} mice (Chang et al., 2004), Rag2$^{-/-}$ mice (Taconic), TCRα$^{-/-}$ mice (Jackson), and SCID mice have been previously described.

Protein and mRNA Analysis

For Western blot analyses, membranes were probed with anti-NFATc1 (7A6; Pharmingen) and anti-actin (Sigma). IP Western was performed using the HA-IP Western kit (Pierce). RNA was purified using the total RNA kit (Amersham). NFATc1^{nuc} was detected by RT-PCR with the NFATc1^{nuc}-specific PCR primers.

NFAT-Luciferase Activity and Cell Cultures

Primary mouse osteoblasts from P4 calvaria were cultured in αMEM with 10% FCS and mineralized with 10 mg/ml β-glycerophosphate and 50 mg/ml ascorbic acid as previously described (Ducy et al., 1999). Primary osteoblasts were transfected with NFAT-GL3 (Shaw et al., 1988) using FuGENE 6 Transfection Reagent (Roche). Transfections were normalized by using renilla luciferase and the Dual Luciferase Kit (Promega). The CCL8 luciferase construct has the murine genomic regions from −600 to +1 and from +1 to +1657 in

the pGL3 luciferase vector (Promega). Bone marrow cells or splenocytes were cocultured with confluent calvarial osteoblasts in the presence of 10^{-8} M 1, 25 dihydroxyvitamin D_3. After 5 days, cells were fixed and TRAP stained and $TRAP^+$ multinucleated cells were counted as described (Glass et al., 2005). RANKL- and M-CSF-induced in vitro osteoclastogenesis was performed as described (Koga et al., 2004).

Histologic and Histomorphometric Analyses

Alcian blue/alizarin red staining was performed as previously described (McLeod, 1980). Vertebrae were von Kossa/van Gieson stained for BV/TV measurement, toluidine blue stained for osteoblasts, and TRAP stained for osteoclasts. Calcein double labeling was performed as described (Vignery and Baron, 1980). Histomorphometric analyses were performed using the Osteomeasure Analysis System (Osteometrics). To turn off transgene expression, $NFATc1^{nuc}$ mice were treated with 200 μg/ml Doxycycline in their drinking water. TRAP staining of whole calvaria was performed as described (Meghji et al., 2001). For in vivo BrdU labeling, timed pregnant mice were injected intraperitoneally with 100 μg/kg bromodeoxyuridine (BrdU; Sigma) and harvested 3 hr later. Paraffin sections (7 μM) were stained with anti-BrdU and were hematoxylin counterstained (Zymed). Deoxypyridinoline crosslinks were measured in morning urine and standardized to creatinine levels (Metra Biosystems). Serum RANKL (R&D Systems), OPG (R&D Systems), ALP, and osteocalcin (Biomedical Technologies) were measured. All statistical differences were calculated by the Student's t test.

Tibial and Femoral Anlage Transplant and μCT Analysis

Tibial and femoral anlagen from E13.5 embryos were transplanted under the kidney capsules of SCID recipients. Transplants were analyzed at 16 μm resolution 4 weeks after implantation by μCT with a μCT40 scanner (Scanco Medical). μCT of P5 tibiae were analyzed at 8 μm resolution.

Isolation of RNA, Hybridization, and Analysis of Microarrays

P4 $NFATc1^{nuc}$ and littermate control calvaria (4–6 calvaria per replicate, three replicates per group) were lysed in Trizol reagent (Invitrogen). cDNA was synthesized (SuperScript Choice synthesis kit; GIBCO-BRL) with $T7\text{-}(dT)_{24}$ primers (Operon). Biotin-labeled cRNA was synthesized (Enzo BioArray kit; Affymetrix) and fragmented according to the manufacturer's instructions. The GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix) were hybridized and scanned by the Stanford Affymetrix Core Facility. Images were analyzed with GCOS software with the all-probe sets scaling strategy. Data analysis and comparisons between arrays were performed with the Data Mining Tool.

Supplemental Data

Supplemental Data include nine figures and two tables and are available at http://www.developmentalcell.com/cgi/content/full/10/6/771/DC1/.

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