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ACTIVATION OF NFATC2 IN OSTEOBLASTS CAUSES OSTEOPENIA*

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Abstract

Nuclear factor of activated T-cells (Nfat)c1 to c4 are transcription factors that play an undisputable role in osteoclastogenesis. However, Nfat function in osteoblastic cells is controversial. Constitutive activation of Nfatc1 and c2 in osteoblasts suppresses cell function, although the study of Nfat *in vivo* has yielded conflicting results. To establish the consequences of Nfatc2 activation in osteoblasts, we generated transgenic mice where a 3.6 kilobase fragment of the collagen type I $\alpha 1$ promoter directs expression of a constitutively active Nfatc2 mutant (*Col3.6-Nfatc2*). The skeletal phenotype of *Col3.6-Nfatc2* mice of both sexes and of sex-matched littermate controls was investigated by microcomputed tomography and histomorphometry. *Col3.6-Nfatc2* mice were born at the expected Mendelian ratio and appeared normal. *Nfatc2* expression was confirmed in parietal bones from 1 and 3 month old transgenic mice. One month old *Col3.6-Nfatc2* female mice exhibited cancellous bone compartment osteopenia secondary to a 30% reduction in bone formation. In contrast, cancellous femoral bone volume and bone formation were not altered in male transgenics, whereas osteoblast number was higher, suggesting incomplete osteoblast maturation. Indices of bone resorption were not affected in either sex. At 3 months of age, the skeletal phenotype evolved; and *Col3.6-Nfatc2* male mice exhibited vertebral osteopenia, whereas femoral cancellous bone was not affected in either sex. Nfatc2 activation in osteoblasts had no impact on cortical bone structure. Nfatc2 activation inhibited alkaline phosphatase activity and mineralized nodule formation in bone marrow stromal cell cultures. In conclusion, Nfatc2 activation in osteoblasts inhibits bone formation and causes cancellous bone osteopenia.

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¹The abbreviations used are: α -MEM, α -minimum essential medium; BGH, bovine growth hormone; bp, base pair; ca, constitutively active; Col1a1, collagen type I $\alpha 1$; FBS, fetal bovine serum; FVB, tropism to Friend leukemia virus strain-B; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; HA, hemagglutinin; IDT, Integrated DNA Technologies; kb, kilobase; μ CT, microcomputed tomography; Nfat, nuclear factor of activated T-cells; pA, polyadenylation; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcription-PCR; Rpl38, ribosomal protein L38; SMI, structure model index.

Keywords

Nfatc2; osteoblasts; bone formation; osteopenia; calcineurin

INTRODUCTION

The fate of mesenchymal cells and their differentiation toward cells of the osteoblastic lineage is defined by the organized regulation of cell-specific gene expression, so that transcription factors play a critical role in the fate and function of osteoblastic cells (Bianco and Gehron, 2000; Canalis, 2005; Canalis et al., 2007). Nuclear factors of activated T-cells (Nfat) are five transcription factors (Nfatc1 to c4 and Nfat5) that are important to the immune response and also regulate the growth and differentiation of multiple cell types (Crabtree and Olson, 2002; Graef et al., 2001). Under basal conditions, Nfatc1 to c4 are highly phosphorylated and reside in the cytoplasm. Activation of the phosphatase calcineurin dephosphorylates specific serine residues in the regulatory domain of Nfat, leading to its nuclear translocation and to the transcription of Nfat target genes (Chow et al., 2008; Hogan et al., 2003; Okamura et al., 2000; Shen et al., 2007).

Nfatc1 through c4 are expressed by skeletal cells, and Nfatc1 is required for osteoclast differentiation (Ikeda et al., 2006; Zanotti et al., 2013). Nfatc2 also plays a role in osteoclastogenesis, although the functions of Nfatc1 and c2 are not necessarily redundant (Aliprantis et al., 2008; Ranger et al., 1998). In contrast to the well-established effects of the calcineurin/Nfat regulatory pathway in osteoclastogenesis, the role of Nfat proteins in osteoblast differentiation and function is less clear. This is in part because of limitations in the genetic manipulations used in former studies to explore the effects of Nfats in osteoblasts (Hodge et al., 1996; Koga et al., 2005; Sun et al., 2005; Winslow et al., 2006; Yeo et al., 2007b). Mouse models harboring either the activation or the downregulation of calcineurin have generated conflicting results (Sun et al., 2005; Yeo et al., 2007b). This is in part because the approach does not discriminate between the independent effects of each Nfat isoform or effects of other calcineurin-dependent signals. Mice expressing a dominant negative Nfatc1 in osteoblasts display increased bone volume due to increased bone formation, suggesting that Nfatc1 inhibits osteoblastic function (Sesler and Zayzafoon, 2013). The global inactivation of *Nfatc2* causes osteopenia and decreased bone formation, but this may be secondary to a hyperproliferation of B and T-cells and dysregulated expression of multiple cytokines (Hodge et al., 1996; Koga et al., 2005; Monticelli and Rao, 2002). In previous work, we demonstrated that expression of constitutively active (ca)Nfatc1 and caNfatc2 in osteoblast cultures inhibits cell differentiation and function, indicating that Nfat proteins suppress the differentiation and function of cells of the osteoblastic lineage (Zanotti et al., 2011; Zanotti et al., 2013).

In the present study, we sought to define the consequences of Nfatc2 activation in the postnatal skeleton by investigating the phenotype of transgenic mice where the collagen type I $\alpha 1$ (*Col1a1*) promoter directs expression of caNfatc2 (*Col3.6-Nfatc2*). The skeletal effects of Nfatc2 activation in osteoblasts were determined by histomorphometric analysis and by microcomputed tomography (μ CT). The mechanisms whereby Nfatc2 exerts its effects in

osteoblastic cells were defined in cultures of bone marrow stromal cells from *Col3.6-Nfatc2* mice.

MATERIALS AND METHODS

Col3.6-Nfatc2 Transgenic Mice

For *in vivo* activation of Nfatc2, a 2.8 kilobase (kb) DNA fragment containing the coding sequence of murine Nfatc2 was used. The fragment contained multiple serine to alanine mutations in the SRR and SPXX repeat motifs of the Nfatc2 regulatory domain, rendering the protein constitutively active (caNfatc2). The caNfatc2 coding sequence was preceded by the sequence coding for amino acids 98 to 106 of the human influenza hemagglutinin. A plasmid containing the described DNA sequences was created by A. Rao (Harvard Medical School, Boston, MA) and obtained from Addgene (Cambridge, MA, Addgene plasmid 11792) (Monticelli and Rao, 2002). A Kozak consensus sequence was inserted by polymerase chain reaction (PCR) upstream of the caNfatc2 coding sequence. The resulting amplification product was cloned downstream of a 3.6 kb fragment of the rat *Colla1* promoter and upstream of the bovine growth hormone polyadenylation signal to generate a *Col3.6-Nfatc2* transgene (Fig. 1) (Kalajzic et al., 2002). Microinjection of linearized DNA into pronuclei of fertilized oocytes from Friend leukemia virus strain B (FVB) mice (Charles River Laboratories, Wilmington, MA) and subsequent transfer into pseudopregnant mice, were carried out by the Gene Targeting and Transgenic Facility of UConn Health Center. Founder mice were identified by PCR analysis of tail DNA with 5'-GAGCAGGAGGCACACGGA-3' forward and 5'-TTCGATAGGGTTCAGATAGTC-3' reverse primers binding to the *Colla1* promoter and caNfatc2 coding sequence, respectively, and amplifying a 460 base pair (bp) fragment of the *Col3.6-Nfatc2* transgene. Forward 5'-TGGACAGGACTGGACCTCTGCTTTCC-3' and reverse 5'-TAGAGCTTTGCCACATCACAGGTCAT-3' primers (all from Integrated DNA Technologies; IDT, Coralville, IA) amplifying a 200 bp fragment of the autosomal gene fatty acid binding protein 1 were used in the same reaction as positive controls. Founders were bred to wild type FVB mice to create *Col3.6-Nfatc2* transgenic lines (Irwin, 1989). To characterize the impact of Nfatc2 activation in osteoblasts, hemizygous *Col3.6-Nfatc2* mice were crossed with wild type FVB mice to generate hemizygous *Col3.6-Nfatc2* transgenics and wild type littermate controls. The presence of the *Col3.6-Nfatc2* transgene in the offspring was documented by PCR in DNA extracted from the tail, as described for the identification of founders. Mice were sacrificed by CO₂ inhalation followed by cervical dislocation prior to the collection of tissues for the characterization of the skeletal effects of Nfatc2 activation or to the harvest of primary cells for culture. To assess mRNA expression in skeletal cells, parietal bones were frozen in liquid nitrogen at the time of harvest and transferred to -80°C for storage before RNA extraction. Experimental protocols were approved by the Institutional Animal Care and Use Committees (IACUC) of Saint Francis Hospital and Medical Center (Hartford, CT).

Microcomputed Tomography

Dissected femurs and the L3 vertebrae were scanned in 70% ethanol at an energy level of 55 kVp, an intensity of 145 μ A, and an integration time of 200 ms on a μ CT 40 scanner (Scanco

Medical AG, Bassersdorf, Switzerland). Femoral length was measured in mm. Trabecular bone volume fraction and microarchitecture were evaluated starting approximately 1.0 mm proximal from the femoral condyles, or 1.0 mm from the cranial side of the vertebral body. For femurs from 1 and 3 month old mice, a total of 140 and 160 consecutive slices, respectively, were acquired at an isotropic voxel size of $216 \mu\text{m}^3$, a slice thickness of $6 \mu\text{m}$ and chosen for analysis. A total of 100 consecutive slices of identical isotropic voxel size and thickness were chosen for analysis of L3 vertebrae from 3 month old mice. Contours were drawn manually every 10 slices a few voxels away from the endocortical boundary to define the region of interest for analysis. The contours of the intervening slices were iterated automatically. Trabecular regions were assessed for bone volume fraction, trabecular thickness, number and separation, connectivity density and structure model index (SMI), using a Gaussian filter ($\sigma = 0.8$, support = 1) and a user-defined threshold (Bouxsein et al., 2010). A total of 100 slices for the cortical region were measured at the mid-diaphysis of each femur with an isotropic voxel size of $216 \mu\text{m}^3$ and a slice thickness of $6 \mu\text{m}$. Contours were iterated across the 100 slices along the cortical shell, excluding the bone marrow cavity, and analysis of cortical bone structure was performed using a Gaussian filter ($\sigma = 0.8$, support = 1) and a user defined threshold (Bouxsein et al., 2010).

Bone Histomorphometric Analysis

Static and dynamic histomorphometry of femurs was carried out after injection of 1 month old mice with calcein 20 mg/kg and demeclocycline 50 mg/kg at an interval of 2 days. Mice were sacrificed 2 days after the demeclocycline injection. Femurs were dissected and fixed in 70% ethanol, dehydrated and embedded undecalcified in methyl methacrylate. Longitudinal femoral sections at a thickness of $5 \mu\text{m}$ were obtained on a microtome (Microm, Richards-Allan Scientific, Kalamazoo, MI) and stained with 0.1 % toluidine blue. Indices of static histomorphometry were measured in a defined area between $360 \mu\text{m}$ and $2160 \mu\text{m}$ from the growth plate of the distal femur using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA) (Gazzerro et al., 2005). For dynamic histomorphometry, mineralizing surface per bone surface and mineral apposition rate were measured on unstained femoral sections under ultraviolet light, using a triple diamino-2-phenylindole fluorescein set long pass filter, and bone formation rate was calculated. Terminology and units used are those recommended in the 2012 update of the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee (Dempster et al., 2013).

Bone Marrow Stromal Cell Cultures

Femurs from 6 week old male and female *Col3.6-Nfatc2* transgenic mice and littermate controls were dissected aseptically, epiphysis removed and primary bone marrow stromal cells recovered by centrifugation, as described (Zanotti et al., 2014). Cells from mice of both sexes were pooled and seeded at a density of 1.25×10^6 cells/ cm^2 in α -minimum essential medium (α -MEM; Life Technologies, Grand Island, NY) containing heat-inactivated 15% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) and cultured at 37°C in a humidified 5% CO_2 incubator. At confluence, cells were exposed to α -MEM supplemented with heat-inactivated 10% FBS, $100 \mu\text{g/ml}$ ascorbic acid and 5 mM β -glycerophosphate (all from Sigma-Aldrich, St. Louis, MO) to induce osteoblastogenesis.

Quantitative Reverse Transcription-PCR (qRT-PCR)

Total RNA was extracted from frozen parietal bones by Trizol (Life Technologies) and phenol/chloroform extraction (Sigma-Aldrich) and from bone marrow stromal cell cultures with the RNeasy mini kit, according to manufacturer's instructions (Qiagen, Valencia, CA). Integrity of the total RNA preparation was ensured by microfluidic electrophoresis conducted with an Experion automated electrophoresis system (BioRad Laboratories, Hercules, CA). 0.5–1 µg of total RNA was reverse-transcribed using the iScript cDNA synthesis kit (BioRad Laboratories) and amplified in the presence of 5'-AGAACAACATGAGAGCCACCATC-3' forward and 5'-AGCTCGATGTCAGCGTTTCG-3' reverse primers for *Nfatc2* (for RefSeq transcripts NM_010899, NM_001037177, and NM_001037178), 5'-CCCCTCTGGAAAGCTGTGGCGT-3' forward and 5'-AGCTTCCCGTTCAGCTCTGG-3' reverse primers for glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*; for RefSeq transcript NM_008084) or 5'-AGAACAAGGATAATGTGAAGTTCAAGGTTC-3' forward and 5'-CTGCTTCAGCTTCTCTGCCTTT-3' reverse primers (all from IDT) for ribosomal protein L38 (*Rpl38*; for RefSeq transcripts NM_001048057, NM_001048058 and NM_023372) and iQ SYBR Green Supermix (BioRad Laboratories) at 60°C for 35 cycles, according to manufacturer's instructions. Transcript copy number was estimated by comparison to a dilution series of *Nfatc2* (A. Rao), *Gapdh* (R. Wu, Ithaca, NY), or *Rpl38* (American Type Culture Collection, Manassas, VA) cDNA (Kouadjo et al., 2007 ; Monticelli and Rao, 2002; Tso et al., 1985). Reactions were conducted in a CFX96 qRT-PCR detection system (BioRad Laboratories), and fluorescence was monitored at the annealing step of every PCR cycle. Specificity of the reaction was confirmed by the presence of a single peak in the melt curve of PCR products.

Cytochemical Assays

Alkaline phosphatase activity was measured in 0.5% Triton X-100 cell extracts (Sigma-Aldrich) by the hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol measured by spectroscopy at 405 nm, according to manufacturer's instructions (Sigma-Aldrich). Total protein content was determined by DC protein assay, according to manufacturer's instructions (Bio-Rad Laboratories). To detect mineralized nodules, bone marrow stromal cells fixed with 3.7% formaldehyde in phosphate buffered saline were stained with 2% alizarin red (all from Sigma-Aldrich) (DAHL, 1952). Images of the stained surfaces were acquired with a Coolpix 995 digital camera (Nikon Inc., Melville, NY). Digital images were imported in ImageJ software v 1.48d (National Institutes of Health, Bethesda, MD), converted to 8-bit grayscale and a threshold applied to define the boundaries of mineralized nodules, which were counted with the analyze particle function of ImageJ (Abramoff et al., 2004).

Statistical Analysis

Data are expressed as means ± SEM. Significance of differences between means were determined by Student's *t*-test or two-way analysis of variance with Student-Newman-Keuls's test analysis for pairwise or multiple comparisons, respectively (Sokal and Rohlf, 1981).

RESULTS

General Appearance of Col3.6-Nfatc2 Transgenic Mice is not Different from Controls

To test the function of Nfatc2 in the skeleton, the effects of the preferential Nfatc2 activation in osteoblasts were investigated in *Col3.6-Nfatc2* transgenic mice at 1 and 3 months of age. Two male *Col3.6-Nfatc2* transgenic founders were obtained, and only one male founder mouse transmitted the transgene to the offspring allowing the establishment of an *Nfatc2* transgenic line. *Col3.6-Nfatc2* transgenics were born at the expected Mendelian ratio, and their general appearance was normal. Analysis of total RNA from parietal bone by qRT-PCR demonstrated that *Nfatc2* mRNA levels were 4 to 11 fold higher in 1 and 3 month old *Col3.6-Nfatc2* transgenic male and female mice than in littermates of the same sex, confirming expression of the transgene (Fig. 2A). No difference in the weight of 1 or 3 month old *Col3.6-Nfatc2* transgenic mice and littermate wild type controls was observed. Femurs of *Col3.6-Nfatc2* female transgenic mice were not significantly different than those of control littermates of the same sex at 1 or 3 months of age (Fig. 2B).

Nfatc2 Activation in Osteoblasts Causes Osteopenia

Histomorphometric analysis of the distal femur revealed that 1 month old *Col3.6-Nfatc2* transgenic female mice had a 45% decrease in cancellous bone volume when compared to littermate controls of the same sex. This effect was secondary to decreased trabecular thickness and possibly to a reduced number of trabeculae (Table 1). Although osteoblast number was not different between *Col3.6-Nfatc2* transgenic female mice and controls, bone formation was suppressed by ~30% in transgenics, indicating that Nfatc2 inhibits the ability of osteoblasts to form bone (Table 1, Fig. 3). In contrast to the phenotype observed in female *Nfatc2* transgenics, 1 month old *Col3.6-Nfatc2* male transgenic mice did not exhibit osteopenia. Osteoblast number was higher than in littermate controls but bone formation was not increased, suggesting that Nfatc2 activation leads to the accumulation of dysfunctional osteoblasts in male mice (Table 1, Fig. 3). Osteoclast number and bone resorption were not affected by Nfatc2 activation in mice of either sex.

In agreement with the histomorphometric analysis, μ CT of 1 month old mice revealed that Nfatc2 activation in osteoblasts causes osteopenia in female but not in male mice (Table 2, Fig. 3). The reduced bone volume in female *Col3.6-Nfatc2* transgenics was associated with a tendency toward decreased connectivity density and an increased ratio of rod-like vs. plate-like trabeculae, indicating that Nfatc2 activation is detrimental to cancellous bone architecture in female mice (Table 2, Fig. 3). Microcomputed tomography of the femoral mid-shaft indicated that Nfatc2 activation in osteoblasts had no impact on cortical bone structure in either sex (Fig. 3, data not shown).

Microcomputed tomography of distal femurs of 3 month old mice revealed that cancellous bone microarchitecture and cortical bone structure were not different between 3 month old *Col3.6-Nfatc2* transgenic male or female mice and controls (data not shown). However, Nfatc2 activation caused a decrease in cancellous bone volume of L3 vertebrae in 3 month old male but not female mice (Table 3). The effect on vertebral cancellous bone was associated with decreased connectivity density and with a higher proportion of rod-like over

plate-like trabeculae. A tendency toward reduced trabecular number and increased trabecular separation were also noted in L3 vertebrae from *Col3.6-Nfatc2* transgenic male mice, but the effects failed to achieve statistical significance (Table 3).

Nfatc2 Activation Impairs Osteoblastogenesis In Vitro

To understand the mechanisms mediating the effects of Nfatc2 activation in osteoblasts, osteoblastogenesis was investigated in bone marrow stromal cells harvested from femurs of 6 week old *Col3.6-Nfatc2* transgenic mice and littermate controls. Gene expression analysis by qRT-PCR revealed that *Nfatc2* transcript levels were 3 fold higher in confluent cells from *Col3.6-Nfatc2* transgenic mice than in those from control mice (Fig. 4A). As the culture progressed, alkaline phosphatase activity and mineralized nodule formation increased in cells from *Col3.6-Nfatc2* transgenic mice to a lesser extent than in those from controls, indicating that Nfatc2 activation suppresses osteoblastogenesis (Fig. 4B and 4C). These findings are in agreement with the inhibitory effects of Nfatc2 on osteoblast function, an effect that could explain the osteoblastic phenotype observed *in vivo* (Zanotti et al., 2013).

DISCUSSION

In this study, we report that activation of Nfatc2 in osteoblasts causes a decrease in cancellous bone volume and is detrimental to trabecular bone microarchitecture, but Nfatc2 does not affect the cortical bone compartment. We created two *Nfatc2* transgenic founders, although only 1 transgenic line was established since one of the founders failed to generate an offspring. This could suggest that excessive levels of Nfatc2 in cells expressing the 3.6 kb fragment of the *Col1a1* promoter are deleterious to embryonic development. The *Col1a1* promoter fragment is not exclusively active in osteoblastic cells, and expression of the constitutively active Nfatc2 mutant in tissues other than bone may have prevented transmission of the transgene (Boban et al., 2006; Scheller et al., 2011). However, hemizygous *Col3.6-Nfatc2* transgenics appeared normal and had normal weight suggesting activation of Nfatc2 within the skeletal compartment.

The skeletal effects of Nfatc2 activation in osteoblasts *in vivo* are consistent with the effects of Nfatc2 in cells of the osteoblastic lineage *in vitro* and confirm an inhibitory role of Nfatc2 on osteoblast differentiation or function (Zanotti et al., 2013). Although the global *Nfatc2* inactivation causes osteopenia secondary to suppressed bone formation, it is important to note that global *Nfatc2* null mice exhibit a systemic inflammatory process that is probably responsible for the osteopenic phenotype (Koga et al., 2005). This would suggest that the downregulation of *Nfatc2* in cells of the immune system, and not in skeletal cells, is responsible for the osteopenia observed (Hodge et al., 1996; Monticelli and Rao, 2002; Wang et al., 2009). It is of interest that stromal cells obtained from *Nfatc2* null mice exhibit enhanced osteoblastic cell differentiation *in vitro*, results that are consistent with an inhibitory effect on Nfatc2 on osteoblastogenesis as reported in this and prior work from our laboratory (Bauer et al., 2011; Zanotti et al., 2013).

Age and sex influenced the skeletal phenotype of *Col3.6-Nfatc2* transgenics. Female mice exhibited osteopenia in the cancellous bone compartment of the femur at 1 month of age because of decreased bone formation, whereas male mice of the same age did not. This was

possibly related to an increase in osteoblast number resulting in the normalization of bone formation in male *Col3.6-Nfatc2* transgenics. However, these cells did not appear fully functional since bone formation was not increased, and the reason for their increased number remains without an obvious explanation. The phenotype in *Col3.6-Nfatc2* transgenics evolved, and at 3 months of age, the femoral trabecular bone architecture was restored in female transgenics but vertebral osteopenia was observed in male mice. These findings reiterate the influence of sex and age on trabecular bone architecture and on the function of skeletal cells (Glatt et al., 2007; Zanotti et al., 2014). They also underscore the importance of studying skeletal phenotypes in mice of both sexes independently and the characterization of mice at various ages. The resolution of the phenotype in female *Col3.6-Nfatc2* mice and the appearance of a vertebral phenotype in male transgenics at 3 months of age do not have an immediate explanation. However, these events do not appear to be related to a decline in the activity of the *Col1a1* promoter since *Nfatc2* mRNA levels in bone extracts remained elevated as the mice matured.

Nfatc2 activation suppressed bone formation in female mice and caused an increase in osteoblast number not accompanied by increased bone formation in male mice, confirming that activation of *Nfatc2* decreases osteoblast function (Zanotti et al., 2013). The reduced capacity of primary bone marrow stromal cells from *Col3.6-Nfatc2* transgenic mice to differentiate into osteoblasts is in agreement with the changes on osteoblast function observed *in vivo*. These findings are consistent with previous work from our laboratory demonstrating that overexpression of a constitutively active form of *Nfatc2* suppresses osteoblastic gene expression *in vitro* (Zanotti et al., 2013).

The DNA binding and regulatory domain of *Nfatc1* and *Nfatc2* have a high degree of sequence homology, and both *Nfat* proteins bind to similar DNA elements (Rao et al., 1997). Therefore, it is not surprising that activation of *Nfatc1* and *Nfatc2* in osteoblasts result in similar effects and that overexpression of a constitutively active form of *Nfatc1* suppresses osteoblastic function *in vitro* (Choo et al., 2009; Yeo et al., 2007a; Zanotti et al., 2011). The increased number of osteoblasts in the context of *Nfatc2* activation in male mice is consistent with the increased osteoblast number and disorganized bone formation reported in mice overexpressing a constitutively active form of *Nfatc1* in osteoblasts, although in this model an increase in bone volume was reported (Winslow et al., 2006). More importantly, expression of a dominant negative form of *Nfatc1* in cells of the osteoblastic lineage increased bone volume, findings that are consistent with a suppressive effect of *Nfat* in osteoblastic function (Sesler and Zayzafoon, 2013). Collectively, these observations suggest that *Nfatc1* and *Nfatc2* act through similar mechanisms to suppress osteoblast function and bone formation.

Nfatc2 activation in osteoblastic cells had no effect on osteoclast number or on bone resorption, suggesting that the effects of *Nfatc2* on the differentiation of cells of the osteoclastic lineage occur by direct targeting of osteoclast precursors (Ikeda et al., 2006). Conversely, mice expressing a constitutively active form of *Nfatc1* in osteoblasts exhibited an increase in the number of osteoclasts and bone resorption, in addition to the direct effects of *Nfatc1* on osteoclast precursors (Winslow et al., 2006). Our findings indicate that the effects of *Nfatc2* activation in cells of the osteoblastic lineage *in vivo* do not recapitulate

those of *Nfatc1* induction in osteoblasts on bone resorption, and that the effects of *Nfatc1* and *Nfatc2* in skeletal cells are not redundant. This contention is also supported by studies on models of global inactivation of *Nfatc1*, resulting in embryonic lethality, and of *Nfatc2* resulting in a distinct generalized inflammatory phenotype (Hodge et al., 1996; Monticelli and Rao, 2002; Ranger et al., 1998).

In conclusion, activation of *Nfatc2* in osteoblasts decreases cancellous bone volume by inhibiting osteoblastic function.

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Fig. 1. Design of the *Col3.6-Nfatc2* transgene

A 3.6-kb fragment of the rat *Col1a1* promoter cloned upstream a Kozak sequence (Kozak), and DNA coding for the human influenza hemagglutinin (HA), murine *Nfatc2* harboring mutations that lead to the expression of a constitutively active protein (ca*Nfatc2*), and the bovine growth hormone polyadenylation signal (BGH pA).

Fig. 2. *Col3.6-Nfatc2* transgenic mice overexpress *Nfatc2* and have a normal general appearance Male or female *Col3.6-Nfatc2* transgenic mice (*Nfatc2*, black bars and filled circles) were compared to littermate wild type controls of the same sex (Control, white bars and open circles). In panel A, total RNA was extracted from parietal bones of 1 and 3 month old mice, and mRNA reverse-transcribed and amplified by qRT-PCR in the presence of specific primers. Data are expressed as *Nfatc2* copy number, corrected for *Gapdh* copy number. Values are means $\pm$ SEM, n = 4. * Significantly different between *Nfatc2* and Control, $p < 0.05$. Panel B, weight and femoral length of 1 and 3 month old mice. Values are means $\pm$ SEM, n = 4 – 9.

Fig. 3. One month old *Col3.6-Nfatc2* transgenic female mice are osteopenic

Microcomputed tomography of proximal trabecular bone (top) and cortical bone at the mid-shaft (middle) of femurs, and toluidine blue staining of proximal femurs sections (bottom), from representative 1 month old male or female *Col3.6-Nfatc2* transgenic mice (Nfatc2), and littermate wild type controls of the same sex (Control). Histological images were acquired at a 400× magnification, and image size was doubled digitally with Photoshop CS6 (Adobe Systems Incorporated, San Jose, CA) after acquisition to achieve an 800× magnification. Red arrows indicate osteoblasts.

Fig. 4. Nfatc2 activation suppresses osteoblastogenesis *in vitro*

Bone marrow stromal cells were harvested from femurs of 1 month old *Col3.6-Nfatc2* transgenic mice (Nfatc2, black bars), or littermate wild type controls (Control, white bars). Cells were pooled from mice of both sexes and cultured under conditions favoring osteoblastogenesis. In panel A, total RNA was extracted, and mRNA reverse-transcribed and amplified by qRT-PCR in the presence of specific primers. Data are expressed as *Nfatc2* copy number corrected for *Rpl38* copy number. Values are means $\pm$ SEM, $n = 4$. In panel B, alkaline phosphatase activity was determined and data are expressed as nanomoles of p-nitrophenol/min/ μ g of total protein. Values are means $\pm$ SEM, $n = 6$. In panel C, cultures were fixed with 4% formaldehyde in PBS and stained with alizarin red. Digital images of the cultures were acquired, and nodules counted with the analyze particles command of ImageJ software. Data are expressed as number of nodules/cm². Values are means $\pm$ SEM, $n = 6$. * Significantly different between Nfatc2 and Control, $p < 0.05$; + significantly different from Day 0 within Nfatc2 or Control cells, $p < 0.05$.

Table 1

Histomorphometry of the distal femur of 1 month old male or female *Col3.6-Nfatc2* transgenic mice (Nfatc2), or littermate wild type controls of the same sex (Control).

Histomorphometry	Males		Females	
	Control	Nfatc2	Control	Nfatc2
Bone Volume/Tissue Volume (%)	6.3 ± 0.9	6.6 ± 0.6	8.5 ± 1.3	4.7 ± 0.7*
Trabecular Thickness (μm)	27.0 ± 1.2	26.8 ± 1.3	30.0 ± 1.3	24.5 ± 1.1*
Trabecular Number	2.3 ± 0.3	2.5 ± 0.5	2.8 ± 0.3	1.9 ± 0.2 ⁺
Osteoblast Surface/Bone Surface (%)	15.2 ± 2.5	28.1 ± 1.6*	27.1 ± 1.7	24.1 ± 1.7
Osteoblasts/Bone Perimeter (mm ⁻¹)	17.8 ± 2.8	32.1 ± 1.8*	30.3 ± 2.0	27.2 ± 2.2
Osteoid Surface/Bone Surface (%)	1.5 ± 0.5	2.4 ± 1.1	2.4 ± 0.6	1.6 ± 0.6
Osteoclast Surface/Bone Surface (%)	11.6 ± 1.0	11.0 ± 0.8	10.2 ± 0.7	11.2 ± 1.2
Osteoclasts/Bone Perimeter (mm ⁻¹)	8.4 ± 0.7	7.9 ± 0.6	7.4 ± 0.5	8.7 ± 0.8
Eroded Surface/Bone Surface (%)	22.2 ± 2.0	21.5 ± 1.5	19.3 ± 1.3	22.2 ± 2.0
Mineral Apposition Rate (μm day ⁻¹)	1.90 ± 0.06	2.16 ± 0.17	2.17 ± 0.16	1.80 ± 0.08 ⁺
Mineralizing Surface/Bone Surface (%)	6.6 ± 0.8	5.5 ± 0.5	6.1 ± 0.4	4.7 ± 0.6 ⁺
Bone Formation Rate (μm ² μm ⁻³ day ⁻¹)	0.125 ± 0.017	0.115 ± 0.004	0.129 ± 0.009	0.089 ± 0.013*

Values are means ± SEM; n = 6 – 9.

* Significantly between Nfatc2 and Control, $p < 0.05$;

⁺ $p < 0.08$.

Table 2

Microcomputed tomography (μ CT) of the distal femur of 1 month old male or female *Col3.6-Nfatc2* transgenic mice (Nfatc2), or littermate wild type controls of the same sex (Control).

μ CT	Males		Females	
	Control	Nfatc2	Control	Nfatc2
Bone Volume Fraction (%)	4.8 ± 0.4	4.4 ± 0.8	4.7 ± 0.5	$3.7 \pm 0.1^*$
Trabecular Separation (μ m)	177 ± 6	202 ± 24	185 ± 7	196 ± 6
Trabecular Number (mm^{-1})	5.7 ± 0.2	5.2 ± 0.6	5.5 ± 0.2	5.2 ± 0.1
Trabecular Thickness (μ m)	19.1 ± 0.3	19.6 ± 0.4	19.0 ± 0.6	18.4 ± 0.2
Connectivity Density (mm^{-3})	263 ± 63	219 ± 69	245 ± 54	$124 \pm 24^+$
Structure Model Index	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	$3.3 \pm 0.1^+$

Values are means $\pm$ SEM; n = 4 – 5.

* Significantly between Nfatc2 and Control, $p < 0.05$;

⁺ $p < 0.08$.

Table 3

Microcomputed tomography (μ CT) of L3 vertebrae of 3 month old male or female *Col3.6-Nfatc2* transgenic mice (Nfatc2), or littermate wild type controls of the same sex (Control).

μ CT	Males		Females	
	Control	Nfatc2	Control	Nfatc2
Bone Volume Fraction (%)	5.7 $\pm$ 0.7	3.0 $\pm$ 0.3*	5.1 $\pm$ 0.1	5.3 $\pm$ 0.2
Trabecular Separation (μ m)	248 $\pm$ 7	283 $\pm$ 16 ⁺	265 $\pm$ 9	242 $\pm$ 18
Trabecular Number (mm^{-1})	4.1 $\pm$ 0.1	3.4 $\pm$ 0.2 ⁺	3.9 $\pm$ 0.1	4.3 $\pm$ 0.3
Trabecular Thickness (μ m)	26 $\pm$ 1	23 $\pm$ 1	25 $\pm$ 2	25 $\pm$ 1
Connectivity Density (mm^{-3})	197 $\pm$ 27	54 $\pm$ 25*	181 $\pm$ 68	215 $\pm$ 12
Structure Model Index	2.5 $\pm$ 0.1	3.0 $\pm$ 0.2*	2.8 $\pm$ 0.2	2.7 $\pm$ 0.1

Values are means $\pm$ SEM; n = 4 – 5.

* Significantly between Nfatc2 and Control, $p < 0.05$;

⁺ $p < 0.08$.