Targeted disruption of nuclear factor erythroid-derived 2-like 1 in osteoblasts reduces bone size and bone formation in mice

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OSTEOPOROSIS IS A DEGENERATIVE bone disease that is a threat to millions of Americans. In patients with osteoporosis, bone mineral density (BMD) is reduced and bone microarchitecture is deteriorated due to the loss of bone volume and minerals (10). A loss of bone density and strength cause bones to become fragile and, in turn, leads to frequent fractures and other serious effects. There are two major known causes of osteoporosis: low peak BMC that is achieved at the end of the adolescent growth period and high bone loss rate that occurs after menopause and during aging. Therefore, studies that focus on the mechanisms regulating bone accretion during active growth periods and bone loss during aging are of considerable importance in the prevention and treatment of osteoporosis.

Nuclear factor erythroid-derived 2-like 1 (NFE2L1), also known as Nrf1/TCF11/LCR-F1, NFE2L2, and NFE2L3 belong to a family of basic leucine zipper proteins (bZIP) and function as transcription factors that bind to the same antioxidant response element (ARE) of DNA in the regulatory regions as the hematopoietic-specific p45NF-E2, as heterodimers with other bZIP proteins like small musculoaponeurotic fibrosarcoma (Maf) (2, 15, 29). The small Mafs include MafF, MafG, and MafK factors, which are widely expressed and can function as activators or repressors of target gene transcription (24, 25). The specificity of NFE2L2 proteins in transcriptional regulation depends on the posttranslational modification and its interactions with other cofactors (5, 19, 34, 41). Transactivation of NFE2L1 and NFE2L2 in hepatocytes by antioxidants or oxidative stress can modulate gene expression of phase II detoxifying enzymes as well as oxidative stress-inducible proteins in different tissues for cellular growth, survival, heme biosynthesis, and antioxidant responses (3, 5, 28, 39, 40). However, activation of NFE2L3 negatively regulates ARE-mediated expression and antioxidant induction of the NAD(P)H-quinine oxidoreductase I gene (32). Targeted disruption of NFE2L1 in every cell type in mice resulted in anemia and embryonic lethality, while conditional knockout (KO) of NFE2L1 in the liver caused nonalcoholic steatohepatitis, apoptosis, fibrosis, and hepatic cancer (3, 39). Loss of NFE2L2 increased susceptibility to the toxicity of electrophiles and reactive oxygen species with normal growth, while the mice with deletion of the NFE2L3 gene failed to reveal obvious phenotypic differences compared with wild-type (WT) animals (4, 7). The findings that mice lacking NFE2L1 exhibit more severe phenotypes than the other two family members of NFE2L2 and NFE2L3 provide evidence that NFE2L1 could have an indispensable role in regulating cell differentiation and/or cell survival, in addition to protection from electrophilic stress.

Both NFE2L1 and NFE2L2 are expressed in bone cells (13, 14, 27). Narayanan et al. (27) found that NFE2L1 interacts with CCAAT enhancer binding protein-β in a phosphorylation-dependent manner and that this interaction is critical for regulation of dentin sialophosphoprotein gene expression during odontoblast differentiation. In contrast to the function of NFE2L1, NFE2L2 has been shown to be a negative regulator of chondrocyte differentiation (14). It also inhibits osteoblast differentiation and maturation via interaction with Runx2 and suppression of Runx2-dependent transcriptional activity (13). The findings from these in vitro studies strongly suggest that NFE2L1 and NFE2L2 may have distinct functions in bone.
However, little is known about the role of NFE2L1 or NFE2L2 in osteoblast differentiation and bone formation in vivo. In previous studies in mice, we and others found that deletion of the gulonolactone oxidase gene, which is involved in the synthesis of an antioxidant ascorbic acid, was responsible for ascorbic acid deficiency and impairment of differentiated functions of osteoblast, bone fracture, and premature death in mice with spontaneous fractures (18, 22). Treatment of the mutant mice with ascorbic acid in drinking water completely rescued the bone phenotypes in vivo and prevented premature death. In further studies on ascorbic acid regulation of transcription factors known to be critical for osteoblast differentiation, we found that ascorbic acid treatment caused an acute increase in osterix expression in bone marrow stromal (BMS) cells via stimulating interaction of NFE2L1 with ARE in the promoter region. Based on our in vitro data that NFE2L1 regulates genes critical for osteoblast differentiation, we hypothesized that NFE2L1 expressed in osteoblasts plays a key role in regulating bone formation. To test this hypothesis, we generated osteoblast-specific NFE2L1 conditional KO mice and examined the role of NFE2L1 in bone formation in vivo. Our findings provide the first experimental evidence that NFE2L1 is involved in regulating osterix expression, osteoblast differentiation, and peak bone mass in mice.

MATERIALS AND METHODS

Plasmid constructs, cell lines, and antibodies. The full-length cDNA of CRE recombinase containing a nuclear localization signaling derived from SV40 T large antigen at the NH2 terminus was amplified by PCR and cloned into the sites of HindIII and XhoI of pAdTrack-CMV (20). Ad293 cells were purchased from Stratagene. Plasmids of pAdTrack-CMV, pAdTrack, and pAdEasy-1 were kindly provided by Dr. Bert Vogelstein of Howard Hughes Medical Institute at Johns Hopkins (Baltimore, MD). Antibodies specific to mouse NFE2L1 and β-actin were from ProteinTech Group (Chicago, IL) and Sigma (St. Louis, MO), respectively.

Generation of mutant mice. The mice that contain two loxp sites flanking the last exon of NFE2L1 (NFE2L1lox/lox) with mixed genetic background of C57BL/6J and 129S4 strains were described previously (39). The Col1α2-iCre transgenic mice expressing improved CRE recombinase (iCre) were also reported (9). NFE2L1 conditional KO mice were generated by crossing NFE2L1lox/lox female with a Cre transgenic male under the control of regulatory sequences of the col1α2 gene to generate Cre+, NFE2L1lox/lox conditional KO mice, and Cre−, NFE2L1lox/lox control mice (Fig. 1A). All mice were housed at the Jerry L. Pettis Memorial VA Medical Center Veterinary Medical Unit (Loma Linda, CA) under standard approved laboratory conditions with controlled illumination (14 h light, 10 h dark) and temperature (22°C) and unrestricted food and water. All of the procedures were performed with the approval of the Institutional Animal Care and Use Committee of the Jerry L. Pettis Memorial VA Medical Center. Genotyping of the NFE2L1 gene and iCre transgene was monitored by PCR using DNA extracted from tail snips as reported (9, 39).

Skeleton staining. Alizarin red and alcian blue staining were performed according to established methods (21). In brief, embryonic day (E) 19.5 embryos were deskinmed, eviscerated, and fixed in 95% ethanol for 5 days. The carcasses were fixed in acetone for 4 days and then stained for 3 days in a solution containing 0.1% alizarin red, 0.3% alcian blue, acetic acid, and 70% ethanol (1:1:1:17, vol/vol/vol/vol). The embryos were then transferred to a solution of 1% KOH in 20% glycerol until clear and then stored in glycerol.

Evaluation of bone phenotypes. Total bone mineral content (BMC) and bone mineral density (BMD) were measured by dual-energy X-ray absorptiometry (DEXA) by using the PIXIms instrument (LunarCorp., Madison, WI) as described previously (11, 12). The PIXIms small animal DEXA system has been reconfigured with lower X-ray energy than in human DEXA machines to achieve optimal contrast in small specimen. The resolution of the PIXIms is 0.18 × 0.18 mm pixels with a usable scanning area of 80 × 65 mm, allowing for measurement of single whole mice and collections of isolated specimens. The volumetric BMD and geometric parameters at the middiaphysis of the femur isolated from 8 wk old mice were determined by peripheral quantitative computed tomography (pQCT; Norland Stratec XCT 960M, Stratec Medizintechnik, Madison, WI) as reported (12). The voxel size was set at 0.07 mm, and a 0.5 mm thick slice was scanned through the entire length of bone. The reference line as a center of scanning was set at the midpoint of the femur; thereafter, the nine slices were scanned symmetrically from the reference line. The data for the middle three slices were used for determination of periosteal and endosteal circumferences. Cortical and trabecular bone microarchitectures of the femur isolated from 8 wk old mice were assessed by using μ-CT (VIVA CT-40, Scanco). The femurs isolated from 8 wk old mice were scanned by X-ray (55–70 kVp volts). The voxel size was 10.5 μm. Reconstruction analysis was performed with SCANCO software (SCANCO Medical, Bruttisellen, Switzerland). The sections of 1 mm at the middiaphysis were analyzed for cortical measurements and a fixed section of 1.8 mm starting at 0.36 mm proximal to the growth plate was analyzed for trabecular measurements by using SCANCO software. The bones analyzed were adjusted for length so that the region of interest chosen for cortical and trabecular bone parameters was anatomically the same between the mutant bone and control littersmates’ bone.

Mechanical strength test. After pQCT measurements, mechanical strength test was performed using a model 8841 mechanical tester (Instron, Canton, MA) as described previously (23). Three-point bending strength was measured at the middiaphysis. Each bone was compressed at a constant rate of 2 mm/min until failure. Breaking force was defined as bending load at failure. Elastic modulus and moment of inertia were calculated as previously described (17).

Dynamic calcein labeling and histomorphometry. Three-week-old mice were injected intraperitoneally with calcein 7 days (20 mg/kg) and 2 days prior to the expected day of euthanization to label mineralizing bone surface. Mouse femurs were fixed in 10% formalin overnight. The bones were washed, dehydrated, and embedded in methyl methacrylate resin for sectioning. Longitudinal sections of comparable anatomic position of the femurs were analyzed by fluorescence microscopy. For analysis of cortical bone formation parameters, middiaphysis of left femurs were used as a sampling site. Evaluation of bone resorption parameters, the right femurs were partially demineralized, embedded in glycomethacrylate, and cut into sections. The sections were stained for tartrate-resistant acid phosphatase (TRAP), and the TRAP-covered surface was measured. All bone histomorphometric parameters were measured as previously described (1, 31). Mineral apposition rate (MAR) and bone formation rate (BFR/bone surface (BS)) were calculated as described previously (30).

Serum assay. Serum osteocalcin levels were measured by a radioimmunoassay using reagents purchased from Biomedical Technologies (Stoughton, MA). We have previously validated this assay for measuring mouse osteocalcin (22, 33). The sensitivity of the assay is 0.5 ng/ml. The inter- and intra-assay coefficients of variation are <8%.

Immunohistochemistry analysis. Immunohistochemistry was used to examine the expression of CRE recombinant protein as described previously (12). Briefly, tissue sections from E19.5 embryos were first heated in citrate buffer (pH 6.0) for 35 min at 95°C for antigen retrieval and blocked with 3% H2O2 and 20% normal goat serum, respectively. The sections were then incubated with an antibody specific to Cre (Novagen Laboratories) at 1:2,000 dilutions at 37°C for 1 h. Subsequent detection procedures, including biotinylated anti-rabbit IgG antibody, streptavidin-horseradish peroxidase (Vector...
Labs, Burlingame, CA), and 3–3′ diaminobenzidine tetrahydrochloride-peroxide (DAB-H2O2) were performed with an automated Ventana ES immunostainer (Ventana Medical Systems, Tucson, AZ) according to the manufacturer’s instructions.

Primary cell culture. Primary osteoblasts were isolated from the calvaria of 4 day to 2 wk old NFE2L1 conditional KO or WT mice by using a modified sequential digestion described previously (6) and plated at 1.5 × 10^5/cm^2 (1.5 × 10^5/well) in 35 mm six-well culture plates in -minimal essential medium (-MEM) containing 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml). The cells were cultured until 80–90% confluent prior to experiments. BMS cells derived from the femurs and the tibias of NFE2L1 conditional KO and corresponding control mice at 3–4 wk old were cultured as reported previously (38). The cells were cultured for 6 days until 80% confluence, followed by treatment and RNA extraction.

Nodule assay. BMS cells were grown to 80% confluence as described above. The cells were treated with mineralization media containing 10 mM β-glycerophosphate, 50 μg/ml of ascorbic acid, and 10% FBS for 24 days. The cells were washed with PBS, fixed in cold 70% ethanol, and stained with 40 mM alizarin red (pH 4.2). The mineralized area was measured with the OsteoMeasure system equipped with a digitizing tablet and a color video camera (Osteometrics, Atlanta, GA).

Adenoviral generation and transduction. Adenoviruses were generated in Ad293 cells as described previously (20). Transient transfection was carried out in Ad293 cells by using LipofectAMINE Reagent according to the manufacturer’s instruction (Invitrogen). Forty-eight hours after transfection, cells were collected in PBS, lysed by four rounds of freeze-thaw cycles, and spun at 10,000 g for 10 min. The supernatant containing adenovirus was then filtered through a
0.45 μm filter and stored at −80°C until use. Titers were determined by infecting HEK-293 cells with serial dilutions and examining green fluorescent protein (GFP) expression of infected cells by flow cytometry 24 h after infection. Primary calvarial osteoblasts were transduced by adding the supernatant containing adenoviral particles into the six-well culture plates for 24 h, followed by washing three times and experimental treatments.

RNA extraction and quantitative PCR. RNA was extracted from primary cultures or bone marrow–free femurs and tibias of 4 wk old NFE2L1 KO and corresponding WT littermates as described previously (37, 38). An aliquot of RNA (2 μg) was reverse-transcribed into cDNA in 20 μl volume of reaction by oligo(dT)12–18 primer. Real-time PCR contained 0.5 μl template cDNA, 1× SYBR GREEN master mix (Qiagen), and 100 nM of specific forward and reverse primers in 25 μl volume of reaction. Primers used for real-time PCR are listed in Table 1.

Western blot analyses. Primary osteoblasts were isolated from 2 wk old mice and cultured in α-MEM containing 10% FBS for 3 days. The cells were then lysed, and the total cellular extracts were used for immunoblotting analyses with specific antibodies to NFE2L1 and β-actin as described previously (38).

Statistical analyses. Data were analyzed by ANOVA or Student’s t-test.

RESULTS

Characterization of osteoblast-specific conditional KO mice. To test our hypothesis, we generated osteoblast-specific conditional NFE2L1 KO mice by using Cre-loxP approach as shown in Fig. 1A. Two generations of breeding NFE2L1loxP mice with transgenic Cre mice yielded 50% Cre+ conditional KO mice with deletion of the last exon of NFE2L1, and 50% Cre− control mice. To examine the specificity of iCre expression in the cells that express Col1α2, we performed immunohistochemistry on the tissue sections of E19.5 conditional KO embryo. Consistent with our previous report (12), we observed the expression of NFE2L1 protein was undetectable in osteoblasts from Cre+ KO mice but was detected in the cells from Cre− WT mice (Fig. 1C).

NFE2L1 deficiency in osteoblasts caused a reduction in bone size, peak bone mass, trabecular number, and mechanical strength. In contrast to total NFE2L1 KO, which led to death prior to birth, mice with conditional disruption of NFE2L1 in osteoblasts, although smaller, survived to adulthood (Fig. 2A). The body weight was reduced by 10% in both male and female mice compared with littermate controls. Skeletal staining of mutant embryo showed reduced bone size and impaired mineralization in calvaria and palmar as indicated by arrows in Fig. 2B. To determine if the targeted disruption of NFE2L1 in type I collagen–producing cells impairs peak bone mass, we measured skeletal parameters in 3, 4, 5, and 8 wk old mice using DEXA. We found that total body BMC was reduced by 8–10%, while total body bone area was reduced by 9–11% in NFE2L1 KO mice compared with the sex-matched littermate controls during the 3–8 wk time course of this study. Although total body areaal BMD was 2–5% less in the KO mice compared with corresponding WT littermates during the entire time course, this difference was not statistically significant (Fig. 2C). We observed the expected sex differences in growth parameters after 4 wk of age with males being larger than females in both KO and WT mice. However, we did not observe a sex-by-strain interaction for either total body BMC or total bone area (data not shown).

Consistent with the reduced body weight, femur length was reduced by 7% in the female and male NFE2L1 conditional KO mice compared with WT controls (Fig. 3, A and B). pQCT analysis of the femurs at the middiaphysis revealed that both peristeal circumference and endosteal circumference were reduced by 6% in the conditional KO mice compared with the littermate controls. The cortical thickness of diaphysis between the KO and WT mice was comparable (Fig. 3, C–E). To determine if lack of NFE2L1 in osteoblasts affects trabecular microstructure, μCT was performed at the distal metaphysis of the femurs. We found that trabecular numbers and trabecular bone volume/total volume were reduced by 56 and 30%, respectively, while trabecular spacing increased by 2.5-

Table 1. Primer sequences for RT real-time PCR

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<th>Gene</th>
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<td>5’-TCAGTGAAGGAAGGATGATTG 5’-TCAGTGAAGGAAGGATGATTG</td>
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<tr>
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<tr>
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<tr>
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fold in the distal femurs isolated from the NFE2L1 KO mice compared with corresponding controls (Fig. 4, A–D).

To identify the target cell types and cellular processes that contribute to reduced peak bone mass in the NFE2L1 conditional KO mice, we performed histomorphometric studies in 3 wk old mice. Figure 4E shows a decreased width of newly formed bone between two calcein labels in the NFE2L1 conditional KO mice compared with control littermates. Consistent with the PIXImus and pQCT data, histology analyses revealed that total bone area and medullary area were reduced by 14 and 16%, respectively, in the mutant mice (Table 2). The periosteal BFR was reduced by 35% in the KO mice compared with corresponding WT mice. Because of reduced bone size in the KO mice, we adjusted BFR for BS. The BFR/BS was reduced by 32% at the periosteum of the femur midshaft in the KO mice (Table 2). To determine if the reduced BFR is caused by reduced activity of osteoblasts, we measured MAR and found a 17% reduction in the MAR in the KO mice compared with the WT littermates that was not statistically significant.

We next determined if loss of NFE2L1 in cells of osteoblast lineage influences bone resorption. Table 2 also shows the data from TRAP staining for periosteal and endosteal surfaces examined at the middiaphysis of the femurs of 3 wk old mice. The percentage of TRAP-labeled surface at both periosteum and endosteum was not affected in the conditional KO mice compared with the littermate controls. The lack of difference in bone resorption between conditional KO and WT mice was not surprising because Cre-LoxP recombination happened primarily in osteoblasts but not in osteoclasts (12).

Deficiency of NFE2L1 in osteoblasts in the KO mice caused a 15 and 45% reduction in load-bearing capacity and area of moment of inertia, respectively, compared with the WT control littermates (Table 3). However, the elastic modulus was not significantly affected in the conditional KO mice compared with the controls.

Lack of NFE2L1 in osteoblasts impaired osterix expression and cell differentiation. To determine the cause for reduced osteoblastic function, we measured serum osteocalcin, a bone formation marker, and expression levels of osterix and Sox9, transcription factors that are critical in osteoblast and chondrocyte differentiation. Total RNA was extracted from the marrow-free femurs and tibias of 4 wk old KO mice and corresponding littermates and used for real-time PCR with specific primers shown in Table 1. We observed that serum osteocalcin was reduced by 20% in the NFE2L1 conditional KO mice compared with control littermates (Fig. 5A). The expression of osterix was decreased by 57%, while Sox9 expression was not changed in the bones of NFE2L1 conditional KO mice (Fig. 5B). To determine if loss of NFE2L1 expression in osteoblasts leads to altered expression of other members of bZIP family,
we examined expression levels of NFE2L family members in bones isolated from KO and WT mice. As expected, mRNA level of NFE2L1 in bone marrow-free bones of the conditional KO mice was decreased by 90%. The loss of NFE2L1 in osteoblasts was associated with an increase in the expression of NFE2L2 mRNA but not NFE2L3 transcript in the bones isolated NFE2L1 conditional KO mice compared with corresponding control mice. In fact, the expression of NFE2L3 was reduced by 60% in the bones of KO mice. The expression of other bZIP family members such as Bach1, Bach2, Maf G, Maf K, and Keap was largely unaffected.

Fig. 3. Reduced femur bone size and bone area in NFE2L1 conditional KO mice. A: femurs of WT and KO mice at 8 wk of age. B: femur length of mixed-sex WT and KO mice at 8 wk of age (n = 10). C: μCT images of cross section at middiaphysis, and cortical thickness (Crt Thk) measured by peripheral quantitative computed tomography (pQCT) (n = 8). D & E: periosteal circumference (PC) and endosteal circumference (EC) of the femurs at middiaphysis, measured by pQCT (n = 8). *Significant difference in KO mice compared with WT mice (P < 0.05).

Fig. 4. Reduced trabecular numbers and periosteal bone formation in NFE2L1 conditional KO mice. Trabecular numbers (Tb. N) (A), trabecular space (Sp.) (B), trabecular bone volume/total bone volume (BV/TV) (C), and representative 3-D images (D) of distal femurs isolated from 8 wk old mice, analyzed by μ-CT. E: images of calcein double labeling of the periostium of the femurs from 3 wk old WT and KO mice (×200). Arrows indicate labeled periosteal bone formation. *Significant difference in KO mice compared with WT mice (P < 0.05, n = 8).
To determine if the reduced bone formation in the NFE2L1 conditional KO mice is caused by impairment in osteoblast differentiation, we used an in vitro bone formation assay with BMS cells derived from NFE2L1 conditional KO and WT control mice. We found that the amount of mineralized nodule was reduced by 68% in BMS cells derived from NFE2L1 conditional KO mice compared with BMS cells from littermate control mice after 24 days of culture in a mineralization medium (Fig. 6, A and B). To further test our model that the ascorbic acid effect on osterix expression involves NFE2L1 in the cells with the same genetic background, we deleted the NFE2L1 gene in neonatal calvarial cells derived from NFE2L1 floxed mice by adenovirus that expresses iCre in vitro. The control cells were infected with the same multiplicity of infection of adenovirus expressing GFP. Infected cells were treated with differentiation medium in the presence or absence of ascorbic acid for 24 h, followed by RNA extraction and RT-PCR to examine the expression of NFE2L1 and genes that are critical for osteoblast differentiation. As shown in Fig. 6C, NFE2L1 expression was almost undetectable in the cells infected with Ad-iCre but was expressed in the control cells that express GFP by RT-PCR. Real-time PCR analyses found the transcript of NFE2L1 was knocked down by 98% in the Ad-iCre infected cells compared with the Ad-GFP transduced cells. Deficiency of NFE2L1 in osteoblast cells diminished ascorbic acid stimulation of osterix expression by 50% in the cells expressing iCre compared with the cells expressing GFP (Fig. 6D). However, the expression levels of other transcription factors that have been predicted to regulate osteoblast differentiation such as Runx2, Dlx3, Dlx5, Msx1, and Msx2 were unaffected either in the presence or absence of ascorbic acid upon treatment of NFE2L1 ioxP osteoblasts with adenoviral Cre (Fig. 6D).

**DISCUSSION**

NFE2L1 has been shown to play important roles in the growth and differentiation of various types of cells in the liver and bone. Although NFE2L1 and its family member NFE2L2 have been implicated in the regulation of bone cell differentiation in vitro, and total loss of NFE2L1 function in somatic cells resulted in perinatal lethality and anemia in mice, little is known on the role of NFE2L1 produced locally by bone cells. In this study, we deleted NFE2L1 gene specifically in the cells of osteoblastic lineage and examined the consequence of conditional disruption of NFE2L1 in bone cells on the skeletal phenotypes in vivo. Our results show that targeted disruption of NFE2L1 gene in osteoblasts resulted in decreased bone size, impaired bone formation and mineralization, reduced BMC, trabecular bone volume, and mechanical strength. The reduced bone formation and bone mineralization in NFE2L1 conditional KO mice were caused by impairment in the differentiated functions of osteoblasts and reduced expression of osterix. Our findings strongly support our hypothesis that NFE2L1 locally produced by osteoblasts is an important regulator of osteoblast activity, bone formation, and peak bone mass.

The strength and tissue specificity of rat type 1 collagen promoter-directed gene targeting in type I collagen producing cells by Cre-loxP approach depend on the integrity of regulatory elements of the promoter and other regulatory sequences and the expression level of Cre recombinase in transgenic mice. In our study, we used a well-characterized transgenic mouse line that expresses a codon-optimized Cre recombinase under the control of a 100 kb promoter/enhancer unit of the collagen-1α2 gene to disrupt NFE2L1 in cells of osteoblastic lineage (9, 12). The Cre was fused to a part of SV40 large T nuclear localization signal sequence at the NH2 terminus so that it localizes in the nuclei of the target cells and efficiently excises NFE2L1 gene in both premature and mature osteoblasts derived from mesenchymal origin in embryonic as well as adult tissues (9, 12). Consistent with our previous studies on the conditional KO of osteoblast-specific IGF-I gene, our conditional disruption of NFE2L1 directed by collagen-1α2 in P1 artificial chromosome in cells of osteoblast lineage was almost complete as evidenced by lack of detectable NFE2L1 protein by Western blot in osteoblasts of conditional KO mice. Targeted disruption of the NFE2L1 gene specifically in cells of osteoblastic lineage was further confirmed by immunohistochemistry of iCre expression. The reduced bone area, trabecular volume, and BFR/BS were significant in the conditional KO mice. However, unlike the 2.3 kb rat collagen-1 promoter, which has been shown to be active in osteoclasts (8), the promoter/enhancer unit of the collagen-1α2 used in our gene targeting system is inactive in osteoclast lineage cells as evidenced by lack of detectable iCre in TRAP-stained bone tissues. Accordingly, the percentage of TRAP-labeled bone surface was not significantly different between NFE2L1 KO and WT mice. In addition, the expression level of NFE2L1 in the spleen cells from the conditional KO mice was comparable to the cells from WT mice (data not shown). Thus, the observed skeletal changes in the NFE2L1 conditional KO mice are likely caused by the disruption of osteoblast-derived, but not osteoclast-derived, NFE2L1.
We chose femurs for histomorphometric analyses because bone size is reduced at this site. Our histomorphometric data revealed that the reduced femur bone size in the conditional KO mice was due to reduced periosteal bone formation rate and not increased bone resorption rate. The reduced bone size in NFE2L1 KO mice was consistent with smaller bones found in osterix KO mice. In addition to reduced bone size, mineralization was affected in bones formed by both intramembranous and endochondral bone formation routes in osterix KO mice. Similarly alizarin red staining of new born NFE2L1 conditional KO mice revealed delayed mineralization in both skull bones formed by intramembranous route and long bones formed by endochondral bone formation route. We, therefore, believe that bone formation through both the intramembranous and endochondral routes is affected in the conditional KO mice.

NFE2L1 and its binding partners including bach1, bach2, Matf, MatK, and knap-1 are expressed in BMS cells and recognize ARE sequence to regulate the target gene in a positive or negative manner. Among osteoblast differentiation genes, osterix has been found to bear a well-conserved ARE in the promoter region to which the activated NFE2L1 can bind in the presence of ascorbic acid (38). Consistent with a model that NFE2L1 is involved in mediating ascorbic acid induction of osterix expression in osteoblasts are the findings of reduced osterix expression in the bones isolated from NFE2L1 KO mice compared with corresponding WT mice, and blockade of ascorbic acid-induced osterix expression in NFE2L1 loxP osteoblasts in which NFE2L1 expression is disrupted by treatment with adenovirus expressing Cre in vitro. While our in vitro studies on BMS differentiation and in vivo data on the skeletal phenotypes of mice lacking NFE2L1 specifically in cells of osteoblast lineage are consistent with our hypothesis that NFE2L1 is involved in mediating ascorbic acid-induced osterix expression, osteoblast differentiation, and peak bone mass, the phenotypes of NFE2L1 conditional KO were modest at best compared with spontaneous fracture phenotype in mice with deletion of gulonolactone oxidase gene that are deficient in vitamin C synthesis (22). Sfx mutant mice are born normal and grow normally until weaning because they get adequate vitamin C from mother’s milk (22). After weaning, bone formation is severely compromised, thus resulting in spontaneous hip fractures. There are a number of potential explanations for the differences in the severity of skeletal phenotype between sfx and NFE2L1 conditional KO mice. First, it is possible that the iCre-mediated deletion of NFE2L1 may not be complete in cells of osteoblastic lineage and that a small amount of NFE2L1 is sufficient to mediate ascorbic acid effects. In this regard, our immunoblot data showed the Cre-mediated deletion of NFE2L1 was almost complete in calvarial osteoblasts derived from conditional KO mice. In addition, our previous studies involving disruption of the IGF-I gene in osteoblasts using the same transgenic iCre line exhibited a drastic skeletal phenotype (12). Thus, it appears unlikely that incomplete deletion of NFE2L1 is the cause for the observed less severe skeletal phenotype in the NFE2L1 conditional KO mice than the sfx mutant mice. Second, other members of NFE2L family could compensate for the loss of NFE2L1 in the conditional KO mice. In this regard, there are six members of the bZip family of transcription factors, including p45 NF-E2, NFE2L1, NFE2L2, NFE2L3, Bach1, and Bach2 that can recognize ARE. Among them, p45 NF-E2 and Bach2 are specifically expressed at low levels in hematopoietic progenitor cells and differentiated cells of the erythroid, megakaryocyte, and mast cell lineages, while NFE2L2, NFE2L3, and NFE2L3 are ubiquitously expressed (2, 16, 29). In our studies, we have found that NFE2L1 was predominantly expressed in the cells of osteoblastic lineage, whereas NFE2L2 and NFE2L3 were expressed at much lower levels (38). However, disruption of NFE2L1 in osteoblasts in vivo resulted in increased expression of NFE2L2 and decreased expression of NFE2L3 in the bone. It is possible that altered expressions of NFE2L2 and NFE2L3 in NFE2L1 conditional KO mice compensate for loss of NFE2L1. Third, vitamin C effects on osterix expression and osteoblast differ-

Fig. 5. Reduced serum osteocalcin and osterix expression in NFE2L1 conditional KO mice. A: serum osteocalcin levels in NFE2L1 conditional KO and WT mice (n = 10). B: expression of osterix, Sox9, and bZip family members in bones from NFE2L1 KO and WT mice. Total RNA was extracted from bones of 4 wk old NFE2L1 conditional KO and WT mice and reverse-transcribed into cDNA for real-time RT-PCR. Data are expressed as percentage of KO mice relative to controls and presented means ± SE. Control values are set as 100. *Significant difference in KO mice compared with WT mice (P < 0.05, n = 10).
entiation may involve other mechanisms besides ascorbic acid induction of ARE-containing genes via NFE2L1. In this regard, our findings reveal that disruption of NFE2L1 only partially abolished the ascorbic acid effect on osterix expression. These data raise the possibility that transcriptional regulation via ARE may not be the only pathway for ascorbic acid regulation of osterix expression and that the remaining osterix expression in these mice may be sufficient for correcting mineralization defect but not the skeletal size difference. In terms of other mechanisms for ascorbic acid regulation of osteoblast expression, the role of collagen-mediated integrin/MAPK signaling in regulating ascorbic acid effects on osterix expression needs to be evaluated based on what is known on the role of ascorbic acid in the regulation of collagen synthesis (26, 35, 36). Furthermore, other transcription factors yet to be identified may also be stimulated by ascorbic acid and may contribute to osteoblast commitment and bone formation.

In our study, we evaluated the skeletal phenotype only up to 8 wk of age. Since peak bone mass continues to accrue until skeletal maturity is attained at the age of ~16 wk, one could raise the possibility of catch-up growth in the NFE2L1 KO mice between 8 and 16 wk of age. In this regard, we found that the magnitude of deficits in total body BMC and total body bone area was maintained in the NFE2L1 KO mice between 3 and 8 wk of age (Fig. 2C). Although these data and the previously published data that nearly 80–90% peak BMD is attained at the age of 8 wk in different inbred strains of mice (1) argue against this possibility, the issue of whether the skeletal phenotype in the NFE2L1 KO mice is maintained after attaining skeletal maturity remains to be determined. Further studies are also needed to determine if NFE2L1 KO mice are more susceptible to bone loss when they are subjected to nutritional or hormonal stress compared with WT mice.

In conclusion, we have demonstrated that targeted deletion of NFE2L1 in osteoblasts resulted in decreased bone size, bone formation, trabecular bone, peak bone mass, and mechanical strength. The impairment of osteoblast differentiation and bone formation in the NFE2L1 conditional KO mice appears in part due to reduced expression of the osterix gene. Our findings provide the first in vivo experimental evidence demonstrating osteoblast produced NFE2L1 is involved in regulating osteoblast differentiation and bone formation.

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No conflicts of interest are declared by the author(s).

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