Targeted disruption of the osteoblast/osteocyte factor 45 gene (OF45) results in increased bone formation and bone mass

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Running title: Increased trabecular bone mass in OF45 knockout mice
SUMMARY

We have previously described osteoblast/osteocyte factor 45 (OF45), a novel bone-specific extracellular matrix protein, and demonstrated that its expression is tightly linked to mineralization and bone formation. In this report, we have cloned and characterized the mouse OF45 cDNA and genomic region. Mouse OF45 (also called MEPE) was similar to its rat orthologue in that its expression was increased during mineralization in osteoblast cultures and the protein was highly expressed within the osteocytes that are imbedded within bone. In order to further determine the role of OF45 in bone metabolism, we generated a targeted mouse line deficient in this protein. Ablation of OF45 resulted in increased bone mass. In fact, disruption of only a single allele of OF45 caused significantly increased bone mass. In addition, knockout mice were resistant to aging-associated trabecular bone loss. Cancellous bone histomorphometry revealed that the increased bone mass was the result of increased osteoblast number and osteoblast activity with unaltered osteoclast number and osteoclast surface in knockout animals. Consistent with the bone histomorphometric results, we also determined that OF45 knockout osteoblasts produced significantly more mineralized nodules in ex vivo cell cultures than did wild type osteoblasts. Osteoclastogenesis and bone resorption in ex vivo cultures was unaffected by OF45 mutation. We conclude that OF45 plays an inhibitory role in bone formation in mouse.

INTRODUCTION

The constant modulation of the balance between skeletal strength and mineral availability in bone is effected by competing cell types in response to physiological needs. Osteoblasts produce, organize and mineralize bone matrix in forming bone. Osteoclasts break down matrix by forming a lytic pocket in which bone is degraded and calcium is released. The generation and activity of these cell types is tightly regulated to provide equilibrium between formation and resorption, therefore an appropriate balance of strength and mineral release. Under certain conditions, such as aging, postmenopausal estrogen deficiency or some pathophysiological states, there can exist an imbalance between bone resorption and bone formation. As a result, skeletal mass and strength are compromised and osteoporotic fractures can occur in the afflicted individuals.
Bone is produced by the organization and mineralization of the extracellular matrix produced by osteoblasts. The major component of the extracellular matrix of these cells is Type I collagen, which functions as a scaffold for new bone. In addition, non-collagenous matrix proteins have been identified that influence the operations of bone turnover, formation and repair. These proteins are generally acidic and highly post-translationally modified by phosphorylation, glycosylation, or sulfation (1).

Targeted deletion of extracellular matrix genes in mice has been a useful method to determine the in vivo functions of several matrix proteins. For example, osteocalcin is an abundant gamma carboxy glutamic acid-containing (GLA) bone matrix protein shown to be highly expressed in osteoblasts and is a biochemical marker of the bone remodeling process. Osteocalcin deletion in mice results in increased cortical bone thickness due to increased osteoblast activity, indicating that osteocalcin has negative effects in vivo on osteoblasts and bone formation (2). Another gene of this category, matrix GLA protein (known as “MGP”), was deleted in mice, resulting in extensive cartilage calcification and in the inappropriate calcification of arteries, leading to blood vessel rupture and lethality in homozygotes (3). Ablation of the extracellular matrix protein osteonectin causes reduced bone formation with a decrease in both osteoblasts and osteoclasts resulting in a net loss of trabecular bone compared to wild type controls (4). Targeted disruption of osteopontin, an RGD-containing protein, results in a defect in the in vitro differentiation of osteoclasts; increased mineral content and maturity in long bones; and resistance to ovariectomy-induced, PTH-induced and mechanical unloading-induced bone resorption in mice (5-8). The functions of matrix proteins can be further elucidated through the characterization of mice deficient in combinations of genes. For example, mice deficient in both osteopontin and MGP exhibit more vascular calcification than the MGP knockout mice, demonstrating that osteopontin can be an inhibitor of ectopic calcification in vivo (9).

In an effort to further characterize the process of bone metabolism and identify the proteins involved, we screened RNA of rat bone marrow cell cultures for novel transcripts specific to bone mineralization (10). This screen produced a novel clone that we designated Osteoblast/Osteocyte Factor 45 (OF45). Rat OF45 encodes an RGD protein having 45% homology to the recently cloned human MEPE as well as loose homology to AG-1/DMP1. In rat, OF45 is highly expressed in the tibial shaft and metaphysis as well as in osteoblasts of induced bone marrow
cultures, calvaria, and the UMR106 osteoblastic cell line. Immunohistochemistry in rat tibia revealed abundant OF45 protein in the osteocytes. Newly embedded rat osteocytes were also positive for OF45 in a marrow ablation model. Interestingly, the temporal expression pattern of OF45 differs from other well-characterized bone markers such as osteocalcin, osteonectin, and osteopontin in both \textit{in vitro} and \textit{in vivo} bone growth models (10). In this report we have cloned and characterized the mouse OF45 cDNA. With the exception of some additional sequence we report at the 5'-prime end, the sequence reported here was identical to that recently published as MEPE (11). In order to demonstrate the functional significance of an elimination or reduction of OF45 expression, we have cloned the mouse OF45 genomic DNA, generated a mouse line with a targeted disruption of the OF45 gene and subjected these mice to phenotypic analysis.

\textbf{EXPERIMENTAL PROCEDURES}

\textit{Cloning of mouse OF45--} A 1255 bp probe encoding most of the rat OF45 cDNA sequence excluding the 3’-UTR was used to screen a mouse 129 genomic lambda phage library (12). A 15 kb clone found to include exon 3 was used to generate PCR primers to clone the complete cDNA, the BAC clone, as well as the targeting construct. The 5’-end of the mRNA was determined by RNA ligase-mediated RACE as described (13). Briefly, 50 µg total RNA from mouse tibia was dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) and the 5’ cap was removed using tobacco acid pyrophosphatase (Epicentre Technologies, Madison, WI). The RNA linker generated from the plasmid pGbx-1 (Gift from Dr. Frohman) was ligated to the decapped RNA using T4 RNA ligase (Epicentre Technologies, Madison, WI). The RNA was converted to cDNA by using Superscript II reverse transcriptase and poly dT(12-18) primer (Gibco/BRL, Gaithersburg, MD). PCR was used to amplify the 5-end of the cDNA using primer pairs NRC-1-288A & 36387.233B and NRC-1-288B & 36387.233A in the first and second round, respectively. A single PCR product of ~550 bp was obtained in the second round PCR. A control sample in which the pyrophosphatase step was omitted did not yield a PCR product, indicating that the ~550 bp product likely resulted from full length mRNA that had been 5’-capped. The second round PCR fragment was isolated and ligated using a TA Cloning Kit (Invitrogen, Carlsbad, CA). Insert containing plasmids were sequenced.
The 3'-end of the mRNA was determined using Gibco/BRL (Gaithersburg, MD) 3' RACE kit as suggested by the manufacturer. Briefly, 5 μg mouse tibia total RNA was reverse transcribed using the AP primer provided. The PCR reaction amplified between OF45 nucleotide 527 (primer 36387.233E) and the AUAP primer provided. Two fragments close in size were cloned with the TA Cloning Kit (Invitrogen, Carlsbad, CA) and sequenced. The sequence of the 2 fragments were identical except for a short stretch of additional sequence at the 3'-end.

Gene specific primers:

36387.233A: 5'-TGTGTCAGGTAGTGAGTGCTCC -3'
36387.233B: 5'-ACTGCCACCATGTCTCTCTC -3'
36387.233C: 5'-CCAGCAGATGTCAATGATGC -3'
36387.233D: 5'-TTGGCAGCATCTGTGTATCC -3'
36387.233E: 5'-CCCAAGAGCAGCAAAGGTAG -3'
36387.233F: 5'-TGCGTGATATTCTGAGGAGG -3'

pGbx-1 RNA linker primers:

NRC-1-288A: 5'-CCAAGACTCACTGGGTACTGC-3'
NRC-2-288B: 5'-CTAGAGGGGCCTGTTGAACC-3'
NRC-3-288C: 5'-GGGAGAGGCCAGCGTATTCC-3'
RC NRC-1-30A: 5'-GCAGTACCCAGTGAGTCTTGG-3'
RC NRC-2-30B: 5'-GGTTCAACAGGCCCCTCTA-3'
RC NRC-3-30C: 5'-GGAATACGCTGGCCTCTCCC-3'

Isolation of BAC clone for mouse OF45-- A 124 bp probe for the 5'-end of the cDNA was generated by PCR with primers to amplify bases 12-135 of the cDNA sequence using primers 36393.80A: 5'-
TTTCAGCAAATGCCCAGAG-3' and 36393.80B: 5'-CCAGGTCATACTGAAGGAGGAGGAC-3'. This probe was sent to Genome Systems, Inc. (St. Louis, MO) for screening of a mouse ES-129/SVIII BAC library. A single clone
was identified and characterized. Chromosomal localization was determined by FISH using the BAC clone by Genome Systems, Inc. (St. Louis, MO) according to their protocols. Intron/exon boundaries were determined by alignment of BAC clone sequence with the OF45 cDNA sequence.

**Mammalian expression of mouse OF45**-- The 1340 bp containing the coding region of the mouse OF45 cDNA (bases 53-1392) was amplified using primers 36393.44C: TTTCTGAAGGTGAATGACG-3' & 36393.44H: 5'-CTAGTCACCAGACTCTCACTAG-3' and subcloned in the CMV mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) for transfection into CHO cells using Lipofectamine Plus (Gibco).

**Antibody generation and western blot analysis**-- High titer polyclonal antiserum was generated by immunization of rabbits with bacterial expressed full length OF45 (Zymed Laboratories Inc., South San Francisco, CA). OF45 antibody was affinity purified by chromatography on OF45-coupled agarose beads by standard methods (14). Protein samples were separated by electrophoresis on Novex (San Diego, CA) 10% NuPAGE gels and transferred to nitrocellulose using a semi-dry transfer. Blots were blocked using 1% Western Blocking Reagent from Roche (Mannheim Germany) for 1 h and 1° antibody diluted in 0.5% block for 1 h. After washing, goat anti-rabbit peroxidase 2° antibody (Roche) diluted into 0.5% block was applied to the blot for 1 h. Signal was detected using an ECL detection kit (Amersham, Buckinghamshire, England).

**Northern blot, RT-PCR and Southern Blot Hybridization**-- Clontech (Palo Alto, CA) multiple tissue northern blots were hybridized with the full-length OF45 cDNA. RNA isolated from mouse tissues was converted to cDNA by using Superscript II reverse transcriptase and poly dT(12-18) primer (Gibco/BRL, Gaithersburg, MD). PCR was used to amplify an internal 483 bp from OF45 mRNA bases 986-1468 using primers 36499-143A: 5'-ACTATCCACAAGTGGCCTCG-3' and 36499-143B: 5'-CTGTTGCTTGTCTGAGTTCC-3'. The cDNA was then hybridized with a radiolabeled cDNA probe specific to bases 160-1020.

**Gene Targeting, ES cell culture and microinjection**-- Genomic DNA fragments from the 15 kb genomic clone were subcloned into the JNS2 targeting plasmid (15) E14Tg2a embryonic stem (ES) cell line derived from the 129sj mouse strain was used for gene targeting of the OF45 locus (16). Electroporation, selection, expansion, and
microinjection of ES cells into C57BL/6 embryos were as described (17). Out of 70 neomycin and Gancyclovir
resistant clones, 4 were positive for the desired recombination event, resulting in a targeting frequency of 1 in 17.
Chimeric animals were mated with C57BL/6 males and females. An 850 bp Spe I/Msc I probe, homologous to a
region directly 3’ of the targeted mutation was used to genotype agouti offspring by Southern blot analysis.
Digestion of tail DNA with Bam HI/Bgl I double digest resulted in a 10 kb hybridizing band in the wild type allele
and a 9 kb hybridizing band in the targeted allele.

Animals-- F2 or F3 129/Bl6 mix heterozygote mice were bred to generate successive populations of littermates used
for in vivo experimentation at either four months or one year of age. The same parent animals were used to generate
both four month and one year age groups. 10 month-old males backcrossed onto a C57/Bl6 background for 9
generations were used for dynamic histomorphometry studies. Animals were maintained on a 12-hr light/12-hr
dark cycle and were provided food and water ad libitum. 12 and 2 days prior to sacrifice, mice were given
subcutaneous injection of the fluorochrome calcein at 10 mg/kg (Sigma Chemical, St. Louis, MO). Animals were
euthanized by cervical dislocation and the femurs placed in 70% ethanol for later pQCT and histomorphometric
analysis. The experiment was conducted according to Pfizer Animal Care and Use-approved protocols and the
animals were maintained in accordance with the ILAR (Institute of Laboratory Animal Research) Guide for the Care
and Use of Laboratory Animals.

Serum Mineral Content-- Calcium and inorganic phosphate levels were measured from blood serum at DNX
Transgenic Sciences (Cranbury, NJ) using the ACE Clinical Chemistry System, (Alfa Wassermann Inc. West
Caldwell, NJ) by the manufacturers protocols. One-year-old animals were used for this study. Sera from 12 male
animals of each genotype were used. 7 wild type and 7 knockout females were also studied.

High Resolution X-Ray-- Femurs were examined at 2X and 3X magnification on a Faxitron model MX-20 Specimen
Radiography System (Buffalo Grove, Illinois) with Kodak Min-R 2000 Mammography film in Min-R 2000 cassettes
with an intensifying screen. Magnification was calculated by SID/SOD = IS/OS where SID=Source to Image
Distance, SOD=Source to Object Distance, IS=Image Size, OS=Object Size. The femurs of 12 to 27 animals of each sex, age and genotype were examined.
Peripheral Quantitative Computerized Tomography (pQCT) Analysis-- Excised femurs were scanned by a pQCT X-ray machine (Stratec XCT Research M, Norland Medical Systems, Fort Atkinson, WI) with software version 5.40. A 1-mm thick cross section of the femur metaphysis was taken at 2.5 mm proximal from the distal end with a voxel size of 0.07 mm. Cortical bone was defined and analyzed using contour mode 2 and cortical mode 4. An outer threshold setting of 340 mg/cm³ was used to distinguish the cortical shell from soft tissue and an inner threshold of 529 mg/cm³ to distinguish cortical bone along the endocortical surface. Trabecular bone was determined using peel mode 4 with a threshold setting of 655 mg/cm³ to distinguish (sub)cortical from cancellous bone. An additional concentric peel of 1% of the defined cancellous bone was used to ensure (sub)cortical bone was eliminated from the analysis. Volumetric content, density, and area were determined for both trabecular and cortical bone. Using the above setting, we have determined that the ex vivo precision of volumetric content, density and area of total bone, trabecular, and cortical regions ranged from 0.99% to 3.49% with repositioning (18). Number of animals examined: Females 4 months, 12 WT, 20 Het, 18 KO, Females, 1 year 12 WT, 17 Het, 27 KO, Males 4 months, 18 WT, 21 Het, 18 KO, Males 1 year 21 WT, 19 het, 21 KO.

Bone histomorphometry-- Following pQCT analysis, the distal half of femur from each animal of 4-month-old and 1-year-old was dehydrated and embedded in methyl methacrylate. Four µm longitudinal sections were prepared with a Reichert-Jung Polycut S microtome (Leica, Deerfield, IL), and stained with modified Masson’s Trichrome stain. Trabecular bone volume, trabecular number and trabecular thickness were determined on the distal femoral metaphysis for these 4-month-old and 1-year-old animals (19, 20). In order to understand the changes in osteoblast and osteoclast on the cancellous bone, a group of wild type (n=8) and knockout (n=10) male mice were necropsied at 10 months of age. The left distal half of femur from these 10-month-old animals was decalcified and 4 µm longitudinal sections were prepared and stained with Toluidine Blue (Sigma, St. Louis, MO). These decalcified sections were used to determine the trabecular bone volume (BV/TV), percent osteoblast surface (Ob.S/BS), number of osteoblast per mm bone surface (N.Ob/BS), percent osteoclast surface (Oc.S/BS), and number of osteoclast per mm bone surface (N.Oc/BS) on the same area of the distal femoral metaphysis as described above (19, 20). The right distal half of femur from 10-month-old animals was dehydrated and embedded in methyl methacrylate. Ten
μm longitudinal sections were prepared and left unstained for the determination of mineral apposition rate (MAR), bone formation rate/bone surface referent (BFR/BS) and bone formation rate/tissue volume referent (BFR/TV) on the distal femoral metaphysis (19, 20). An Image Analysis System (Osteomeasure, Inc., Atlanta, GA) was used for all histomorphometric analysis. Histomorphometric measurements were performed on cancellous bone tissue of the distal femoral metaphyses between 0.5 mm and 2 mm proximal to the growth plate-epiphyseal junction, and extended to the endocortical surface in the lateral dimension (21).

**Bone Marrow Harvest and Culture**-- Long bones were isolated from 3-6 month old animals. Femur and tibia bone marrow cells harvested by centrifugation were plated at a density of 15x10⁶ cells per 100 mM plate in α MEM (Gibco), with 10% FBS and 50 μg/ml Gentamycin (Gibco). 50 μg/ml L-Ascorbic Acid, and 10 mM β-glycerophosphate were added at day 10 of culture. Cultures were fed 3 times per week, maintained for 4 weeks, and then stained by the Von Kossa method (22).

**Calvaria Harvest and Culture**-- Calvaria were dissected from postnatal day 3 mice. 0.2mg/ml Collagenase P/0.25% Trypsin digestions were performed as described (23). The cells liberated in the second digest were plated at 2X10⁴ cells /cm². At confluence, cultures were supplemented with 50 μg/ml L-ascorbic acid and 10 mM β-glycerophosphate. Cells were later harvested for RNA or Alizarin Red staining (24). Stain solubilization and quantitation were as described (25).

**Osteoclast Cultures**-- Bone marrow cultures were established as described above, plated at 1X10⁶ cells per well in a 24 well plate. Cells were stimulated with 10 nM 1α,25-(OH)₂D₃ and TRAP stained as described (26). Bone resorption was assessed by culturing bone marrow cells on bone slices for 21 days and counting the resorption pits as has been described by Grasser et al (27).

**Statistical Methods**-- Statistical significance of *in vivo* and *in vitro* parameters was determined by two-tailed student’s T-Test. A p-value of less than 0.05 was considered statistically significant.

**RESULTS**
OF45 cDNA cloning and characterization-- Mouse OF45 was cloned using low stringency hybridization of a rat OF45 sequence to a murine 129 strain lambda phage genomic library. The full-length cDNA was then cloned by rtPCR and 5’ and 3’ rapid amplification of cDNA ends (RACE) from mouse tibia RNA. The complete cDNA was 1741 bp in length. An additional 1679 bp cDNA was cloned which was identical to the longer, more abundant message with the exception of a shorter 3’ extension. Three early methionine codons allow alternative start sites for the OF45 protein, the first of which would result in a 441 amino acid protein. A Kyte-Doolittle hydrophilicity plot revealed that the peptide sequence contained a hydrophobic leader sequence followed by a hydrophilic protein (Fig. 1A). Analysis of the amino acid sequence with PSORTII software, which predicts the subcellular localization sites of proteins from their amino acid sequences, indicated that this cDNA likely encodes an extracellular protein (28-30). The predicted site of cleavage would be between Ala24 and Ala25, yielding a 417 amino acid secreted peptide with a calculated molecular weight of 44247 Da. Experimental confirmation of this analysis was obtained by Western blot analysis of media from transiently transfected CHO cells. An OF45 specific antibody detected a secreted protein of ~44 kDa (Fig. 1B). An OF45 peptide product of lower molecular weight was also detected in variable amounts suggesting that proteolytic processing or degradation occurred in the media. The predicted amino acid composition of the basic OF45 peptide (predicted pI 9.17) was rich in serine, glycine and charged residues.

Several consensus protein kinase C, casein kinase II, tyrosine kinase, and cAMP-dependant kinase phosphorylation sites, one N-glycosylation site, and a SDGD glycosaminoglycan binding site offer the potential for post-translational modifications to increase the acidic character of the protein.

Comparison of OF45 sequence to known genes-- Based on sequence and expression analysis, we believe that the mouse OF45 cDNA reported here represents the mouse orthologue of the rat OF45 gene (10) (GenBank Accession #260922). Alignment with the rat OF45 amino acid sequence demonstrated 67% identity. Importantly, key structural features were conserved. Both proteins were rich in serine, glycine and charged amino acids and included an amino terminal hydrophobic signal sequence that targeted the peptides for secretion. The RGD sequence at amino acids 183-185 is an element traditionally involved in cell-matrix interactions through integrin binding and signaling, and was also conserved between species. Comparison of the OF45 cDNA sequence to GenBank using the BLAST 1.4 algorithm (31) showed 45% amino acid identity to the recently identified human matrix extracellular
phosphoglycoprotein (MEPE) gene (32). The macaque MEPE gene showed similar identity to OF45 (GenBank Accession number AB05025). The homology between mouse OF45 and human MEPE was distributed throughout the sequences and the RGD motif was conserved. MEPE was cloned as a candidate gene for a tumor-secreted phosphaturic factor responsible for tumor-induced osteomalacia. Like MEPE, OF45 shares structural features common to a class of extracellular matrix phosphoglycoproteins that includes osteopontin, dentin sialophosphoprotein, dentin matrix protein 1, and bone sialoprotein II. These genes are highly expressed in bone or dentin and play significant roles in mineralization (33-35). The mouse OF45 sequence was also 100% identical to the mouse MEPE cDNA recently published by L’Argiro et al. (11), although the cloned OF45 cDNA we report contained additional sequence at both the 5’ and 3’ ends. Comparison of mouse OF45, rat OF45, macaque MEPE and human MEPE show several tightly conserved regions across all four species. These include the RGD motif, the signal sequence cleavage site, the putative N-glycosylation site, the SGDG glycosaminoglycan binding site, and several putative phosphorylation sites. Based on the sequence comparison and the restricted tissue expression, we concur with L’Argiro et al. that the sequence we report as mouse OF45 is the ortholog of human MEPE.

**OF45 gene expression**—The expression of mouse OF45 was analogous to that reported for rat OF45 (10). Mouse OF45 mRNA was induced during mineralization in primary mouse calvarial cultures, appearing at 2 weeks and maintained throughout 3 weeks of culture (Fig.1C). Expression correlated with increasing differentiation of the osteoblast lineage. Mouse OF45 mRNA expression was highly bone-specific. A high level of OF45 message was detected in total RNA prepared from mouse tibia, but all other tested tissues were negative, even by Northern blot analysis of polyA+ mRNA (Fig.1D). Using highly sensitive RT-PCR, low levels of OF45 mRNA were detected in brown fat, white fat, testes, brain, and aorta (Fig. 1E).

Localization of OF45 protein was determined by immunohistochemical analysis of longitudinal tibia sections (Fig. 2). Mouse OF45 protein was expressed in all osteocytes, cells of osteoblast lineage embedded within bone matrix. In contrast to the OF45 mRNA expression observed in cell culture, osteoblasts and lining cells observed in the bone sections were negative for OF45 immunostaining. There was no specific staining detected in osteoclasts, chondrocytes or periosteal cells. Also, OF45 was not detected in hematopoietic cells of the marrow compartment.
OF45 genomic organization and targeting-- The genomic DNA encoding mouse OF45 was subcloned from a hybridizing mouse 129 BAC clone and mapped. The mouse OF45 gene was comprised of three exons, two small 76 and 78 bp exons separated from the third 1597 bp exon by 10.5 kb (Fig. 3A). Fluorescence in situ hybridization (FISH) analysis was used to determine that the OF45 gene is located on mouse chromosome 5, region 5E3-E5.

A targeting vector was constructed to delete the third and largest exon of OF45 and replace it with the neomycin selectable marker gene (Fig. 3A). This deletion removed the DNA encoding Met27 through the end of the translated sequence including the final Asp441. This targeting construct was electroporated into E14Tg2a embryonic stem cells and then screened for positive recombinants. Blastocyst microinjection of a targeted cell line resulted in chimeric animals that were subsequently bred to C57/Bl6 mice to yield mixed-background offspring heterozygous for the OF45 mutation. Heterozygote intercrosses produced litters with Mendelian ratios of wild type, heterozygous and homozygous pups (Fig. 3B). Northern analysis of mRNA prepared from tibia detected no OF45 message in knockout animals and reduced expression in heterozygous animals (Fig. 3C). Immunohistochemistry of tibia sections confirmed the absence of OF45 protein expression in osteocytes of homozygous mutant mice (Fig. 2).

Phenotypic analysis of OF45 knockout animals-- In order to examine the phenotypic effects of OF45 mutation, heterozygote parents were bred to generate groups of at least twelve animals of each sex and genotype to be examined at 4 or 12 months of age. Mice heterozygous and homozygous for the OF45 mutations were normal, fertile, and healthy, with no obvious pathology. Serum chemical analysis revealed no statistically significant alterations in blood phosphate or calcium content (Table 1). Whole body X-rays revealed normal skeletal patterning and structure (not shown). However, high resolution X-rays of femurs revealed a pronounced increase in the amount of trabecular bone in both heterozygote and knockout animals at 1 year (Fig. 4). This difference was observable with high resolution X-rays at 4 months, but was less dramatic (not shown). Quantitative analysis of the distal femoral metaphysis by peripheral quantitative computerized tomography (pQCT) demonstrated significantly increased volumetric trabecular bone content and density in both males and females (Table 2). There was no consistent change in the volumetric cortical bone content at this site. The periosteal and endosteal circumference...
was increased in the knockout animals with no significant change in cortical thickness (Table 2). Caliper measurement revealed no difference in femur length between genotypes (Table 3).

To further investigate the trabecular bone structure of these mice, we performed cancellous bone histomorphometric analysis at the distal femoral metaphysis (19). We observed a 50% increase in trabecular bone volume [BV/TV(%)] in 4 month old female knockout mice compared to wild type mice (Fig. 5). In males, there was a 32% greater trabecular bone volume in knockout mice at 4 months. The effect of OF45 knockout mutation was more pronounced at 1 year with >2-fold and >3-fold more trabecular bone volume in male and female knockout mice, respectively. The increased trabecular bone volume was reflective of both increased trabecular number and increased trabecular thickness (Fig. 5).

The loss of even one OF45 allele and the resulting reduced gene expression (Fig. 3C) caused significant phenotypic effects in bone. As measured by both pQCT and histomorphometry, heterozygous animals exhibited the phenotypic effect of increased trabecular bone volume. Trabecular bone volume in 4 month males and 1 year females was equivalent in knockout and heterozygous animals with both being significantly increased over wild type mice. Heterozygote phenotype appears to be influenced by sex and age, appearing in only older females, but in both young and old males.

An additional important observation was that both heterozygous and knockout mice exhibited less aging associated bone loss than wild-type animals. The male and female 1-year old wild-type groups possessed approximately 40% and 70%, respectively, less trabecular bone volume versus the matched 4-month-old groups (Fig. 5). In knockout animals, however, males exhibited no trabecular bone loss at 1 year. Female trabecular bone volume was reduced by only 15% in the 1-year old mice. Thus, OF45 mutation appeared to have protected against age-related bone loss. This protective effect of OF45 mutation was also observed in heterozygote animals.

The net increase in trabecular bone volume observed in the heterozygous and knockout animals could theoretically arise from either 1) decreased osteoclastic bone resorption, 2) increased osteoblast-mediated bone formation or 3) a combination of osteoblast and osteoclast effects. In order to investigate the effect of OF45 mutation on bone
resorption and bone formation, we performed static and dynamic bone histomorphometric analysis of distal femoral metaphyseal cancellous bone using wild type and knockout male mice at 10 months of age. Percentage of bone surface occupied by osteoblasts (Ob.S/BS) and the osteoblast number per mm bone surface (N.Ob/BS) were significantly increased by approximately 2-fold in knockout animals compared with wild type controls (Table 4). A trend toward slightly but non-significantly decreased osteoclast surface (Oc.S/BS) and osteoclast number (N.Oc/BS) was observed (Table 4). Dynamic histomorphometry measurements utilizing calcein double-labeled bones to quantitatively measure the bone formation rate within a 10 day labeling interval demonstrated significantly increased mineral apposition rate (MAR, an index of osteoblast activity), bone formation rate /bone surface reference (BFR/BS) and bone formation rate/tissue volume referent (BFR/TV) in knockout animals (Table 4). These data demonstrate that the increased cancellous bone mass in OF45 knockout mice is a result of increased osteoblast-mediated bone formation with unchanged osteoclast-mediated bone resorption.

**Ex-vivo cell culture**-- In order to further examine the cellular mechanisms behind the increased trabecular bone volume in heterozygote and knockout animals, we examined the *in vitro* mineralization and osteoclastogenesis potential of cultures derived from wild type and mutant animals. Primary cultures of bone marrow cells from all three genotypes were grown in the presence of ascorbate and ß-glycerophosphate to induce osteoblast differentiation and promote bone nodule formation. After three weeks, mineralization was detected by Von Kossa stain and quantitated by image analysis (Fig. 6A). In several separate experiments, heterozygote- and knockout-derived cultures gave rise to significantly more bone nodules than those from wild type bone marrow, suggesting the presence of more osteoblastic precursor cells in the marrow (36). In addition, the mineralized area per nodule was increased in knockout cultures. A second culture system derived from mouse calvarial bone of 3-day-old mice produced comparable results over multiple experiments. In this more highly osteoblast enriched system, calvarial cultures from OF45 knockout mice produced 60% more mineralized matrix as assayed by quantitation of solubilized Alizarin Red stain (Fig. 6B). Importantly, this experiment indicated that the osteoblastic cells derived from knockout mice at only 3 days after birth already exhibited increased mineralization potential.
In order to determine if a deficiency in the formation or activity of osteoclasts contributed to the increased bone phenotype, bone marrow cultures from wild type and knockout animals were established under osteoclast promoting conditions. In multiple cultures, the ability of marrow cultures to form multinucleated tartrate-resistant alkaline phosphatase (TRAP) positive cells was unimpaired (Fig. 7A and 7C). In fact, knockout bone marrow cultures exhibited a trend toward higher numbers of TRAP positive multinucleated cells than wild type derived cultures. The osteoclasts generated in the marrow culture system had the ability to resorb bone when culture on bone slices (Fig. 7B). Resorption pits appeared identical between cells from wild type and knockout mice. No difference in the number of resorption pits was observed (Fig 7C).

**DISCUSSION**

We successfully generated a strain of mice with a deletion in the OF45 locus and ablated OF45 protein expression. This mutation did not affect the gross skeletal morphology or overall health of these animals. Refined examination of long bone revealed significantly increased cancellous bone mass in both male and female knockout animals, with differences in magnitude based on sex and age. These differences were visible by high resolution X-ray, and quantifiable by both pQCT and histomorphometry. In addition, analysis of sex-matched populations at 4 months and 1 year revealed that deletion of OF45 prevented the loss of trabecular bone characteristic of aged mice. The bone loss normally observed in aged mammals is thought to be due to the reduced abundance and reduced survival of osteoblasts, with no change in the abundance or activity of osteoclasts (37-39). The increased bone mass in OF45 ablated mice was due to increased abundance and activity of osteoblasts as indicated by the increased N.Ob/BS and BFR/BS observed relative to wild type mice. *Ex vivo* cell culture from bone marrow indicated increased number osteoblast precursors in OF45 knockout animals. Although not specifically addressed in this study, it is possible that OF45 deletion also affected osteoblast survival or lifetime.

Heterozygous mice also exhibited significantly increased trabecular bone. Therefore, even a reduction of OF45 levels markedly impacted bone structure suggesting that OF45 is a limiting control point in the regulation of bone. The haploinsufficiency nature of OF45 suggests that genetic variation in OF45 expression levels could result in
variation of peak bone mass or aging-related bone loss in individuals. It will be of interest to determine if polymorphisms in the OF45 gene contribute to differences in bone mass within the human population.

The spectrum of tissue expression of mouse OF45 closely matched that of rat, with abundant production restricted to bone. In differentiating calvarial osteoblast cultures, OF45 expression increased with progressive differentiation and maintained a steady state of expression in mature cultures. Immunohistochemistry of tibial bone sections revealed abundant staining of the embedded osteocytes. We did not observe OF45 immunostaining of osteoblasts in either rats (10) or mice despite the abundant mRNA expression in osteoblast-rich ex vivo cultures. This discrepancy could be explained as a technical artifact in the immunostaining methods. For example, perhaps only the embedded cells accumulate sufficient amounts of this secreted protein to allow detection. However, as we previously reported, Northern blot analyses of mRNA isolated from the rat marrow ablation model support the conclusion that OF45 mRNA is expressed only by cells embedded within bone and not by the osteoblastic cells that are not imbedded (10). Taking these data together, we believe that OF45 protein is expressed in vivo in mature osteoblasts during the process of embedding in new bone and maintained throughout osteocyte development.

In addition to bone expression, we report low mRNA levels in other organs including testes, fat and the aorta. The significance of these low levels of mRNA is unclear. Adipose tissue expression may be meaningful given the common mesenchymal stem cell origin of both adipocytes and osteoblasts. Expression of OF45 in the aorta may also have significance, especially under pathophysiological conditions of vascular calcification (40). A recent report has demonstrated OF45 mRNA expression in odontoblasts, the tooth cells analogous to osteoblasts (41). Further, the human tissue expression of OF45 (called MEPE) was reported in brain as well as bone (32). We have not been able to detect OF45 mRNA in mouse brain, while we have been able to confirm relatively abundant OF45 expression in human brain mRNA samples (TAB, not shown). To determine if the differences in expression pattern between humans and mice translate to additional functions for OF45 in humans will require further investigation.

The OF45 sequence described here is the mouse orthologue of the human MEPE gene. The human MEPE gene was cloned as a candidate for the long sought after phosphaturic factor secreted by tumors causing oncogenic osteomalacia (32). A parallel field of research on a phenotypically similar condition, X-linked hypophosphatemic
rickets, has suggested that the phosphaturic factor is processed or degraded by the PHEX endoprotease. However, subsequent investigation has implicated FGF23 as a phosphaturic factor in both tumor-induced osteomalacia and autosomal-dominant hypophosphatemic rickets (ADHR) (42, 43). Further, it has been demonstrated that FGF23 is a substrate for the PHEX endoprotease (42). It has been reported that MEPE, in contrast to FGF23 does not exhibit phosphaturic activity on renal cells (42) and is not a direct substrate of the PHEX endoprotease (44). Our observation that OF45 knockout animals exhibited no statistically significant differences in serum calcium or phosphate also argues against MEPE being a critical phosphaturic factor. Nonetheless, it is still significant that OF45/MEPE was identified due to its overexpression by osteomalacia-inducing tumors and there remains a link between PHEX and OF45/MEPE. While Bowe et al. (44) have claimed that OF45/MEPE is not a direct substrate of PHEX, Argiro et al. have demonstrated that the osteoblasts of PHEX deficient Hyp mice do express markedly higher levels of Mepe mRNA than do those from wild type mice (11). We have demonstrated that co-transfection of OF45 and PHEX resulted in decreased OF45 protein detected in the media than observed with OF45 alone (TAB, unpublished results). Conversely, in a cell-free system, Guo et. al. demonstrated that Phex inhibits the cleavage of OF45/MEPE (45). It seems increasingly clear that FGF23 and PHEX are members of a phosphate regulatory pathway. MEPE may be a downstream target of this pathway and a member of a bone regulatory branch of this system.

In summary, we have determined that reduction or total ablation of the OF45 gene in mouse resulted in increased bone mass. While the molecular mechanism of OF45 action is unknown, the etiology of this increased bone mass cannot be attributed to a failure in the differentiation or abundance of osteoclasts. Bone histomorphometry and primary cell culture experiments did not detect any impairment in the osteoclast cells that mediate bone resorption. The increased osteoblast number and activity (mineral apposition rate) observed by bone histomorphometry and the mineralization potential observed in both marrow and calvarial cultures indicate that the phenotypic effect in the knockout animals resulted from an increased number and activity of osteoblasts and osteoblastic precursors. One interpretation of these results is that OF45 functions \textit{in vivo} as a negative regulator of osteoblast number and activity. The concept that regulation of bone mass can be controlled by negative regulators has recently been demonstrated with the characterization of osteocalcin (2) and leptin deficient mice (46). Further experimentation
will be needed to determine if OF45 acts directly on osteoblasts or through indirect mechanisms. The expression of OF45 within osteocytes, the terminally differentiated osteoblasts that become encased in the mineralized matrix during bone formation (47), opens the possibility that secreted OF45 may act locally on osteoblasts and osteoblast precursors. Osteocytes can communicate through extensive dendritic processes and have been theorized to be the primary sensors of mechanical strain within bone (48-51). OF45 may function as an integral component of the intimate connection between the sensing and signaling of osteocytes and the osteoblast response.
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**Figure Legends**

Fig. 1. **OF45 encoded a secreted protein and was expressed primarily in bone.**

A. A Kyte-Doolittle hydrophilicity plot of the predicted amino acid sequence of OF45 indicated a hydrophobic leader sequence followed by a hydrophilic peptide. The complete cDNA nucleotide sequence was deposited as GenBank Accession #AF298661.

B. Western blot analysis of OF45 expression in the media from CHO cells transfected with pcDNA3-OF45 expression vector. Media from CHO cells transfected with the pcDNA3 vector control is also shown. The major band corresponded to ~44 kDa as predicted from the cDNA sequence. Lower molecular weight species, likely the product of proteolysis, were also detected in media from OF45 transfected cells.

C. Northern blot analysis of 20 µg total RNA from mouse calvarial bone cultures stimulated to mineralize with ascorbate and β-glycerophosphate. RNA samples were taken over a time course of 1 day to 3 weeks as indicated and analyzed for OF45 mRNA expression.

D. Tissue specific expression of OF45 mRNA as measured by northern blot analysis of 5 µg poly A+ RNA from various mouse tissues (Clontech) and 20 µg total RNA from mouse tibial metaphysis. Top panel: mouse OF45, bottom panel GAPDH control probe.

E. Tissue specific expression of OF45 mRNA as measured by Southern blot analysis of cDNA amplified from poly A+ RNA. Top panel: Southern blot of RT PCR with OF45 specific primers. Bottom panel: Ethidium bromide stain of control PCR reaction with actin specific primers.

Fig. 2. **OF45 protein localized to osteocytes.**

Immunohistochemical analysis of OF45 protein localization in bone sections of mouse tibia indicated OF45 expression in the osteocytes embedded in mineralized bone of wild type animals. Arrows shown in
indicate positive staining osteocytes with the cortical bone of a wild type mouse. OF45 immunoreactivity was absent in bones from knockout mice. IgG control with secondary antibody alone indicated the absence of non-specific signal in bone derived from either wild type or knockout mice. Bar: 200 µm.

CB=cortical bone, MC=marrow cavity

Figure 3. **Genomic organization and targeting of OF45.**

A, Schematic representation of genomic organization and targeting strategy, including schematic of targeted locus. Exon sizes and intron/exon boundaries of the mouse OF45 gene are diagrammed in the top figure. Exons are indicated by gray boxes. Recombination of the targeting vector with the OF45 locus resulted in the replacement of the third and largest exon with the neomycin selectable marker gene. A Bam HI site is introduced via neomycin integration. Successful targeting was detected with both external (probe 1) and internal (probe 2) probes. X=XbaI, Bg=Bgl II, Sp=SpeI, M=MscI, B=Bam HI

B, Detection of successful OF45 targeting. Left panel- Southern blot analysis of tail DNAs digested with Bam HI and Bgl II and hybridized with probe 2. A 10 kb band is detected at a wild type allele and a 9 kb band is detected at a targeted allele.

C, Northern blot analysis of 20 µg total RNA prepared from tibia of wild type, heterozygous, and knockout animals. Note the absence of OF45 expression in the RNA from knockout mice and the reduction of expression in the RNA from the heterozygous mice.

Fig. 4. **High resolution X-ray indicated that OF45 mutant mice had increased trabecular bone.**

Femurs from male and female mice at 1 year of age were subjected to high resolution X-ray imaging. In the magnification of the distal femur increased trabecular bone can be observed in both heterozygous and knockout mice when compared to the wild type mice. The increased trabecular bone was evident in both males and females at 1 year of age.
Fig. 5. **OF45 mutant mice had increased trabecular bone volume, trabecular number and trabecular thickness.**

Graphical representation of trabecular parameters measured by histomorphometry of stained sections of the distal femur. Trabecular bone volume, trabecular number and trabecular thickness are shown for each sex, age and genotype. 10 animals of each sex and genotype were processed for histomorphometry. Error bars denote standard error. (*) indicates statistical significance at p<0.05.
Fig. 6. **Increased mineralization in OF45 mutant ex vivo cell cultures.**

A, Primary bone marrow cultures from wild type, heterozygote and knockout animals were cultured under mineralizing conditions with ascorbate and β-glycerophosphate. Mineralization was detected by Von Kossa staining. The black stained mineralized nodules were quantified using Optimas image analysis software. Graphs depict quantitation of nodule number, total nodule area and average area per nodule.

B, Primary calvarial cultures derived from wild type and knockout animals at 3 days postnatal were cultured under mineralizing conditions with ascorbate and β-glycerophosphate. Alizarin Red stain was used to detect mineralization. Graph depicts concentration of alizarin red stain quantitated by stain solubilization and optical density reading. Error bars denote standard error. (*) indicates statistical significance at $p<0.05$.

Fig. 7. **Bone marrow cells from OF45 mutant mice were fully capable of osteoclast formation and osteoclast-mediated bone resorption.**

A, Osteoclastogenesis in bone marrow cells derived from wild type and knockout mice. Tartrate resistant acid phosphatase (TRAP) cytochemical stained cultures from wild type and knockout animals. TRAP positive cells with three or more discrete nuclei were considered osteoclasts.

B, Bone resorptive activity of OF45 wild type and knockout osteoclasts. Representative resorption pits formed on bone slices by osteoclasts from wild type and knockout animals.

C, Osteoclastogenesis was assessed by counting TRAP-positive multinucleated cells. Bone resorption was assessed by counting number of resorption pits per bone slice. Error bars denote standard error. No statistically significant differences were observed.
### Table 1. Serum mineral content in one year old animals

| Analyte | Calcium | Phosphorus |
|---------|---------|------------|
|         |         | Wild Type  | Heterozygote | Knockout | Wild Type | Heterozygote | Knockout |
| **Males** |         | 9.98±0.8  | 9.8±1.5     | 9.94±1.1 | 7.49±1.6  | 6.9±2.6     | 7.32±2.0 |
| **Females** | N/D    | 9.24±0.65 | N/D        | 9.24±1.7 | 4.93±1.0  | N/D        | 6.04±1.9 |

All values +/- standard error.

N/D = not determined
Table 2. Selected parameters from pQCT analysis of distal femoral metaphysis.

|                  | Trabecular Content | Trabecular Density |
|------------------|--------------------|--------------------|
| Females          | 4 months           | 1 year             | 4 months           | 1 year             |
| WT               | 0.23±/−0.01        | 0.25±/−0.02        | 196±/−8            | 146±/−9            |
| HET              | 0.27±/−0.01        | 0.32±/−0.03        | 208±/−6            | 167±/−15           |
| KO               | 0.27±/−0.01*       | 0.35±/−0.02*       | 207±/−6            | 178±/−8*           |
| % increase KO/WT | 15%                | 29%                | 5%                 | 18%                |
| Males            | 4 months           | 1 year             | 4 months           | 1 year             |
| WT               | 0.29±/−0.02        | 0.41±/−0.03        | 216±/−7            | 193±/−12           |
| HET              | 0.36±/−0.02*       | 0.49±/−0.04        | 242±/− 7           | 195±/−14           |
| KO               | 0.36±/−0.02*       | 0.65±/−0.05*       | 281±/−7*           | 242±/−18*          |
| % increase KO/WT | 20%                | 37%                | 14%                | 20%                |

|                  | Cortical Content  | Cortical Thickness | Periosteal circumference | Endosteal circumference |
|------------------|-------------------|--------------------|--------------------------|-------------------------|
| Females          | 4 months          | 1 year             | 4 months                 | 1 year                 | 4 months                | 1 year                 |
| WT               | 1.35±/−0.06       | 1.61±/−0.08        | 0.38±/−0.01              | 0.31±/−0.01            | 6.27±/−0.10             | 6.12±/−0.08            | 3.86±/−0.05            | 4.18±/−0.11            |
| HET              | 1.40±/−0.05       | 1.67±/−0.06        | 0.40±/−0.01              | 0.32±/−0.01            | 6.52±/−0.10             | 6.37±/−0.09            | 4.03±/−0.06            | 4.38±/−0.10            |
| KO               | 1.40±/−0.05       | 1.54±/−0.05        | 0.39±/−0.01              | 0.31±/−0.01            | 6.56±/−0.08*            | 6.43±/−0.06            | 4.10±/−0.08*           | 4.49±/−0.08            |
| Males            | 4 months          | 1 year             | 4 months                 | 1 year                 | 4 months                | 1 year                 |
| WT               | 1.05±/−0.04       | 1.35±/−0.08        | 0.33±/−0.01              | 0.31±/−0.01            | 6.49±/−0.10             | 6.39±/−0.10            | 4.39±/−0.08            | 4.47±/−0.07            |
| HET              | 1.24±/−0.05       | 1.39±/−0.04        | 0.38±/−0.01*             | 0.27±/−0.01*           | 6.90±/−0.10*            | 6.80±/−0.06*           | 4.55±/−0.10            | 5.08±/−0.09*           |
| KO               | 1.13±/−0.05       | 1.46±/−0.05        | 0.36±/−0.01              | 0.28±/−0.01            | 6.79±/−0.07*            | 7.01±/−0.08*           | 4.52±/−0.08            | 5.23±/−0.09*           |

All values ± standard error.

* indicates statistical significance p<0.05
Table 3. Femur Length in one year old animals.

|         | Wild Type | Heterozygote | Knockout |
|---------|-----------|--------------|----------|
| **Male**| 16.38±0.43| 16.74±0.42   | 16.70±0.37|
| **Female**| 16.58±0.39| 16.72±0.42   | 16.57±0.39|

All values +/- standard error.
Table 4. Selected parameters from distal femoral metaphyseal cancellous bone histomorphometry.

|                  | BV/TV % | Ob.S/BS % | N.Ob/BS #/mm | Oc.S/BS % | N.Oc/BS #/mm | MAR µm/d | BFR/BS µm³/µm²/d | BFR/TV %/y |
|------------------|---------|-----------|--------------|-----------|--------------|----------|-----------------|-------------|
| Wild type        | 13.2+/-1.4 | 9.3+/-1.2 | 0.84+/-0.08  | 1.22+/-0.13 | 0.078+/-0.01 | 0.75+/-0.11 | 0.14+/-0.03    | 0.09+/-0.02 |
| Knockout         | 18.8+/-1.7* | 19.8+/-1.9* | 1.68+/-0.152* | 1.08+/-0.16 | 0.061+/-0.01 | 1.00+/-0.05* | 0.22+/-0.02*   | 0.17+/-0.02* |
| Percent Difference KO/WT | 42%*     | 113%*     | 99%*         | -11%      | -22%         | 34%*      | 56%*           | 93%*        |

* indicates statistical significance at p < 0.05
A. Genomic Organization

Exon 1 Exon 2
("500 bp") (78 bp)
Intron 1 (1879 bp)

Exon 3
(1597 bp)
Intron 2 (~18.5kb)

Endogenous Locus

Targeting Vector

X

Targeted Allele

B. Wild Type Heterozygote Knockout

C. Wild Type Heterozygote Knockout
A. 

Wildtype  
Heterozygote  
Knockout  

Number of Bone Nodules  

Total Nodule Area  

Average Area per Nodule  

B. 

Wildtype  
Knockout  

Alizarin Red Stain

Concentration (μM)  

Wild Type  
Knockout
Targeted disruption of the osteoblast/osteocyte factor 45 gene (OF45) results in increased bone formation and bone mass

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