Impaired Osteoblast Function in GPRC6A Null Mice

Min Pi,1 Lishu Zhang,2,3 Shu-Feng Lei,3 Min-Zhao Huang,1 Wenyu Zhu,1 Jianghong Zhang,4 Hui Shen,3 Hong-Wen Deng,2,3 and L Darryl Quarles1

1Department of Medicine, University of Tennessee Health Science Center, Memphis, TN, USA
2College of Life Sciences and Bioengineering, Beijing Jiaotong University, Beijing, People’s Republic of China
3School of Medicine, University of Missouri–Kansas City, Kansas City, MO, USA
4Vanderbilt University, Center for Bone Biology, Clinical Pharmacology, Division/Medicine, Nashville, TN, USA

ABSTRACT

GPRC6A is a widely expressed orphan G protein–coupled receptor that senses extracellular amino acids, osteocalcin, and divalent cations in vitro. GPRC6A null (GPRC6A−/−) mice exhibit multiple metabolic abnormalities including osteopenia. To investigate whether the osseous abnormalities are a direct function of GPRC6A in osteoblasts, we examined the function of primary osteoblasts and bone marrow stromal cell cultures (BMSCs) in GPRC6A−/− mice. We confirmed that GPRC6A−/− mice exhibited a decrease in bone mineral density (BMD) associated with reduced expression of osteocalcin, ALP, osteoprotegerin, and Runx2-II transcripts in bone. Osteoblasts and BMSCs derived from GPRC6A−/− mice exhibited an attenuated response to extracellular calcium-stimulated extracellular signal-related kinase (ERK) activation, diminished alkaline phosphatase (ALP) expression, and impaired mineralization ex vivo. In addition, siRNA-mediated knockdown of GPRC6A in MC3T3 osteoblasts also resulted in a reduction in extracellular calcium-stimulated ERK activity. To explore the potential relevance of GPRC6A function in humans, we looked for an association between GPRC6A gene polymorphisms and BMD in a sample of 1000 unrelated American Caucasians. We found that GPRC6A gene polymorphisms were significantly associated with human spine BMD. These data indicate that GPRC6A directly participates in the regulation of osteoblast-mediated bone mineralization and may mediate the anabolic effects of extracellular amino acids, osteocalcin, and divalent cations in bone. © 2010 American Society for Bone and Mineral Research.

KEY WORDS: GPRC6A; G PROTEIN–COUPLED RECEPTOR (GPCR); OSTEOBLAST; BONE MINERAL DENSITY; GENE POLYMORPHISMS

Introduction

GPRC6A is a recently identified member of family C of G protein–coupled receptors (GPCRs) that senses extracellular cations, osteocalcin, and amino acids.1–4 Transcripts for GPRC6A are expressed in many tissues and organs, including lung, liver, spleen, heart, kidney, blood vessels, skeletal muscle, testis, brain, and bone.1–5 Consistent with its broad expression, ablation of GPRC6A is associated with multiple abnormalities, including glucose intolerance, hepatic steatosis, abnormal steroid biogenesis, and osteopenia, suggesting that GPRC6A may directly or indirectly regulate anabolic responses in multiple organs.6

Bone has been proposed to be a special tissue compartment where the combination of calcium, osteocalcin, and amino acids might constitute important extracellular signals regulating bone formation.6 While osteoblast-mediated bone formation is coupled to osteoclast-mediated bone resorption through the production by osteoblastic stromal cells of osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL),7 there is also emerging evidence for reverse coupling by factors released from osteoclast-mediated degradation of mineralized bone matrix, such as amino acids and calcium, that act on osteoblasts to fill in the resorptive cavity.8 Indeed, several mouse models with a primary increase in osteoclast-mediated bone resorption, including OPG and calcitonin null mice,9,10 have a secondary increase in osteoblast-mediated bone formation. Conversely, osteopetrotic disorders caused by a primary decrease in bone resorption are often accompanied by decreased bone formation, possibly through the loss of signals from osteoclasts.8 In addition, high ambient Ca2+ concentrations (in the range of 8 to 40 mM) and amino acids are present at sites of bone resorption,11 and a positive correlation exists between lumbar and femoral bone mass and the intake of protein and calcium.12 Since dietary protein-derived chemical signals may be derived from their metabolism into free amino acids,13 circulating levels of amino acids and calcium also may modulate signaling pathways in bone. Finally, both osteoblasts and osteoclasts respond to extracellular calcium in vitro through a putative extracellular amino acid and calcium-sensing GPCR.11,14,15
GPRC6A is expressed in osteoblasts, but its function in bone is not clear. Preliminary characterization of the skeleton of GPRC6A−/− mice indicates that loss of this receptor is associated with decreased bone mineral density (BMD) and impaired mineralization of bone, but the presence of other abnormalities confounded the ability to ascertain the direct and indirect effects of GPRC6A on skeletal function. To determine if GPRC6A is a potential candidate for the purported extracellular calcium-sensing receptor in osteoblasts, we performed a more detailed assessment of the skeletal phenotype of GPRC6A−/− mice, examined the function of primary osteoblasts and bone marrow stromal cells derived from these mutant mice ex vivo, and evaluated whether polymorphisms in GPRC6A are associated with skeletal abnormalities in humans by a gene association analysis.

**Materials and Methods**

**GPRC6A knockout mice**

The GPRC6A-deficient mouse model was created by replacing exon 2 of the GPRC6A gene with the hygromycin resistance gene, as described previously. Mice were maintained and used in accordance with recommendations of the National Research Council's (1985) *Guide for the Care and Use of Laboratory Animals* (DHHS Publication NIH 86-23, Institute on Laboratory Animal Resources, Rockville, MD, USA) and following guidelines established by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

**RT-PCR and real-time RT-PCR**

Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using two-step RNA PCR (Perkin-Elmer, Waltham, MA, USA). In separate reactions, 2.0 μg of DNase-treated total RNA was reverse transcribed into cDNA with the respective reverse primers specified below and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Rockville, MD, USA). Reactions were carried out at 42°C for 60 minutes followed by 94°C for 5 minutes and 5°C for 5 minutes. The products of first-strand cDNA synthesis were directly amplified by PCR using AmpliTaq DNA polymerase (Perkin-Elmer). The primer sets used to amplify various gene transcripts with intron spanning are as follows: mGPRC6A.189F: GGCGAT CCAAGACGACCACAATCCG and mGPRC6A.539R: CCAAGCTTGATTCAATACTCACCTGTGGC; mALP.905F: AAACCAAGACACGATCTTCC and mALP.1458R: CTGGGCCCTGTAGTTGTTGT, G3PDH.F143: GACCCCTTCATTGAC- CATG; and G3PDH.R1050: GGTCTTACTCCATTGAGGCC- CATGT for control RNA loading.

For quantitative real-time RT-PCR assessment of bone marker expression, we isolated and reverse transcribed 2.0 μg of total RNA from the long bones of 8-week-old mice as described previously. PCR reactions contained 100 ng of template (cDNA or RNA), 300 nM each of forward and reverse primer, and 1 x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in 50 μL. Samples were amplified for 40 cycles in an iCycler iQ Real-Time PCR Detection System (Bio-Rad) with an initial melt at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. PCR product accumulation was monitored at multiple points during each cycle by measuring the increase in fluorescence caused by the binding of SybrGreen I to dsDNA. The threshold cycle (Ct) of tested-gene product from the indicated genotype was normalized to the Ct for cyclophilin A. Dissociation analysis was used to confirm the presence of a single transcript and lack of primer-dimer amplification in all PCR reactions.

**PIXImus bone densitometer analysis, X-ray, and bone histology**

Bone mineral density (BMD) values of whole skeletons and femurs were assessed at 6, 8, 12, and 16 weeks of age using a PIXIImus bone densitometer (Lunar Corp., Madison, WI, USA) as described previously. Femurs were dissected free of muscle and X-rayed with a Faxitron model MX-20 specimen radiography system (Faxitron X-Ray Corp., Lincolnshire, IL, USA). In addition, length and width (measured at 50% of the femur length) were assessed in isolated femora. Skeletons of mice were prelabeled twice with calcine (Sigma C-0875, 30 μg/g of body weight; Sigma Chemical Company, St. Louis, MO, USA) by intraperitoneal injection at 8 and 3 days prior to sacrifice. Tibiae and femora were removed from 8- and 16-week-old mice, fixed in 70% ethanol, prestained in Villanueva stain, and processed for methyl methacrylate embedding. Then 10-μm Villanueva-pretained sections were evaluated under fluorescent light, as reported previously by our laboratory.

**Micro-computed tomographic (μCT) analysis**

The distal femoral metaphyses were scanned using a μCT 40 device (Scanco Medical AG, Wayne, PA, USA); 167 slices of the metaphysis under the growth plate, constituting 1.0 mm in length, were selected. The 3D images were generated using the following values for a gauss filter (σ = 0.8, support 1) and a threshold of 275. A 3D image analysis was performed to determine bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). Cortical bone was measured on the midshaft region of cortical bone in 50 slices of the diaphysis, constituting 0.3 mm in length. The mean cortical thickness (Ct.Th) was determined at eight different points on the cortical slice.

**Primary bone marrow stromal cells and osteoblastic cells culture**

The femora and tibiae from 8-week-old wild-type and GPRC6A−/− mice were dissected, the ends of the bones were cut, and marrow was flushed out with 2 mL of ice-cold α modified essential medium (α-MEM) containing 10% fetal bovine serum (FBS) by using a needle and syringe. A suspension of bone marrow cells was obtained by repeated aspiration of the cell preparation through a 22G needle, and nucleated cells were counted with a hemocytometer. Cells were seeded into 6-well plates at a density of 3 x 10^3 cells/mL and cultured for 3 days in α-MEM supplemented with 10% FBS, 100 kU/L of sodium penicillin G, and 100 mg/L of streptomycin sulfate in a humidified incubator with 5% CO2 and 95% air at a temperature of 37°C. On day 3, all nonadherent cells were removed with the first medium change, and then the adherent cells [representing bone marrow–derived mesenchymal stem cells (BMSCs)] were grown for additional
periods of up 3 days in the same medium. After overnight quiescence, the cells were stimulated for 5 minutes with calcium, NPS-R568, and arginine at the concentrations indicated.

We used modifications of a nonenzymatic method for obtaining the osteoblastic cell lines. A fragment of the frontal and/or parietal bone from the single calvarium was aseptically removed from a 3- to 7-day-old mouse. Suture lines and endosteum were dissected away, and the bone fragment was placed in a culture dish. One or two metal strips were positioned on the endocranial surface and incubated for 3 to 4 days in DMEM nutrient mixture F-12 (Invitrogen, Carlsbad, CA, USA) containing 10% (v/v) FBS, 100 kU/L of sodium penicillin G, and 100 mg/L of streptomycin until the outgrowth of osteoblasts. The metal strips were removed, and the cells were allowed to grow until approximately 60% confluent. The cells were subcultured and propagated by incubation in α-MEM (Invitrogen) containing 10% FBS, 100 kU/L of sodium penicillin G, 100 mg/L of streptomycin, and 50 μg/mL ascorbic acid in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Agonist stimulation and Western blotting
Agonist stimulation was performed in quiescent cells. Quiescence was achieved in subconfluent cultures by removing the medium and washing with Hank’s balanced salt solution (Invitrogen) to remove residual serum, followed by incubation for an additional 24 hours in serum-free medium. After agonist treatment at the specified concentrations and durations, cells were washed twice with ice-cold PBS and scraped into 250 μL of lysis buffer (25 mM Hepes; pH 7.2, 5 mM MgCl₂, 5 mM EDTA, 1% Triton X-100, and 0.02 tablet/mL of protease inhibitor mixture). Equal amounts of lysates were subjected to 10% SDS-PAGE and transferred onto nitrocellulose paper. The transferred membranes were incubated at room temperature for 1 hour with antibody to digoxigenin conjugated to horseradish peroxidase (Boehringer Mannheim) and then incubated with antibody to phospho-ERK1/2 mitogen-activated protein kinase antibody (Cell Signaling Technology Inc., Beverly, MA, USA). The membranes were washed three times with 1× TBS and once with 50% TBS. The membranes were incubated with 1:10,000 dilution of horseradish peroxidase-labeled anti-rabbit IgG and then washed. The membranes were incubated with diaminobenzidine (DAB) and then developed with 0.02 tablet/mL of protease inhibitor mixture. Equal amounts of lysates were subjected to 10% SDS-PAGE and transferred onto nitrocellulose paper. The transferred membranes were incubated at room temperature for 1 hour with antibody to ERK 1/2. The membranes were washed three times with 1× TBS and once with 50% TBS. The membranes were incubated with 1:10,000 dilution of horseradish peroxidase-labeled anti-rabbit IgG and then washed. The membranes were incubated with diaminobenzidine (DAB) and then developed with 0.02 tablet/mL of protease inhibitor mixture. Equal amounts of lysates were subjected to 10% SDS-PAGE and transferred onto nitrocellulose paper. The transferred membranes were incubated at room temperature for 1 hour with antibody to phospho-ERK1/2 mitogen-activated protein kinase antibody (Cell Signaling Technology Inc., Beverly, MA, USA). The membranes were washed three times with 1× TBS and once with 50% TBS. The membranes were incubated with 1:10,000 dilution of horseradish peroxidase-labeled anti-rabbit IgG and then washed. The membranes were incubated with diaminobenzidine (DAB) and then developed with 0.02 tablet/mL of protease inhibitor mixture.

Mineralization assay
The formation of in vitro mineralization nodules was determined by alizarin red S histochemical staining (15). The 14-day cultured cells in the α-MEM containing 10% FBS, 100 kU/L of sodium penicillin G, 100 mg/L of streptomycin sulfate, 50 μg/mL of ascorbic acid, and 4 mM of arginine were fixed for 24 hours in a solution of 10% Formalin, methanol, and water (1:1:1.5); the fixative was removed; and the fixed cells and matrices were stained for 15 minutes with a 2% (w/v) solution of alizarin red S and 5% SDS for 30 minutes at 70°C. The stained samples were washed three times with water and then air-dried.

Immunohistochemistry for osteocalcin (OC) and measurement of alkaline phosphatase (ALP) activity
Anti-osteocalcin antibody was obtained from Dr Larry Fisher (NIDCR, National Institutes of Health). After deparaffinization and rehydration, the bone sections were immersed in 3% hydrogen peroxide to quench endogenous peroxidase and further digested with 1 μg/mL trypsin for 30 minutes at 37°C. Sections then were blocked with 1% bovine serum albumin at room temperature for 2 hours. The primary antibodies were added to the sections and incubated overnight at 4°C. After washing, the sections were coated with biotinylated second antibody (Vector Laboratories Burlingame, CA, USA) at a dilution of 1:200 and then incubated at room temperature for 60 minutes. The sections were washed again and incubated with the ABC reagent (Vector Laboratories, Burlingame, CA, USA) at room temperature for 60 minutes. The 3,3′-diaminobenzidine substrate was used to visualize immunoreaction sites. Sections were counterstained with hematoxylin and mounted on glass slides. Negative controls were obtained by substituting the primary antibody with normal IgG.

Alkaline phosphatase (ALP) activity was detected by an enzyme-substrate assay in frozen sections of bone. Briefly, fresh nondecalcified spines from 8-week-old wild-type and Gprc6a knockout mice were dissected and embedded with Optimum Cutting Temperature (OCT, Sakura Finetek, USA, Inc., Torrance, CA, USA) medium. ALP activity was detected in 12-μm-thick cryostat sections at alkaline pH by adding ALP substrate 4-nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) directly on the slides according to the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN, USA). Endogenous ALP activity was visualized as a purple-blue color reaction with methyl green (nuclei) counterstain.

In situ hybridization for osteocalcin mRNA expression
A mouse osteocalcin cDNA plasmid, EcoRV/Pst I subclone inserted into pSP65, was obtained from Dr Stephen Harris (University of Texas Health Science Center, San Antonio, TX, USA). A digoxigenin (DIG)-labeled cRNA probe was prepared by using the RNA Labeling Kit (Roche Applied Science). To make the antisense probe, a partial mouse osteocalcin cDNA (0.5 kb) was cleaved by Hind III and labeled with Sp6 polymerase. Sections of the tibia from a 8-week-old mouse were dewaxed, dehydrated, fixed with 4% paraformaldehyde, treated with 2% glycine and proteinase K, acetylated using an acetic anhydride–TEA solution, washed again and incubated with the ABC reagent (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:200 and then washed. The sections were coated with biotinylated second antibody (Vector Laboratories, Burlingame, CA, USA) at room temperature for 60 minutes. The sections were washed again and incubated with the ABC reagent (Vector Laboratories, Burlingame, CA, USA) at room temperature for 60 minutes. The 3,3′-diaminobenzidine substrate was used to visualize immunoreaction sites. Sections were counterstained with hematoxylin and mounted on glass slides. Negative controls were obtained by substituting the primary antibody with normal IgG.

Serum and urine biochemical measurements
Serum was collected using a retro-orbital bleeding technique. For urine samples collection, mice were placed in metabolic cages (Hatteras Instrument Cary, NC, USA), and urine was collected for 24 hours. The urine volume was measured before storage at −70°C.
Serum and urinary calcium were measured by the colorimetric cresolphthalein-binding method, and phosphorus was measured by the phosphomolybdate–ascorbic acid method. Serum tartrate-resistant acid phosphatase (TRACP) was assayed with the ELISA-based SBA Sciences mouseTRAP assay (Immutoxics, Inc., San Clemente, CA, USA). Serum parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D were measured with an Immunodiagnostic Systems device (Immunodiagnostic Systems, Ltd., Scottsdale, AZ, USA). Serum Fgf23 levels were measured using an FGF-23 ELISA kit (Kainos Laboratories, Inc., Tokyo, Japan) following the manufacturer’s protocol. Creatinine was measured by the colorimetric alkaline picrate method (Sigma Kit 555). Urinary protein and deoxypridinolme (Dpd) were measured by Bio-Rad and Metra Biosystems (Hercules, San Diego, CA, USA) devices, respectively.

siRNA transfection

Two siRNAs (siRNA.m1638: CCAACACAGCTTGGCTAT and siRNA.m2553: GCAGAAGACTAACACCAAA) specific for marine GPRC6A (GenBank Accession Number NM_153071), identified using the ‘‘siRNA Target Finder’’ at the Web site (www.ambion.com/techlib/misc/sirna_target_finder.html), were used as a template for synthesizing siRNA. Double-stranded siRNA was synthesized from DNA oligonucleotides using the Silencer siRNA construction kit (Ambion, Austin, TX, USA) according to manufacturer’s instructions.

For transfection, MC3T3 cells were plated at a density of 1.0 × 10⁴ cells per well in a 6-well dish and grown overnight. Cells were transiently transfected with 25 nM of siRNA using 4 μL of Lipofectamine 2000 (Invitrogen) for 4 hours, after which growth medium was replaced. Then, 48 hours after transfection, cells were quiescence overnight and then stimulated by 10 nM of calcium for 5 minutes and lysed for Western blot analysis. Scrambled siRNA (Select Negative Control 1 siRNA, Ambion) was used as negative control.

Study populations and genotyping

The 1000 unrelated American Caucasians of European origin studied were identified from our established and expanding genetic repertoire at UMKC. Signed informed-consent documents were obtained from all study participants before they entered the study. The population genetic research on GPRC6A was approved by the Institutional Review Board. The inclusion and exclusion criteria are the same as those described elsewhere. The basic characteristics of the studied subjects are listed in Supplemental Table 1. Briefly, the sample includes 251 elderly men (age range at recruitment 52.9 to 87.9 years), 250 younger men (age range at recruitment 19.1 to 49.9 years), 250 postmenopausal women (age range at recruitment 54.3 to 80.6 years), and 249 premenopausal women (age range at recruitment 19.2 to 53.2 years). Area BMD (g/cm²) at the spine and hip region was measured by dual-energy X-ray absorptiometry (DXA) with Hologic QDR 4500W densitometers (Hologic, Inc., Bedford, MA, USA). The scanners were calibrated daily. The coefficient of variation (CV) values of the DXA measurements were about 1.98% and 1.87% for spine and hip BMD, respectively.

Results

Skeletal imaging

By X-ray analysis, we observed no significantly gross developmental abnormalities in the skeleton (Fig. 1A). Consistent with prior reports, BMD as assessed by dual-energy X-ray absorptiometry (DEXA) was significantly lower in 16-week-old GPRC6A−/− mice compared with wild-type mice (Fig. 1B). We observed no difference in femur length (Fig. 1C, D), but there was a significant reduction in femur width between GPRC6A−/− and wild-type mice (Fig. 1C, E). In addition, we found that BMD was reduced by μCT in both the metaphyseal area, which predominately consists of trabecular bone, and the midshaft region, which is composed of cortical bone (Fig. 1F). These differences were not due to effects of a mixed genetic background because these animals were crossed onto C57BL/6 mice for more than six generations. There were no demonstrable changes, however, in bone structural parameters, including bone volume (BV/TV) and cortical thickness (CT.Th), between GPRC6A−/− and wild-type mice, as assessed by μCT, suggesting that the decreased BMD was not due to structural changes in bone architecture.

Biomarkers/immunohistochemistry/gene expression

We found no evidence for GPRC6A effects on osteoclast function. In this regard, the osteoclastic marker tartrate-resistant acid

DELETION OF GPRC6A CAUSES OSTEOPENIA
phosphatase (TRACP) was not significantly different between wild-type and GPRC6A/C0/C0 mice (Table 1), consistent with our prior findings that neither urinary Dpd/creatinine ratio nor serum TRACP levels were different between GPRC6A/C0/C0 and GPRC6A+/+ mice.(6) To better understand the effect of GPRC6A on osteoblasts, we examined gene expression profiles of whole-bone samples from GPRC6A/C0/C0 mice. Assessment of expression of osteoblast markers in bone from 16-week-old GPRC6A/C0/C0 mice revealed reductions in osteocalcin, ALP, osteoprotegerin, and Runx2-II message levels compared with wild-type mice by real-time RT-PCR (Table 1), consistent with a reduction in mineral apposition rates, as reported previously.(6) We also confirmed by in situ hybridization and immunohistochemical staining that osteocalcin expression (Fig. 2A–D) and ALP activity (Fig. 2E, F) were reduced in bone from 16-week-old GPRC6A/C0/C0 mice. The chondrocyte marker ColII and adipocyte markers αP2 and Lp1 were not significantly different between wild-type and GPRC6A/C0/C0 mice (Table 1).

Impaired extracellular calcium response in BMSCs and calvarial-derived osteoblastic cells cultures from GPRC6A/C0/C0 mice

To explore whether the observed skeletal abnormalities are due to a primary osteoblastic abnormality owing to loss of GPRC6A in

![Image](https://example.com/image.png)

**Fig. 1.** Characterization of the bone phenotype of GPRC6A−/− mice. (A) X-ray appearance for GPRC6A+/+ and GPRC6A−/− mice at 16 weeks of age. (B) Comparison of the total BMD analysis by PIXImus analysis in GPRC6A+/+ and GPRC6A−/− mice at ages ranging from 6 to 16 weeks. (C) Abnormalities in femur bone of GPRC6A−/− mice by X-ray. (D, E) Comparison of femur length (D) and femur width (E) in GPRC6A+/+ and GPRC6A−/− mice at 16 weeks of age. (F) Comparison of the structure and BMD of the femur as assessed by µCT scanning in 16-week-old GPRC6A+/+ and GPRC6A−/− mice. Data represent the mean ± SEM from 6 to 10 mice in each group. Significant difference from GPRC6A+/+ and GPRC6A−/− mice at p < .05.

**Table 1.** Gene Expression Profile in Bone From GPRC6A+/+ and GPRC6A−/− Mice

| Gene             | Accession number | GPRC6A+/+ | GPRC6A−/− |
|------------------|------------------|-----------|-----------|
| ALP              | NM_007431        | 0.493 ± 0.096 | 0.194 ± 0.0045* |
| Osteocalcin      | NM_007541        | 1.101 ± 0.068 | 0.411 ± 0.1* |
| Osteoprotegerin  | MMU94331         | 0.0755 ± 0.021 | 0.0159 ± 0.0045* |
| Runx2-II         | NM_009820        | 0.156 ± 0.034 | 0.0563 ± 0.0061* |
| Osterix          | AF184902         | 0.00187 ± 0.00078 | 0.00136 ± 0.00047 |
| RANKL            | NM_011613        | 0.000987 ± 0.00011 | 0.00124 ± 0.00037 |
| TRACP            | NM_007388        | 0.793 ± 0.188 | 0.742 ± 0.12 |
| Coll1            | NM_031163        | 0.457 ± 0.219 | 0.186 ± 0.056 |
| αP2              | NM_024406        | 1.229 ± 0.305 | 1.624 ± 0.342 |
| Lp1              | NM_008509        | 0.0803 ± 0.0149 | 0.118 ± 0.054 |

Data are mean ± SEM from 8-week-old mice. Values are expressed relative to the housekeeping gene cyclophilin A. ALP = alkaline phosphatase; RANKL = receptor activator for nuclear factor κB ligand; TRACP = tartrate-resistant acid phosphatase; αP2 = adipocyte fatty acid–binding protein 2; Coll1 = collagen type II; and Lp1 = lipoprotein lipase.

*Denotes significant difference between GPRC6A+/+ and GPRC6A−/− mice at p < .05.
these cells, we evaluated the response to calcium, calcimimetic, and arginine of cultured bone marrow stromal cell cultures (BMSCs) and calvarial osteoblasts obtained from wild-type and GPRC6A/−/− mice (Fig. 3). In both BMSCs and primary osteoblasts, we observed a reduced ability of extracellular calcium to stimulate ERK activity in GPRC6A/−/− mice compared with wild-type cells (approximately 50% reduction; Fig. 3A, B). In addition, a receptor allosteric modulator, the calcimimetic NPS-568, and the amino acid receptor ligand L-arginine both had an attenuated ERK activation in BMSCs and primary osteoblasts obtained from GPRC6A/−/− mice compared with wild-type cells (Figs. 3C, D).

To further evaluate osteoblast dysfunction in GPRC6A/−/− mice, we examined the impact of loss of GPRC6A on the capacity of cultured osteoblasts and BMSCs to undergo differentiation in vitro, as assessed by culture duration–dependent changes in alkaline phosphatase expression and activity. We found that wild-type osteoblasts and BMSC cultures increased alkaline phosphatase expression and activity during differentiation, but this increase was attenuated in GPRC6A/−/− cells (Fig. 4A, B). These results are consistent with the gene expression data by real-time RT-PCR (Table 1) and immunochemical staining (Fig. 2E, F) in intact bone. Moreover, the addition of arginine increased mineralization in wild-type BMSC cultures but not in GPRC6A/−/− cells (Fig. 4C). These data indicate that the lack of GPRC6A impairs the ability of osteoblasts to sense calcium and amino acids as well as their ability to undergo differentiation and form a mineralized extracellular matrix.

Response of osteoblastic cell line MC3T3 to extracellular calcium through GPRC6A

To confirm the role of GPRC6A as a relevant calcium- and amino acid–sensing receptor, we examined the MC3T3 clonal osteoblastic cell line, which is known to express a putative calcium-sensing receptor.(4,24) We examined the function of GPRC6A by siRNA-mediated knockdown in MC3T3 osteoblasts. We transfected MC3T3 cells with either GPRC6A siRNA.m2563 or siRNA.m1638. Mock-transfected cells and transfected cells with a random negative control siRNA plasmid were used as the control. Transfected MC3T3 cells GPRC6A siRNA.m2563 and siRNA.m1638 successfully downregulated the levels of mRNA expression of GPRC6A in MC3T3 cells compared with wild-type cells (Figs. 5A, B).

Next, we performed a gene association analysis to test whether GPRC6A gene polymorphisms are associated with the variation in human BMD.(25) Nine SNPs located from 21 kb downstream to
Fig. 3. Decreased response to extracellular calcium, NPS-R568, and amino acid in BMSCs or osteoblasts from GPRC6A−/− mice. (A, B) Bone marrow stromal cell cultures (BMSCs) and osteoblasts from GPRC6A−/− mice displayed impaired responses to extracellular calcium-mediated ERK activation. (C) ERK activation in response to the calcimimetic NPS-R568 also was impaired in BMSCs derived from GPRC6A−/− mice. (D) Osteoblasts derived from GPRC6A−/− mice also showed impaired ERK activation in response to the amino acid arginine. ERK phosphorylation was assessed by Western blot analysis using an anti-phospho-ERK antibody. Data represent three to four independent experiments.

Fig. 4. Characterization of temporal maturational sequence in osteoblasts or BMSCs from GPRC6A+/+ and GPRC6A−/− mice. (A) RT-PCR of alkaline ALP from 4- and 10-day cultured cells derived from 8-week-old GPRC6A+/+ and GPRC6A−/− calvaria. (B) ALP activity. The GPRC6A−/− BMSCs had significantly lower ALP activity at days 10 and 14 of culture compared with age-matched wild-type mice. (C) Quantification of mineralization. Alizarin red S was extracted with 10% cetylpyridinium chloride and quantified as described under “Materials and Methods.” Data represent the mean ± SEM from three to four independent experiments.
42 kb upstream of the GPRC6A gene were tested for their association with hip and spine BMD variation in the human subjects studied. All nine SNPs are in HWE ($p > .01$) with minor allele frequency (MAF) greater than 5%. The basic information on these SNPs is shown in Table 2.

Four SNPs, rs686708, rs571296, rs6938235, and rs17078405, showed nominal significant associations with hip BMD ($p < .05$; Table 2), but these associations became nonsignificant after multiple-testing correction (significance-threshold $p$ values were set at .0074 by the program SNPSpD based on Nyholt’s method(23)). All nine SNPs showed nominal significant associations with spine BMD ($p < .05$; Table 2), but after multiple-testing correction (threshold $p = .0074$), only the association of SNPs rs686708 and rs571296 with spine BMD remained significant ($p = .0010$ and $p = .0068$, respectively).

For the SNP rs686708 (A/G), which showed the strongest evidence of association with spine BMD, its allele A was associated with lower spine BMD values in the studied subjects. The subjects with the AA or AG genotypes had significant lower spine BMD values than those with the GG genotype (raw spine BMD values were 1.016, 1.032, and 1.055 g/cm$^2$ for the AA, AG, and GG genotypes, $p = .029$), showing an allele dose effect of 0.02 g/cm$^2$ lower spine BMD value per copy of the A allele. For the SNP rs571296 (A/G), its minor allele G was associated with lower spine BMD values. Subjects with the GG or AG genotypes had significant lower spine BMD values than those with the AA genotype (raw spine BMD values were 1.061, 1.037, and 1.019 g/cm$^2$ for AA, AG, and GG genotypes, $p = .048$), showing an allele dose effect of 0.021 g/cm$^2$ lower spine BMD value per copy of the G allele. Linkage disequilibrium (LD) analysis based on the

### Table 2. Associations of the Analyzed GPRC6A SNPs With Hip and Spine BMD

| No. | SNP name | Position (bp) | Role | Major/minor alleles | MAF$^a$ | $p$ Value,$^b$ | $p$ Value,$^b$ |
|-----|----------|---------------|------|---------------------|--------|---------------|---------------|
| 1   | rs686708 | 117198376     | Downstream | A/G                 | 0.453  | .0280         | .0010         |
| 2   | rs571296 | 117199251     | Downstream | A/G                 | 0.344  | **.0231**     | **.0068**     |
| 3   | rs587771 | 117212122     | Downstream | G/T                 | 0.205  | .9168         | .0713         |
| 4   | rs6924002| 117220916     | CDS        | A/T                 | 0.307  | .058          | .0909         |
| 5   | rs1707833 | 117230654     | Intron     | C/T                 | 0.058  | .9099         | .0400         |
| 6   | rs6938235| 117254639     | Intron     | G/T                 | 0.330  | **.0268**     | **.0139**     |
| 7   | rs17078405| 117260764    | Upstream   | A/G                 | 0.061  | **.0400**     | **.0244**     |
| 8   | rs339319 | 117295428     | Upstream   | G/A                 | 0.277  | .6473         | .483         |
| 9   | rs339321 | 117298883     | Upstream   | T/C                 | 0.276  | .6769         | .4000         |

$^a$MAF in our sample.

$^b$p Values in bold indicate nominal significant associations ($p \leq .05$). $p$ Values in bold italic indicate significant associations after adjusting for multiple testing ($p < .0074$).

For the SNP rs686708, the adjusted BMD values for the three genotypes AA, AG, and GG in the samples studied were as follows: $-0.015$, $0.000$, and $0.022$ ($p = .023$); For the SNP rs571296, the adjusted BMD values for the three genotypes AA, AG, and GG in the samples studied were as follows: $0.025$, $0.005$, and $-0.012$ ($p = .048$).
Discussion

GPRC6A is a widely distributed member of the amino acid– and calcium-sensing receptor family that appears to function as an nutrient-sensing anabolic receptor. In this study we have demonstrated direct effects of GPRC6A on regulating osteoblast function. The predominant effect of GPRC6A deficiency in vivo was to impair bone mineralization, which was associated with a reduction in osteoblast gene expression markers without changing bone-resorptive markers in vivo. We show that these changes are due, at least in part, to direct effects of the loss of GPRC6A in osteoblasts. In this regard, a primary GPRC6A-dependent effect on osteoblasts is suggested by the abnormal differentiation and attenuated response to calcium and arginine stimulation of osteoblasts derived from GPRC6A null mice in vitro. A direct role of GPRC6A in osteoblasts also was confirmed by siRNA-mediated knockdown of this receptor in MC3T3-E1 osteoblasts that was associated with reduction in calcium-stimulated ERK activation. The human relevance of these observations is supported by the association between SNPs rs686708 and rs571296 and reductions in spinal BMD in Caucasians, raising the possibility that GPRC6A polymorphisms may contribute to human osteopenia.

Several limitations of this study prevent more definitive conclusions. First, since GPRC6A null mice represent a global knockout of this receptor, our in vivo findings are still confounded by the potential actions of concomitant alterations in the ratio of testosterone and estrogens known to be present in these mice or to other undefined secondary consequences of the generalized loss of GPRC6A. The absence of increased bone resorption in GPRC6A null mice, however, along with the direct in vitro effects of GPRC6 on osteoblast function in two different models (GPRC6A null and GPRC6A knockdown), suggests that direct effects from receptor loss rather than alterations in sex hormones may account for the osteopenia in GPRC6A null mice. Indeed, testosterone deficiency typically leads to increased osteoclast-mediated bone resorption, which was not observed in GPRC6A null mice. While the attenuation of calcium and amino acid response in isolated osteoblasts from GPRC6A null mice supports a direct role, further studies that compare the effects of testosterone replacement with administration of GPRC6A ligands (e.g., calcium, strontium, amino acids, and osteocalcin) to restore bone mass in GPRC6A null mice will be needed to determine the relative contribution of secondary alterations in sex hormones and primary loss of calcium-sensing receptor responses to the observed bone phenotype in GPRC6A null mice. Second, with regard to the role of GPRC6A in humans, SNPs rs686708 and rs571296 are not in the coding region of GPRC6A and therefore are not definitely the actual disease-causing variants. Fine mapping of SNPs tightly linked to SNPs rs686708 and rs571296 and functional studies will be required to identify the causal variants in the GPRC6A region responsible for human osteopenia.

Our data also support the presence of more than one calcium-sensing mechanism in osteoblasts. Indeed, the full complement of physiologically relevant receptors mediating the changes in amino acids, calcium, and osteocalcin released by osteoclastic-mediated bone resorption continues to be explored. Recent data support a role for the amino acid– and calcium-sensing receptor CASR in regulating osteoblast function. Conditional deletion of the 7-TM domain of CASR using Col1-2.3-Cre results in a severe skeletal phenotype. In addition, some studies have implicated CASR in the differentiation of osteoblasts and growth plate chondrocytes. On the other hand, the original CASR knockout mouse model (which lacks exon 5) fails to display a bone phenotype when hyperparathyroidism is corrected by either performing a “molecular parathyroidectomy” or ablating PTH receptor signaling. Also, the anabolic bone effects of strontium ranelate on osteoblast replication and survival are independent of CASR. The explanation for disparity in bone phenotype between the conditional and global CASR–/– lacking exon 5 knockout mouse models is not clear. One possibility is that global CASR–/– mice lacking exon 5 are hypomorphic owing to the persistent function of an alternatively spliced exon 5–deleted CASR in bone and cartilage. Although CASR null osteoblasts retain their calcium-sensing capabilities, so far no signal-transduction activity of the transfected alternatively spliced exon 5–deleted CASR has been identified. Another theoretical possibility is that the actions of cre-recombinase in the conditional CASR model creates a secreted extracellular domain of CASR that acts as a dominant-negative “decoy” receptor to disrupt CASR function in nearby tissues. Alternatively, the expression of GPRC6A in bone and osteoblasts and the resulting bone phenotype raise the possibility that GPRC6A is a candidate for another osteoblastic calcium-sensing receptor that is distinct from CASR and which could account for some of these disparate observations. The residual calcium-sensing functions in GPRC6A null mice, after silencing of GPRC6A in MC3T3 cells, leaves open the possibility of more than one calcium-sensing receptor in osteoblasts.

Nevertheless, the wide expression of GPRC6A also raises the possibility that GPRC6 could have a physiologic function in coordinating the responses of bone with other organ systems to changing nutritional cues. For example, there may be a physiologic need to coordinate renal calcium excretion and osteoblast-mediated bone formation. Theoretically, a primary decrease in bone formation and decreased buffering capacity for calcium, with consequent increased urinary expression of dietary calcium, could account for the relationship between osteopenia and hypercalciuria in GPRC6A–/– mice. Conversely, activation of GPRC6A would be predicted to stimulate osteoblast-mediated bone calcium accretion and renal calcium conservation to meet the need for bone mineralization. There are some enigmatic clinical disorders with features similar to those of GPRC6A–/– mice that support the possibility of coordinated effects between bone formation and renal conservation of calcium. In this regard, a subset of male patients with idiopathic osteoporosis described by Zerwekh and Pak35 has the combined features of decreased osteoblast-mediated bone formation and hypercalciuria without...
evidence of hypogonadism, secondary hyperparathyroidism, or abnormal vitamin D levels. Some patients with what appears to be primary hyperparathyroidism and nephrolithiasis also have concomitant low bone density and low bone turnover without increased PTH levels that would be expected from a sole defect in renal calcium handling. There are also examples of primary bone defects that are associated with hypercalciuria without increased PTH. These include osteogenesis imperfecta type 1 caused by Col1A1 and Col1A2 mutations, infantile hypophosphatasia caused by inactivating mutations of ALP, and McCune-Albright syndrome caused by activating mutations of GNAS1. It will be interesting to determine if gene polymorphisms of GPRC6A are associated with osteopenic and hypercalciuric clinical disorders.

These findings also expand the repertoire of GPCRs that regulate the functional activity and provides greater insights into the function of Gαi in bone. In this regard, GPRC6A is predominantly a Gαi-coupled receptor. Compared with other well-characterized anabolic receptors, such as the PTH receptor, which is coupled to Gαs and Gαq-dependent signaling pathways that regulate proliferation and differentiation of osteoblasts, these studies suggest that Gαi-dependent pathways play a distinct role in regulating osteoblast gene expression, mainly affecting the mineralization of bone. The classic effect of Gαi activation in inhibiting adenylyl cyclase activity might be expected to oppose signaling of Gαs-coupled GPCRs in osteoblasts. There is little available information on the role of osteoblast signaling by Gαi, but the pertussis toxin–dependent proliferative actions of fluoride and strontium on osteoblasts in vitro, Gαi-coupled apelin receptor stimulation of osteoblast proliferation in vitro, development of osteoporosis in Gαi-coupled CB2 cannabinoid receptor knockout mice, as well as our findings in GPRC6A null mice, suggest that Gαi signaling is important. Potential anabolic actions of Gαi-coupled receptors, including GPRC6A, might be mediated through activation of mitogen-activated protein kinase (MAPK) pathways.

In contrast to our studies, Wellendorph and colleagues generated a GPRC6A null mouse by targeting exon VI of GPRC6A, which encodes the seven transmembrane domain and C-terminal tail. Analysis of these mice failed to identify any skeletal abnormalities in 13-week-old mice. At present, we have no explanation for these disparate findings, but similar to the differences observed between existing models described earlier, this may represent another example where disrupting the extracellular domain results in no expression of the GPCR, whereas disrupting the transmembrane domain may lead to partial translation of the extracellular domain and functional effects. Regardless, there are other instances of multiple laboratories independently targeting the same GPCR and failing to find similar phenotypes that have been attributed to a variety of different mechanisms.

In summary, we have shown that GPRC6A has a direct function in osteoblasts. The ligand profile of GPRC6A, which includes extracellular calcium, calcimimetics, amino acids, and osteocalcin, along with the complex phenotype of GPRC6A null mice, suggests that GPRC6A may represent an anabolic receptor that responds to a variety of nutritional and hormonal signals and may serve to coordinate the functions of multiple organs, including bone, to changes in the local and systemic concentrations of these ligands.

**Disclosures**

LDQ serves as a consultant for Amgen, Shire, Cytochroma, Novartis, and Osteometrics and receives research support from Amgen, Genzyme, Servier, and VasoGenix. All the other authors state that they have no conflicts of interest.

**Acknowledgments**

This work was supported by NIH Grant R01-AR37308 (LDQ), COBRE Grant P20 RR017686 (MP), and NIH Grants R01 AR05 0496-01, R21 AG027110, R01 AG026564, and P50 AR055081 (HWD, SFL, H5, and LSZ).

**References**

1. Kuang D, Yao Y, Lam J, Tsushima RG, Hampson DR. Cloning and characterization of a family C orphan G protein–coupled receptor. J Neurochem. 2005;93:383–391.
2. Wellendorph P, Hansen KB, Balsgaard A, Greenwood JR, Egebjerg J, Brauner-Osborne H. Deorphanization of GPRC6A: a promiscuous L-α-amino acid receptor with preference for basic amino acids. Mol Pharmacol. 2005;67:589–597.
3. Wellendorph P, Brauner-Osborne H. Molecular cloning, expression, and sequence analysis of GPRC6A, a novel family C G protein–coupled receptor. Gene. 2004;335:37–46.
4. Pi M, Faber P, Ekmna G, et al. Identification of a novel extracellular cation-sensing G protein–coupled receptor. J Biol Chem. 2005;280:40201–40209.
5. Harno E, Edwards G, Geraghty AR, et al. Evidence for the presence of GPRC6A receptors in rat mesenteric arteries. Cell Calcium. 2008;44:210–219.
6. Pi M, Chen L, Huang MZ, et al. GPRC6A null mice exhibit osteopenia, feminization and metabolic syndrome. PLoS ONE. 2008;3:e3858.
7. Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Arch Biochem Biophys. 2008;473:139–46.
8. Martin TJ, Sims NA. Osteocalcin-derived activity in the coupling of bone formation to resorption. Trends Mol Med. 2005;11:76–81.
9. Busay N, Sarosi I, Dunstan CR, et al. Osteoporotic-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev. 1998;12:1260–8.
10. Hoff AO, Catala-Lehnen P, Thomas PM, et al. Increased bone mass is an unexpected phenotype associated with deletion of the calcitonin gene. J Clin Invest. 2002;110:1849–57.
11. Silver IA, Murrills RJ, Etherington DJ. Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. Exp Cell Res. 1988;175:266–76.
12. Bonjour JP, Ammann P, Chevalley T, Rizzoli R. Protein intake and bone growth. Can J Appl Physiol. 2001;26 Suppl: S153–66.
13. Adibi SA, Mercier DW. Protein digestion in human intestine as reflected in luminal, mucosal, and plasma amino acid concentrations after meals. J Clin Invest. 1973;52:1586–94.
14. Shahboub V, Grisanti M, Padaghas J, et al. In vitro studies with the calcimimetic cinacalcet HCl on normal human adult osteoblastic and osteoclastic cells. Crit Rev Eukaryot Gene Expr. 2003;13:89–106.
15. Pi M, Garner SC, Flannery P, Spurney RF, Quarles LD. Sensing of extracellular cations in CasR-deficient osteoblasts: evidence for a novel cation-sensing mechanism. J Biol Chem. 2000;275:3256–63.

16. Wellendorph P, Johansen LD, Jensen AA, et al. No evidence for a bone phenotype in GPRC6A knockout mice under normal physiological conditions. J Mol Endocrinol. 2009;42:215–23.

17. Xiao ZS, Simpson LG, Quarles LD. IRES-dependent translational control of Cbfα1/Runx2 expression. J Cell Biochem. 2003;88:493–505.

18. Tu Q, Pi M, Karsenty G, Simpson L, Liu S, Quarles LD. Rescue of the skeletal phenotype in CasR-deficient mice by transfer onto the Gcm2 null background. J Clin Invest. 2003;111:1029–37.

19. Ecarot-Charrier B, Glorieux FH, van der Rest M, Pereira G. Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. J Cell Biol. 1983;96:639–43.

20. Liu YJ, Liu XG, Wang L, et al. Genome-wide association scans identified CTNNBL1 as a novel gene for obesity. Hum Mol Genet. 2008;17:1803–13.

21. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005;21:263–5.

22. Gabriel SB, Schaffner SF, Nguyen H, et al. The structure of haplotype blocks in the human genome. Science. 2002;296:2225–9.

23. Nyholt DR. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. Am J Hum Genet. 2004;74:763–9.

24. Pi M, Quarles LD. Osteoblast calcium-sensing receptor has characteristics of ANF/7TM receptors. J Cell Biochem. 2005;95:1081–92.

25. Niu T, Rosen CJ. The insulin-like growth factor-I gene and osteoporosis: a critical appraisal. Gene. 2005;361:38–56.

26. Reim NS, Breig B, Stahr K, et al. Cortical bone loss in androgen-deficient aged male rats is mainly caused by increased endocortical bone remodeling. J Bone Miner Res. 2008;23:694–704.

27. Dvorak MM, Siddiqua A, Ward DT, et al. Physiological changes in extracellular calcium concentration directly control osteoblast function in the absence of calciotropic hormones. Proc Natl Acad Sci U S A. 2004;101:5140–5.

28. Chang W, Tu C, Chen TH, Bikle D, Shoback D. The extracellular calcium-sensing receptor (CaSR) is a critical modulator of skeletal development. Sci Signal. 2008;1:ra1.

29. Yamaguchi T, Sugimoto T. Impaired bone mineralization in calcium-sensing receptor (CaSR) knockout mice: the physiological action of CaSR in bone microenvironments]. Clin Calcium. 2007;17:1567–73.

30. Garner SC, Pi M, Tu Q, Quarles LD. Rickets in cation-sensing receptor-deficient mice: an unexpected skeletal phenotype. Endocrinology. 2001;142:3996–4005.

31. Boldyreff B, Wehling M. Rapid aldosterone actions: from the membrane to signaling cascades to gene transcription and physiological effects. J Steroid Biochem Mol Biol. 2003;85:375–81.

32. Fromigue O, Hay E, Barbara A, et al. Calcium sensing receptor-dependent and -independent activation of osteoblast replication and survival by strontium ranelate. J Cell Mol Med. 2009.

33. Oda Y, Tu CL, Pillai S, Bikle DD. The calcium sensing receptor and its alternatively spliced form in keratinocyte differentiation. J Biol Chem. 1998;273:23344–52.

34. Oda Y, Tu CL, Chang W, et al. The calcium sensing receptor and its alternatively spliced form in murine epidermal differentiation. J Biol Chem. 2000;275:1183–90.

35. Zerwekh JE, Sakhaee K, Breslau NA, Gottschalk F, Pak CY. Impaired bone formation in male idiopathic osteoporosis: further reduction in the presence of concomitant hypercalcemia. Osteoporos Int. 1992;2:128–34.

36. Giannini S, Nobile M, Sella S, Dalle Carbonare L. Bone disease in primary hypercalcemia. Crit Rev Clin Lab Sci. 2005;42:229–48.

37. Hsiao EC, Boudignon BM, Chang WC, et al. Osteoblast expression of an engineered Gs-coupled receptor dramatically increases bone mass. Proc Natl Acad Sci U S A. 2008;105:1209–14.

38. Morley P, Whitfield JF, Willick GE. Design and applications of parathyroid hormone analogues. Curr Med Chem. 2005;6:1095–106.

39. Lau KH, Baylink DJ. Molecular mechanism of action of fluoride on bone cells. J Bone Miner Res. 1998;13:1660–7.

40. Xie H, Tang SY, Cui RR, et al. Apelin and its receptor are expressed in human osteoblasts. Regul Pept. 2006;134:118–25.

41. Ofek O, Karsak M, Leclerc N, et al. Peripheral cannabinoid receptor, CB2, regulates bone mass. Proc Natl Acad Sci U S A. 2006;103:696–701.

42. Marinissen MJ, Gutkind JS. G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol Sci. 2001;22:686–76.

43. Ho C, Conner DA, Pollak MR, et al. A mouse model of human familial hypocalciuric hypercalcaemia and neonatal severe hyperparathyroidism. Nat Genet. 1995;11:389–94.

44. Rohrer DK, Kobilka BK. G protein-coupled receptors: functional and mechanistic insights through altered gene expression. Physiol Rev. 1998;78:35–52.

45. Lee NK, Sowa H, Hinoi E, et al. Endocrine regulation of energy metabolism by the skeleton. Cell. 2007;130:456–69.