DEFICIENCY OF THE G PROTEIN α-SUBUNIT Gsα IN OSTEObLASTS LEADS TO DIFFERENTIAL EFFECTS ON TRABECULAR AND CORTICAL BONE
Akio Sakamoto‡, Min Chen‡, Takashi Nakamura§, Tao Xie‡, Gerard Karsenty¶, and Lee S. Weinstein‡

From the ‡Metabolic Diseases Branch, National Institute of Diabetes, Digestive, and Kidney Diseases and §Craniofacial Developmental Biology and Regeneration Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland 20892 and ¶Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030

Address correspondence to: Lee S. Weinstein, Metabolic Diseases Branch, NIDDK/NIH, Bldg 10 Rm 8C101, Bethesda, Maryland 20892-1752, USA, Tel: 301-402-2923; Fax: 301-402-0374; E-mail: leew@amb.niddk.nih.gov

The G-protein α-subunit Gsα is required for the intracellular cAMP responses to hormones and other agonists. Gsα is known to mediate the cAMP response to parathyroid hormone and other hormones and cytokines in bone and cartilage. To analyze the in vivo role of Gsα signaling in osteoblasts, we developed mice with osteoblast/osteocyte-specific Gsα deficiency (BGsKO) by mating Gsα floxed mice with collagen Iα1 promoter-Cre recombinase transgenic mice. Early skeletal development was normal in BGsKO mice, as formation of the initial cartilage template and bone collar were unaffected. The chondrocytic zones of the growth plates also appeared normal in BGsKO mice. BGsKO had a defect in formation of the primary spongiosa with reduced immature osteoid (new bone formation) and overall length which led to reduced trabecular bone volume. In contrast, cortical bone was thickened with narrowing of the bone marrow cavity. This was likely due to decreased cortical bone resorption, as osteoclasts were markedly reduced on the endosteal surface of cortical bone. In addition, expression of alkaline phosphatase, an early osteoblastic differentiation marker, was normal, while expression of the late osteoblast differentiation markers osteopontin and osteocalcin was reduced, suggesting that the number of mature osteoblasts in bone are reduced. Expression of the osteoclast-stimulating factor receptor activator of NF-κB ligand was also reduced. Overall our findings have similarities to parathyroid hormone null mice and confirm that the differential effects of parathyroid hormone on trabecular and cortical bone are primarily mediated via Gsα in osteoblasts. Osteoblast-specific Gsα deficiency leads to reduced bone turnover.

Gsα is a ubiquitously expressed G protein α-subunit that couples receptors to adenylyl cyclase and is required for receptor-stimulated cAMP generation (1). cAMP is an important second messenger in osteoblasts for several hormones and other extracellular factors, such as parathyroid hormone (PTH) and prostaglandin E2 (PGE2). Both PTH and PTH-related peptide (PTHrP), a paracrine regulator of chondrocyte differentiation in growth plates, activate a common receptor (PTH/PTHrP receptor, PPR) that activates Gsα as well as other G proteins (2-4). PTH stimulates bone formation and bone resorption, resulting in high bone remodeling (5).

Different bones are formed by one of two different mechanisms, either intramembranous or endochondral ossification. Intramembranous ossification, which leads to formation of many of the flat bones, occurs when pluripotent mesenchymal cells directly enter an osteoblast lineage and differentiate into osteoblastic cells. Long bones develop by endochondral ossification, in which a cartilage template is formed, chondrocytes undergo hypertrophic differentiation, and are then replaced by osteoblasts that form the primary spongiosa which eventually forms the trabecular bone. Simultaneously perichondrial cells surrounding the hypertrophic chondrocytes differentiate into osteoblasts to form the bone collar, which eventually becomes the cortical bone. It has been suggested that PTH may be
important for regulation of fetal skeletal development (6). PTH null mice have defective formation of primary spongiosa and reduced trabecular bone and increased cortical bone thickness (6), while an activated PPR in osteoblasts produces opposite effects in trabecular and cortical bone (7).

Bone mass is tightly regulated by the balance between bone formation by osteoblasts and bone resorption by osteoclasts (8). Receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) are expressed by osteoblasts and play an important role in the regulation of osteoclasts. RANKL activates osteoclasts by binding to the RANK receptor, while OPG is a secreted decoy receptor for RANK that binds RANKL and therefore inhibits osteoclast recruitment and activity. PTH has been shown to stimulate RANKL and inhibit OPG expression in osteoblasts, which promotes osteoclastic bone resorption (9).

The gene encoding Gs \( \alpha \) (Gnas) is a complex imprinted gene that generates multiple gene products through the use of alternative promoters and first exons (1). Mice with homozygous knockout of Gnas die during early embryonic development (10), rendering this model useless for the study of the effects of Gs \( \alpha \) deficiency on skeletal development or function. We therefore took advantage of the Cre-loxP system to generate osteoblast/osteocyte-specific Gs \( \alpha \) knockout mice (BGsKO mice) by mating Gs \( \alpha \)-floxed mice with collagen I \( \alpha \),1 promoter-Cre recombinase transgenic mice (11). BGsKO mice had reduced formation of primary spongiosa and reduced trabecular bone volume, but had increased cortical bone thickness, primarily due to a decrease in RANKL expression and osteoclastic bone resorption. Gene expression studies suggest that the number of mature osteoblasts in bone is reduced. Overall our findings suggest that osteoblast-specific Gs \( \alpha \) deficiency leads to reduced bone turnover and that the differential effects of PTH on trabecular and cortical bone are mediated via Gs \( \alpha \) signaling pathways in osteoblasts.

**EXPERIMENTAL PROCEDURES**

*Mice* - The generation of collagen I\( \alpha \),1 promoter-Cre (Col1-Cre) transgenic mice was previously described (11). Cre expression patterns in Col1-Cre mice were assessed by mating with Rosa26-R (R26R) reporter mice provided by P. Soriano. We have generated mice with loxP recombination sites surrounding Gs \( \alpha \) exon 1 at positions -1601 and +419 relative to the Gs \( \alpha \) translational start site (E1\( ^{fl/fl} \)) (12,13). Mice with osteoblast/osteocyte-specific Gs \( \alpha \) deficiency (BGsKO; Col1-Cre\( ^{E1^{fl}} \)) were generated by repeated matings of Col1-Cre\( ^{E1^{fl}} \) mice with E1\( ^{fl/mol} \) mice. Col1-Cre\( ^{E1^{fl}} \) mice and E1\( ^{fl/mol} \) mice were in FVB and Black Swiss genetic backgrounds, respectively. The E1\( ^{fl} \) allele has no effect on Gs \( \alpha \) expression or phenotype (data not shown) and therefore Col-Cre\( ^{E1^{fl}} \) littermates were used as controls. Mouse studies were approved by the NIDDK Animal Care and Use Committee.

**Genotyping** - The presence of wild type Gs \( \alpha \) (E1\( ^{+} \)), E1\( ^{fl} \), and recombined E1\( ^{fl} \) (E1\( ^{+} \)) alleles was determined in mouse tail DNA samples by multiplex PCR using the common upstream primer 5’-GAGAGCGAGAGGAAGACAGC-3’ (G1) and downstream primers 5’-TCG GGCCCTCTGGCGGAGCTT-3’ (G2) and 5’-AGCCCTACTCTGTCGCAGTC-3’ (G3), respectively. The PCR mixture included 25% (v/v) betaine (Sigma, St. Louis, MO). The presence or absence of the Col1-Cre transgene was determined by duplex PCR analysis with Cre-specific primers (5’-CCT GTTTTGTGCACGTTCACCG-3’ and 5’-ATGCTTCTGTCGAGCTC-3’ (G2) and 5’-AGCCCTACTCTGTCGCAGTC-3’ (G3), respectively. The PCR mixture included 25% (v/v) betaine (Sigma, St. Louis, MO). The presence or absence of the Col1-Cre transgene was determined by duplex PCR analysis with Cre-specific primers (5’-CCT GTTTTGTGCACGTTCACCG-3’ and 5’-ATGCTTCTGTCGAGCTC-3’) and \( \alpha \)-tubulin-specific primers (5’-AGACCATTGGGGGAGAGGAT-3’ and 5’-GTGGGTCCAGCTCAGA-3’), respectively. The PCR mixture included 25% (v/v) betaine (Sigma, St. Louis, MO). The presence or absence of the Col1-Cre transgene was determined by duplex PCR analysis with Cre-specific primers (5’-CCT GTTTTGTGCACGTTCACCG-3’ and 5’-ATGCTTCTGTCGAGCTC-3’) and \( \alpha \)-tubulin-specific primers (5’-AGACCATTGGGGGAGAGGAT-3’ and 5’-GTGGGTCCAGCTCAGA-3’) which was included in each reaction as a positive control. PCR reactions were performed with the following cycling profile: 94°C for 5 min, followed by 32-35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and a final cycle with 7 min of extension at 72°C.

**Skeletal staining** - Skeletal preparation stained with alcian blue and alizarin red staining was based on the method of McLeod (14). Dissected skeletons were fixed in 90% ethanol for 1-2 days and stained with alcian blue (0.015% Alcian blue 8GX [Sigma], 20% acetic acid, 75% ethanol) overnight at room temperature. Samples were then stained with alizarin red (0.005% alizarin acid red [Sigma], 1% potassium hydroxide). Samples were cleared with potassium hydroxide and stored in glycerol: ethanol (1:1).

**Histology** - Dissected samples were fixed overnight in 4% paraformaldehyde, embedded in paraffin using standard procedures,
and stained with hematoxylin and eosin, Masson-Trichrome or von Kossa stain.

Tartrate-resistant acid phosphatase (TRAP) staining - Deparaffinized sections were incubated at 37°C in 0.1M acetate buffer (pH5) (Sigma) containing 220 µM naphthol AS-MX phosphate/dimethyl formaldehyde solution (Sigma), 2 mM fast red violet LB salt (Sigma), 50mM L(+)-sodium tartrate (Sigma), and 1 M MgCl₂ for 30 min. Sections were then counterstained with hematoxylin.

Immunoblotting - Flat skull bones, liver and whole brain from newborn mice were homogenized in lysis buffer (T-PER; Pierce, Rockford, IL) on ice using a 200 µl micro tissue grinder (Wheaton, Millville, NJ). For flat skull bones, as much surrounding soft tissue was removed as possible and bones were immediately frozen in liquid nitrogen. Protein concentrations of whole tissue homogenates were determined using a dye assay (Bio-Rad, Hercules, CA). Homogenates (25 µg protein/lane) were separated in 8% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transfered to nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Membranes were incubated with an anti-Gs (15) and horseradish peroxidase-conjugated goat anti-rabbit (Amersham, Piscataway, NJ) and bands were detected by chemiluminescence using SuperSignal West Pico chemiluminescent substrate (Pierce). Membranes were then stripped as directed (Pierce) and reprobed with an anti-α-tubulin antibody (CP06; Calbiochem, La Jolla, CA) and goat anti-mouse antibodies (Santa Cruz, Santa Cruz, CA).

Reverse transcription and real-time PCR analysis - Flat skull bones were snap frozen with liquid nitrogen and total RNA was extracted (RNaseasy Fibrous Tissue Mini kit, Qiagen, Germantown, MD) then treated with DNaseI (Invitrogen) at room temperature for 15 min. Reverse transcription was performed using SuperScript III (SuperScript III First-Strand Synthesis System for RT-PCR, Invitrogen). Gene expression levels were measured by quantitative RT-PCR using a real time PCR machine (MxP3000, Stratagene, La Jolla, CA). PCR reactions (25 µl total volume) included cDNA, 100 nM primers and 10 µl of SYBR Green MasterMix (Applied Biosystems, Foster City, CA). To get relative quantification, standard curves were simultaneously generated with serial dilutions of cDNA and results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in each sample, which were determined simultaneously by the same method. Specificity of each RT-PCR product was indicated by its dissociation curve and the presence of a single band of expected size on acrylamide gel electrophoresis. 5-6 pairs of BGsKO mice and control littermates were used for each assay. The primers used for Gs (16), collagen 1 (17), alkaline phosphatase (18), osteopontin (19), osteocalcin (20), RANKL (21), and osteoprotegrin (18) have been previously published. GAPDH primers were obtained from Applied Biosystems.

RESULTS

Generation of mice with osteoblast/osteocyte-specific Gs deficiency- Col1-Cre transgenic mice were previously shown to specifically express the Cre recombinase transgene in osteoblasts and osteocytes (11). To confirm the tissue expression patterns of the Col1-Cre transgene, Col1-Cre mice were mated with R26R reporter mice in which cre-mediated recombination leads to β-galactosidase expression (22). Embryos at days E12.5, E15.5 and E17.5 were stained with X-gal to visualize the tissue distribution of Cre-recombinase activity. At E12.5 Cre expression was limited to the anterior craniofacial components (Fig. 1A). At days E15.5 and E17.5 Cre activity was also observed in osseous components of skull, ribs and long bones. These results were similar to those previously described in similar experiments (11).

We have generated E1fl/fl mice, in which loxP recombinase sites were inserted upstream and downstream of the Gs promoter and first exon (12). Cre-mediated recombination at these sites disrupts Gs expression without disrupting expression of other Gnas gene products, such as NESP55 and XLαs. These mice were repeatedly mated with Col1-Cre mice to generate Col1-Cre:E1fl/fl mice in which the Gs gene is disrupted in osteoblasts and osteocytes (BGsKO mice). Genotyping was performed by PCR using a single upstream primer (G1) and two downstream primers (G2 and G3) in the same reaction (Fig. 1B). Amplification with primers G1 and G2 produces a 330 bp band for the normal Gs (E1⁺) allele and a 390 bp band for the E1⁻ allele (Fig. 1, B and C). Because the tail samples include bone tissue, samples from BGsKO mice also produce a 250 bp band resulting from amplification of
ossification is the result of Gα protein levels were reduced in skull bones in newborn BGsKO as compared with control littermates while Gα expression was relatively unaffected in liver and brain (Fig. 1E). Gα mRNA levels were also decreased by 80% in skull bones (Fig. 4A). Given that these samples are also composed of other non-osteoblastic cells, this result indicates that the vast majority of osteoblasts in BGsKO mice are Gα deficient.

All BGsKO newborn pups were unable to nurse and died within 6-8 h after birth. In addition, BGsKO mice were born with subcutaneous edema, a finding also observed in other models with germline disruption of the maternal Gnas allele (10,23) (Fig. 1D). BGsKO mice had notable skeletal abnormalities, including small skulls with shortened craniofacial diameter and short limbs. In contrast, Col1-Cre;E1flox/+ mice with heterozygous Gα exon 1 deletion had no obvious phenotype (data not shown).

**Craniofacial abnormalities in BGsKO mice** - Skeletal preparation of newborn BGsKO mice showed hypoplastic craniofacial bones, particularly in the anterior components such as the maxilla and mandible and thickening of the zygomatic arch (Fig. 1, G-J). These craniofacial abnormalities correspond to the region of early Cre expression in Col1-Cre mice (Fig. 1A), and suggests an important role for Gα signaling pathways in craniofacial development. Meckel’s cartilage, a prominent feature of the developing mandible was ossified in newborn BGsKO mice with the most severe craniofacial abnormalities (Fig. 1, H and I). Histology showed premature occurrence of endochondral ossification of the cartilage at E17.5 (data not shown). Based on the observed pattern of Cre expression (Fig. 1A) and the fact that chondrocytes of Meckel’s cartilage may have a greater propensity to express collagen 1 than other chondrocytes (24), it is likely that the premature endochondral ossification is the result of Gα deficiency in Meckel’s cartilage chondrocytes. Premature ossification of Meckel’s cartilage has also been observed in PTHrP knockout mice (25).

Cleft palate was present in 58% (33 out of 57 mutants) of BGsKO mice (Fig. 1F) and was absent in all control littermates (data not shown). It is unclear whether cleft palate in BGsKO mice results from a primary defect in skeletal development or is a secondary effect resulting from a large protuberant tongue relative to the hypoplastic skeletal structures.

**Skeletal development in BGsKO mice** - Skeletal preparations showed no obvious other changes in overall skeletal structure between BGsKO and control mice (Fig. 1G). There was a small but significant decrease in length of long bones in BGsKO (tibial length in BGsKO mice was 90.6% of that in control mice). Histological sections showed no obvious differences in the cartilaginous templates of femurs of BGsKO and control mice at E13.5 or E14.5 (Fig. 2, A-D). Von Kossa staining showed no calcification within the cartilaginous templates on day E13.5 in either BGsKO or control mice (data not shown). Calcification or bone collar induction of cartilaginous templates on day E14.5 was similar between BGsKO and control mice (Fig. 2, E and F), indicating that osteoblast-specific Gα deficiency had no effect on the time of initiation of osteogenesis.

Femurs of BGsKO mice on day E16.5 were shorter than control femurs, with shortening of the metaphysis and diaphysis and relatively little change in the epiphysis. Although at this stage cortical bone formation is similar in BGsKO and control mice, formation of primary spongiosa from endochondral ossification was markedly reduced in BGsKO mice as compared to controls (Fig. 2, G-J). Shortening of the metaphysis and diaphysis of the femur in BGsKO mice was more obvious on day P0.5 (Fig. 3, A and B). At this stage the zone of primary spongiosa formation was shorter in the distal femur of BGsKO mice than controls (Fig. 3, C and D). Moreover, Masson-Trichrome staining showed that the primary spongiosa of BGsKO mice was primarily composed of more mature and calcified matrix (dark blue) while in control mice the primary spongiosa has a greater amount of immature osteoid, indicative of new bone formation (Fig. 3, E and F). These differences in endochondral ossification were limited to primary spongiosa formation, as the cartilaginous zones of the growth plate appeared normal in BGsKO mice. Overall, BGsKO appear to have reduced formation of primary spongiosa, which may lead to bone shortening due to reduced endochondral ossification.

Long bones contain a collar of cortical bone which is formed from perichondrium by
mice (Fig. 4, F-G). However by day P0.5 BGsKO mice have a significantly increased thickness of cortical bone and narrowing of the bone marrow space within the diaphysis of the femur based upon both radiographic and histological analysis (Fig. 3, A,B,G and H). Histology showed this region of bone to have increased thickness and connectivity in BGsKO mice (Fig. 3, G and H). Tartrate-resistant acid phosphatase (TRAP) staining, which is a marker for osteoclasts, was markedly reduced on the inner surface of cortical bone in BGsKO mice, indicating that G\(\alpha\) deficiency in cortical osteoblasts and osteocytes reduces the recruitment of osteoclasts (Fig. 3, I and J). Reduced osteoclastic bone resorption is likely to be an important contributing factor to increased cortical bone thickness in BGsKO mice. In contrast, we did not observe an obvious difference in TRAP staining within the trabecular bone.

Expression of osteoblast differentiation markers and RANKL/OPG – We next examined the expression of osteoblastic differentiation markers and osteoblast-derived factors which affect osteoclast recruitment and activity by quantitative RT-PCR of skull bone samples. While there was no difference in expression of alkaline phosphatase (ALP), an early osteoblastic differentiation marker between BGsKO and control mice (Fig. 4B), expression of other osteoblast markers, such as collagen 1, osteopontin and osteocalcin, were significantly decreased in BGsKO mice (Fig. 4, C-E), indicating that G\(\alpha\) deficiency inhibits osteoblast maturation and bone forming ability. The differences were greatest for osteocalcin, a late marker of osteoblast differentiation. Osteoblasts secrete both RANKL and OPG, which stimulate and inhibit osteoclast resorption, respectively. Expression of both RANKL and OPG was significantly reduced in bone tissue of BGsKO mice (Fig. 4, F and G), which may be the result of poor osteoblastic maturation in these mice. Low RANKL may be one contributing factor to the low level of osteoclast recruitment observed in BGsKO mice. Overall, BGsKO mice appear to have reduced bone turnover, with reduced osteoclast recruitment secondary to a primary defect in osteoblast maturation.

DISCUSSION

Early embryonic lethality of germline Gnas knockout mice does not allow us to examine the effects of G\(\alpha\) deficiency in skeletal development, which begins later in gestation. To circumvent this problem, we made use of the Cre-loxP recombination system and used a collagen Ia1-Cre transgenic mouse line to generate mice with G\(\alpha\) deficiency in osteoblasts and osteocytes. In situ hybridization analysis confirmed that collagen Ia1 is expressed in both osteoblasts and osteocytes at similar levels in control and BGsKO (data not shown). BGsKO are born with subcutaneous edema and die soon after birth, features that have been observed in other germline Gnas knockout models (10,23). As there is no obvious explanation for how a skeletal defect could lead to subcutaneous edema, we suspect that this is due to Cre expression, leading to G\(\alpha\) deficiency, in one or more non-skeletal tissues (eg. placenta). We have no obvious explanation for the perinatal lethality, although one possibility is poor nutritional intake due to severe craniofacial malformation. Perinatal lethality is also observed in PTHrP and PPR knockout mice (3,4). This lethality allows us to only examine the effects of G\(\alpha\) deficiency on bone development during gestational development.

Long bones develop by endochondral ossification, a process by which mesenchymal cells differentiate into chondrocytes to form cartilaginous template. Chondrocytes at the center become hypertrophic, followed by invasion of blood vessels, formation of a mineralized matrix and osteoblastic replacement (primary spongiosa) which eventually becomes the central trabecular bone. This process proceeds in both directions, forming the growth plates which lead to bone elongation. Simultaneously, perichondrial cells surrounding the hypertrophic chondrocytes directly differentiate into osteoblasts to form the bone collar, which eventually becomes the cortical bone (2). BGsKO mice have no apparent defect in the initial formation of the cartilage template or hypertrophic differentiation of chondrocytes, which is not surprising given that G\(\alpha\) deficiency in this model is limited to cells of the osteoblastic, rather than chondrocytic lineage. This is also explains why BGsKO show no significant changes within the proliferative and hypertrophic zones of the epiphyseal growth
plate at later stages. Chondrocyte-specific $\alpha$ knockout and other knockout models suggest that regulation of the endochondral growth plates is mediated through PTHrP-PPR-$\alpha$ pathways (3,4,12,26).

BGsKO also did not show any changes in the gestational age at which bone collar formation began, suggesting that $\alpha$ deficiency in osteoblasts does not affect the initiation of cortical bone formation. Consistent with this, the number of ossification centers in metacarpal bones (bone age) in newborn pups was the same between BGsKO and control mice (data not shown). In addition, the expression level of alkaline phosphatase, an early differentiation marker of osteoblasts, is similar in skull bones between BGsKO and control mice. Collagen Iα1 is an early differentiation marker of osteoblasts (27,28), which is not expressed in osteoblastic precursors (bone-marrow stromal or perichondrial cells; data not shown). This is likely to explain the lack of effect on the early stages of osteoblastic development and the relatively moderate skeletal phenotype in BGsKO mice compared to other relevant mouse models.

Differences in skeletal development of long bones between BGsKO and control mice became most apparent on day E16.5, when it was clear that primary spongiosa in BGsKO mice had less immature bone matrix (osteoid) area, indicative of slower bone formation in this region which resulted in less metaphyseal trabecular bone. Similar findings have been observed in PPR (29) and PTH (6), but not PTHrP (29), knockout mice, suggesting that PTH is a major prenatal stimulator of bone formation within the primary spongiosa. PTH is also known to act as an anabolic agent for trabecular bone (30), and for this reason is presently used as a therapy for osteoporosis. Although we found no differences in expression of alkaline phosphatase, an early osteoblastic differentiation marker, expression of other differentiation markers (collagen I, osteopontin and osteocalcin) were significantly reduced in bone of BGsKO mice. This may be due to lower bone formation, reduced osteoblastic differentiation, or both. PTH and other cAMP stimulators have been shown to stimulate osteocalcin expression in cultured osteoblastic cells in some (31), but not all (32,33) studies, while PGE2, another factor which stimulates $\alpha$ pathways in bone, was shown to have little effect on osteocalcin expression (34). The present results, as well as results in other knockout and cell culture models, suggest that PTH, mediating its action through $\alpha$ pathways in osteoblasts, may be an important regulator of trabecular bone formation.

In contrast to the effects on trabecular bone content, BGsKO had a significant increase in the thickness of cortical bone, leading to a reduced marrow space. PTH (6) and PPR (29) knockout models also had reduced trabecular and increased cortical bone, while mice with osteoblast-specific expression of an activated PPR had opposite effects on trabecular and cortical bone (7). Moreover, patients with primary hyperparathyroidism with excess circulation PTH levels tend to have greater loss of cortical bone and relative sparing of trabecular bone (35). The findings in this study, along with prior observations in both mouse and human, suggest that PTH through $\alpha$ activation has effects on osteoblasts in that lead to differential effects on cortical and trabecular bone volume (Fig. 5). Prior studies suggest that reduced osteoblast apoptosis due to loss of PTH action on osteoblasts may contribute to the observed increase in cortical bone (6,36). Although reduced osteoclast recruitment to cortical bone in BGsKO mice is likely a reflection of reduced osteoblast function in cortical bone, we have not ruled out the possibility that thickening of cortical bone in these mice is partially due to increased rates of cortical bone formation.

A more likely contributor to the increased cortical thickness observed in BGsKO mice is reduced bone resorption by osteoclasts, as there was markedly reduced TRAP staining on the endosteal surface of cortical bone in BGsKO mice. TRAP staining in trabecular bone was relatively unaffected. It is possible therefore that the different effects we observed in cortical vs. trabecular bone in BGsKO mice could be due to differential effects of $\alpha$ deficiency in cortical vs. trabecular osteoblasts on bone formation, osteoclast resorption, or both.

Osteoclast recruitment and activation are stimulated by RANKL and other factors, such as interleukin 6, which are released from osteoblasts. PTH (9) and PGE2 (37,38) have both been shown to upregulate RANKL expression in osteoblasts through stimulation of cAMP-dependent protein kinase (PKA), and both PPR (29) and prostaglandin receptor knockout (39) mice have increased bone thickness and reduced osteoclastic bone

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resorption. Moreover, somatic G\(_{\alpha}\) mutations which lead to constitutive activation in fibrous dysplasia of bone results in osteoclast recruitment and invasion into cortical bone (40). It is unclear why we also observed OPG expression to be low in BGsKO mice, as PTH is known to reduce OPG expression (9). This may indicate that PTH affects OPG expression through an alternative signaling pathway or reflect the fact that osteoblast number or maturation is reduced in skull bones of BGsKO mice. It is also possible that this observed effect, and possibly the differences observed in other osteoblastic markers is due to are not due to osteoblast-autonomous effects, but rather result from changes in the interaction of osteoblasts with matrix or other cellular components of bone.

The low bone formation and osteoclast recruitment observed in BGsKO suggest that these mice overall have a low bone remodeling or turnover rate. PTH is one agent known to increase bone remodeling (5), and therefore osteoblast-specific G\(_{\alpha}\) deficiency would be predicted to result in a low turnover state. As BGsKO mice only survive until birth, direct histomorphometric studies to directly assess bone turnover in this model is difficult. Further study in bone-specific knockouts using inducible or other more bone-specific transgenes will be required to examine this question. Overall this initial study examining the effects of G\(_{\alpha}\) deficiency in osteoblastic cells shows that these pathways play an important role in bone formation and turnover by mediating the actions of PTH, prostaglandins, and other circulating factors on osteoblasts. G\(_{\alpha}\) may also have a more specific role in the development of the craniofacial skeleton.

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FOOTNOTE

The abbreviations used are: PTH, parathyroid hormone; PGE2, prostaglandin E2; PTHrP, PTH-related peptide; PPR, PTH/PTHrP receptor; RANKL, receptor activator of nuclear factor-κB ligand; OPG, osteoprotegerin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gα, stimulatory G protein α-subunit; BGsKO, osteoblast/osteocyte-specific Gα knockout; Coll1-Cre, collagen Iα1 promoter-Cre recombinase transgene, TRAP, tartrate-resistant acid phosphatase; ALP, alkaline phosphatase; PKA, protein kinase A.

FIGURE LEGENDS

FIG. 1. Generation of osteoblast-specific Gα knockout (BGsKO) mice. A, Coll1-Cre+R26R embryos at days E12.5, E15.5 and E17.5 stained with X-gal (green) to visualize tissues expressing Cre-recombinase. B, schematic (not to scale) showing the relative location of PCR primers, loxP sites (triangles), and Gα exon 1 (rectangle; Ex1) and the expected products amplified from E1*, E110, and E1- alleles, respectively. C, results of PCR using primers G1, G2, and G3 on genomic DNA isolated from tails of newborn mice with indicated genotypes. D, gross appearance of a newborn BGsKO (right) and control (left) mouse. E, immunoblots using Gα-specific (upper row) and α-tubulin-specific (lower row) antibodies on homogenates of skull bones (left panel), liver (center panel), and brain (right panel) from newborn BGsKO and control mice. F, photograph of palate of BGsKO and control mice from below. Arrows indicate cleft palate in BGsKO mice. G, skeletal preparation of newborn BGsKO (right) and control mice (left), stained with Alcian blue (cartilage) and Alizarin red (bone). H, lateral view of mandible in situ (above) and dissected mandible viewed from above (lower left) and side (lower right) from control mouse. Arrows indicate Meckel's cartilage. I, similar views of mandible from BGsKO mouse. J, caudal view of base of skull of control (left) and BGsKO (right) mouse after alizarin red/alcian blue staining.

FIG. 2. Skeletal histology of long bone in embryonic BGsKO and control mice. A-D, G, I, hematoxylin & eosin staining of femur from (A, C, G) control and (B, D, I) BGsKO mice at days E13.5 (A, B), E14.5 (C, D), and E16.5 (G, I), respectively. E, F, H, J, von Kossa (silver) staining of femur of control (E, H) and BGsKO (F, J) at days E14.5 (E, F) and E16.5 (H, J), respectively.

FIG. 3. Skeletal phenotype of long bone in new born (day P0.5) BGsKO and control mice. A, B, radiographs of the femur of (A) control and (B) BGsKO mice. C, D, Masson-Trichrome staining of primary spongia from area of endochondral ossification of distal femur from (C) control and (D) BGsKO mice. E, F, higher magnification views of areas outlined in red in panels C and D, respectively. Osteoid (non-calcified bone matrix), which is stained light blue, is indicated by arrows. G, H, hematoxylin & eosin staining of femoral diaphysis of (G) control and (H) BGsKO mice. I, J, TRAP staining (red) of femoral diaphysis of (I) control and (J) BGsKO mice.
FIG. 4. Gene expression in skull bone samples from control and BGsKO mice. Expression of A, Gsα, B, alkaline phosphatase (ALP), C, collagen 1 (col 1), D, osteopontin, E, osteocalcin, F, RANKL, and G, OPG mRNA, all normalized to GAPDH mRNA expression, in skull bone samples from control and BGsKO mice. Results are expressed in arbitrary units as mean ± S.E.M.(n=5-6 per group, *p<0.05, **p<0.01).

FIG. 5. Loss of Gsα signaling in osteoblasts leads to differential effects on trabecular and cortical bone. PTH stimulation of Gsα in osteoblasts stimulates trabecular (T) bone formation leading to increased trabecular bone volume. This same pathway in cortical (C) bone osteoblasts leads to reduced cortical bone volume, most likely due to reduced recruitment of osteoclasts on the endosteal surface. These effects are important for maintenance of the bone marrow space and for longitudinal and transverse bone growth. The right panel shows the effects of osteoblast-specific Gsα deficiency in long bones. Compared to wild-type mice, BGsKO mice have reduced trabecular bone area and thickening of the cortical bone which is associated with reduced osteoclastic resorption (represented as black circles). These effects lead to reduced bone length and width and narrowing of the bone marrow cavity.
FIG. 1

A

E12.5  E15.5  E17.5

B

G1 → G2 → G3
E1* → E2
330 bp (G1/G2)

E1* → G2 → G3
380 bp (E1*/G1)

E1 → G1 → G3
250 bp (G1/G3)

C

390 bp (E1*)
330 bp (E1*)
250 bp (E1)

D

Control  BGsKO

E

Bone  Liver  Brain

BGsKO  Control  BGsKO  Control

F

Control  BGsKO

G

Control  BGsKO

H

Control  BGsKO

I

Control  BGsKO

J

Control  BGsKO

FIG. 2

A  B

E13.5  E14.5

C  D

Control (E16.5)  BGsKO (E16.5)

E  F

G

Control

H

I

J

11
FIG. 5

PTH

\[ G_s \alpha \]

Trabecular (T) bone volume
Cortical (C) bone volume

Bone marrow (BM) cavity
Length & width of long bone

Wild-type

BGsKO

Growth Plate

\[ G_s \alpha \] (PTH)

BM

\[ T \]

\[ C \]
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Akio Sakamoto, Min Chen, Takashi Nakamura, Tao Xie, Gerard Karsenty and Lee S. Weinstein

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