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# G protein and its signaling pathway in bone development and disease

**Mengrui Wu[1,2], Lianfu Deng[2], Guochun Zhu[2], Yi-Ping Li[1,2,3,4]**

*[1]Life Science College, Zhejiang University, 388 Yuhang Road, Hongzhou 310058, People's Republic of China, [2]Shanghai Institute of Traumatology and Orthopaedics, Ruijin Hospital, School of Medicine, Jiao Tong University, 197 Ruijin er Road, Shanghai 200025, People's Republic of China, [3]Department of Cytokine Biology, The Forsyth Institute, 140 The Fenway, Boston, Massachusetts 02115, USA, [4]Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine, 188 Longwood Avenue, Boston, Massachusetts 02115, USA*

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## 1. ABSTRACT

G protein signaling is comprised of G protein coupled receptors (GPCRs) that detect ligands or sense cations, heterotrimeric G proteins, and downstream effectors and regulators. G protein signaling plays important roles in bone development, remodeling, and disease. In human cases, mutations of certain GPCRs and G proteins impair bone development and metabolism, resulting in bone diseases. This review focuses on the functions of G proteins and GPCRs in osteoblasts and osteoclasts, their signaling pathways, and their gene mutations in mouse models and human diseases. We have discussed the roles of all four types of G proteins (i.e. Gs, Gq/11, Gi/o, and G12/13) and assessed the roles of the GPCRs, such as type 1 Parathyroid hormone receptor (PTH1R), calcitonin receptor, cation sensing receptor (CaSR), relaxin family peptides, cannabinoid receptor, frizzleds, and proton sensing receptor in normal bone formation and remodeling. The roles of regulators of G protein signaling (RGS) and GTPase-activating proteins (GAP) in G-protein signaling pathways are also reviewed.

Lastly, we give perspective for the research of G protein signaling in bone development and disease.

## 2. INTRODUCTION

Bone modeling/development and bone remodeling/turnover are the two most important physiological processes that establish and maintain the normal morphology of bone. Bone development occurs during the fetal stage by two courses: intramembranous ossification and endochondral ossification. The former mainly occurs in formation of flat bones such as the skull, while the latter takes place on long bones, such as the limbs. Intramembranous ossification begins with the condensation of mesenchymal cells and ossification occurs by direct bone matrix deposition-forming plates (1). Undifferentiated mesenchymal cells differentiate into osteoprogenitor cells, which further differentiate into mature osteoblasts (1). These osteoblasts then either undergo apoptosis or are embedded in the matrix, becoming osteocytes. Endochondral ossification also begins with mesenchymal condensation, but relies on the growth of a chondrocyte center/cartilaginous template (2). Firstly, condensed mesenchymal cells differentiate into colII chondrocytes at center and colI perichondrial osteoblasts at periphery. Then, the colII chondrocyte center differentiates and forms the cartilaginous template, with colII proliferating chondrocytes at the two ends (named epiphyseal plates), and colX proliferating chondrocytes at the center (named primary ossification center). Proliferating chondrocytes then supply cells to hypertrophic chondrocytes, which grow towards the distal end of the epiphyseal plate to lengthen the bone. Finally, hypertrophic chondrocytes undergo apoptosis and are replaced by osteoblasts. Neovascularization also takes place in primary ossification center at this stage.

Bone remodeling occurs throughout the whole life. The process is initiated by the recruitment of osteoclast precursors to bone, where they mature into resorptive osteoclasts (3). The differentiated osteoclasts generate a resorption lacunae ('pit'), which is then replaced by osteoblasts that deposit new bone in the cavity (3). Osteoclasts, the principal bone-resorbing cells, are multinuclear giant cells developed from hematopoietic stem cells and belonged to monocyte/macrophage family (4). Mature osteoclasts polarize to form ruffled membrane juxtaposed to bone, and secretes protons and specific enzymes like CathepsinK to degrade the bone (5). Osteoblasts, developed from mesenchymal stem cells, are responsible for bone formation by synthesis and secretion of most proteins of the bone extracellular matrix (6). Imbalance between bone formation and bone resorption leads to bone diseases such as osteoporosis and osteopetrosis.

Heterotrimeric G proteins are molecular switches of intracellular signaling, which is 'on' when binding to GTP (guanosine triphosphate) and off when binding to GDP (guanosine diphosphate). G proteins are 'turned on' by seven-time-transmembrane G-protein-coupled receptors (GPCRs) in response to extracellular stimuli (7). It has been proven that Heterotrimeric G proteins act as mediators of proliferation, differentiation and apoptosis in multiple cell types. G protein signaling has also been reported to be involved in cancer and embryonic development (7). It has been found that certain mutations in G protein subunits and GPCRs are responsible for human bone diseases, highlighting the role of G protein signaling in bone development. Mouse models with deficiency in G proteins or GPCRs also reveal the role of G protein signaling in bone development (Table. 1). GPCRs have been the molecular targets for nearly half of the therapeutic drugs that are prescribed worldwide (8). Accordingly, drugs targeting GPCRs in bone, such as PTH/PTHrP (Parathyroid hormone/parathyroid hormone related protein) and calcitonin, have also been used widely in treating bone diseases, like osteoporosis.

## 3. G PROTEINS IN BONE

G proteins are composed of three non-identical subunits, Gα (33-35kDa), Gβ (~35kDa) and Gγ (~15kDa) (37). In humans, there are 21 Gα subunits encoded by 16 genes, 6 Gβ subunits encoded by 5 genes, and 12 Gγ subunits (38). Gα subunits are typically divided into four subgroups, Gαs, Gαi/o (including Gαi1, Gαi2, Gαi3, GαoA,B, Gαz), Gαq (including Gαq, Gα11, Gα14, 16) and Gα13 (including Gα12, Gα13) (39). The structures of the Gα subunit reveal a conserved protein fold composed of a GTPase domain and a helical domain (38). The GTPase domain contains three flexible loops that undergo conformational changes when switching binding of GTP and GDP (38).

G proteins switch between active and inactive isoforms, binding to GTP or GDP. GDP bound Gα complexed with Gβγ is the inactive state. Agonist-activated GPCRs act as GEF (guanine nucleotide exchange factor) to catalyze the exchange of GDP for GTP on the Gα subunit. After binding with GTP, Gα dissociates from Gβγ and activates downstream effectors, including AC (adenylate cyclase), PLC (phospholipase C), and RhoGEF (Rho guanine-nucleotide exchange factors), to turn on different signaling pathways (7, 40, 41). The signaling is turned off by GTPase domain on Gα protein. This interference catalyzes the hydrolysis of GTP to GDP and the recombination of heterotrimeric G protein (7, 40, 41) (Figure 1). Other proteins, such as RGS (regulator of G protein signaling) (42), GRK (G protein coupled receptor kinase) (43), arrestins (43), NHERF (Na+/H+ exchange regulatory factor) (44, 45) are involved in termination and regulation of the signaling (refer to "regulation of G protein signaling") (Figure 1).

Specific inhibitors or activators, constitutive active mutant or dominant negative mutant isoforms, and knockout or knockin mouse models provide useful approaches to understanding the function and mechanism of G protein signaling. For example, Pertussis toxin specifically inhibiting Gαi/o subunits (46) and cholera toxin specifically activating Gαs subunits (47), are applied to study Gαi/o and Gαs signaling, respectively. Recently, the RASSL system (receptors activated solely by synthetic

**Table 1. Mouse models with skeletal defects**

| Gene | Model | Phenotype | | | | | | | Ref |
|---|---|---|---|---|---|---|---|---|---|
| | | Bone resorption | Bone formation | Chondrocyte differentiation | BMD change | Cortical thickness | Trabecular thickness | others | |
| Gs/ Gαs /GNAS | Gs+/- | Nd | Nd | Nd | nd | Nd | Nd | Resistant to PTH | (9) |
| | Gsflox/rennin-cre | Nd | Nd | ↓ | ↓ | Nd | Nd | Ectopic ossification | (10) |
| | Gsflox/col1-cre | ↓ | ↓ | Nd | nd | ↑ | ↓ | | (11) |
| | Col1-Rs | ↑ | ↑ | Nd | ↑ | Nd | Nd | | (12) |
| | Gsflox/Col2a1-cre | Nd | Nd | ↑ | nd | Nd | Nd | Short stature, lethal | (13) |
| Gq/ Gαq /GNAQ | Col1-Q209LGq (constitutive active) | — | ↓ | Nd | ↓ | ↑ | ↑ | | (14) |
| Gi | Col1-Ro1 | ↓ | ↓ | Nd | nd | — | ↓ | Reduced bone mineralization | (15) |
| PTH/PTHrP | PTH-/- | ↓ | ↓ | — | nd | ↑ | ↓ | | (16) |
| | PTHrP-/- | ↓ | ↓ | ↑ | nd | ↑ | ↑ | High mineralization | (16) |
| | PTH-/-PTHrP-/- | ↓ | ↓ | ↑ | nd | ↑ | ↓ | | (16) (17) |
| | Knock in PTHrP (1-84) | ↓ | ↓ | Proliferation (down) | ↓ | ↓ | ↓ | Increase apoptosis | (18) |
| PTH1R | DMP1-caPTH1R | ↑ | ↑ | Nd | ↑ | Nd | Nd | | (19) |
| CALCR/CTR | CTR+/- | — | ↑ | Nd | ↑ | — | ↑ | | (20) |
| CT/CGRP | CT/CGRP-/- | — | — | Nd | — | Nd | Nd | Over response to PTH | (21-23) |
| CGRP | CGRP-/- | — | ↓ | Nd | nd | Nd | Nd | | (24) (25) |
| amylin | Amylin-/- | ↑ | — | Nd | ↓ | ↓ | ↓ | | (26) |
| CaSR | CaSR-/- | ↑ | ↑ | ↑ | ↓ | Nd | Nd | Hypercalcemia, decreased mineralization | (27) |
| GPRC6A | GPRC6A-/- | ↑ | ↓ | Nd | ↓ | Nd | Nd | Impaired mineralization | (28) |
| CB2 | CB2-/- | ↑ | ↑ | Nd | ↓ | — | ↓ | | (29) |
| Gpr55 | GPR55-/- | ↓ | ↑ | Nd | ↑ | — | ↑ | | (30) |
| Rxfp2 | Rxfp2-/- | ↓ | ↓ | Nd | ↓ | Nd | Volume (up) | Induced mineralization | (31, 32) |
| RGS10 | RGS10-/- | ↑ | ↑ | Nd | ↓ | Nd | Nd | | (33, 34) |
| GPR30/GPER1 | GPR30-/- | Nd | Nd | Growth plate closure | — | — | — | | (35, 36) |

This list summarizes mouse models deficient in G proteins, G protein coupled receptors, and G signaling regulators important in bone development. ↑, promotion; ↓, inhibition; —, not changed; nd, not detected

ligands) was developed to examine the function of a specific G protein signal *in vivo*. RASSL is an engineered receptor coupled to a certain G protein which responds specifically to synthetic agonists (12, 15).

### 3.1. G$\alpha_s$/Gs/GNAS signaling

#### 3.1.1. Human diseases

G$\alpha_s$/Gs/GNAS, encoded by a complex imprinted gene, is one of the first G proteins to be identified and expressed universally (48). Imprinted genes refer to a group of genes that undergo epigenetic modification (such as DNA methylation and histone acetylation) in either paternal or maternal allele, in order to achieve monoallelic expression. GNAS mutations lead to a number of human diseases. MAS (McCune-Albright syndrome) is caused by the spontaneous activation of a GNAS mutant: the substitution of Arg201 with either Cys or His (48-50). The classical triad of MAS includes polyostotic fibrous dysplasia (FD), skin hyperpigmentation (cafe-au-lait spots), and endocrine dysfunction, frequently seen in females as precocious puberty (50). G$\alpha_s$ activating mutations are also found in patients with non-MAS FD (51), marked by unmineralized osteoids with a nonlamellar structure, reduced mineral content in mineralized bone within FD lesions, and hyperparathyroidism (52), which indicates the role of Gαs in bone mineralization. Conversely, Gαs–inactivating mutations cause AHO (Albright hereditary osteodystrophy), a disease characterized by obesity, short stature, and subcutaneous ossifications (53, 54). Gs-inactivating mutations are also related to non-AHO heterotropic ossifications, such as progressive osseous heteroplasia (POH), due to Cbfa1 misexpression (55, 56). The relationship between GNAS mutation and human disease strongly indicates the anabolic role of GNAS in bone formation and mineralization.

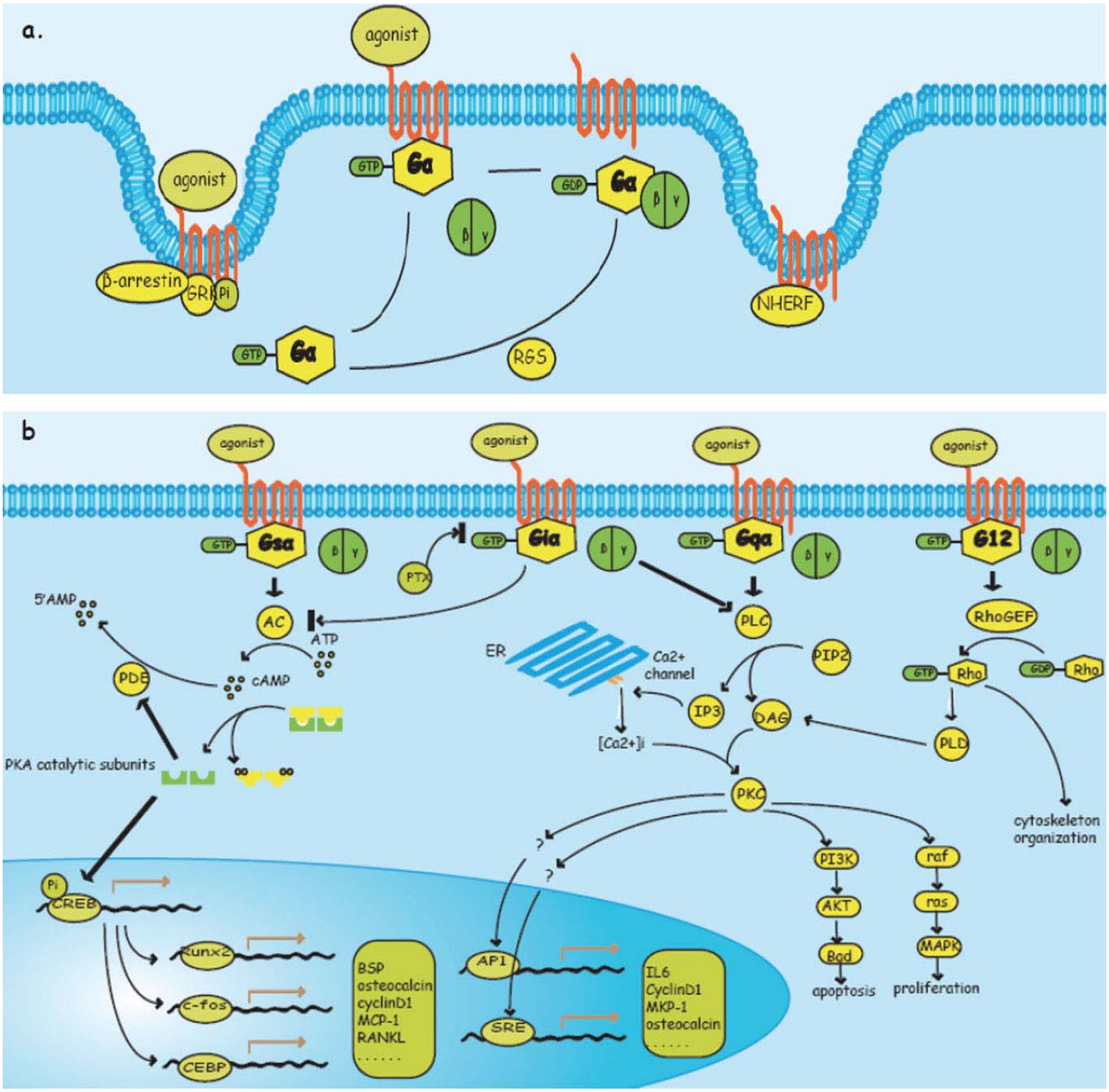


**Figure 1.** G protein signaling in osteoblasts. **A.** G protein switches between GTP-binding active isoform, and GDP-binding inactive isoform to control intracellular signaling pathways. G protein is a heterometric complex comprised of α, β, and γ subunits. G protein is activated by GPCR (G protein coupled receptor) in response to external stimuli, which acts as a guanine exchange factor (GEF). Gα has internal GTPase activity, which can be enhanced by RGS (regulator of G protein signaling). Internalization of GPCR is regulated by the beta-arrestin-GRK (G protein receptor kinase) system, is dependent on agonist interaction and NHERF (Na/H exchange regulatory factor), and independent of agonist interaction. **B**. there are four groups of G proteins transducing different signaling pathways in osteoblasts: Gαi, Gαs, Gαq, Gα12/13. G protein signaling controls the differentiation, proliferation, apoptosis, and function of osteoblasts. BSP, bone sialoprotein; MCP-1, monocytechemoattractonprotein-1; MKP-1, mitogen-activated kinase protein-1; AC, adenylate cyclase; PLC, phospholipase C; PLD, phospholipase D; DAG, diacylglycerol; PDE, cyclic nucleotide phosphodiesterases; SRE, serum responsive element; ER, endoplasmic reticulum

### 3.1.2. Mouse models

Patients with AHO display variable inheritance and tissue specific resistance to multiple hormones, including PTH (57). The resistance to PTH is termed Pseudohypoparathyroidism (PHP, lack of response to parathyroid hormone). The development of the mice model with AHO—mice with heterozygous Gs deficiency—explains this phenomenon (9). Firstly, the homozygote genotype is embryonically lethal, suggesting that AHO patients should be heterozygous. Secondly, as opposed to

maternal (m-/+) inheritance, paternal (+/p-) inheritance of Gs nullifies the disease phenotype, demonstrating that GNAS is mainly expressed from the maternal allele Considering GNAS is an imprinted gene, it should be imprinted in the paternal allele. Thirdly, PHP of maternal (m-/+) inheritance exhibits a tissue specific manner, indicating that GNAS should be imprinted in a tissue-dependent manner.

Patients with AHO are also characterized by short stature. This may be caused by impaired long bone development, otherwise known as endochondral bone development. Skeletal malformations of mice with human rennin (hren) promoter-Cre driven Gs deficiency further support the roles of Gs in endochondral bone development. These mice are fertile and viable, but exhibit a shortened skeleton, fused extremity of bone, and ectopic ossification of subcutis and skin (10). As aforementioned, chondrocyte differentiation is an important stage of endochondral bone development. Accordingly, chondrocyte-specific Gs deficiency (Gsflox/Cal2a1-Cre) in mice is lethal and results in severe epiphyseal growth plate defects, shortening of the proliferative zone, and accelerated hypertrophic differentiation (13).

To study the role of Gs in bone remodeling, conditional knock-out and knock-in models in osteoblast were developed. Mice with collagen1α (col1α) promoter-Cre driven osteoblast-specific Gαs deficiency (BGsKO), developed by Sakamoto A. *et al*. (2005) (11), exhibit reduced bone turnover, thickened cortical bone, and narrowed bone marrow cavity. Based on the RASSL system and using col1 promoter, Edward C. Hsiao *et al*. (2008) (12) expressed an engineered Gαs-coupled receptor (Rs1) in mice osteoblasts. Rs1, a constitutively active GPCR specifically coupled to Gs, is generated by introducing the D100A mutation into the human 5HT4b receptor. Rs1 expression results in significantly elevated bone volume, cellularity, areal bone mineral density, osteoblast gene markers, and serum bone turnover markers. The BGsKO together with the Rs1 mice model demonstrates that Gs not only promotes osteoblast differentiation, but that it is also essential to bone resorption through osteoblasts.

**3.1.3. Signaling pathway and molecular mechanism.**

In osteoblasts and other cells, activated Gs activate AC (adenylate cyclase), which then catalyzes the production of cAMP (cyclic adenosine monophosphate) from ATP (adenosine triphosphate). cAMP binds to the regulatory subunit PKA (protein kinase A) and releases the catalytic subunits, so as to activate PKA (Figure 1). PKA phosphorylates nearby effectors, including Raf, to induce MAPK signaling, and CREB (cAMP-response element binding protein) to mediate transcription (41). In osteoblasts PKA also activates cyclic nucleotide phosphodiesterases (PDEs) to terminate cAMP-PKA signaling, which catalyze cAMP hydrolysis to the inactive form 5' AMP (58).

People diagnosed with AHO suffer from the resistance to multiple-hormones, including PTH (parathyroid hormone) (54), demonstrating the role of Gs in PTH signaling. *In vivo* and *in vitro* studies confirm that Gs-cAMP is coupled to PTH/PTHrP signaling and accounts for most physiological function of PTH (59). Gs is also coupled to other bone-related hormones besides PTH, like IGF-1 (insulin-like growth factor) (60), PGE-2 (Prostaglandin E2) (61), and calcitonin (62).

As mentioned above, Gs promotes bone formation *in vivo*. Congruently, Gs promotes osteoblast differentiation and proliferation *in vitro*. Transcription factors, including CREB (63), Runx2 (Runt-related transcription factor2) (64), c-fos (63) and CCAAT enhancer-binding protein delta (C/EBPδ) (61), were shown to be induced by Gs-cAMP signaling in osteoblast. CREB stimulates expression of clock genes, which mediate the antiproliferative function by inhibiting G1 cyclin expression, and activator protein 1 (AP1) genes, which stimulate proliferation of osteoblasts (65). Runx2, a master switch for inducing osteoblast differentiation, has been reviewed (6), C/EBPδ activate osteocalcin expression and synergize with Runx2 to promote osteoblast-specific expression (66). c-fos, an AP1 factor, is best known as a key regulator in osteoclastogenesis, but it is also a cofactor of Runx2 (67). Gs-cAMP signaling also induces transforming growth factor-beta type III receptor (TGFβRIII) expression (61) and pathogenic phosphaturic factor FGF-23 (fibroblast growth factor-23) expression in FD (fibrous dysplasia) lesions (68). TGFβ is a critical cytokine promoting bone remodeling. FGF-23 is a newly identified member of FGF family, and is supposed to be involved in phosphate homeostasis (69).

Reduced bone marrow cavity in BGsKO mice and enhanced bone remodeling in Rs1 mice support that Gs-cAMP signaling in osteoblasts also promotes osteoclastogenesis. IL-6 (interleukin-6), an osteoclastogenesis cytokine, is expressed in fibrous dysplasia lesions in the bone of MAS patients, as well as in Gs activating mutant transfected MC3T3-E1 cells (70). It is also proposed to be the major factor contributing to osteoclast differentiation and function in MAS patients. In addition, osteoclastogenesis cytokine RANKL (receptor activator nuclear factor kappa B ligand) is upregulated (71), and osteoclastogenesis inhibiting factor OPG (osteoprotegerin) is downregulated in osteoblasts regulated by hormones through cAMP-PKA activity (72).

Recent studies show that HSPC (Hematopoietic stem and progenitor cells) renewal and engraftment of bone marrow is also regulated by Gs (73-75). Gs-activating-mutant HSPCs are diminished with age, shown by detection of mutated CFU-Fs (colony-forming unit-fibroblast) from FD lesions (73). HSPC derived from Gs deficient mice demonstrates reduced homing to, and enhanced mobilization from bone marrow (74, 75). Understanding the mechanism may shed light on HSPC transplantation.

**3.2. $G_{q/11}$/$G\alpha_{q/11}$ signaling**

Gαq/11 is responsible for PLC□phospholipase C□signaling in osteoblasts. In the canonical pathway (41), Gαq/11 activates PLC, which then catalyzes the production of IP3 (inositol 1,4,5-triphosphate) and DAG

(diacylglycerol) from PIP2 (phosphatidylinositol 4,5-bisphosphate). Afterwards, IP3 activates (Ca2+)i influx and CaMKII (Ca2+/calmodulin-activated protein kinase II). Accordingly, Gq is responsible for calcium mobilization in osteoblasts, in most situation. For example, CGPR (calcitonin gene-related peptide) (62) and Calcitrol (76) induces IP3 formation and calcium mobilization in osteoblasts which are insensitive to PTX, implying the involvement of Gq.

The increased calcium concentration mediates PKC (protein kinase C) activation. Then PKC phosphorylates nearby effectors to induce downstream signaling (41). (Figure 1) Gβγ released by Gi is also able to activate PLCβ-PKC signaling. However, Gβγ-activated PLCβ and Gq-activated PLCβ can be distinguished by Gi specific inhibitor PTX (pertussis toxin) (77, 78), and Gq inhibitor Galpha (q)- (305-359) minigene (79).

### 3.2.1. $G_{q/11}$ and osteoblast proliferation

PKC is able to activate mitogenic signaling, promoting cell proliferation. Accordingly, *in vitro* assay shows that $G_{q/11}$-PKC signaling preferentially promotes osteoblast proliferation. Extracellular calcium (($Ca^{2+}$)e) mainly promotes osteoclast proliferation through cation sensing receptor, including calcium-sensing receptor (CasR) and GPRC6A (G protein coupled receptor, family C, group 6, member A). Examination of the kinetics of the CasR-mediated increase in Ins (1,4,5)P3 and ($Ca^{2+}$)i indicates the involvement of Gq signaling (80). Expression of dominant negative Galpha (q)- (305-359) minigene inhibits GPRC6A activation, indicating that GPRC6A is coupled to Gq signaling (81). Another extracellular cation, strontium, stimulates osteoblast proliferation, but is dependent on Gq-mediated SRE (serum response element) activation (82). PTH is coupled to Gs, Gq, and Gi (83). As determined by (3H) thymidine and bromodeoxyuridine incorporation and augmented osteogenic colonies, PTH increases osteogenic proliferation through PKC-induced MAPK signaling, showing that $G\alpha_{q/11}$ may be involved in the process (84). Other extracellular regulators of bone, including LPA (lipoproteinA) and PGF2α (prostaglandin F2α), are also coupled to Gq for mitogenic signaling to promote osteoblast proliferation (85).

### 3.2.2. $G_{q/11}$ and osteoblast differentiation

The regulation of osteoblast differentiation by $G_{q/11}$ is a controversial matter. The hormones coupled to Gq, as mentioned above, more or less promote osteoblast differentiation (80) (81). However, this may be attributed to the increased number of preosteoblasts mitigated by Gq and the osteogenic function of other G proteins. Other observations contradict the anabolic role of Gq in osteoblast differentiation. Transgenic mice transfected with constitutively activated $G\alpha_q$ in osteoblast exhibit severe osteopenia, including reduced length of long bone, thinner cortices, fewer trabeculae, reduced osteoblast number, and lowered BMD (bone marrow density (14). *In vitro* cultured osteoblasts from the transgenic mice show impaired differentiation, which is confirmed by ALP (alkaline phosphatase) activity detection, alizarin red staining, and marker gene assay. The impaired phenotype can be partially rescued by adding PKC inhibitor bisindolymaleimide (GF109203X) (14). PMT (Pasteurella multocida toxin), a mitogen and intracellular acting toxin, stimulates growth signaling and inhibits osteoblast differentiation, by targeting Rho and Gq. PMT targets Gq for phosphorylation, which facilitates its activation by GPCR and leads to activation of PLC and PKC (86, 87). Verified by Gq (305-359) minigene, Gq is also involved in mFZ1 inhibition of wnt3a/catenin pathway, thus causing the inhibition of osteoblastogenesis (79).

### 3.3. Gi signaling: Gi and Gi bound Gβγ

After activation, Gi and Gβγ (i) is split apart. Gαi antagonizes the function of Gs by inhibiting AC activity. Gβγ (i) activates PLCβ and induces signaling similar to Gq (41). (Figure 1)

### 3.3.1. Gi and osteoblastogenesis, *in vitro* and *in vivo*

In many cases, Gi is a positive regulator in osteoblast differentiation, proliferation, and survival. Gi influences osteoblast differentiation which is induced by CXCR4 (CXC chemokine receptor 4) (88), melatonin (89), and CNP (C-type natriuretic peptide) (90). Also, Gi stimulates osteoblast proliferation and survival, which is induced by LPA (lysophophatidic acid) (91), 1,25 (OH)2D3 (77, 78), and epinephrine (92). Gβγ-PLCβ-DAG-PKC-raf-ras-MAPK (93) signaling and downstream ERK (93, 94) activation has been implicated in Gi-regulated osteoblastogenesis and proliferation. Additionally, caspase cascade induced by Gβγ-PLCβ-DAG-PKC-PI3K (phosphatidylinositol 3-kinase)–Akt (serine/threonine kinase) signaling has been denoted in Gi-regulated anti-apoptosis (77, 78). Gi is also responsible for PTX-sensitive calcium mobilization in osteoblasts through Gβγ-PLCβ-IP3-calcium influx (80).

Inconsistent with what is observed *in vitro*, *in vivo* studies show that hyperactivition of Gαi/o inhibits osteoblastogenesis through inhibiting Gαs-AC signaling. J. Peng et. al. (2008) (15) expressed a Gi-coupled RASSL receptor Ro1 in osteoblasts by mating Ro1 transgenic mice with col1α transgenic mice. Ro1 specifically activates Gi and is activated by doxycycline. Expression of Ro1 from conception results in neonatal lethality because of reduced bone mineralization. Expression of Ro1 at late embryogenesis results in a severe trabecular bone deficit. The expression of Ro1 for 8 weeks after mice are 4-weeks old results in reduction of trabecular bone volume, rates of fraction, rates of bone formation, and rates of mineral apposition. One may wonder why this discrepancy exists between the *in vitro* assays and the *in vivo* experimentation. It is possibly due to the stimulatory role played by Gβγ subunits and the inhibitory role played by Gi subunit. If activated normally, the inhibition of Gi is antagonized by Gs subunits. However, if Gi stands alone in an abnormal state, the inhibitory role of Gi is much more significant than the stimulatory role of Gβγ subunits.

### 3.3.2. Gi is coupled to receptor sensing non-biomolecular stimuli

Gi responds to a wide range of stimuli besides hormones and cytokines, including cation concentration,

ultrasound, mechanical loading, and mechanical stretch in bone, for which most receptors have not been identified (95). A group of Cation sensing receptors signal in response to extracellular cations, including calcium (80, 81), strontium (81), Aluminin (96, 97), zinc (98) and lanthanum (99). As mentioned in Gq signaling, calcium sensing receptor GPRC6A and CasR in osteoblasts transduce signaling both through Gs and Gq. Aluminin had strong inhibitory actions on PTH-dependent cAMP production by ROS cells, while PTX treatment prevents the inhibitory effect of Aluminin (97). PTX completely blocks the chemotactic response of osteoblasts to Zn, which is released from bone-resorptive sites and plays an important role in the recruitment of osteoblasts and bone renewal (98). La3+ treatment enhanced osteoblast differentiation *in vitro*, whose function is dependent on Gi and ERK activation as evidenced by the inhibitory effect of the PTX and ERK inhibitor U0126 (99). Ultrasound treatment, especially Pulsed Signal Therapy, is administered to treat arthritis and repetitive stress injuries, to relieve pain and inflammation, and to speed healing. Ultrasound promotes osteogenic transcription, including Cbfa/Runx2 and osteocalcin expression through Gi signaling and ERK activation (94). Mechanical loading of bone, including interstitial fluid flow leads to bone remodeling and PGE2 (prostaglandin2) production. The latter is confirmed at least partially by Gi regulation, in a pathway involved in PLA2 (100, 101). However, the frequency of stretch regulates the release of nitric oxide in bone, while PTX reverses this high frequency-inhibited release (102).

### 3.4. G12/13 signaling

In the canonical pathway, Gα12 and Gα13 activate Rho proteins through RhoGEF (Rho guanine-nucleotide exchange factors) (Figure 1), which either mediates gene expression through the MEKK-MEK-JNK pathway or regulates cytoskeleton organization (41). To date, there is little study of G12/13 in bone, and their relationship to osteoclasts, osteoblasts, and bone is unknown. It was reported that Gα12/13 is involved in PTH-mediated PLD (phospholipase D)-regulating phospholipid hydrolysis through RhoA activation, and is theorized to play a role in cell shape changes and cell survival (103). PLD generated Diacylglycerol (DAG) contributes to PKC membrane translocation in response to PTH. In this study, exoenzyme C3 (an inhibitor of Rho family small G proteins), dominant-negative RhoA, and pcDNA-G12 and pcDNA-G13 minigene vectors inhibit PTH-mediated PLD activation (103). Constitutively active G12 and G13 and dominant-negative RhoA promote TH-mediated PLD activation (103).

### 3.5. G proteins in osteoclasts

Though the significance of heterotrimeric G proteins has been indicated in many cell types, our knowledge about their function in osteoclasts is rather limited, unclear, and controversial.

Gs and Gi are both coupled to Calcitonin receptor (CTR) in osteoclasts, but functions in different procedures. Calcitonin (CT) inhibits osteoclastic bone resorption and disrupts actin ring formation, but increases TRAP (tartrate-resistance acidic phosphatase) secretion (104-106). While Gs activator (cholera toxin) or PKA activator (forskolin) mimic all the three conditions (105, 106), Gi inhibitor PTX inhibits bone resorption significantly but doesn't influence TRAP secretion or actin ring formation (105, 106). CT induces down-regulation and de-sensitization of CTR in osteoclasts, which is regulated by cAMP-PKA pathway but not by the PKC activity (107).

Gi is also involved in αvβ3 integrin production, and CCR1 (chemokine receptor1) and FSH (Follicle-Stimulating Hormone) function in osteoclasts. αvβ3 integrin is essential for activation of osteoclast function. αvβ3 integrin expression in human leukemic preosteoclastic cell line (FLG 29.1 cells) induced by extracellular matrix protein fibronectin (FN) is sensitive to PTX (108). CCR1 is a chemokine receptor gene that acts downstream of NFAT2 and is induced by RANKL in RAW264.7 or bone marrow cells. CCR1 promotes osteoclast progenitor migration and osteoclastogenesis, which is blocked by PTX (109). FSH has been long known as primary stimulus for estrogen production, acting on FSHR (FSH receptor) in ovarian. However, a recent study shows that FSHR is also expressed in osteoclasts, and FSH directly promotes osteoclast maturation and function (110). Gi2α is coupled to FSHR to activate MEK/Erk, Nf-κB, and Akt in osteoclast (110). This study provides explanation for hypogonadal bone loss caused by high circulating FSH.

Gs-cAMP-PKA signaling may be involved in mediating the activity of CatK (cathepsin K) in osteoclasts. CatK is the major cysteine protease produced and secreted by osteoclasts to degrade the extracellular matrix. The cAMP antagonist, Rp-cAMP, and the protein kinase A (PKA) inhibitors KT5720 and H89 (111) prevent intracellular maturation of CatK.

## 4. G PROTEIN COUPLED RECEPTORS AND LIGANDS IN BONE

Heterotrimeric G proteins are activated by a group of seven-transmembrane receptors named G protein coupled receptors (GPCRs). All GPCRs have seven transmembrane-spanning αhelices, an extracellular N terminus, an intracellular C terminus and three interhelical loops on each side of the membrane (112). GPCRs can be divided three groups, A, B, and C (113). Some GPCRs have specificity for certain G proteins. For example, group A GPCR preferentially activates Gs proteins, and 5HT1B serotonin receptor specifically activates Gi proteins (114). The GPCRs present in bone discussed in this review have been summarized in Table 2.

### 4.1. PTH/PTHrP and PTH1R in bone

#### 4.1.1. Physiological role of PTH/PTHrP in bone

Parathyroid hormone (PTH) is an 84-amino-acid straight-chain calcitropic peptide known to be released from the parathyroid glands in response to a hypocalcemic stimulus (115). PTHrP (parathyroid hormone related peptide) is a genetically-related peptide that shares homology with PTH within the amino-terminal domain,

**Table 2. GPCRs (G protein coupled receptor) in bone**

| GPCR | Ligands | Location | Heterotrimeric G protein (s) | Role |
|---|---|---|---|---|
| PTH1R | PTH/PTHrP | Osteoblast | Gs, Gq, Gi, G12/13 | Normal chondrocyte development in growth plate, calcium homeostasis (up), mineralization (down), bone resorption (up), bone formation (up) |
| Calcium sensing receptor | Cations | osteoblast, osteoclast | Gq, Gi | Normal chondrocyte development in growth plate, calcium homeostasis (down), mineralization (up), bone resorption (up), bone formation (up) |
| Calcitonin receptor | calcitonin | Osteoclast | Gs, Gq, Gi | Protects from PTH-induced hypercalcemia and bone loss, protect from ovariectomy-induced bone loss |
| Calcitonin gene-related peptide receptor | CGRP (calcitonin gene-related peptide) | Osteoblast | | Promotes bone formation, inhibits osteoclastogenesis (only *in vitro*) |
| Cannabinoid receptor type 1 | Cannabinoids including AM251 | osteoclast, Central neural system | | Promotes osteoclastogenesis, inhibits osteoblastogenesis through inhibiting β2AR ligand release from nerve terminals |
| Cannabinoid receptor type 2 | Cannabinoids including HU308 | osteoclast, osteoblast | | Inhibits osteoclast formation and function, protect from ovariectomy-induced bone loss<br>SNPs is associated with post-menopausal osteoporosis |
| GPR55 | Cannabinoids including O-1602, LPI | osteoclast, osteoblast | | promotes osteoclast function |
| Relaxin family peptide1 | Relaxin | Osteoclast | | Tumorigenesis |
| Relaxin family peptide2 | ILSR3 (insulin like factor 3) | Osteoblast | Gs | bone remodeling and mineralization |
| OGR1/GPR68 | Proton | Osteoclast | Gq, Gi | Proton sensing; tumorigenesis |
| GPR30/GPER1 | estrogen | osteoblast, osteoclast, osteocyte, Chondrocyte | | Growth plate closure |

which is the domain required for binding of PTH/PTHrP to its corresponding receptor PTH1R (115, 116). PTH1R (type 1 PTH/PTHrP receptor), is expressed in PTH/PTHrP target cells such as osteoblasts and renal tubular cells. These receptors belong to the Secretin family of B-type 7-TM GPCR which consists of 15 members and an extracellular domain for peptide hormone binding (113). Calcitonin and calcitonin-like receptors and growth-hormone-releasing hormone receptor (GHRHR) are also members of the secretin family (113). It has long been known that the recessive loss-of-function mutation of PTH1R is associated with Blomstrand osteochondrodysplasia (BOCD), a lethal form of short-limbed dwarfism (117). And three PTH1R mutations were reported recently as the cause of a human disease PFE (primary failure of tooth eruption), which is associated with several syndromes primarily affecting skeletal development (118).

#### 4.1.1.1. Calcium homeostasis

PTH/PTHrP is well known as a hormonal regulator of calcium homeostasis. Both PTH and PTHrP lead to an increase in serum calcium, mainly through calcium resorption from the kidney and paracellular regions (116, 119). PTH expression is downregulated with increased serum calcium in the parathyroid, forming a negative feedback loop. However, since PTHrP is expressed universally, how it is regulated by serum calcium is uncertain (discussed in the part of cation sensing receptor). Enlargement of the parathyroid glands is also observed in the PTH-ablated mice, a finding that is consistent with fetal hypocalcemia. PTHrP–/– mice have reduced capacity to maintain normal placental calcium transport and therefore develop hypocalcemia (16). Knock-in mice expressed with a phosphorylation deficient PTH1R, which can not be internalized, have dramatically exaggerated cAMP and calcemic responses to PTH administration (120). Though the serum calcium level is normal, the PTH levels in the experimental mice are only one third of the PTH levels in the control mice (120). All these observations are consistent with the role of PTH/PTHrP in calcium homeostasis and the causal increase in serum calcium.

#### 4.1.1.2. Anabolic and catabolic regulator of bone formation

PTH/PTHrP is also a hormonal regulator of bone formation, expressing opposing effects. These effects are anabolic and catabolic. On one hand, the binding of PTH to osteoblasts results in upregulated expression of the receptor activator nuclear factor kappa B ligand (RANKL), and reduced expression of soluble decoy receptor of RANKL called osteoprotegerin (OPG). This combined upregulation and decreased expression enhances osteoclastic osteolysis and mobilization of calcium from the skeleton (121, 122). On the other hand, PTH (1-84), PTH (1-32) and PTHrP (1-36) augment bone marrow density if administered intermittently (123, 124). PTH is widely used as an anabolic agent for osteoporosis (125), signifying the anabolic role of PTH in bone development. Moreover, PTH/PTHrP has been implicated in the commitment of mesenchymal cells to osteoblasts (126-128), osteoblastogenesis (129, 130), osteoblast proliferation, osteoblast/osteocyte apoptosis (131, 132), and bone mineralization (133).

Since PTH/PTHrP has both anabolic and catabolic effects in bone development, the ultimate influence relies on which effect is predominant. Various mouse models have been generated, depicting the significant psychological role of the endogenous PTH/PTHrP-PTH1R system. Firstly, PTH/PTHrP,

especially PTHrP, is essential to chondrocyte differentiation since endochondral ossification and body size are influenced in PTHrP/PTH deficient mice. PTH-/- mice exhibit shorter metatarsal bones, long bones, and smaller vertebral bodies compared with control mice (16). PTHrP-/- mice exhibit short-limbed dwarfism and a much smaller whole skeleton, significantly shortened long bone. They also display evidence of a reduced proliferating chondrocyte zone compared to the hypotrophic zone, as well as an increased chondrocyte apoptosis, even compared to PTH-/- mice (16). PTH-/- and PTHrP-/- mice suffer from more serious skeletal malformation (16). Knock-in mice expressed with a mutant PTH1R, which only induces AC signaling but not PKC signaling, display defects in ossification of the tail, digital, and metatarsal bones due to delayed chondrocyte hypertrophy, that is, impaired chondrocyte differentiation, with increased proliferation (17). Secondly, PTH and PTHrP deficiency impairs both bone formation and bone resorption, while it increases mineralization. PTH-/-, PTHrP-/-, and double knockout mice all have thickened cortical bone due to decreased bone resorption They also have a reduction in the number of osteoblasts and an increase in osteoblast apoptosis (16). However, as observed in PTHrP-/- mice, there is an excessive degree of mineralization as delineated by staining with alizarin red S (133). Thirdly, PTH signaling cooperates with Wnt signaling to enhance bone remodeling. Expressing constitutively active PTH1R (caPTH1R) in osteocytes, which is driven by osteocyte specific promoter DMP1, leads to a remarkable and progressive increase in bone mass in the appendicular and axial skeleton and to an increase of both osteoclast and osteoblast perimeters, compared to wild-type littermates (19). Further study shows that the DMP1-caPTH1R osteocytes overexpress Sost and Sclerostin, which are ligands for Wnt signaling that contribute to increased bone mass, but not to bone resorption. Increased bone resorption may be attributed to enhanced secretion of osteoclastogenic cytokines by caPTH1R osteocytes. Lastly, the mouse model shows that PTHrP is more than a secreted hormone. It also contains a NLS (nuclear location sequence). Knockin of PTHrP (1-84), which is a mutant capable of inducing PTH1R signaling, but lacking its NLS domain, results in skeletal growth retardation and premature osteoporosis due to the evident repression of proliferation and increase in the apoptosis of chondrocytes and osteocytes/osteoblasts (18). In conclusion, PTH and PTHrP are essential to chondrocyte survival and proliferation, osteoblastogenesis, and bone resorption.

#### 4.1.2. Signaling pathway and molecular effectors regulated by PTH/PTHrP in osteoblasts

PTH/PTHrP couples to all four Gα subunits in osteoblast/osteocyte: Gi/o, Gq/11, G12/13 (83, 134) and Gs (83). In most cases, PTH activates the Gs subunit, leading to the AC-cAMP-PKA signaling pathway (135), or it activates Gq/11 subunits, leading to PLC-IP3/DAG-PKC signaling pathway (136). PTH also induces DAG production by PLD (phospholipase D)-regulating phospholipid hydrolysis, signaled through G12/13-RhoGEF-RhoA activation (103, 134).

PTH1R in response to PTH/PTHrP stimulates a wide range of molecules (Table 3). According to both *in vitro* and *in vivo* studies, the psychological roles of PTH1R can be summarized as follows: (1) regulates mesenchymal commitment, (2) promotes osteoblast differentiation, (3) regulates osteoblast proliferation, (4) downregulates osteoblast mineralization, (5) inhibits osteoblast apoptosis, and (6) promotes osteoclast differentiation through osteoblasts. (Figure 2) The identification of effectors in PTH/PTHrP signaling provides molecular mechanisms to explain its psychological functions.

Mesenchymal commitment to osteoblasts rather than to adipocytes is promoted by PTHrP/PTH, through different signaling pathways. PTHrP coupled with BMP-2 (bone morphogenic protein-2), enhances osteoblastogenesis and inhibits adipogenesis dependent PKC activity (126). Intermittent treatment, rather than continuous treatment, with PTH also inhibits adipocyte differentiation and favors osteoblast differentiation, and it is predominantly dependent on PKA signaling (137). PTH/PTHrP promotes osteoblast differentiation through activation of key transcription factors of osteoblasts including ATF-4 (cAMP-dependent transcription factor) (138), Runx2 (129), CREB (139) and AP-1 (140), and induces mature osteoblast formation including indicators such as collagen I (141), osteocalcin (142), osteonectin (143) and BSP (bone sialoprotein) (144). Recently, EphrinB2 is also found to be upregulated by PTH in osteoblasts. Usually EphB4/EphrinB2 are found on two adjacent cells (osteoblasts and osteoclasts) and transduce a two-way signaling pathway. This new finding shows that ephrinB2/EphB4 signaling within the osteoblast lineage has a paracrine role in osteoblast differentiation (145, 146). PTH/PTHrP promotes proliferation of early osteoblasts, but inhibits proliferation of mature osteoblasts by regulating the activity and expression of cyclin D1 (132, 147, 148) in a mechanism that involves both CREB and AP-1 (149). Cyclin D1 is a positive regulator of progression through the G1 phase of the cell cycle. The expressions of Cyclin D1, Cyclin D1 kinase, activator CDK (cyclin dependent kinase) 4 and 6, and CDK inhibitor p27, p21, and p16 are all regulated by PTH (132, 147, 148, 150). PTH/PTHrP upregulates MGP (matrix gla protein) (151), a negative regulator of mineralization, and down regulates osteonectin (143), a positive regulator of mineralization. PTH inhibits osteoblast/osteocyte apoptosis through activation of anti-apoptosis proteins like Bcl-2, Bcl-xl, and Bad, and through the inhibition of proapoptosis Bcl-2 family members (131, 152). Crosstalk between PTH and Wnt signaling also contributes to the inhibition of apoptosis (19, 153).

As mentioned earlier, PTH/PTHrP promotes osteoclastogenesis and bone resorption through osteoblasts. PTH up-regulates RANKL expression and inhibits OPG expression in osteoblasts. PTH/PTHrP also promotes the secretion of other osteoclastogenic cytokines, like IL6 (154-156) and MCP-1 (monocyte chemoattractant protein-1) (157). IL6 is an inflammatory-related protein which promotes osteoclast formation. MCP-1 is a chemoattractant protein that recruits osteoclast precursors to bone resorption sites to facilitate its maturation and fusion. *In vivo*, growth factors FGF-2 (fibroblast growth factor-2) (158, 159), IGF-1 (160, 161), and TGF-β (162, 163) involved in bone

**Table 3. Molecules regulated by PTHrP/PTH-PTH1R signaling in bone**

| Classification | Protein name | Function | Mechanism | Refe |
|---|---|---|---|---|
| Growth factors, Hormones | FGF-2 | required for anabolic effect of PTH, essential for PTH-mediated Bcl-2/Bax, Runx2 and CREB activation in osteoblast and osteoclastogenesis, *in vivo* | Released from bone matrix by activated osteoclasts, or induces the expression of ligands or receptors, or crosstalk between signaling pathways | (158, 159) |
| | IGF-1 | Required for anabolic effect of PTH, confirmed *in vivo* (IGF-1 receptor null mice) and *in vitro* | | (160, 161) |
| | BMPs | PTH plus BMPs induces bone development by MSCs (mesenchymal stem cells) | | (164) |
| | TGF-β | Bone remodeling; regulates PTHrP expression through AP1 | | (162, 163) |
| | Wnt | Bone remodeling, anti-apoptotic | targeting GSK-3β (166), β-catenin[43], LRP5 ligands (19) | (19, 153, 165, 166) |
| Transcription factors | ATF4 | Fundamental to anabolic effect of PTH, convinced by ATF-/- mice; osterix expression | | (138) |
| | Runx2/Cbfa1 | Osteoblast specific transcription factor | PKA-dependent activation, expression upregulated by CREB | (129) |
| | CREB | cAMP response element binding proteins | Gs-PKA signaling | (139) |
| | AP1 (fos, JunB) | | Gq-PKC signaling | (140) |
| Targeted molecules with upregulated expression | EphrinB2 | Communication between osteoblast and osteoclast | | (146, 167) |
| | osteocalcin | Osteoblast indicator | PKA-, PKC-dependent | (142) |
| | BSP | early marker of osteoblast | CREB | (144) |
| | CDK1 | Cell cycle, cyclin D1 kinase and activator (early osteoblastic cell) | | (132) |
| | Cyclin D1 | G1 Cell cycle (early osteoblastic cell) | CREB-, AP-1 dependent | (132) |
| | p16, P21 | Cell cycle | | (148) (147) |
| | p27Kip1 | Cell cycle | PKA-dependent | (150) |
| | MKP-1 | ERK1/2 inactivation, cyclin D1 downregulation, growth arrest of osteoblast | PKA-, PKC-dependent | (147, 168) |
| | Bcl-2, Bcl-xl | Anti-apoptosis | | (131) |
| | MGP | a known inhibitor of mineralization | | (151) |
| | Bad | Anti-apoptosis | PI3K-Akt-Bad phsosphorylation/activation | (152) |
| | IL-6 | Osteoclastogenic cytokine interleukin-6 | PKCβ-, MAPK-dependent | (154-156) |
| | RANKL | promotes osteoclastogenesis | | (141, 169) |
| | MCP-1 | able to recruit osteoclast monocyte precursors and facilitate the RANKL-induced osteoclastogenesis and fusion | cAMP-dependent | (157) |
| Targeted molecules with downregulated expression | osteonectin | matricellular calcium-binding glycoprotein, major noncollagenous constituent of bone, positive factor in the mineralization process | | (143) |
| | OPG | Inhibits osteoclastogenesis | | (72, 141, 169) |
| | CDK4,6,1,2 | Cell cycle, cyclin D1 kinase and activator | | (148) (150) |
| | Cyclin D1 | G1 Cell cycle. | MAPK-, PKA-JunB-dependent | (147, 148) |
| | Bax, PUMA | Pro-apoptosis | | (131) |
| | p27Kip1 | Cell cycle (early osteoblastic cell) | | (132) |

emodeling is released from the bone extracellular matrix by osteoclast activation in response to PTH/PTHrP and act on osteoblasts, in turn, so as to form a positive feedback loop.

### 4.2. Calcium sensing receptor (CaSR/CaR)

CaR (calcium sensing receptor/CaSR) has 1078 amino acids and belongs to C type GPCR. Although Ca2+ is the physiological ligand for the CaR, it can also sense and respond to other divalent and polyvalent cations, including ionized magnesium (Mg2+), beryllium (Be2+), barium (Ba2+), strontium (Sr2+), lanthanum (La3+), and gadolinium (Gd3+). The CaR is expressed in organs controlling (Ca2+)e homeostasis, including thyroid C-cells, kidney, osteoclasts, osteoblasts, osteocytes, chondrocytes, etc. (170, 171).

CaR gene mutations are related with a number of human diseases. Loss-of-function mutations of the CaR cause familial hypocalciuric hypercalcemia (FHH, formerly known as familial benign hypercalcemia) and neonatal revere hyperparathyroidism (NSHPT). FHH shows mild-to-moderate hypercalcemia, while NSHPT displays life-threatening hypercalcemia and under-mineralized skeleton with subperiosteal bone resorption (172). Conversely, the rare genetic hypocalcemic disorder autosomal dominant hypocalcemia (ADH) is caused by activating the gain-of-function mutation in CaR. Patients with ADH reveal low to normal PTH levels and related hypercalciuria despite hypocalcemia, while phenotypically, the skeleton is still defined (173). These symptoms indicate CaR plays a role opposing to PTH: increasing bone mineralization and decreasing serum calcium.

In addition to hyperparathyroidism, hypercalcemia, dwarfism, increased mineralized bone formation, and increased bone resorption, CaR-/- mice also display serious rickets, as evidenced by a widened zone of hypertrophic chondrocytes, impaired growth plate calcification, disorderly deposition of minerals, excessive osteoid accumulation, and prolonged mineralization lag

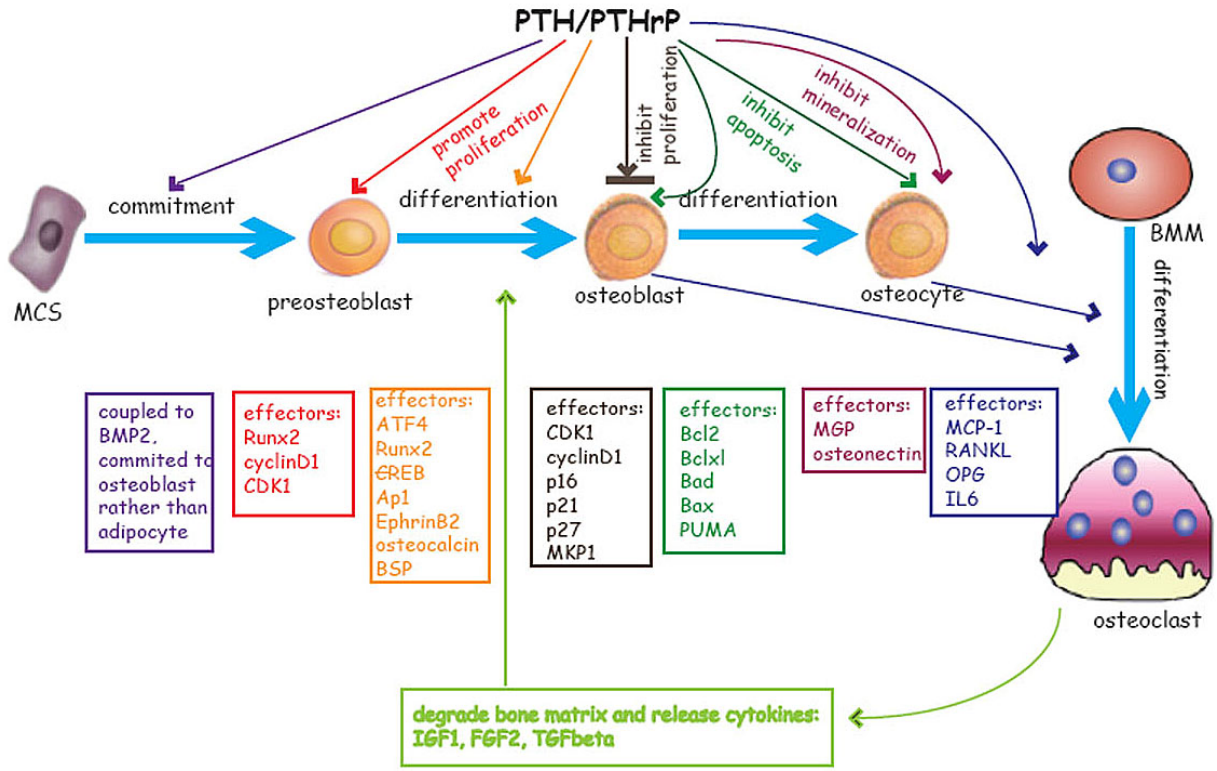


**Figure 2.** Role of PTH/PTHrP in bone remodeling. PTH/PTHrP controls the commitment of MCS (mesenchymal stem cells). PTH also controls differentiation, proliferation, apoptosis, and mineralization of osteoblasts/osteocytes. Additionally, PTH promotes osteoclastogenesis through osteoblasts, which in turn influences osteoblastogenesis. Effectors written in a specific color correspond to a specific aspect of PTH/PTHrP function and are marked by an arrow. CDK, cyclinD1 kinase; BSP, bone sialoprotein; MKP1, Mitogen-Activated Protein Kinase Phosphatase 1; MGP, matrix Gla protein; MCP-1, monocytechemoattractonprotein-1.

time in metaphyseal bone (27). CaR in the parathyroid gland inhibits PTH expression, which in turn regulates bone remodeling. Thus, the direct role of CaR in bone can not be fully illuminated by CaR deficient mice. Using the osteocalcin promoter, Dvorak *et al* (174) created a transgenic mouse with constitutively active CaR targeted to osteoblasts. RANKL was upregulated in osteoblasts cultured from mutant calvaria and femora, and histomorphometry confirmed an increase in osteoclasts. It is supposed that locally activated CaR upregulates the expression of PTHrP (175), which in turn promotes the bone formation and RANKL expression in osteoclasts to enhance bone resorption.

*In vitro* assay indicates that activated calcium sensing receptor promotes osteoblast proliferation (176, 177) and regulates osteoclast resorption (178). Further study shows that CaR is coupled to Gq and Gi signaling. Calcium activated CaR stimulates IP3 production, calcium influx, and ERK phosphorylation (179). CaR is also demonstrated as the receptor of strontium, which is an anabolic agent to bone formation and used as a treatment of osteoporosis (180). Strontium activates IP3, ERK, PKC, and PKD signaling, significantly increasing early osteoblast genes including c-fos and egr-1 (early growth response factor-1), and promotes osteoblast proliferation, which can be attenuated by overexpressing dominant negative CaR (179, 181). Unlike calcium, which has a maximal effect after a 5 minute exposure, strontium induces a delayed signaling in addition an immediate reflection, indicating an involvement of autocrine growth factor release by strontium (181). High extracellular calcium concentration (3 to 5 mM) and the CaSR agonist neomycin inhibits 1,25 (OH)2D3- or human parathyroid hormone (hPTH) (1-34)-induced osteoclast-like cell (Ocl) formation from osteoclast precursor cells derived from spleen cells (182). Calcium induces mature osteoclast apoptosis, dependent on PLC activation and Nfκβ nuclear translocation (183, 184). Like calcium, strontium is also shown to promote osteoclast apoptosis through CaR, dependent on PKCβ2 signaling and independent of IP3 action (185). However, MC3T3-E1 osteoblasts that lack CASR respond to millimolar concentrations of strontium (180), and CasR-/- osteoblasts continue to respond to extracellular cations including aluminum and the CasR agonists gadolinium and calcium (186), indicating the existence of other cation sensing receptors in osteoblasts.

Both *in vivo* and *in vitro* assay indicate the existence of other cation sensing receptors in bone besides CaSR. GPRC6A, a receptor with conserved calcium and calcimimetic binding sites is identified in

osteoblasts (81). GPRC6A is a cation (including calcium, magnesium, strontium, aluminum, gadolinium,), calcimimetic, and osteocalcin sensing receptor, and is widely expressed in mouse tissues, including bone, calvaria, kidney, testes, and liver. GPRC6A is coupled with Gi and Gq, and may also coupled to G12/13 as indicated by the RhoA inhibitor C3 toxin which inhibits activation of GPRC6A by extracellular cations (81). GPRC6A (-/-) mice exhibits hepatic steatosis, hyperglycemia, glucose intolerance, insulin resistance, and a decrease in bone mineral density (BMD) in association with impaired mineralization of bone (28).

### 4.3. Calcitonin

Calcitonin (CT) has been discovered as a hypocalcemic hormone, opposing the function of PTH, produced by thyroidal C cells. Due to its anti-resorptive property, Salmon calcitonin has been available as a therapeutic agent for more than 30 years, utilized clinically for the treatment of metabolic bone diseases including osteoporosis and Paget's disease, and potentially in the treatment of osteoarthritis (187, 188). The receptor for Calcitonin, CTR/CALCR (Calcitonin receptor/calcitonin gene related peptide receptor) is a B type 7-TM receptor. Another three proteins of the calcitonin family of secreted polypeptides, calcitonin-gene-related peptide (CGRP), mylin, and adrenomedullin, and their receptors are also expressed in bone and may serve as potential agent targets (189).

#### 4.3.1. Calcitonin and the calcitonin receptor

Calcitonin receptor/calcitonin gene related peptide receptor (CTR/CALCR), a peptide with 490-amino acids (190). CTR has been found in several cell types, including neurons of the central nervous system, placental cells, lymphocytes, and bone. Two CTR isoforms are expressed in mouse and rat osteoclasts, predominantly C1a and C1b (191). CTR is not expressed in bone marrow cells, but it is significantly induced by RANKL (192). In contrast, there is no evidence showing that CTR is expressed in osteoblasts. Human CTR SNP is related to bone density (193-195). For example, polymorphism C1377T is associated with BMD at the femoral neck in postmenopausal women (195). CTR+/- mice display high bone mass due to an increase in bone formation and normal bone resorption (26), which is rather perplexing, considering the anti-resorptive function of CT and the absence of CTR in osteoblasts. Calcitriol (1,25 (OH) (2)D (3))-induced hypercalcemia is greater in CTR deficient mice than wild-type mice, demonstrating that CTR plays a modest physiological role in the regulation of calcium homeostasis (20).

Calcitonin/CT, the ligand of CTR, is a 32-amino acid 3.4-kD protein produced from a 141-amino acid 15.5-kD precursor protein (196, 197). The precursor protein is comprised of leader sequence, calcitonin sequence, and a C-terminal peptide. In regulating osteoclastogenesis, CTR has recently been shown to couple with multiple trimeric G proteins and to activate several signaling proteins, including protein kinase C, cAMP-dependent protein kinase, and calcium/calmodulin-dependent protein kinase (198). However, calcitonin is not as efficient as bisphosphonates as an anti-resorptive drug. Continuous treatment of osteoclasts with calcitonin results in acquired resistance, due to rapidly reduced calcitonin receptor mRNA expression induced by CT signaling (199). Recently, calcitonin was reported to induce expression of inducible cAMP early repressor (ICER) in osteoclasts, which is encoded by the CREM gene (cAMP response element modulator gene). Compared to the control mice, calcitonin-treated mice exhibit inhibition of osteoclastogenesis of CREM-/- BMM to a more significant extent and do not show reduced expression of the calcitonin receptor (200). Thus, it can be supposed that CT-CTR-Gs-cAMP-PKA-ICER signaling is involved in CTR down-regulation as to form a negative feedback loop.

CT and CGRP are encoded by the same gene. CT/CGRP knockout mice exhibit significant trabecular bone volume and a 1.5- to 2-fold increase in bone formation at the ages of 1 and 3-months (22). Additionally, these mice maintain bone mass following ovariectomy, and their bone resorption is unaffected (22). However, bone resorption of CT/CGRP-/- mice increases with age, confirmed by dynamic histomorphometry and increasing urinary collagen degradation products (21). Moreover, compared with the control, CT/CGRP-/- mice also exhibit greater hypercalcemia, more bone mineral content loss, and longer recovery time when treated with PTH, while application of CT prevents these conditions (22, 23). Thus it can be implicated that CT induces and exerts its bone resorption function only in stressful conditions, like excessive bone loss and hypercalcemia,.

#### 4.3.2. Other calcitonin family peptides and their receptors

Calcitonin receptor-like receptor (CRLR), which is 55% similar to CTR, also belongs to the type B G protein coupled receptor. RAMP1, 2, and 3, are single-transmembrane-domain proteins, which form complexes with CRLR or CTR to modulate their receptor specificity and function. CGRP1 consisting of RAMP1 and CRLR, serves as a receptor for CGRP (187, 188). AM1 and AM2, consisting of RAMP2 or 3 and CRLR, serves as receptor for adrenomedullin (187, 188). RAMP1-3 and CRLR are also expressed in BMM cells and osteoclasts, and CRLR and RAMP1 expression is upregulated by RANKL (192), indicating a potential role of CGRP in osteoclasts. Though CTR is expressed in osteoclasts, but not osteoblasts, receptors for CGRP and adrenomedullin are also expressed in osteoblasts (201).

CGRP (calcitonin gene-related peptide), CGRP-α, and CGRP-β are vasoactive neuropeptides encoded by the same gene as calcitonin. Unlike calcitonin, CGRP is also expressed in the brain, such as in neurons and many other tissues (202). In contrast to CT/CGRP-/- mice, CGRP-α deficient mice with normal calcitonin expression show osteopenia due to impeded bone formation. This expression indicates that CGRP-α is a physiologic activator of bone formation. Osteoclastogenesis and bone resorption is not altered in CT/CGRP-α-/- mice (24). CGRP-α-/- mice do not exhibit obvious bone resorption inhibition, and are

resistant to polyethylene particle-induced osteolysis compared with wild-type mice (25). *In vitro* assay demonstrates that CGRP promotes osteoblast differentiation, as well as inhibits osteoclast differentiation directly, by inhibiting RANKL induced NFkB activation (203). Gq/11 and Gs signaling may be involved in the process (62). Since Calcitonin-gene-related peptide (CGRP) is a neuropeptide richly distributed in sensory neurons innervating the skeleton, understanding the role of CGRP sheds light on the regulatory role of the neural system on bone development.

Adrenomedullin is mitogenic to osteoblastic cells *in vitro* and promotes bone growth *in vivo* (201, 204). Amylin and adrenomedullin target not only CTLR but also tyrosine kinase receptor for IGF1 (insulin-like growth factor1) (205). However, the exact mechanism is unclear. Amylin-/- mice (26) exhibit osteopenia due to increasing bone resorption, implicated by increasing osteoclast surface as demonstrated by trap staining. However, bone formation is not influenced. *In vitro* culture of BMM in the presence of amylin also demonstrates that amylin inhibits osteoclastogenesis, which depends on ERK activation

**4.4. Cannabinoid receptor**

Cannabinoid receptor is a G-protein-coupled receptor for a collection of lipid compounds named cannabinoids, such as endocannabinoids anandamide, or 2-AG, and synthesized cannabinoid cannabis (206). The cannabinoid receptor-endocannabinoid system is an emerging target of pharmacotherapy (207, 208). There are four members of cannabinoid receptors, CB1 (cannabinoid receptor type 1, mainly expressed in the central nervous system), CB2 (cannabinoid receptor type 2), GPR55 (G protein coupled receptor 55), and GPR119 (G protein coupled receptor 119), which has been cloned recently (209). CB1, CB2, and GPR55 have been reported to regulate bone development. CB1 and CB2 are coupled primarily to Gi/o, and CB1 is also coupled to Gq/11 in cultured hippocampal neurons (208). However, the signaling in bone cells induced by cannabinoid is unclear.

Cannabinoid receptor type 1/CB1, the first cloned cannabinoid receptor, is mainly expressed in the central nervous system, and also expressed in osteoclasts, but not in osteoblasts (208). The most significant role of CB1 is in coordinating brain-skeleton communication: CB1 controls osteoblast function through negatively regulating noradrenaline, a ligand of osteoblastic β2AR, which is released from sympathetic nerve terminals in the immediate vicinity of these cells (208). CB1 also regulates osteoclastogenesis directly. CB1 deficient mice have increased bone mass and are protected from ovariectomy-induced bone loss (210). An antagonist to the CB1 receptor, synthetic cannabinoid AM251 inhibits osteoclast formation and bone resorption *in vitro*, and protects from ovariectomy-induced bone loss *in vivo*. Conversely, endogenous cannabinoid agonist anandamide and synthetic agonist CP55940 enhance osteoclast formation *in vitro* (210).

Cannabinoid receptor type 2/CB2, a 360-amino acid 7-transmembrane-spanning protein, shares 44% similarity with CB1 (211) and is expressed in the immune system (211), atherosclerosis plaques (29), osteoblasts, osteocytes, and osteoclasts (212). CB2 is implicated as a potential pharmacological target for pain (213), osteoporosis (29), and atherosclerosis (212). SNPs of Human CB2 gene located on chromosome 1p36 is associated with postmenopausal osteoporosis, according to a systematic genetic association study in 388 French women with post-menopausal osteoporosis and matched female controls (214). CB2 deficient mice exhibit osteoporosis characterized by markedly accelerated age-related trabecular bone loss and cortical expansion, but unchanged cortical thickness (29). CB2 deficient mice also have increased trabecular osteoblast activity and increased osteoclast number (29). CB2-specific agonist HU308 attenuates ovariectomy-induced bone loss, and enhances endocortical osteoblast number and activity. HU308 also inhibits trabecular osteoclastogenesis by inhibiting proliferation of osteoclast precursors and RANKL expression in osteoblasts/stromal cells (29). The phenotype of CB2 deficient mice and the function of CB2 agonist, suggest a negative role of CB2 in osteoclastogenesis. Thus, it can be presumed that the CB2 antagonist, which inhibits the function of CB2, may have a positive role in osteoclastogenesis. However, CB2-specific antagonists SR144528 and AM630 inhibit osteoclast formation and resorption *in vitro* and prevent mice from ovariectomy-induced bone loss *in vivo* (210).

GPR55, a receptor activated by O-1602 and L-α-lysophosphatidylinositol (LPI) and inhibited by CBD (cannabidiol), is expressed in both osteoclasts and osteoblasts. The bone phenotype of GPR55-/- mice differs between sexes (30). 12-week-old male GPR55-/- mice have significantly increased bone volume in the tibia and femur and increased trabecular number due to plump but inactive osteoclasts, though bone formation is not influenced. However, 12-week-old female GPR55-/- mice show considerably reduced osteoclast numbers without change in trabecular bone volume. CBD application inhibits bone resorption *in vivo*. It is hypothesized that GPR55 inhibits osteoclast formation but promotes osteoclast function since the formation of osteoclasts is increased by CBD treatment or in GPR55-/- osteoclasts. LPI and O-1602 induce polarization and bone resorption in osteoclasts through a pathway involving Rho activation and ERK phosphorylation (30). Since GPR55 has been reported to couple to G12/13 in other cells (207), it is likely that GPR55 regulates Rho activity through G12/13 signaling in osteoclasts.

**4.5. RXFP/relaxin family peptide**

RXFP (relaxin family peptide), is a G-protein-coupled receptor for relaxin, an insulin-like hormone, with multiple functions including extracellular matrix remodeling, collagen degradation, and up-regulation of matrix metalloproteinases (215). It has been proven that Relaxin induces osteoclastogenesis by targeting RXFP-1. RXFP-1 is expressed in osteoclasts and is induced by RANKL (216, 217). Silencing RXFP-1 results in impeding osteoclastogenesis (216). Relaxin also promotes clustering, migration, and activation states of mononuclear myelocytic

cells (218). Since Relaxin has been implicated as an autocrine/paracrine factor for tumor biology and osteoclasts are the major mediators of osteolysis during tumor skeletal metastases, relaxin is a candidate contributor to tumor skeletal metastases (219).

INSL3 (Insulin-like factor) is produced primarily by testicular leydig cells and specifically targets G-protein-coupled receptor RXFP2**.** Osteoblast stimulation with INSL3 shows a dose-dependent cell proliferation and cAMP accumulation. Young men with RXFP T222P mutation have significantly reduced BMD, and Rxfp2-/- mice show a decrease in bone mass, mineralizing surface, bone formation, and osteoclast surface, suggesting the role of INSL3/RXFR2 signaling in bone remodeling and mineralization (31, 32). Since RXFR2 is not expressed in osteoclasts (216), osteoclastogenesis may be indirectly impacted by RXFR2 through osteoblasts.

Both RXFP1 and RXFP2 modulate cAMP accumulation. Initially, both RXFP1 and RXFP2 couple to Gαs and an inhibitory Gαi/o pathway. RXFP1, but not RXFP2, is then able to recruit Gαi3 and release G-βγ subunits; thereby, activating a delayed PI3K-PKCζ pathway to further increase cAMP accumulation (220). However, the signaling pathway by RXFP-1/2 in bone is unclear.

**4.6. non-canonical Wnt-G protein signaling in bone**

Wnts are a family of 19 secretory proteins that mediate important processes including cell proliferation, fate specification, polarity, and migration. In the Wnt/β-catenin canonical pathway, Wnt targets a receptor complex, comprised of a seven-transmembrane Frizzled (FZD) and a low density lipoprotein receptor related protein 5 or 6 (LPR5,6) (221, 222). The Wnt canonical signaling pathway plays a substantial role in controlling bone development and regulating osteoblast apoptosis (223, 224).

Wnt has also been proven to couple to G protein signaling in some cells. For example, Wnt signaling through PKA mediated CREB activation controls myogenesis (225), and Wnt-5A and Rfz-2 is sufficient to activate two known $Ca^{+}$-sensitive enzymes: CaMKII ($Ca^{+}$/calmodulin-dependent protein kinase II) and PKC (226). Xiaolin Tu *et al.* (2007) demonstrates that Wnt3a signals through $G_{q/11}$-PKCδ signaling to promote bone formation, which is independent of β-catenin and the canonical pathway. PKCδ knockout results in less bone in the early embryonic skeleton, delayed chondrocyte maturation in long bones, and delayed osx expression in the early osteoblast lineage (227). However, the function of noncanonical Wnt signaling coupled to G protein in bone has yet to be explored.

**4.7. Proton sensing OGR1/ovarian cancer G-protein coupled receptor (GPR68**)

Proton sensing OGR1/ovarian cancer G-protein coupled receptor (GPR68) belongs to a small subfamily of G-protein-coupled receptors that transduce signals in response to pH changes. The subfamily is comprised of four members: OGR1/GPR68, GPR4, G2A, and TDAG8 (228, 229). Proton sensing is important for bone since the skeleton participates in pH homeostasis as a buffering organ; osteoblasts respond to pH changes in the physiological range. However, the mechanism remains unclear. GPR68 is expressed in both osteoclasts (230) and osteoblasts (228). In other cells, OGR1 has been proven to selectively bind both protons and bioactive lipids and act through Gi and Gq proteins. The expression of OGR1 in osteoclasts is induced by CSF-1 or RANKL after osteoclasts have been cultured for two days, and it peaks when they are cultured for four days. Anti-OGR1 antibody and siRNA targeting of OGR1 both block osteoclastogenesis (231). However, OGR1 deficient mice do not show any gross abnormalities of skeleton, with normal osteoclast/osteoblast numbers, trabecular thickness, and bone marrow cavity (230). The OGR1-/- mice exhibit reduced tumorigenesis but are otherwise normal and viable. It may be explained that other OGR1 subfamily genes play similar and redundant roles *in vivo*. Double knockout may confirm whether there is a redundant function in the OGR1 subfamily. However, it can still be expected that OGR1 may exert its osteoclastogenesis promoting function in certain pathological conditions if the survival and calcium signaling of osteoclasts are significantly enhanced by acidification of the medium in an OGR1-depedent manner

**4.8. G protein-coupled receptor 30/G protein-coupled estrogen receptor: GPCR for estrogen**

Estrogen affects multiple aspects of human physiology, including the normal growth and development of female reproductive tissues, bone integrity, and cardiovascular and central nervous system functions. Estrogens promote bone mass and close the bone growth plate in both genders (232). The best characterized signaling mechanism of estrogen function involves two nuclear receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ). ERα is the major ER in bone and plays roles in both functions of estrogen (117, 233). Only very recently was it found that estrogen signals through GPCR in bone (234).

GPR30/GPER1 (G protein-coupled receptor 30/G protein-coupled estrogen receptor) was identified as a receptor for estrogen in 2005 (235, 236) and was reported to couple to Gs and Gi signaling (237). GPR30 estrogen receptor is expressed in the growth plate and declines as puberty progresses (234). Heino *et al*. (2008) found that GPR30 is also expressed in osteoblasts, osteocytes, and osteoclasts (231). Further study shows that GPR30 regulates growth plate closure and longitudinal bone growth, but not bone density. GPR30 deficient mice display reduced growth correlated with a proportional decrease in skeletal development (35). Compared with ovariectomized (OVX) wild-type mice, OVX GPR30-/- mice have similar changes in bone mass (body BMD, spine BMD, trabecular BMD and cortical bone thickness) in response to estrogen (36). However, estrogen treatment reduced longitudinal bone growth, reflected by decreased femur length and distal femur growth plate height, in the WT mice, but not in the GPR30-/- mice (36).

## 5. REGULATING G PROTEIN SIGNALING IN BONE

### 5.1. RGS/regulator of G protein signaling

RGSs (regulator of G protein signaling) are multifunctional, GTPase-activating proteins (GAP) that inactivate G-protein signaling pathways by increasing the intrinsic GTPase activity of the G-protein α subunits through stabilization of the transition-state conformation of GTPase domain in Gα (7, 38, 238). RGS proteins are capable of accelerating GTPase activity up to 1000-fold, so as to speed up the deactivation of a system once stimulation has ceased (239, 240). Additionally, RGS proteins also act as effector antagonists that prevent G proteins from binding to their effectors by physically blocking this interaction, and by altering the number of free βγ subunits available to interact with their effectors through enhancing the affinity of Gα subunits for βγ subunits (238). RGS proteins are themselves highly regulated, undergoing post-translational modifications, including phosphorylation, palmitoylation, and sumoylation, which modulates GAP activity (240). The RGS protein family contains at least 25 members in mammals, all of which share a characteristic RGS-homology domain of about 130 amino-acid residues (239). Based on their protein structure homology, RGS protein members are divided into eight subfamilies, A/RZ, B/R4, C/R7,. D/R12, E/RA, F/GEF, G/GRK, H/SNX, RGS22, and D-AKAP2 (240). The role of RGS2, RGS18, RGS12, and RGS10 in regulating the signaling pathway in osteoclasts or osteoblasts has been studied.

RGS2, a member of the B/R4 subfamily, is a 211-amino-acid protein with a RGS domain and a conserved N-terminal for membrane association (240). RGS2 specifically targets Gq to block PLCβ activation, but lacks GAP activity for Gi. RGS2 also suppresses certain isoforms of adenylyl cyclase activation (III, V, and VI, but not I and II), but the mechanism is unclear (241). RGS2 mRNA expression is induced by PTH in osteoblasts both *in vivo* and *in vitro* (242, 243), especially by pulsatile PTH administration (244). Further investigation shows that RGS2 mRNA is the cross-talk regulator between Gs and Gq signaling in osteoblasts. Most studies supports that RGS2 is upregulated by Gs signaling and inhibits Gq signaling (242, 245-247). There is a study suggesting that RGS2 is also upregulated by Gq signaling and inhibits Gs signaling (247). However, other studies counter this argument. RGS2 is not activated by Gq signaling activators PMA, PTH (3-34) and PTH (7-34), or inhibited by the Gq signaling inhibitor bisindolylmaleimide I (243, 245, 246). Also overexpression of RGS2 significantly inhibits PKC induced IL-6 expression by fluprostenol treatment, but not cAMP-induced IL-6 expression by PTH treatment (242).

RGS18, a 234–amino-acid protein (248) that also belongs to the B/R4 subfamily, is isolated from hematopoietic stem cells and is highly expressed in bone marrow fetal liver, spleen, and lung (249). In bone marrow, RGS18 levels are highest in long-term and short-term hematopoietic stem cells, and they are decreased as the cells differentiate into more committed multiple progenitors. RGS18 is expressed both in RAW264.7 and BMM, and it is downregulated by RANKL. Though purified RGS18 specifically interacts with both Gq and Gi (249), it acts only on Gq signaling *in vivo*. Transient overexpression of RGS18 attenuated IP3 via the angiotensin receptor, and it attenuated transcriptional activation through the cAMP-responsive element via the M1 muscarinic receptor in hematopoietic stem cells (249). In addition, RGS18 inhibits osteoclastogenesis through the OGR1/NFAT signaling pathway by controlling Gq but not Gi (250).

RGS12 (251) and RGS10A (33, 34) are involved in RANKL-evoked PLCγ–calcium channels– (Ca2+)i oscillation–NFAT2 pathway for terminal differentiation of osteoclasts *in vivo*. RGS12 is induced by RANKL and interacts with N-type calcium channels in BMM or RAW264.7 (251). RGS12 silencing results in impaired phosphorylation of PLCγ activation and blocks calcium oscillations (251). RGS10-/- mice exhibit severe osteoporosis (34). RGS10 deficiency or RGS10A silencing results in the absence of calcium oscillations and the loss of NFATc1, while ectopic expression of RGS10 leads to PLCγ activation. (33, 34) These studies propose the crosstalk between RANKL signaling and G protein signaling

### 5.2. GRK-beta-arrestin system

GRK-beta-arrestin desensitizes the signaling by regulating activity of the 7-TM receptor. Activated by G protein signaling, GRK specifically binds and phosphorylates agonist-occupied or activated conformation of the receptor to facilitate its internalization by beta-arrestin (239), There are seven GRK members (GRK1-7) and four beta-arrestin members (beta-arrestin 1-4), which show different preferences for different receptors.

Two GRK members are expressed in osteoblasts: GRK2 and GRK3. However, the expression of GRK2 is predominantly higher than GRK3, and the expression of GRK2 is increased with the maturation of osteoblasts (252). GRK2 (252-257) regulates PTH desensitization and the internalization of PTH1R. GRK2 inhibits cAMP generation induced by PTH (256), while GRK inhibitor transfected mice exhibit increased BMD in trabecular-rich lumbar spine, cAMP activity, and GPCR signaling activity (255, 257).

Beta-arrestin2 appears to have more important roles in bone development, though both beta-arrestin1 and beta-arrestin2 are found in osteoblasts. Beta-arrestin2 (252, 253, 258) negatively regulates PTH-PTH1R signaling as GRK2. Expression of internalization-impaired PTH/PTHrP receptor mutant dramatically exaggerates the cAMP and calcemic response (120). Similarly, beta-arrestin2 knockout osteoblasts exhibit increased and sustained intracellular cAMP in response to PTH and lowered RANKL expression. The anabolic response of PTH in cortical and trabecular bone is more pronounced in beta-arrestin knockout mice compared to wide-type mice (258). Beta-arrestin2 also triggers G protein signaling to MAPK signaling in response to PTH (259).

### 5.3. NHERF

NHERF (Na/H exchange regulatory factor) is a cytoplasmic adapter protein with a PDZ domain that

regulates trafficking and signaling of several G protein-coupled receptors (GPCRs) including PTH1R. Both NEHRF1 and NEHRF2 are expressed in osteoblasts and are able to bind PTH1R through a PDZ-domain interaction *in vitro* and in PTH target cells (44, 45). While NHERF2 acts as a switch between Gq signaling and Gs signaling, NHERF1 is involved in internalization and desensitization of the PTH receptor. NHERF2 simultaneously binds PLCβ1 and PTH1R through PDZ1 and PDZ2, activating PLCβ and inhibiting AC through stimulation of Gi/o (45). Ligand-binding, activation-independent PTH1R internalization is regulated by NHERF1. However, ligand-induced endocytosis is blocked by NHERF1 (260, 261). The binding of NHERF1 with PTH11R inhibits beta-arrestin2 binding to PTH1R and thus inhibits desensitization (44).

## 6. DRUG DEVELOPMENT BASED ON GPCRS

G-protein-coupled receptors may be the most popular target in pharmacology. The GPCR superfamily represents one of the largest and most diverse groups of proteins encoded in the genome, and regulates critical cellular processes in almost all cell types. Furthermore, GPCRs are expressed on the membrane and drugs do not need to filter into cells. In 2000, nearly half of all pharmaceuticals in American markets targeted GPCRs (8).

Osteoporosis is a skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone, leading to increased susceptibility to bone fractures. With increasing age, or with reduction of estrogen levels postmenopause, bone loss exceeds the bone remodeling, so as to increase the risk of osteoporosis (262-264). Pharmacological treatment of osteoporosis mainly falls into four classes of drugs: calcitonins, bone calcium regulators, parathyroid hormones (PTHs), and selective estrogen-receptor modulators (SERMs) (262). Of the four classes of drugs, two target G protein-coupled receptors in bone: calcitonins and PTH (262)(Table 4).

Calcitonin is an anti-resorptive peptide hormone. The clinically used calcitonin is either genetically engineered from human calcitonin or derived from salmon (265). The major problem with this kind of drug is its inefficiency due to the downregulation of CTR induced by CT (200). Anti-fracture efficacy has been shown at only a single dose and only in vertebrae, and the BMD increase is modest. Similarly, calcitonin by injection has also been effective in Paget's disease and in hypercalcemia of malignancy, but with problems with efficiency (262, 265).

Full length PTH (1-84) and teriparatide (PTH (1-34)) are now clinically used drugs. Teriparatide approved since 2002, induces robust increases in BMD and substantial reductions in vertebral and non-vertebral fractures when injected once daily in clinical trials over a 21-month period (266). The incidence of hypercalcemia or hypercalciuria is considered to be low (262). However, the catabolic role of PTHs still cannot be overlooked. PTH must be administered intermittently, via subcutaneously daily injections, and limited to 24 months. Then another therapy is required to maintain the bone density gained with PTHs. Anti-resorptive agents, like calcitonin, bisphosphonates, and strontium may be used (266, 267). Another drawback of PTH is that it can not be used in patients with hypercalcemia, hyperthyroidism, or Paget's disease (268).

Strontium-containing compounds (strontium-ranelate) are useful for osteoporosis (262). Unlike calcitonin, which inhibits bone resorption and turnover, and PTH, which promotes bone formation and bone resorption, strontium enhances bone formation as well as inhibits bone resorption (262). Strontium application decreases the risk of morphometric vertebral fractures and reduces the risk of nonvertebral fractures in postmenopausal osteoporosis (269, 270). However, in clinical trials, strontium ranelate caused a higher incidence of diarrhea and is also associated with an increase risk of thromboembolic events (269, 270).

A potential target for osteoporosis may be CB2 (208). Like Strontium, CB2 promotes bone formation while inhibiting bone resorption (29). The selective CB2 agonist HU-308, but not CB1, attenuates ovariectomy-induced bone loss and markedly stimulates cortical thickness through the suppression of osteoclast number and stimulation of endocortical bone formation (208). Furthermore, HU-308 dose dependently increases the number and activity of endocortical osteoblasts and restrained trabecular osteoclastogenesis by inhibiting proliferation of osteoclast precursors (208).

## 7. SUMMARY AND PERSPECTIVES

The relationship between G protein and GPCR mutations with human bone diseases reveals the importance of G protein signaling in bone development and bone remodeling. Gs mutations are the cause of MAS (McCune-Albright syndrome) (50), non-MAS FD (fibrous dysplasia) (51), AHO (Albright hereditary osteodystrophy) (53, 54), and non-AHO ectopic ossification (55, 56). PTH1R mutations are the cause of BOCD (Blomstrand osteochondrodysplasia) (117) and PFE (primary failure of tooth eruption) (118). CaSR mutations are the cause of FHH (familial hypocalciuric hypercalcemia), NSHPT (neonatal severe hyperparathyroidism) and ADH (autosomal dominant hypocalcemia) (172, 173). In addition, SNP of several G protein coupled receptors, including CB2 (214), RXFP (32) and calcitonin receptor (193-195), are associated with reduced bone density in human beings.

The function of each of the four subgroups of G proteins has been studied in osteoblasts. Gs promotes osteoblast proliferation and differentiation and regulates bone mineralization and formation. Though data obtained from *in vitro* cell culture shows an anabolic role of both Gi and Gq in osteoblast differentiation and proliferation, specific activation of Gi and Gq in osteoblasts *in vivo* leads to hypomineralization and osteopenia, respectively. Conditional knockout mice should be developed to more thoroughly understand the role of Gi and Gq. G12/13 is studied less and its function needs to be further clarified.

**Table 4**. Clinically useful drugs based on GPCRs

| Drugs | Target | Skeletal disorder | Drawbacks |
|---|---|---|---|
| Strontium ranelate | CaSR and similar receptors in bone | Osteoporosis | An incidence to develop Diarrhea and thromboembolic events |
| Salmon Calcitonin | Calcitonin receptor | Osteoporosis, Paget's disease, hypercalcaemia of malignancy | Insufficiency |
| rPTH | PTH1R | Osteoporosis | Catabolic function, restricted use in patients with hypercalcemia, hyperthyroidism, or Paget's disease |
| Teriparatide (PTH (1-34)) | PTH1R | Osteoporosis | |

Diverse G-protein-coupled receptors are expressed in bone cells and enable bone tissue responses to a wide range of stimuli, including peptide hormones, lipid compounds, cations, and pH changes. The existence of GPCR for neuropeptides, such as CGRP (calcitonin gene-related peptide), indicates a role of GPCR in CNS (central nervous system)-mediated bone formation. Many GPCRs cooperate with each other to modulate internal environment homeostasis. PTH/PTHrP is a multifunctional hormone that promotes bone remodeling, chondrocyte differentiation, and mesenchymal stem cell commitment. It also regulates calcium and phosphate homeostasis. PTH or PTHrP deficient mice display hypocalcium and bone skeletal abnormalities. While PTH/PTHrP acts to increase serum calcium, CaR and calcitonin antagonize the function of PTH/PTHrP to decrease serum calcium. Coupled to serum calcium changes, these hormones also regulate bone mineralization.

Attributing to the anabolic or anti-resorptive properties of the GPCR ligands, some of the ligands have been developed as clinically usable drugs to treat bone diseases. For instance, strontium, PTH, or PTH (1-34) is used to treat osteoporosis, while calcitonin is used to treat Paget's disease and osteoporosis. All drugs have pros and cons, thus better therapies should be explored. CB2, which inhibits resorption as well as promotes bone formation, has been proposed to be a potential target for osteoclastogenesis.

A number of GPCRs are located on the osteoclast membrane, like CT1, CaR, CB1, RXFR1 and GPR30, and all have been shown to regulate osteoclast differentiation and function. However, the role of G proteins in osteoclasts is poorly identified. Recently, RGS10 regulated PLCγ–calcium oscillation has been shown to be induced by RANKL, and it targets NFAT2 to regulate terminal differentiation of osteoclasts. However, there are seldom reports concerning G protein signaling with canonical RANKL signaling in osteoclasts. Mice models with conditional deletion of G proteins in osteoclasts need to be developed to depict the signaling pathway of G protein signaling in osteoclasts.

Although large progress has been made in understanding the roles of G proteins and GPCRs in bone development and diseases, much remains unknown. Due to the embryonic lethality that results from many G protein and GPCR mutations, the number of mouse models available to study G proteins are limited. Fortunately, the conditional knockout approach has emerged, which will enable further study of the mechanisms related to G proteins and GPCRs in the mouse model. Another reason for the gaps in our understanding of G proteins and GPCRs is that there are many complications in their signaling pathways and patterns of expression in osteoclasts and osteoblasts. Based on new data available from microarrays and protein arrays, an increasing number of important GPCRs remain to be characterized. After the addition of mouse models and more characterized GPCRs, their signaling pathways and mechanisms will be revealed.

Currently, it is still a big challenge to develop a drug with low toxicity and side-effects for osteoporosis and other bone related diseases. There is a need for safer and more effective therapies since renal toxicity and osteopetrosis of the jaw are potential complications associated with bisphosphonate, which is one of most common drugs used for treatment of osteoporosis and tumor bone metastasis. Characterization of the G protein signaling mechanism of osteoclast-mediated bone resorption is likely to reveal critical information that will enable the creation of a novel drug that will treat bone diseases without sacrificing healthy bone resorption or bone formation. This is very important as osteoporosis, osteoarthritis, and rheumatoid arthritis are becoming more prevalent and problematic in an aging society. In the future, the detailed mechanisms that involve G proteins and GPCRs in bone formation and bone resorption will be characterized. Then, translational medicine will be undertaken to transform the discoveries of basic science from the bench to the bedside because GPCRs are considered one of best drug targets known. We expect that some of the new drugs will not only be able to prevent bone loss, but rebuild bone as well.

Building on the extensive research and marvelous progress that has been achieved in the last decade, new technology, such as conditional knockout mice and high-throughput screening, promises to rapidly advance our understanding about how G proteins and GPCRs work. These new technologies have dramatically improved bone disease treatment and will ultimately conquer bone diseases.

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**Send correspondence to:** Yi-Ping Li, Department of Cytokine Biology, The Forsyth Institute, 140 The Fenway, Boston, MA 02115, Tel: 617-892-8260, Fax: 617-262 4021, E-mail: ypli@forsyth.org

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