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Role of regulator of G protein signaling proteins in bone

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1. ABSTRACT

Regulators of G protein signaling (RGS) proteins are a family with more than 30 proteins that all contain an RGS domain. In the past decade, increasing evidence has indicated that RGS proteins play crucial roles in the regulation of G protein coupling receptors (GPCR), G proteins, and calcium signaling during cell proliferation, migration, and differentiation in a variety of tissues. In bone, those proteins modulate bone development and remodeling by influencing various signaling pathways such as GPCR-G protein signaling, Wnt, calcium oscillations and PTH. This review summarizes the recent advances in the understanding of the regulation of RGS genes expression, as well as the functions and mechanisms of RGS proteins, especially in regulating GPCR-G protein signaling, Wnt signaling, calcium oscillations signaling and PTH signaling during bone development and remodeling. This review also highlights the regulation of different RGS proteins in osteoblasts, chondrocytes and osteoclasts. The knowledge from the recent advances of RGS study summarized in the review would provide the insights into new therapies for bone diseases.

2. INTRODUCTION

Bone is a mineralized connective tissue consisting of 33% organic matrix. Bone remodeling, cycles of bone apposition and resorption, continues throughout life (1). Osteoblasts are mononeclear cells, which arise from osteoprogenitor cells located in the deep layer of perosteum and the bone marrow and are responsible for bone formation. In contract, osteoclasts are highly specialized multinucleated cells derived from the hematopoietic cell precursors in the bone marrow and peripheral blood, and are capable of breaking down both the inorganic and organic matrix of bone to resorb the bone. Both osteoblasts and osteoclasts participate in bone remodeling and are affected by a variety of signals during their differentiation and function (1). Signal transduction from the extracellular environment across the plasma membrane and into the intracellular milieu is a fundamental aspect of cellular regulation, and coordinates essential aspects of cell and organ homeostasis. G Protein Coupled Receptors (GPCRs), also known as seven trans-membrane domain receptors, constitute a large protein family of receptors. Those receptors perceive many extracellular signals (*e.g.* neurotransmitters, hormones, phospholipids and growth

Table 1. Classification of RGS protein subfamilies and their members (11)

Subfamily	Members
RZ	RGS17,RGS19,RGS20,RET-RGS1
R4	RGS1,RGS2,RGS3,RGS4,RGS5,RGS8,RGS13,RGS16,RGS18,RGS21
R7	RGS6,RGS7,RGS9,RGS11
R12	RGS10,RGS12,RGS14
RA	Axin, Conductin
GEF	P115-RhoGEF, PDZ-RhoGEF□LARG
GRK	GRK1,GRK2,GRK3,GRK4,GRK4,GRK5,GRK6,GRK8
SNX	RGS-PX1.SMX14,SNX25
D-AKAP2	D-AKAP2

factors) and transduce them to heterotrimeric G proteins, which further transduce those signals intracellularly to appropriate downstream effectors and thereby play an important role in various signaling pathways (2). GPCRs represent important specific targets for a variety of physiological functions and therapeutic approaches, such as the control of blood pressure, allergic response, kidney function, hormonal disorders, neurological diseases, bone development, and bone remodeling (3, 4). By binding with specific agonists/ligands, GPCRs leads to the rapid activation of heterotrimeric G-proteins (binding of GTP) and the regulation of intracellular second messengers (*e.g.* cAMP and intracellular Ca^{2+} levels). The expressions of multiple GPCRs have been found in osteoblasts, osteocytes, and osteoclasts which regulate local and systemic signaling (3).

G proteins are a family of intracellular proteins that act as mediators of proliferation, differentiation, and apoptosis in multiple cell types (5). G proteins are composed of three non-identical subunits, Gα (33-35 kDa), Gβ (~35 kDa) and Gγ (~15 kDa) (6). The kinetics of G-protein signaling are regulated by regulators of G protein signaling (RGS) proteins that act as scaffolds to help assemble signaling complexes (7) and as GTPase activating proteins (GAPs) on Gα subunits. RGS proteins regulate heterotrimeric G proteins by increasing the rates at which their subunits hydrolyze bound GTP and return to the inactive state (8). Hence, the G-protein activity cycle is sequentially regulated by RGS proteins, and is critical for the rapid activation and inactivation of cellular responses (8).

The RGS protein family has more than 30 family members (9). Sequence and structure analysis suggests that mammalian RGS proteins are divided into nine subfamilies including RZ/A, R4/B, R7/C, R12/E, GEF/F, GRK/G, SNX/H and D-AKAp2/I (8, 10) as shown in Table 1 (11). Those proteins specifically and selectively regulate G protein mediated receptors, ion channels, and other signaling events (11). Many studies have demonstrated that RGS proteins serve as key modulators in neurons (12, 13), the cardiovascular system (14, 15), inflammatory disorders (16, 17), and cancer (18). The importance of RGS proteins in bone has been addressed in recent years. Here, we reviewed current findings outlining the regulation of RGS gene expression, and RGS proteins in the regulation of G protein and GPCRs. We further summarized the various signaling pathways that are either dependent upon or related to RGS function in bone development and remodeling, and the role of RGS proteins in the regulation of bone cell differentiation and function.

3. REGULATION OF RGS GENE EXPRESSION

Increasing evidence has demonstrated that RGS proteins are regulated through multiple mechanisms, including the regulation of GPCR signaling, bacterial LPS, proinflammatory cytokines, and cell stress conditions. Additionally, the detrimental effects of cell stress under certain conditions such as hyperoxia, hypoxia/ischemia, mechanical stress, and drug treatment also influence the RGS expression (19).

3.1. G-protein-mediated pheromone signaling

Some studies showed that RGS gene expression is negatively and/or positively regulated by G-protein-mediated pheromone signaling. For example, Gq/Gi-coupled agonists can increase the expression of several RGS genes, such as RGS2, RGS4, and RGS16 (19). Interestingly, angiotensin II (Gq/Gi-coupled agonists) has a dual effect on RGS2 expression. Short-term angiotensin II (ANG II) stimulation for one day in fibroblasts increases RGS2 levels. In contrast, prolonged ANG II stimulation (3–14 days) markedly decreases RGS2 (20). Similarly, stimulation of opioid receptors can induce a rapid and persistent (8 hours) increase in RGS4 mRNA (21). While Gs/cAMP-coupled agonists increase RGS2 expression, but inhibit the expression of RGS3 (22), RGS4 (22, 23), RGS5 (24), and RGS16 (25) in various cell types. Additionally, cAMP reduces mRNA levels of RGS13, and its PKA-mediated phosphorylation and subsequent stabilization maintains high protein levels of the RGS13 protein, likely due to protection from proteasomal degradation (26).

3.2. Bacterial LPS and proinflammatory cytokines

In addition to above mentioned proteins, bacterial LPS and proinflammatory cytokines (important mediators of the immune response and inflammation) also regulate RGS gene expression (19). In immune cells, LPS variably affects the expression of RGS proteins, depending on the RGS and the cell type (19). For example, LPS down-regulates RGS2 mRNA in macrophages (27, 28), but up-regulates RGS1 expression in dendritic cells and macrophages (27, 29). IFN-β induces the expression of RGS1 in peripheral blood mononuclear cells, monocytes, T cells, and B cells (30), and stimulates the expression of RGS2 and RGS16 in mononuclear leukocytes (31, 32).

3.3. PTH and TSH

The expression of RGS proteins is also regulated by PTH signaling (33, 34). For example, RGS2 expression is increased by PTH stimulation in osteoblasts. Moreover, the expression of RGS2 mRNA is rapidly and transiently increased 2-to 5-fold by injection of PTH in femoral

metaphyseal spongiosa of young male rats (33). Thyrotropin (TSH) signaling is also involved in the RGS regulation (35). The expression of RGS2 mRNA is significantly up-regulated within 1 hour after TSH stimulation in human thyroid primary cultures and reaches a maximum after 4 hours (35). Co-expression of RGS2 and TSH receptor in COS-7 cells inhibits TSH-G(q)alpha signaling, implying that RGS 2 is involved in TSHR-induced G(q) signal transduction (35).

4. RGS PROTEINS IN THE REGULATION OF SIGNALING PATHWAYS

4.1. RGS proteins and GPCR-G protein signaling

GPCRs transduce signals by coupling to heterotrimeric (α-, β-, γ-subunit-containing) GTP-binding (G) proteins that regulate effector molecules (36). RGS proteins have a conserved RGS core domain of approximately 120 amino acids that is necessary and sufficient for binding to Gα subunits (9). There are four principal Gα proteins (G_s, G_i, $G_{q/11}$, $G_{12/13}$). The structures of G subunit reveal a conserved protein fold composed of a helical domain and a GTPase domain that undergoes conformational changes when binding GTP and GDP (37). One critical regulatory point in the G-protein cycle is the deactivation of G-proteins by GTP hydrolysis that is enhanced by GTPase activating proteins (GAP) – RGS proteins (8). RGS proteins are capable of accelerating GTPase activity up to 1000-fold to promote GTP hydrolysis by α subunit of heterotrimeric G proteins, thereby inactivating G protein and rapidly switching off GPCR signaling (38). In addition to functioning as a deactivator for G proteins, Zhong *et al.* found that high concentrations of receptors lead to saturation of GDP-GTP exchange making GTP hydrolysis rate-limiting and local depletion of inactive heterotrimeric G-GDP, which is reversed by RGS GAP activity. RGS enhances receptor-mediated Gi protein activation even as it deactivates the Gi protein (39). Therefore, the authors proposed that RGS can constrict the spatial range of active G protein around some receptors to limit the spill-over of G protein signals to more distant effector molecules, thus enhancing the specificity of Gi protein signals (39, 40). Beside the GAP role of RGS proteins, RGS12 was recently found to function as a Ras/Raf/MEK scaffold to regulate nerve growth factor (NGF)-mediated neurite outgrowth in PC12 cells by facilitating the coordination of H-Ras, B-Raf, and MEK2 and prolonging extracellular regulated kinases (ERK) activation (24). Additionally, RGS4 (41) and RGS3/5(42) were also found to regulate activation of ERK as well as Gq-mediated PLCβ. Gβγ released by Gi is also able to activate PLCβ-PKC signaling and can be distinguished from Gq-activated PLCβ by Gi specific inhibitor PTX (pertussis toxin) (43) and Gq specific inhibitor (44). PLCβ seems to have paradoxical roles, being both an effector and a GAP for Gαq/11 (2, 45).

Some studies have shown that mutation of upstream genes of RGS proteins such as G protein or GPCR have severe skeleton phenotypes (36). Deficiency of Gαs in osteoblasts impairs bone formation due to the reduced differentiation of osteoblasts through inhibiting adenylyl cyclase in cAMP-PKA pathway (46), suggesting that Gαs stimulates adenylyl cyclase. In contrast, Gαi inhibits the activity of adenylyl cyclase by antagonizing Gαs-mediated increases in adenylyl cyclase activity in osteoblasts(47). Transgenic expression of a constitutively active Gi-coupled GPCR in osteoblasts leads to marked trabecular osteopenia (48), consistent with the concept of Gi-coupled signaling and opposing Gs-mediated trabecular bone formation. Millard *et al.* (49) investigated the skeletal effects of blocking Gi-coupled signaling in osteoblasts *in vivo* by transgenic expression of the catalytic subunit of pertussis toxin (PTX) under control of the collagen Iα 2.3-kb promoter. The mice show increased periosteal bone formation and cortical thickness which correlates with associated with expanded mineralizing surfaces.

4.2. RGS proteins and Wnt signaling

Mounting studies have shown that RGS proteins such as Axin play critical roles in the regulation of Wnt signaling pathway (50-53). Wnt signaling cascade regulates cell proliferation, differentiation, and motility, and plays a critical role in development (54). Wnts/β-catenin pathway is commonly called the canonical Wnt pathway. In bone, the canonical pathway plays a substantial role in the control of bone formation and bone remodeling (55). This pathway is mediated by β-catenin which goes into phosphorylation and ubiquitination when there is no Wnt binding. Inhibition of β-catenin phosphorylation prevents its degradation, and thus results in an increase in its cytoplasmic concentration (50). Wnts/β-catenin subsequently contribute to the proliferation and survival of osteoblasts (56).

Axin2, an atypical RGS protein that serves as a molecular scaffold for a β-catenin destruction complex, has been shown to be a key negative regulator of bone remodeling (50-53). Yan *et al.* reported that deletion of axin2 causes a significant increase in bone mass that results from a significant increase in osteoblast proliferation and differentiation, and a decrease in osteoclast differentiation (50). They further found that axin2 negatively regulates osteoblast differentiation and bone remodeling through the beta-catenin-BMP2/4-Osx signaling pathway in osteoblasts. Hey *et al.* recently found that axin interacts with N-cadherin to negatively regulate Wnt signaling through β-catenin degradation, resulting in impaired osteoblast differentiation. By analyzing the N-cadherin transgenic mice using a Col12.3-N-cadherin-construct, they further found that increased β-catenin degradation induced by N-cadherin-LRP5 interaction inhibits osteoblast function and bone formation and delays bone mass acquisition *in vivo* (57). Additionally, Regard *et al.* reported that activating Gα mutations can interact with axin to potentiate Wnt/β-catenin signaling. Furthermore, they found that removal of Gαs led to reduced Wnt/β-catenin signaling and decreased bone formation (58). It was also found that Wnt signaling is negatively influenced by GRK2 activity in bone-forming osteoblasts and therefore may be suggested for increasing bone formation (59).

4.3. RGS proteins and PTH signaling

Of several systemic hormones and local factors affecting bone remodeling during adult life, PTH may be

one of the most important factors in bone homeostasis (60, 61). RGS proteins are instrumental in the regulation of PTH. It is well known that PTH regulates cell differentiation and function through GPCRs in two principal target organs: kidney and bone (62, 63). By binding with its GPCR, PTH can modulate both $G\alpha_s$-regulated adenylate cyclase and Gαq-regulated phospholipase C, resulting in the activation of cAMP/protein kinase A (PKA) and protein kinase C (PKC)/Ca^{2+} dependent cellular processes and transcriptional responses (64, 65). The relative activation of adenylate cyclase and phospholipase C depends mainly on the duration and magnitude of activation of the Gαs and Gαq protein subunits. RGS proteins, as GAP proteins, have been shown to accelerate the inactivation of G proteins by enhancing their GTPase activity (10), and therefore play critical role in PTH signaling.

Accumulating evidence has shown that PTH-induced RGS2 expression in osteoblasts involves PTH signal transduction via its GPCRs (33, 66, 67). PTH binds to its PTH/PTHrP receptor (68) to activate cAMP/PKA- and PKC/Ca^{2+}- dependent signal transduction via Gs-and $G_{q/11}$- proteins (67). Pulsatile PTH treatment causes A 4- to 10-fold lower RGS2 abundance, compared to a continuous PTH exposure (69). Moreover, functional analysis with RGS2 overexpression suggests that RGS2 down-regulates PTH- and forskolin-induced cAMP production in osteoblasts (34) and limits Gs signaling (70). Further studies showed that the basal levels of endogenous RGS2 do not appear to regulate Gs or Gq signaling in osteoblasts (70). Using Forskolin treatment of wild-type osteoblasts to activate Gs signaling, suppresses Gq-dependent accumulation of inositol phosphates and nucleotide-stimulated calcium release that does not occur in RGS2 (-/-) osteoblasts. Conversely, activation of Gq signaling suppresses PTHrP-dependent cAMP accumulation in wild type but not in rRGS2 (-/-) osteoblasts. Those findings demonstrate that up-regulation of RGS2 contributes to cross-desensitization of Gs- and Gq-coupled signals (70). Additionally, Miles *et al.* (33) confirmed that the expression of RGS2 mRNA is rapidly and transiently increased by human PTH in both metaphyseal (4-to 5-fold) and diaphyseal (2- to 3-fold) bone, as well as in cultured osteoblast cultures (2- to 37-fold). *In vitro*, forskolin and dibutyryl cAMP can similarly elevate RGS2 mRNA. They further found, *in vivo*, different PTH analogs affect RGS12 gene expression. PTH analogs, such as 1-31 and 1-38 (which stimulate intracellular cAMP accumulation, PTHrP, and prostaglandin E2) induce RGS2 mRNA expression; whereas PTH analogs, such as 3-34 and 7-74 (which do not stimulate cAMP production) have no effect on RGS2 expression. In tissue distribution analysis, RGS2 is widely expressed in all examined tissues including heart, spleen, liver, skeletal muscle, kidney, and testis, and significantly expressed in two nonclassical PTH-sensitive tissues: brain and heart. After PTH injection, RGS2 mRNA expression is up-regulated in rat bone but not in any of the other examined tissues. Those findings demonstrate that RGS2 is regulated by PTH, prostaglandin E2, and PTHrP, suggesting increased RGS2 expression in osteoblasts may be one of the early events influencing PTH signaling(33).

Besides PTH signaling, some studies have shown that other factors also regulate PTH-mediated RGS2 expression and function. Ueno *et al.* (31) reported that RGS2 gene expression markedly increases with PTH infusion and its expression is dose-dependently suppressed by treatment of $1,25(OH)_2$ D3. Their results suggest that $1,25\ (OH)_2$ D3 inhibits PTH-mediated G protein signaling. Homme *et al.* (71) further studied the influence of vitamin D and dexamethasone on PTH-induced RGS2 expression in osteoblast-like cells. By determining RGS2 gene transcription rate, they found that the transcription rate is increased by 35% with 1, 25-$(OH)_2$D3 and decreased by 63% with dexamethasone pretreatment, demonstrating that glucocorticoids and vitamin D inversely regulate PTH-induced RGS2 expression via a transcriptional mechanism. Additionally, PTH secretion by parathyroid cells responds to changes in extracellular calcium through signaling by the calcium-sensing receptor (CaR) (72, 73). Koh *et al.* (74) found that RGS5 is selectively up-regulated in parathyroid adenomas relative to normal glands. Transient expression of RGS5 in cells stably expressing CaR results in dose-dependent abrogation of calcium-stimulated inositol trisphosphate production and ERK1/2 phosphorylation. Furthermore, they found that RGS5 knockout mice display significantly reduced plasma PTH levels, which is consistent with attenuated opposition to CaR activity. Collectively, their data suggest that RGS5 can act as a physiological negative regulator of CaR in the parathyroid gland. Similarly, PTH-related peptide (PTHrP) was found to increase osteogenic proliferation through activation of Ras and MAPK signaling pathway, showing that Gq is likely involved in that process (75).

4.4. RGS proteins and calcium (Ca^{2+}) oscillations

How Ca^{2+} oscillations regulate tissue specific gene expression and cell differentiation has been an unsolved question in cell biology. Several lines of evidence have shown that RGS proteins play pivotal roles in controlling Ca^{2+} oscillations and cell differentiation in T lymphocytes, neurons, and cardiac myocytes (76-79). Interestingly, different RGS proteins have different receptor preferences in the regulation of calcium signaling and cell differentiation and function (80). In the pancreas, for example, the amino-terminal residues allow selective interaction between RGS4 and the muscarinic acetylcholine receptor (76). Many RGS proteins, including RGS1, RGS2, RGS4 and GAIP, as terminators of the active state of G proteins, can accelerate the GAP activity of Gαq (8, 38) to inactive Ca^{2+} reuptake and delay Ca^{2+} spikes for several seconds or even minutes. Additionally, the amplitude and frequency of changes in cytoplasmic Ca^{2+} concentrations influence the nature of the cellular response. Dolmetsch *et al.* (81, 82) reported that Ca^{2+} oscillations regulate gene expression and cell differentiation in T cells. Rapid oscillations stimulate three transcriptional factors (NFAT, Oct/OAP, NF-κB), whereas infrequent oscillations activate only NF-κB. By differentially controlling the activation of distinct sets of transcription factors and the expression of different genes, oscillation frequency may direct cells along specific developmental pathways (83, 84). These finding highlighted that RGS proteins play critical role in the regulation of Ca^{2+} oscillations.

Some studies have showed that the components of the Ca^{2+} signal, including IP_3 production and Ca^{2+} fluxes across the ER and plasma membranes, regulate the frequency and amplitude of Ca^{2+} oscillations (85). The activation of Gα by against activates PLCβ to generate IP_3. IP_3 releases Ca^{2+} from the endoplasmic reticulum (ER), which is followed by the activation of store-operated Ca^{2+} channels in the plasma membrane and Ca^{2+} influx. The increase in Ca^{2+} leads to activation of the plasma membrane Ca^{2+} ATPase (PMCA) and sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps to remove Ca^{2+} from the cytosol (86). During this process, RGS proteins, as the negative regulators of GPCRs signaling, play a central role in determining the duration of the stimulated state and controlling Ca^{2+} oscillations by regulating GTPase activity of G protein (87). The RGS domain accelerates GTP hydrolysis on the G alpha subunit to uncouple receptor stimulation from inositol 1,4,5-trisphosphate (IP_3) production. The C-terminus mediates the interaction with accessory proteins in the complex and the N-terminus acts in a receptor-selective manner to confer regulatory specificity(88). Hence, RGS proteins have both catalytic and scaffolding function in regulating Ca^{2+} signaling. Further study from Wang *et al.* (86) showed that deletion of RGS2 regulates the kinetics of IP_3 production without affecting the peak level of IP_3, but increases the steady-state level of IP_3 at all agonist concentrations. Deletion of RGS2 decreases the expression of IP_3 receptor 1 and IP_3 receptor 3, and then reduces the sensitivity for IP_3 to release Ca^{2+} from the ER to the cytosol. Thus, Sarco/endoplasmic reticulum Ca^{2+} ATPase 2b is up-regulated to more rapidly remove Ca^{2+} from the cytosol of RGS2-deleted cells. Furthermore, they found that deletion of RGS2 reduced the response of the cells to changes in the ER Ca^{2+} load and to an increase in extracellular Ca^{2+}. These findings concentrate the central role of RGS proteins in Ca^{2+} oscillations.

Ca^{2+} oscillations were first found playing critical roles in NFATc1 activation and osteoclast differentiation (89) in 2002. Takayanagi *et al* found that RANKL evokes Ca^{2+} oscillations that lead to calcineurin-mediated activation of NFATc1 and therefore triggers a sustained NFATc1-dependent transcriptional program during osteoclast differentiation. Inhibiting NFATc1 activity using dominant negative alleles blocks osteoclastogenesis, whereas overexpression of the wild-type protein stimulates osteoclast development from embryonic stem cells in a RANKL-independent manner. Both the transient initial release of Ca^{2+} from intracellular stores and the influx through specialized Ca^{2+} channels control the dephosphorylation of the cytoplazmic components (NFATc1 proteins) and lead to their nuclear localization to activate osteoclast specific genes(90). These results indicate that NFATc1 may represent a master switch for regulating terminal differentiation of osteoclasts and functioning downstream of RANKL. Further study (91) showed that mice lacking immunoreceptor tyrosine-based activation motif (ITAM)-harboring adaptors, Fc receptor common γ subunit (FcRγ) and DNAX-activating protein (DAP) 12, exhibit severe osteopetrosis owing to impaired osteoclast differentiation. In osteoclast precursor cells, FcRγ and DAP12 associate with multiple immunoreceptors and activate calcium signaling through phospholipase Cγ (PLCγ).

Based on those studies, we proposed that some regulators such as RGS proteins might exist to regulate Ca^{2+} oscillations during osteoclast differentiation. By using differential screening, we found that RGS10 and RGS12 are both prominently expressed in osteoclast-like cells. Regulator of G-protein signaling 10A (RGS10A), but not the RGS10B isoform, is specifically expressed in human osteoclasts. The expression of RGS10A is also induced by RANKL in osteoclast precursors and prominently expressed in mouse osteoclast-like cells. RGS10A silencing by RNA interference blocks intracellular Ca^{2+} oscillations, the expression of NFATc1, and osteoclast terminal differentiation in both bone marrow cells and osteoclast precursor cell lines. Reintroduction of RGS10A rescues the impaired osteoclast differentiation. RGS10A silencing also results in premature osteoclast apoptosis (92). By generating and characterizing the RGS10 knockout model, we found that RGS10-deficient ($RGS10^{-/-}$) mice exhibit severe osteopetrosis and impaired osteoclast differentiation. The deficiency of RGS10 results in the absence of Ca^{2+} oscillations and loss of NFATc1. Ectopic expression of RGS10 increases the sensitivity of osteoclast differentiation to RANKL signaling. Additionally, ectopic expression of NFATc1 rescues the impaired osteoclast differentiation from deletion of RGS10 (93). Our results further reveal a mechanism that RGS10 competitively interacts with Ca^{2+}/calmodulin and phosphatidylinositol 3,4,5-trisphosphate (PIP3) in a Ca^{2+}-dependent manner to mediate PLC activation and Ca^{2+} oscillations, and that RGS10 specifically regulates the RANKL-Ca^{2+} oscillation-NFATc1 signaling pathway during osteoclast differentiation.

Compared to RGS10, we found that knockdown of RGS12, the largest protein in the RGS family, could also block Ca^{2+} oscillations, NFATc1 expression and osteoclast differentiation (93). Furthermore, we found that calcium sensing receptor (CaR) is expressed in preosteoclasts and osteoclasts and that RGS12 interacts with N type Ca^{2+} channels and CaR during osteoclast differentiation (94). Interestingly, we found RGS10 knockout mice exhibit a severe osteopetrosis phenotype, impaired Ca^{2+} oscillation, and osteoclast differentiation, which cannot be rescued by RGS12. Unlike RGS10 (93), RGS12 does not binds with Ca^{2+}/calmodulin (CaM) and PIP3 in a Ca^{2+}-dependent manner during osteoclast differentiation. It acts on N-type Ca^{2+} channels and CaR. Most recently, by specific deletion of RGS12 in osteoclast lineage also caused osteopetrosis phenotype. The further study is going on. Those results demonstrated that RGS proteins may play different roles in regulating Ca^{2+} oscillations and osteoclast differentiation.

5. REGULATION OF DIFFERENTIATION AND FUNCTION OF BONE CELL BY RGS PROTEINS

5.1. Osteoblasts

Osteoblasts are the mononucleate bone forming cells, which derive from the bone marrow. Osteoblasts

produce new bone called "osteoid" made of bone collagen and other protein, and are responsible for mineralization of this matrix. Many studies have shown that numerous GPCRs express in osteoblasts, including frizzled, the parathyroid hormone (PTH)/parathyroid hormone-related peptide (PTHrP) receptor type 1 (PTH1R), CaR, calcitonin gene-related peptide receptor, and relaxin family peptide 2 (36). Those proteins are important for the differentiation and function of osteoblasts (36). RGS proteins selectively interact with G proteins and their corresponding GPCR (95). Transgenic mice with a constitutively active mutant CaR (Act-CaR), which target to mature osteoblasts by the 3.5 kb osteocalcin promoter, show reduced bone volume and density of cancellous bone, accompanied by a diminished trabecular network. Constitutive signaling of the CaR in mature osteoblasts increases expression of receptor activator of nuclear factor-kappaB ligand (RANKL), the major stimulator of osteoclast differentiation and activation, which is the likely underlying mechanism for the bone loss (96). Moreover, it is proven that CaR promotes osteoblast proliferation (97, 98) likely through coupling to Gq and Gi signaling to stimulate IP3 production, calcium influx, and ERK phosphorylation (99), suggesting the involvement of RGS proteins. Recently, a newly CaR, GPRC6A, is found to be widely expressed in bone, calvaria, and osteoblastic cell line MC3T3-E1. Overexpression of RGS2 or RGS4 and pretreatment with pertussis toxin (PTX) inhibits activation of GPRC6A by extracellular cations (100).

RGS2 has been implicated in the regulation of G protein signaling in bone. RGS2 is expressed in rat metaphyseal and diaphyseal bone, mouse calvarial organ cultures, and cultured osteoblasts. The levels of RGS2 mRNA are up-regulated in response to PTH, PTHrP, and prostaglandin E2 or by the promoters of protein kinase A activity, such as forskolin, cholera toxin, and cell-permeant cAMP analogues in osteoblasts (18, 19). Some studies have showed that the basal levels of endogenous RGS2 do not regulate Gs or Gq signaling in osteoblasts (70). Functionally, RGS2 increases the rate of GTP hydrolysis on the Gq subunit (9, 23) and also inhibits Gq-stimulated activation of PLC (24). In contrast, RGS2 has no effect on the rate of GTP hydrolysis by the Gs subunit (8, 23), but inhibits intracellular cAMP accumulation (8,13–16). Both Gq and Gs recruit RGS2 to the plasma membrane in mammalian cells (8), and bind directly to RGS2 (16, 20, 21, 25). Further study found that forskolin-activated Gs signaling up-regulates RGS2 expression and suppresses Gq-dependent accumulation of inositol phosphates and nucleotide-stimulated calcium release in wild-type osteoblasts, but not in RGS2 deficient osteoblasts. Conversely, pretreatment with ATP to activate Gq signaling suppresses PTHrP-dependent cAMP accumulation in wild type but not in RGS2 deficient osteoblasts. Further studies showed that endogenously expressed RGS2 can limit Gs signaling. Thus, these studies imply that up-regulation of RGS2 by Gq signaling desensitizes Gs signals. Noticeably, up-regulation of RGS2 contributes to cross-desensitization of Gs- and Gq-coupled signals (70). For example, up-regulation of RGS2 by agents that activate the Gs signaling pathway, inhibits P2Y and endothelin receptor-stimulated Gq signals; conversely, Gq -mediated up-regulation of RGS2 inhibits PTH1R-stimulated Gs signals, revealing cross-talk between these pathways. Those studies demonstrate that RGS2 expression is regulated by both Gs and Gq signaling in osteoblasts.

Besides these studies, overexpression of osteoblast-specific transcription factor Runx2 directly leads to a stimulation of RGS2 promoter activity to promote RGS2 expression (34). Additionally, PGE2 rapidly and transiently increases the level of RGS2 mRNA in bone cells *in vivo* through the activation of cAMP signal transduction (33). The effect of RGS2 on osteoblasts is primarily mediated by PTH, resulting in increased osteoblast activity and inhibition of the osteoclast activation (31, 101, 102). Runx2 sensitizes cAMP-related GPCR signaling by activating Gpr30 and repressing RGS2 gene expression in osteoblasts to increase responsiveness to mitogenic signals (103). This is also in accordance with the findings from Tsingotjidou *et al.* (101). They demonstrate that RGS2 is a PTH-induced primary response gene in murine osteoblasts, and that RGS2 is induced primarily through the cAMP-PKA pathway and specifically inhibits Gαq-coupled receptors.

5.2. Chondrocytes

Chondrocytes are only cells found in cartilage which produce and maintain the cartilaginous matrix, made mainly of collagen and proteoglycans. James *et al.* found that RGS2 can induce the increased growth and matrix apposition of chondrocytes (104). Addtionally, overexpression of RGS2 in the chondrogenic cell line ATDC5 can accelerate chondricytic hypertrophic differentiation (104), as well as advance production of glycosaminoglycans and ALP, and induce parallel increases in the expression of the chondrogenic marker genes Fgfr3 and Ibsp (104), indicating RGS proteins are also involved in chondrocytic differentiation and function. It has been known that RGS proteins are closely involoved in CaR regulation. Some studies have shown that deletion of CaR in chondrocytes (cartilage-producing cells) results in mouse lethality before embryonic day 13 (E13). Mice in which chondrocyte-specific deletion of CaR is induced between E16 and E18, are viable, but show the delayed growth plate development (105). This study raises further question about whether RGS proteins are involved in this CaR regulation during chondrocytic differentiation and function.

5.3. Osteoclasts

Osteoclasts are bone-resorbing cells by removing bone mineralized matrix and breaking up the organic bone. Those cells exert an important impact on skeletal metabolism. Disorders of skeletal insufficiency, such as osteoporosis, are characterized by enhanced osteoclastic bone resorption relative to bone formation. In recent years, significant progress in the mechanisms of osteoclast formation and activation, have been made, however, how GPCR/G proteins and RGS proteins regulate osteoclasts is largely unknown. Additionally, although numerous kinds of G-protein–coupled receptors and effectors such as calcitonin receptor, CaR, relaxin family peptide 1, GPR55

and cannabinoid receptor type 1 (36) have been identified in osteoclasts, the regulation of RGS protein has been relatively neglected. Calcium is an important signal for osteoclast motility downstream of tyrosine kinase signals (106). Osteoclasts, as calcium-mobilizing cells, have an active calcium regulatory system, including a membrane Ca^{2+}-ATPase (107) and an endoplasmic Ca^{2+}-ATPase (108). The basolateral membranes of osteoclasts are quite sensitive to elevated Ca^{2+}; cytosolic Ca^{2+} rises, with cell–matrix detachment and cessation of bone resorption (109). Takayanagi *et al.* (89) found that NFATc1, a member of the NFAT (nuclear factor of activated T cells) family of transcription factor genes, is the most strongly induced transcription factor gene following RANKL stimulation, and that RANKL stimulation results in the induction of Ca^{2+} oscillation and contributes to the sustained activation of NFATc1 (89). The NFATc1 expression is dependent on both the TRAF6 and c-Fos pathways (89). Our studies (92, 110) as described previously, revealed that RGS10 and RGS12 are both essential for the terminal differentiation of osteoclasts induced by RANKL. Interestingly, recent study showed that RGS18 also play an important role in osteoclast differentiation (111). Here, we review their function and regulatory mechanism in following sections.

5.3.1. RGS10

Human RGS10 was first characterized by Hunt *et al.*(112), as a member of the RGS protein family. They found that RGS10 associates with the activated forms of two related G-protein subunits, $G\alpha_{i3}$, and $G\alpha_z$, but fails to interact with $G\alpha_s$ subunit. The mRGS10 gene is expressed predominantly in brain and testis (113, 114). It also expressed in atrial myocytes (115), B lymphocytes (114, 116), and human monocyte-derived dendritic cells (29). By differential screening of a human osteoclastoma cDNA library, we found that the RGS10A isoform, but not the RGS10B isoform, is specifically expressed in human osteoclasts. The expression of RGS10A is also induced by RANKL in osteoclast precursors and is prominently expressed in mouse osteoclast-like cells. *RGS10A* silencing by RNA interference blocks intracellular Ca^{2+} oscillations and osteoclast differentiation. Reintroduction of *RGS10A* rescues the impaired osteoclast differentiation. *RGS10A* silencing also causes premature osteoclast apoptosis. *RGS10A* silencing affected the RANKL-Ca^{2+} oscillation-NFATc1 signaling pathway (92). To further understand the function of RGS10 *in vivo*, we generated RGS10-deficient (RGS10-/-) mice. Our result showed that RGS10-/- mice exhibit severe osteopetrosis and impaired osteoclast differentiation, which cannot be rescued by RGS12 (93). Ectopic expression of RGS10 dramatically increases the sensitivity of osteoclast differentiation to RANKL signaling. Deficiency of RGS10 results in the absence of Ca2+ oscillations and NFATc1. Ectopic NFATc1 expression rescues impaired osteoclast differentiation from deletion of RGS10. We further found that RGS10 competitively interacts with Ca2+/calmodulin and PIP3 in a Ca2+-dependent manner to regulate Ca2+ oscillations. Thus, our results reveal a mechanism by which RGS10 specifically regulates the RANKL-evoked RGS10/calmodulin-Ca2+ oscillation-calcineurin-NFATc1 signaling pathway in osteoclast differentiation.

5.3.2. RGS12

RGS12 is the largest protein in the RGS protein family based on its protein molecular weight. It was first identified by Snow *et al* (117). *RGS12* mRNA is expressed in rat spleen, lung, prostate, testis, ovary, kidney and brain (117). The expression of RGS12 is also detected at different embryonic stages during mouse development (118). Due to its multi-domain architecture, RGS12 protein has the potential to regulate multiple signaling pathway components. It contains a RGS domain, which is responsible for GAP activity (119), and another Gα-interaction region, the GoLoco motif, which has guanine nucleotide dissociation inhibitor (GDI) activity toward Gαi subunits (120, 121). RGS12 also has a pair of Ras-binding domains (RBDs) (122), suggesting that RGS12 may integrate signaling pathways involving both heterotrimeric and monomeric G-proteins. The long RGS12 splice variant has an N-terminal PDZ (PSD-95/Dlg/ZO-1) domain capable of binding the interleukin-8 receptor B (CXCR2) or its own C-terminal (119) and a phosphotyrosine-binding (PTB) domain that associates with tyrosine-phosphorylated N-type calcium channel (123). Several lines of evidences showed that cytosolic Ca^{2+} oscillations are generated mainly by influx of extracellular Ca^{2+} through multiple channels, which include L- and N-type channels, and Ca^{2+} influx is necessary for maintenance of oscillations(123-126). Additionally RGS12 is capable of direct interaction with the N-type calcium channel through its PTB domain and modulates channel activity directly (123, 125, 126). Interestingly, by analyzing RGS12 gene expression pattern, we found that RGS12 is also prominently expressed in RANKL induced osteoclasts. Silence of RGS12 expression using RNA interference impairs phosphorylation of PLCγ and blocks Ca2+ oscillations, NFATc1 expression, and osteoclast differentiation. We further found that N-type calcium channels are expressed in RANKL induced osteoclasts and that RGS12 directly interacts with the N-type calcium channels. Compared to RGS10 (93), RGS12 does not binds with Ca^{2+}/calmodulin and PIP3 in a Ca^{2+}-dependent manner during osteoclast differentiation. Instead of these bindings, it acts on N-type Ca^{2+} channels and CaR. Most recently, by specific deletion of RGS12 in osteoclast lineage by breeding $RGS12^{flox/flox}$ mice with CD11b-cre and Mx1-cre also cause osteopetrosis phenotype and impaired Ca^{2+} oscillations, which cannot be rescued by RGS10 (Manuscripts submitted). We also found that overexpression of RGS12 PTB domain dramatically promotes osteoclast differentiation. Those results demonstrate that RGS10 and RGS12 proteins play different roles in regulating Ca^{2+} oscillations and osteoclast differentiation. Our data support that RGS12 regulates osteoclast differentiation through calcium channel-Ca^{2+} oscillation-NFATc1 pathway.

5.3.3. RGS18

Another RGS protein, RGS18, has been reported to play an important role in RANKL-induced osteoclast differentiation as a negative regulator (127). Iwai *et al.* analyzed RGS18 expression in the macrophage/monocyte lineage cell line and the primary osteoclast precursor monocytes derived from mouse bone marrow, and found that both cell lines express mRNA for 10 different

Table 2. RGS proteins in the regulation of bone

Gene name	Function	Mechanism	References
RGS2	Increased osteoblastic activity	Regulation of GPCR, PTH levels and Ca^{2+} oscillations	(31, 33, 34, 66, 67, 69-71, 86, 101-104, 130)
Axin2	A key negative regulator of bone remodeling	Regulation of Wnt signaling	(50-53)
RGS5	Osteoblast differentiation	Regulation of PTH levels	(74)
RGS10	Osteoclast differentiation	Regulation of Ca^{2+} oscillations and NFATc1	(92)
RGS12	Osteoclast differentiation	Regulation of calcium channel, Ca^{2+} oscillations and NFATc1	(93, 94)
RGS18	Inhibiting osteoclastogenesis	Regulation of acid-sensing OGR1/NFAT signaling pathway	(127)

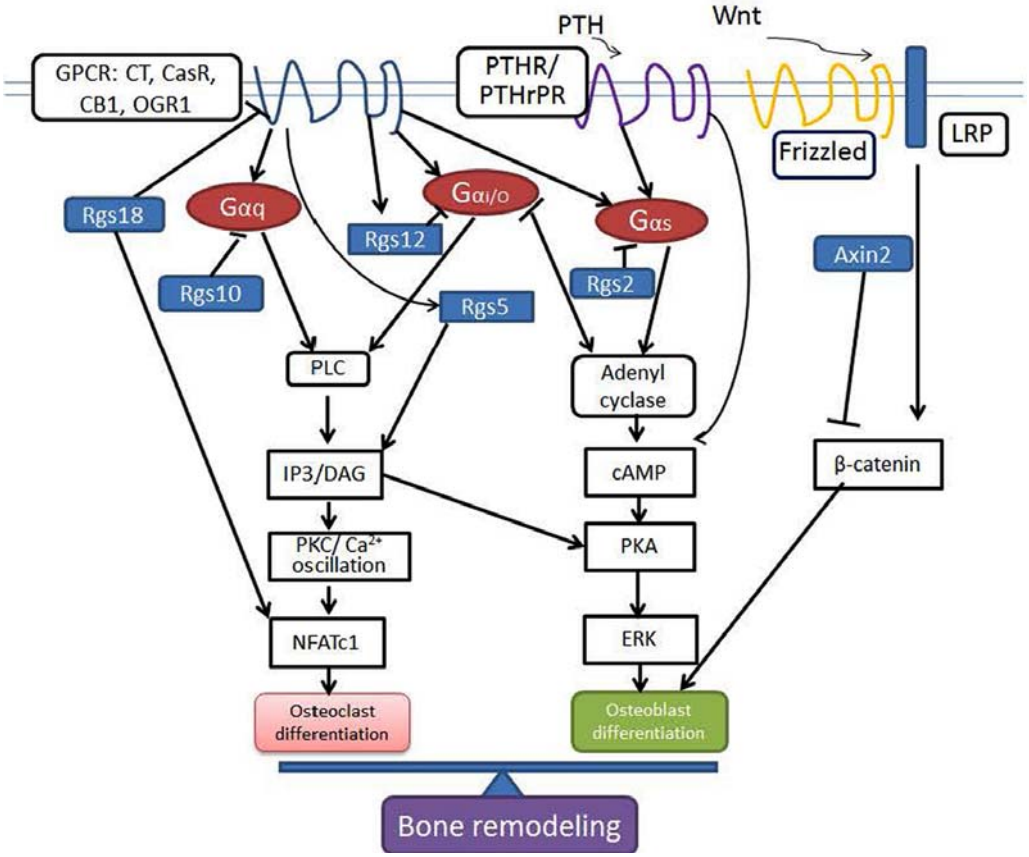

Figure 1. RGS proteins in the regulation of GPCR, PTH, and Wnt signaling pathways during bone remodeling. Binding of Wnt to the FZD receptor induces ß-catenin accumulation, which translocates to the nucleus to activate target gene transcription, which is important for osteoblast differentiation. Axin2, a member of the RGS family, is found to form a stable complex with β-catenin to prevent its activation without Wnt. In osteoblasts, binding of PTH to its G-protein-coupled receptor (PTHR/PTHrPR) induces the expression of RGS proteins such as RGS2 and RGS5 via activation of the cAMP/PKA- pathway. RGS2 predominately binds to the Gq-subunit of the G-protein complex and inhibits activation of the PKC/Ca^{2+} dependent signaling cascade, which is involved in osteoblast differentiation. RGS5 likely regulates osteoblast differentiation through the regulation of PTH levels. In osteoclasts, PKC/Ca^{2+}-induced NFATc1 is crucial for osteoclast differentiation. PLC is suggested to regulate the NFATc1 expression through IP3/DAG. RGS10 and RGS12 regulate the activity of Gαq and Gαi/o to activate PLC, which is critical for calcium oscillation and the activation of NFATc1. NFATc1 activation leads to osteoclast differentiation. RGS18 negatively regulates osteoclast differentiation through acidosis-induced osteoclastogenic OGR1/NFAT signaling pathway.

mammalian RGS, including RGS18. Interestingly, they found that expression of RGS18 is down-regulated by RANKL. Silence of RGS18 using RNA interference prominently promotes RANKL induced osteoclastogenesis. Without RANKL stimulation, treatment with RGS18 siRNA does not induce osteoclast formation, suggesting that RGS18 absence is not sufficient to stimulate osteoclastogenesis. Blocking of an ovarian cancer G-protein–coupled receptor (OGR1), they found, can reverse the effect of RGS18 inhibition for osteoclastogenesis. Meanwhile, RGS18 is not involved in control of Gi signaling. Hence, they suggest that RGS18-targeted inhibition is dependent on Gq-protein signaling pathway via OGR1. Further study showed that overexpression of exogenous RGS18 inhibits the acidosis-induced NFATc1 activation (127). Thus, the authors conclude that RGS18

acts as a negative regulator of the acidosis-induced osteoclastogenic OGR1/NFAT signaling pathway, and RANKL stimulates osteoclastogenesis by inhibiting RGS18 expression.

6. SUMMARY AND PERSPECTIVES

The importance of GPCR and G protein signaling for determining bone cell growth and differentiation has been recognized for many years (129). After their discovery in the mid-1990s, RGSs were quickly appreciated as key players in the regulation of GPCR/G protein signaling. Many studies have been performed in osteoblasts, osteoclasts, as well as chondrocytes, each focusing on one or a few RGS proteins (Table 2). They have provided a wealth of information regarding the function and mechanisms of RGSs as modulators and integrators for GPCR and G-protein signaling as we described above and in Figure 1. Those studies not only enhance the understanding of RGS proteins in bone biology; but also open up a new approach to targeting RGS proteins in drug discovery. As more reports are being published on the function of this group of proteins, increasing attention would focus on RGS proteins as exciting new candidates for therapeutic intervention and drug development**.**

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Abbreviations: CaM – calmodulin; CaR - calcium-sensing receptor; CB1-cannabinoid receptor type1; CT-calcitonin receptor; ERK - extracellular regulated kinases; GPCR- G protein coupling receptors; GAPs - GTPase activating proteins; IFN-β- interferon- β; IP_3 – inositol trisphosphate; M-CSF – macrophage colony stimulating factor; NFAT – nuclear factor of activated T-cells; OGR1- ovarian cancer G-protein–coupled receptor 1; PGE_2- prostaglandin E_2; PKA - protein kinase A; PKC - protein kinase C ; PLC-phospholipase C; PMCA - plasma membrane Ca^{2+} ATPase; PTH- parathyroid hormone; PTHR-parathyroid hormone receptor; PTHrP - parathyroid hormone-related peptide; RANKL – receptor activator of nuclear factor kappa-B ligand; RGS - regulators of G protein signaling; PTX - pertussis toxin; SERCA - sarco/endoplasmic reticulum Ca^{2+} ATPase; TSH – thyrotropin

Key Words: Osteoblasts, Osteoclasts, Bone, Heterotrimeric G protein, G protein coupled receptor, PTH, PTHrP, Calcium Sensing Receptor, Regulator of G Protein

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