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Interplay between CaSR and PTH1R Signaling in Skeletal Development and Osteoanabolism

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Abstract

Parathyroid hormone (PTH)-related peptide (PTHrP) controls the pace of pre- and post-natal growth plate development by activating the PTH1R in chondrocytes, while PTH maintains mineral and skeletal homeostasis by modulating calciotropic activities in kidneys, gut, and bone. The extracellular calcium-sensing receptor (CaSR) is a member of family C G protein-coupled receptor, which regulates mineral and skeletal homeostasis by controlling PTH secretion in parathyroid glands and Ca^{2+} excretion in kidneys. Recent studies showed the expression of CaSR in chondrocytes, osteoblasts, and osteoclasts and confirmed its non-redundant roles in modulating the recruitment, proliferation, survival, and differentiation of the cells. This review emphasizes the actions of CaSR and PTH1R signaling responses in cartilage and bone and discusses how these two signaling cascades interact to control growth plate development and maintain skeletal metabolism in physiological and pathological conditions. Lastly, novel therapeutic regimens that exploit interrelationship between the CaSR and PTH1R are proposed to produce more robust osteoanabolism.

Keywords

1. Introduction

Maintaining normal Ca^{2+} homeostasis is essential for all cellular functions in our body. Land vertebrates develop large bony skeleton to store excess Ca^{2+} in the form of hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$ and releases it to meet systemic demands at the time of Ca^{2+} deficiency. The parathyroid gland (PTG) also evolves to coordinate the calciotropic activities in the skeleton with those in the gut and kidney, by secreting the parathyroid

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hormone (PTH). The current working model for the regulation of serum Ca^{2+} concentration (sCa^{2+}) emphasizes: (i) the ability of parathyroid cell (PTC) to respond to subtle changes in sCa^{2+} that promptly alter PTH secretion (Figure 1A, ①); (ii) the ability of PTH to activate its receptor, PTH1R (②), in the kidney to promote Ca^{2+} reabsorption (③) and stimulate 1,25dihydroxyvitamin D (1,25D) production to increase intestinal Ca^{2+} absorption (④); (iii) the ability of PTH to enhance bone turnover (⑤-⑨, see detailed descriptions in Section 8 Actions of PTH and PTH1R in bone) to release Ca^{2+} into the circulation (⑩); and (iv) the negative feedback of increasing sCa^{2+} and serum 1,25D (s1,25D) to suppress PTH secretion by activating the extracellular Ca^{2+}-sensing receptor (CaSR) and vitamin D receptor (VDR) (①) to close this regulatory loop [1–3]. Defects at any point in this pathway disturb mineral balance and produce endocrine and skeletal dysfunction.

Skeletal development begins in the embryo and continues throughout adolescence until a peak bone mass is attained in early adulthood. The mature skeleton is then maintained by continuous bone turnover (or remodeling) through balanced bone-forming activities of osteoblasts (OBs) and bone-resorbing activities of osteoclasts (OCLs) (Figure 1A, ⑥-⑨) in the bone-remodeling units (BRUs). Excessive bone resorption due to aging, post-menopause, use of glucocorticoids, and metabolic diseases, like hyperparathyroidism (HPT), produces osteoporotic skeleton with increased risk of fracture [4–13].

Ca^{2+} availability critically impacts skeletal development and bone turnover [14]. Ca^{2+} deficiency produces rickets and osteomalacia, characterized by inadequate cartilage or bone matrix mineralization, in patients [15–18]. Supplementation of the diet with Ca^{2+} and vitamin D, and in some cases with Ca^{2+} alone, completely heals those cartilage and bone defects [14, 15, 19–21]. Similarly, rachitic changes in bone and cartilage in VDR knockout (KO) mice are prevented by a high Ca^{2+} diet [22–24]. The above observations underscore the importance of adequate Ca^{2+} supply to normal cartilage and bone development. Although Ca^{2+} could contribute passively to bone mineralization as an essential substrate, recent discoveries of CaSR in chondrocytes, OBs, and OCLs have prompted investigations for direct Ca^{2+} actions on those cells as a critical "growth factor".

Inactivating mutations in the *CASR* gene reduce the responsiveness of PTC to changes in sCa^{2+} and produce familial hypocalciuric hypercalcemia (FHH) and neonatal severe HPT (NSHPT) in patients, who show elevated serum PTH (sPTH), s1,25D, and sCa^{2+} levels and in the severest forms a growth-retarded and under-mineralized skeleton [25–28]. Skeletal defects in NSHPT patients are likely caused by aberrant PTH secretion and the associated mineral and hormonal disturbances. Direct effects of mutant CaSR in chondrocyte and bone cell, however, cannot be ruled out [29–38].

Prolonged elevation of sPTH produces catabolic effects on bone [39–42], but once-daily (or intermittent) injections of supra-physiological doses of PTH1–34 or PTH1–84 increase trabecular bone mass in normal and osteoporotic animal models and in osteoporosis patients [43–47]. The anabolic effect of intermittent PTH (iPTH) appears to rely on its ability to promote bone-forming activities of OBs to a greater extent than the bone-resorbing activities of OCLs at the beginning of the treatment -- creating a so-called "anabolic window", but its underlying cellular and molecular mechanisms remain unclear.

In the past decades, investigations using genetically manipulated mouse models and cell cultures have confirmed essential roles for the CaSR and PTH1R signaling in controlling pre-and post-natal skeletal development and maintenance of adult skeleton. However, perspectives on how these two signaling pathways interact in cartilage and bone are lacking. In light of two recent review articles that provide comprehensive updates on general systemic and local actions of the CaSR and PTH on bone and mineral metabolism [30, 48], this review emphasizes the interplay between the CaSR and PTH1R signaling in chondrocytes, OBs, and OCLs and proposes novel regimens exploiting this receptor interaction to enhance osteoanabolism for treatment of skeletal disease.

2. Endochondral bone formation

In vertebrates, all weight-bearing axial and appendicular skeletons are formed by endochondral bone formation that begins in early embryos. This process starts with the condensation of mesenchymal progenitors and their commitment to the chondrocytic lineage to form cartilaginous anlagen that later becomes a growth plate (GP). In the GP, chondrocytes proliferate, mature, and hypertrophy sequentially within single cell columns and then begin to deposit Ca^{2+}/phosphate-containing minerals into the surrounding matrix after they reach terminal differentiation (Figure 2A). Within this mineralized matrix, the terminally differentiated chondrocytes produce matrix metalloproteinases to remodel surrounding matrix [49–51] and release growth factors to induce vascular invasion and promote OB differentiation at the chondro-osseous junction. It was originally proposed, mainly based on histological observations, that terminally differentiated hypertrophic chondrocytes in the GP undergo cell death and OBs arise from the osteoprogenitors delivered by the invading vasculature to replace the dying chondrocytes and produce new bone. This classic scheme of chondro-to-osteo transition has just undergone a significant paradigm shift [52–54]. By using protein-based fluorescent probes to label chondrocytes in vivo and by following the fate of the labeled cells in the bone using time-lapse cell/tissue imaging, it has been clearly shown that the majority of hypertrophic chondrocytes can trans-differentiate directly into OBs in the GP during endochondral bone formation or in the healing callus of fractured bone [53, 55–57]. GPs exist throughout adolescence to support longitudinal bone growth by repeating the above cell differentiation programs until the chondroprogenitor pool is exhausted at the time of GP closure in early adulthood. Aberrant acceleration or delay in chondrocyte differentiation produces disorganized GPs and impede bone growth [58].

3. PTHrP and PTH1R in endochondral bone formation

Many transcription [58–69] and autocrine/paracrine factors [56, 70–83] were found to induce the commitment of progenitors to the chondrocytic lineage and to pace their differentiation [49, 56, 76, 84, 85]. Among them, the parathyroid hormone–related protein/ Indian hedgehog (PTHrP/Ihh) feedback loop is the best-established pathway that prevents aberrant acceleration of chondrocyte differentiation and early closure of the GP [58, 86, 87]. According to the current model, PTHrP produced by perichondral cells in embryonic skeleton or by maturing/prehypertrophic chondrocytes in postnatal GPs diffuses into the proliferation zone where it activates the PTH1R and downstream signaling cascades to

sustain the proliferative activities of the cells and delay their further differentiation [88] (Figure 2A). When chondrocytes eventually mature, they increase the production of Ihh to simulate its receptor Patched in the neighboring cells and increase PTHrP production via mechanisms that remain to be determined, thus constituting a feedback loop to slow down cell differentiation [87, 89] (Figure 2A). *Pth1r, Pthrp*, and *Ihh* gene KOs in mice all led to accelerated chondrocyte differentiation, early GP closure, and dwarfism [87]. Transgenic mice overexpressing PTHrP specifically in chondrocytes also presented short-limbed dwarfism, but their growth plates were composed exclusively of proliferating cells and lacked endochondral ossification [90], confirming the role of PTHrP/PTH1R signaling in preventing an early entry of proliferating chondrocyte into terminal differentiation.

Several elegant investigations explored signaling events underlying the actions of PTHrP on cartilage development with emphases on its ability to activate different heterotrimeric GTP-binding proteins (G-proteins) and multiple down-stream effectors in chondrocytes [86]. In fibroblastic COS-7 cells expressing exogenous PTH1Rs, binding of PTHrP to the receptor stimulated Gs-mediated cAMP synthesis as well as Gq-mediated intracellular Ca^{2+} releases, indicating the multifaceted actions of PTHrP/PTH1R signaling [91]. To determine the impact of Gs-mediated signaling responses on GP development, chimeric mice with GPs comprising mixed populations of normal and Gsα-deficient chondrocytes were studied [92]. In the chimeric GPs, Gsα-deficient chondrocytes appeared to stop proliferating and become hypertrophic prematurely [91] -- phenotypes similar to those of chondrocyte-specific PTH1R KO mice [93, 94]. It was, therefore, concluded that PTHrP activates Gs-mediated signaling responses to sustain chondrocyte proliferation [87].

In cultured chondrocytes, pharmacological stimulation of Gq-coupled protein kinase C (PKC) and mitogen-activated protein kinase kinase (MEK) pathway suppressed proliferation, enhanced cell hypertrophy, and increased expression of type X collagen [95, 96], supporting a role for Gq-mediated signaling in promoting chondrocyte terminal differentiation. But there has been no report on the study of mice with targeted ablation of Gαq specifically in chondrocytes to clearly define its impact on GP development *in vivo*. Instead, studies of mice with a knock-in of an engineered *Pth1r* mutant gene, which encodes a mutant PTH1R that retains the ability to activate Gs, but not Gq, signaling pathway, showed delayed GP ossification and increased chondrocyte proliferation [97]. Though, the effects were modest, likely due to the relatively restricted expression of PTH1R in the proliferation zone. Nevertheless, the investigators of the study concluded that the PTH1R-mediatd Gs and Gq signaling cascades constitute a "Yin-Yang" relationship to control the pace of cell differentiation [86, 87]. However, the fact that chondrocyte-specific PTH1R KO mice presented profoundly accelerated chondrocyte differentiation and early GP closure indicates the existence of other, perhaps Gq-coupled, mediators that can promote the terminal differentiation of chondrocyte and engage in a "tug-of-war" relationship with the PTHrP/PTH1R/Ihh feedback loop to control the pace of GP development. Recent studies suggest that Ca^{2+} and its receptor, CaSR, constitute a critical signaling pathway that instigates such "pro-differentiation" activities in chondrocytes.

4. Signaling transduction of the CaSR

The CaSR is a member of family C G-protein coupled receptor (GPCR), which consists of a large extracellular domain (ECD; ≈450–600 amino acids) for ligand binding, a seven-transmembrane domain (7-TMD) for G protein coupling, and a long intracellular C-terminal tail (≈250 amino acids) for recruitment of signaling molecules and for receptor binding to cytoskeletons [98, 99]. Members of family C GPCRs function exclusively in the form of multimeric complex [100, 101]. The CaSR can form homodimers [100, 101] or heterodimerize with other members of family C GPCRs, including metabotropic glutamate receptors [102] and type B gamma-aminobutyric acid receptors (GABA$_B$R1 and GABA$_B$R2) [103, 104]. Like other GPCRs, the CaSR activates multiple downstream signaling cascades by coupling to 3 major groups of G proteins, Gq/11, Gi/o and G12/13 [99]. Through coupling to the Gq/11, the CaSR activated different subtypes (β, γ, δ, ε, ζ, η) of phospholipase C (PLC) to cleave the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into diacyl glycerol (DAG), which activates protein kinase C, and inositol 1,4,5-trisphosphate (IP$_3$), which releases Ca^{2+} from intracellular stores by binding to IP$_3$ receptors in the stores [99]. This signaling cascade has been demonstrated in most of the cell systems tested, including parathyroid cells [105, 106], keratinocytes [107], chondrocytes, osteoblasts [29], and transformed cells expressing the CaSR exogenously [108], to regulate diverse cell functions ranging from PTH secretion [109], osteoblast migration [35], and cell growth, survival, and differentiation [33]. By coupling to the pertussis toxin-sensitive Gi/o, the CaSR suppressed adenylyl cyclase activities and cAMP production in PTCs [110] and OBs [29]. Activation of Gi/o also activated the extracellular-signal-regulated kinases (ERK1/2) in PTCs [111, 112], OBs [113, 114], and HEK-293 cells expressing exogenous CaSRs [115–117]. Through the activation of G12/13, the CaSR enhanced Wnt3a-βcatenin signaling to promote osteoblast differentiation [118], but inhibited osteoclastogenesis by suppressing the expression of the receptor activator of nuclear factor kappa-B ligand (RANKL) and increasing osteoprotegerin (OPG) expression [119]. The CaSR could also activate phospholipase D though coupling to G12/13 in Madin-Darby canine kidney cells [120].

5. CaSR in chondrocyte differentiation and cartilage development

Ca^{2+} deficiency produced rickets in childhood [18] and in VDR and Cyp27b1 KO mice [24, 121] by delaying chondrocyte differentiation and blocking matrix mineralization in their GPs. The ability of dietary Ca^{2+} supplements to reverse the GP defects [21] signifies the importance of Ca^{2+} availability to GP development. The expression of CaSR first appears in maturing chondrocytes in the GP and increases in hypertrophic chondrocytes (Figure 2B) [29] including those being released from the cartilage matrix at the chondro-osseous junction (Figure 2C, red arrowheads) and adjacent OBs [29]. This expression pattern supports a role for the CaSR in mediating the terminal differentiation of hypertrophic chondrocytes and their transformation into osteoblastic lineage, according to the newly established paradigm [52–54].

Direct actions of Ca^{2+} and CaSR on chondrocyte differentiation have been confirmed by studies of primary cells cultured from cartilage of different species, chondrogenic cell lines, and metatarsal bone rudiments explants. Chondrocytes in a high-density culture exhibited

spontaneous differentiation that recapitulates keep steps of chondrogenesis as seen in vivo (Figure 3A) [29, 103, 122–124]. For example, mouse GP chondrocytes proliferated robustly and produced a proteoglycans (PG)-rich matrix immediately after plating (Figure 3A). Mineral deposition appeared to start in the matrix surrounding the hypertrophic chondrocytes (Figure 3A, 7-day post-confluence, insert). As mineral deposition increased in the cultures, PG accumulation declined and the cells lost their chondrocytic morphology (Figure 3A, 21-day post-confluence, insert). Along with those morphological changes, RNA levels for early differentiation markers -- aggrecan (Agg) and type II collagen $\alpha 1$ subunit [α_1(II)] -- were highest in early cultures and decreased in later cultures (by >90%), while the expression of late differentiation markers -- alkaline phosphatase (ALP) and type X collagen α_1 subunit [α_1(X)], and putative OB markers --osteopontin (OPN), osteocalcin (OCN) and osteonectin (ON) increased with time of culture (Figure 3B). The above changes in cell morphology, gene expression, and matrix protein synthesis recapitulate steps of chondrogenesis and indicate time-dependent transformation of the cultured chondrocytes into the osteoblastic lineage.

Changes in [Ca^{2+}]$_e$ profoundly impacted the differentiation of cultured chondrocytes. In tibio-tarsal chondrocytes cultured from chicken embryos, high [Ca^{2+}]$_e$ increased the expression of α_1(X) [125]. In mouse GP chondrocyte cultures, raising [Ca^{2+}]$_e$ dose-dependently suppressed PG accumulation, increased mineral accumulation (Figure 3C and 3D -PTHrP), reduced expression of chondrocyte markers [Agg, α_1(II), and α_1(X)], and increased expression of OPN (Figure 3E, Control) [29, 103, 122, 123]. Similar effects of high [Ca^{2+}]$_e$ were seen in cultures of non-transformed chondrogenic RCJ3.1C5.18 (or C5.18) cells, cloned from fetal rat calvarias [29, 124, 126]. The ability of high [Ca^{2+}]$_e$ to increase intracellular Ca^{2+} mobilization and promote terminal differentiation in C5.18 cells could be blocked by overexpression of a dominant-negative CaSR or anti-sense RNA in the cells [29, 124, 126]. In cultures of fetal rat metatarsal bone explants, administration of CaSR agonist (or calcimimetics) increased their longitudinal growth by enhancing chondrocyte differentiation in the GP [127]. The above studies confirm the actions of Ca^{2+} and CaSR in promoting chondrocyte differentiation and mineralizing functions, and perhaps to speed up their transformation to acquire osteogenic phenotypes.

A global CaSR KO ([Exon5]CaSR–/–) mouse model was generated by inserting a neomycin gene cassette into the exon 5 of the gene, which encodes 77 amino acids in the ECD of the receptor. The [Exon5]CaSR–/– mice manifested severe phenotypes of human disorder NSHPT -- HPT, hypercalcemia, hypocalciuria, hypophosphatemia, parathyroid hyperplasia, and failure to thrive and the mice died before 3–4 weeks of age. Analyses of their bones reveled severe rickets with delayed formation of secondary ossification center, expanded and disorganized growth plate, and impaired bone formation [36, 128]. Interestingly, the growth and skeletal defects and early death of the [Exon5]CaSR –/– mice could be rescued by preventing the development of HPT after breeding the mice with PTH–/– mice lacking PTH gene or Gcm2–/– mice lacking the development of PTG [129, 130]. The reversal of skeletal defects in the [Exon5]CaSR –/–;PTH–/–and [Exon5]CaSR –/–;Gcm2–/– double KO mice led to the conclusion that the CaSR is not essential for skeletal development [129, 130] and prompted searches for other Ca^{2+}-sensing mechanism(s) [131, 132]. However, it was not realized at the time that the exon 5 gene-targeting strategy allowed an in-frame gene-splicing

event to exclude the exon 5 along with the inserted neomycin cassette from the full-length transcript, producing a truncated CaSR lacking 77 amino acids in its ECD. This truncated receptor was expressed in the skin, growth plate, and bone of the ^{Exon5}CaSR $-/-$ mice [122, 133] and is sufficient to render Ca^{2+}-resposiveness in GPCs cultured from the mice [122].

To clearly define the role of CaSR in skeletal development, a floxed-CaSR mouse model was generated by flanking the exon 7 of the *Casr* gene with two loxP sites. The exon 7 encodes the entire 7-TM domain and the C-terminal tail, which are absolutely required for the coupling of the receptor to downstream signaling cascades [33]. The utility of this floxed-CaSR model was validated by the generation of PTC-specific CaSR KO mice (^{PTC}CaSR $^{flox/flox}$) through breeding the floxed-CaSR mice with PTH-Cre mice expressing Cre-recombinase under the control of PTH promoter [33]. Analyses of genomic DNA, RNA, and protein extracted from different tissues of the ^{PTC}CaSR $^{flox/flox}$ mice showed completely deletion of the exon 7 of the gene in PTGs, but not in other vital organs [33]. ^{PTC}CaSR $^{flox/flox}$ mice presented severe HPT hypercalcemia, skeletal and growth phenotypes, and early death as seen in the ^{Exon5}CaSR $-/-$ mice, except that the ^{PTC}CaSR $^{flox/flox}$ mice developed hypercalciuria, but not hypocalciuria, due to the preservation of normal renal CaSR functions, which enhances Ca^{2+} excretion in response to hypercalcemia [33].

To determine the role of CaSR in GP development, the floxed-CaSR mice were bred with Col(II)-Cre mice, which express Cre recombinase under the control of α_1(II) gene promoter [134]. Unexpectedly, the resulting ^{Cart}CaSR $^{flox/flox}$ embryos died before embryonic day 13 (E13), with severely under-mineralized skeleton [33]. The cause for the early death of ^{Cart}CaSR $^{flox/flox}$ embryos remains unclear [33]. As chondrogenesis also takes place during the development of heart valve [135–138], defective cardiac functions could have caused the death of ^{Cart}CaSR $^{flox/flox}$ embryos. These observations also support the ability of exon5-less CaSR to sustain the development of ^{Exon5}CaSR $-/-$ embryos. An additional mouse model was made to study the impact of CaSR function at later stages of GP development by breeding floxed-CaSR mice with Tam-Col(II)-Cre mice, which express tamoxifen-inducible Cre recombinase under the control of α_1(II) gene promoter [139] to achieve time-dependent chondrocyte-specific CaSR gene ablation. The resulting $^{Tam-Cart}$CaSR$^{flox/flox}$ mice developed normally until adulthood in the absence of tamoxifen. Induction of CaSR KO in E18–19 embryos by a single maternal injection of tamoxifen profoundly ablated CaSR expression in the GPs of the newborn $^{Tam-Cart}$CaSR $^{flox/flox}$ mice, which presented short stature with expanded and under-mineralized GPs and delayed chondrocyte terminal differentiation as seen in rickets [33]. These in vivo studies confirm a non-redundant role for the CaSR in mediating chondrocyte differentiation and GP development.

6. Interplay between Ca^{2+}/CaSR and PTHrP/PTH1R signaling in chondrocytes

Studies of cultured GP chondrocytes revealed a close interaction between PTHrP/PTH1R and Ca^{2+}/CaSR signaling pathways in controlling the pace of chondrocyte differentiation. Raising $[Ca^{2+}]_e$ profoundly inhibited PTH1R and PTHrP expression in cultures of mouse

GP chondrocytes (Figure 3E, Control and unpublished observations) [123]. Conversely, treating those cultures with PTHrP(1–34) significantly blunted the ability of high $[Ca^{2+}]_e$ to suppress PG accumulation and promote mineral deposition (Figure 3D; -PTHrP vs +PTHrP). In cells maintained at 0.5 mM Ca^{2+}, treatment with PTHrP significantly increased Agg expression and markedly reduced the expression of $\alpha_1(X)$ and ALP -- markers of maturing and hypertrophic chondrocytes (Figure 3E). Incubation with PTHrP also blocked the ability of high $[Ca^{2+}]_e$ to inhibit Agg and $\alpha_1(II)$ expression and to increase OPN RNA levels (Figure 3E), suggesting that increased PTHrP/PTH1R signaling can counteract the effects of high $[Ca^{2+}]_e$ on cell differentiation.

In the GPs of $^{Tam-Cart}$CaSR $^{flox/\ flox}$ mice, the expression of IGF1 and IGF1R was profoundly reduced [33], suggesting that Ca^{2+}/CaSR could promote chondrocyte differentiation at least in part by enhancing IGF1 signaling (Figure 2A). This scenario is supported by the ability *of Igf1r* gene knockdown to suppress the ability of high $[Ca^{2+}]_e$ to promote terminal differentiation and matrix mineralization in cultured chondrocytes [33]. Furthermore, ablating the *Igf1r* gene specifically in GP chondrocytes in mice increased their expression of PTHrP, but not PTH1R [140], indicating a negative regulation of PTHrP expression by IGF1R signaling. These observations support a paradigm in which Ca^{2+}/CaSR signaling counteracts PTHrP/PTH1R signaling by suppressing PTH1R expression independently of IGF1/IGF1R signaling and by inhibiting PTHrP expression via the IGF1R-dependent pathway to support normal progression of chondrocyte differentiation and growth plate development (Figure 2A).

7. Bone modeling and remodeling

At the end of chondrogenesis in the GP, vascular invasion recruits OCL precursors to the chondro-osseous junction where they differentiate and resorb mineralized cartilage matrix to facilitate the release of GP-derived OB precursors [53, 55–57]. The vasculature may also provide a migratory pathway for osterix-expressing osteoprogenitors from the periosteum to future bone sites [141, 142]. The relative contributions of various sources of OB precursors to overall bone development remain unclear.

In the primary spongiosa beneath the GP, osteoprogenitors progress though the stages of pre-OBs, committed OBs, mature OBs, and osteocytes, which are characterized by the expression of specific marker proteins, osterix (Osx), type I collagen [Col(I)], OCN, and dentin matrix protein 1 (DMP1), respectively. The immature OBs produce a large quantity of Col(I), which constitutes the majority of protein matrix (or osteoid), while mature OBs exert mineralizing functions to deposit Ca^{2+} and phosphate into the protein matrix to increase its mechanical strength. At the end of bone-forming activity, OBs, which are embedded in the mineralized matrix, become osteocytes, while others turn into inactive flattened bone-lining OBs. Upon stimulation by calcemic factors, like PTH, bone-lining OBs are reactivated and OCLs are recruited to the BRUs, which serve to liberate matrix Ca^{2+} to meet systemic demands of Ca^{2+} and repair micro damages of the bone.

8. Actions of PTH and PTH1R in bone

Comparison of the skeletal phenotypes in PTH–/– mice and PTH–/–;PTHrP–/– double KO mice indicated PTH-dependent bone-forming activities in the primary spongiosa of long bone [143]. Bone cell-specific PTH1R KO mouse models are, however, required to further define cell-autonomous actions of the receptor. Thus far, there is no report on study of mice with PTH1R KO at early stages of osteoblast differentiation. Mice with osteocyte-specific PTH1R KO showed increases in bone mineral density and trabecular and cortical bone volume and thickness, along with a low bone turnover state due to suppressed OB and OCL activities [144]. Interestingly, mice with osteocyte-specific overexpression of a constitutively active PTH1R also showed increased trabecular and cortical bone mass, but in the state of high bone turnover [145, 146]. These studies support a role for the osteocytic PTH1R in controlling bone turnover, but other factors are involved in balancing bone forming and resorbing activities and determining overall bone accrual.

Direct actions of PTH on OBs were deduced from in vitro studies using cultures of bone marrow-derived osteoprogenitors, osteoblasts/osteocytes released from bone fragments, and osteogenic cell lines, and in vivo studies of mice injected with PTH [44–47]. Those studies together support the scheme that PTH increases osteoblastic activities by recruiting osteoprogenitors and sustaining the proliferation and survival of the committed OBs (Figure 1A, ⑤). Although, iPTH could increase bone accrual and more importantly bone mineralization in vivo [44–47], PTH actually inhibits terminal differentiation of primary OBs or OB-like cell lines and their mineralizing functions in culture [147–151]. This paradox between in vivo and in vitro observations suggests that iPTH in vivo must produce other changes in bone microenvironment that are needed to promote terminal differentiation of the newly recruited OBs by iPTH. This effect is not recapitulated in OB cultures, likely due to the absence of OCL activity.

In vivo, PTH enhances osteoclastic activities by increasing osteoblastic expression of RANKL, macrophage colony stimulating factor (M-CSF), and other cytokines to recruit osteoclast precursors and promote their growth and differentiation through activation of RANK, c-fms (M-CSF receptor), and other signaling pathways in the cells (Figure 1A, ⑥) [152–155]. The above PTH actions on OBs are mediated by cell-autonomous responses as well as by locally produced growth factors and cytokines, including IGF1 [155–157], fibroblast growth factor-2 (FGF-2) [158, 159], Wnt signaling-related agonists and antagonists [160–163], periostin [164], and sympathetic tone [165]. These sequential effects of PTH on OBs and then OCLs provide not only a cellular basis for iPTH to increase bone turnover rate, but also a time window to produce anabolic effects before its catabolic effects catch up [44–47].

The importance of OCL activity in producing osteoanabolism is shown by studies of antiresorptives, such as bisphosphonates and the humanized monoclonal antibody denosumab, which binds to and neutralizes the activity of RANKL and therefore suppresses osteoclastogenesis and bone resorption [166–170]. Antiresorptive agents not only block bone resorption, but also impede bone-forming activities in the BRUs. The exact mechanisms for coupling bone resorption to formation remain unclear. It is proposed that

OCLs interact with OBs directly through binding of their cell-surface receptors (e.g., the RANK/RANKL and Ephrin/Eph systems) [152–155] to promote mutual cell differentiation and functions. Alternatively, OCLs actively release growth factors (e.g., IGF1 and TGFβ) [171, 172] and other constituents (e.g., Ca^{2+}) from the matrix that may serve as anabolic signals to recruit osteoprogenitors and promote their differentiation. It has been shown that local $[Ca^{2+}]$ can rise to >40 mM at sites of active resorption [173] and that Ca^{2+} can function as a strong anabolic signal for OB recruitment, growth, survival, and differentiation [29, 31, 33, 174–178]. Based on this coupling mechanism, inhibition of bone resorption by antiresorptives is expected to limit local Ca^{2+} availability and thereby slow down OB differentiation and bone formation. On the other hand, increasing bone turnover by iPTH is anticipated to increase local $[Ca^{2+}]$ bathing the OBs and promote their differentiation (Figure 1A, ⑧).

9. Actions of Ca^{2+} and CaSR in bone

9.1 Osteoblastogenesis

Studies of primary OBs and osteocytes and osteoblastic cell lines in culture demonstrated the ability of extracellular Ca^{2+} to stimulate acute signaling responses and enhance the migration, proliferation, survival, expression of terminal differentiation markers, and mineralizing functions of the cells by activating the CaSR [29, 32, 33, 35, 37, 174, 178–181]. In bone, the CaSR was found in active OBs, inactive bone-lining cells, and osteocytes ([29] and Figure 2B, 2E). As seen in cultured chondrocytes, CaSR activation with specific agonists stimulated Gq-mediated PLC activity, increased production of IP_3, elevated $[Ca^{2+}]_i$, and opened Ca^{2+}-dependent K^+ channels to promote chemotaxis and cell proliferation in cultures of osteoblastic MC3T3-E1 [35, 182]. In calvarial OBs, CaSR activation (i) stimulated ERK 1/2 and downstream Akt and glycogen synthase kinase 3β (GSK3β) signaling cascades to promote cell growth, survival, and matrix mineralization [178, 180] and (ii) activated PLC and store-operated Ca^{2+} entry to increase $[Ca^{2+}]_i$ to support cell proliferation [183]. The above observations are just few of many studies demonstrating the multifaceted actions of the CaSR in mediating OB proliferation, survival, and terminal differentiation.

While the above in vitro studies support a role for the CaSR in OB differentiation, its role in vivo had been controversial. This was due to the ability of concurrent *Pth* or *Gcm2* gene KO to rescue the skeletal defects in the global Exon5CaSR–/– mice [184]. It was concluded at the time that the development of HPT due to defective CaSRs in PTCs was the main cause for skeletal defects observed in the Exon5CaSR–/– mice [129, 130]. Again, follow-up studies revealed the expression of the truncated exon 5-less CaSR in the cartilage and bone of the Exon5CaSR–/– mice. This truncated CaSR appeared to be sufficient to support overall skeletal development. The latter notion was further supported by studies of mice with OB-specific ablation of the exon 7 of the *Casr* gene KO [185].

Deletion of the exon 7 of *Casr* at the early stage of OB differentiation in vivo by crossing the floxed-CaSR mice with mice expressing Cre under the control of two different versions (2.3 and 3.6 kb) of the Col(I)-α1 gene promoter produced the OBCaSR flox/ flox mice [185], which died before 3–4 weeks of age with severely blunted growth. μCT images reveled their

severely under-mineralized skeletons with multiple unhealed bone fractures [33, 175]. Histomorphometric analyses of the CaSR-deficient bones showed reduced bone formation rates and bone volume and large quantifies of unmineralized osteoid deposited in both trabecular and cortical bone. Gene expression profiling showed profoundly reduced expression of OB differentiation markers, but increased expression of IL-10 gene -- an inducer of cell apoptosis. The up-regulation of the latter gene was consistent with an increased number of apoptotic OBs and osteocytes in the bones of the KO mice [33, 175]. The above skeletal defects were presented in the presence of lower serum PTH levels, further supporting cell-autonomous effects of the gene KO. These data together confirm an essential role for the CaSR in mediating OB proliferation, survival, and mineralizing functions.

Mice with transgenic overexpression of a constitutively active CaSR mutant cDNA under the control of a 3.5-kb OCN gene promoter were also made to examine the impact of the CaSR in mature OBs [176, 177]. The transgenic mice displayed mild osteopenia due to increased number and activity of osteoclast as a result of increased RANKL expression, supporting a role for the CaSR in mediating the coupling between osteoblastic and osteoclastic activities.

9.2 Osteoclastogenesis

OCLs responded to changes in $[Ca^{2+}]_e$ in culture [186–189]. CaSR expression has been detected in monocytes and macrophages freshly isolated from human bone marrow [190], and in osteoclasts cultured from bone marrow and spleen [37, 38]. In situ hybridization and immunohistochemistry (Figure 2C, green arrowheads) confirmed the expression of CaSR mRNA and protein, respectively, in bone marrow cells and osteoclasts in resorbing pits [29]. High $[Ca^{2+}]_e$ and/or CaSR agonists stimulated PLC, elevated $[Ca^{2+}]_i$ [187–189, 191], and enhanced the translocation of nuclear factor NF-κB [189] in cultured OCLs. Some of those signaling responses were blunted in OCLs cultured from ^{Exon5}CaSR–/– mice [189]. High $[Ca^{2+}]_e$ also inhibited the differentiation [37], secretion of acid phosphatase [188], and bone-resorbing functions in cultured OCLs [38] and increased apoptosis of mature OCLs [189]. The above in vitro data support the scheme that CaSR activation inhibits bone-resorbing activities by suppressing differentiation and secretory function of OCL and promoting cell apoptosis. Interestingly, allosteric activator of the CaSR (e.g., cinacalcet HCl) at the concentration, which suppresses PTH section in PTCs, had no effect on resorbing functions of human OCLs in culture [192]. Similarly, the inhibitory effect of the allosteric inhibitor of the CaSR, NPS 2143, on resorbing functions of cultured human OCLs could be seen only at concentrations that are 250 fold higher than those required to block the CaSR in PTCs [193]. These data indicate different pharmacological profiles of the CaSR in OCLs vs PTCs. As decreasing pH renders the CaSR a right-shifted Ca^{2+} set-point [194], so the CaSR in OCLs are predicted to be less responsive to its ligand (i.e., Ca^{2+}) and perhaps its modulators (calcimimetics and calcilytics) in actively resorbing pits. By using a combination H^+ and Ca^{2+} double-barreled electrode, Silver et al. showed that the pH reached a lower limit of 4.7 and the $[Ca^{2+}]_e$ rose to a maximum of 40 mM in the erosion sites of the bone. It is plausible that the OCL CaSRs could be operational under such high $[Ca^{2+}]_e$ environments despite the low pH. Future in vitro studies of OCL cultures with better controls of $[Ca^{2+}]_e$ and pH that

mimic in vivo conditions and in vivo studies of OCL-specific CaSR KO mice are required to clearly define the CaSR actions in OCLs.

10. Interplay between Ca²⁺/CaSR and PTHrP/PTH1R signaling in bone

Based on the studies reviewed above, we propose the following models for the regulation of mineral and bone metabolism by the interactions between Ca^{2+}/CaSR and PTHrP/PTH1R signaling. Under a physiological state (Figure 1A), a normal sCa^{2+} level (①) maintains a steady supply of PTH from PTCs (②) to support basal Ca^{2+} reabsorption in the kidney (③) and Ca^{2+} absorption in the gut (via stimulation of renal 1,25D production) (④), together maintaining Ca^{2+} homeostasis at the level that meets the systemic demand. This level of PTH also supports steady bone forming activities by recruiting OB progenitors, activating bone-lining OBs, and sustaining their survival to maintain an adequate number of bone-forming cells (Figure 1A, ⑤) [44, 157, 158, 195–200]. Through production of growth factors and/or direct physical interactions, the OBs aid in the recruitment of osteoclast precursors and their survival and differentiation (⑥) in the BRUs. The resulting bone resorbing activities liberate matrix Ca^{2+} into fluid bathing OBs and OCLs (⑦). Under conditions of Ca^{2+} sufficiency, low systemic demand of Ca^{2+} (⑩) allows retention of the liberated Ca^{2+} to increase local $[Ca^{2+}]$ that stimulates OB maturation and their mineralizing functions to redeposit the Ca^{2+} into newly formed matrices (⑧). The increasing $[Ca^{2+}]_e$ also feeds back to OCLs to prevent their further expansion and aberrant bone resorption (⑨). These balanced bone-forming and bone-resorbing activities sustain a steady bone turnover rate to continuously remodel the skeleton without bone loss.

In conditions of chronic Ca^{2+} deficiency (Figure 1B), e.g., insufficient Ca^{2+} and/or vitamin D intakes, reduced sCa^{2+} levels increase PTH secretion (①) in PTGs to enhance (②) renal Ca^{2+} reabsorption (③) and 1,25D production to increase intestinal Ca^{2+} absorption in an attempt to restore sCa^{2+} levels to normal. But inadequate intestinal Ca^{2+} intakes (④) prevent such normalization and lead to chronic hypocalcemia and sustained elevation of sPTH, which drastically increases bone turnover rates (⑤-⑦). As a result, excessive bone resorption releases a large amount of Ca^{2+} (⑦), which is shunted into the circulation (⑩) to further meet the systemic demands of Ca^{2+}. Consequently, the decreasing $[Ca^{2+}]_e$ in the BRUs retards the maturation and differentiation of the OBs as well as their mineralizing functions (⑧), leading to accumulation of unmineralized osteoid and osteomalacia. Inability of low $[Ca^{2+}]_e$ to check on the osteoclastogenesis (⑨) further increases bone resorption and exacerbates the catabolic effects of chronic HPT.

11. Skeletal anabolism by targeting the PTH1R and CaSR in bone

Osteoporosis is a growing epidemic that afflicts aging men and women across the world [201]. iPTH is the only FDA-approved therapy that produces skeletal anabolism [43–47], but its dosing is limited to the lowest level that produces anabolic effects with an acceptable rate of hypercalcemia as an adverse effect[43, 45]. A better understanding of the mechanism underlying the anabolic effects of iPTH is needed to improve the therapy. Based on the current data, we propose that daily injections of supra-physiological doses of PTH1–34 (Figure 1C, ②) in addition to the endogenous PTH1–84 transiently enhance calcemic

activities in the kidney ($\circled{3}$) and the gut ($\circled{4}$) to a degree that can cause hypercalcemia and perhaps shunting of Ca^{2+} into the bone ($\circled{10}$). As a result, local $[Ca^{2+}]_e$ is elevated in the BRUs and promotes the differentiation and mineralizing functions ($\circled{8}$) of the OBs recruited by the injected PTH ($\circled{5}$). Although the increased OB activity is also anticipated to promote osteoclastogenesis ($\circled{6}$) in the BRUs, this effect could be transient due to a short half-life of PTH (in minutes) and a negative feedback of the increasing $[Ca^{2+}]$ ($\circled{9}$) at least before the Ca^{2+} is redeposit into the matrix. The above events together give an anabolic window for a bone gain. We hypothesize that continuous infusion of PTH eventually allows osteoclastic activities to surpass the osteoblastic activities due to the enhancement of RANKL/RANK signaling, therefore producing catabolic effects on bone. According to this scheme, the efficacy of iPTH treatment will highly depend on the availability of Ca^{2+} and calcemic functions in the kidney and the intestine. This may explain for the considerably variable efficacies of the treatment in patients. This regulatory scheme also critically relies on a functional CaSR in OBs. Indeed, a blunted anabolic effect of iPTH was recently observed in the $^{OB}CaSR$ $^{flox/\ flox}$ mice [202]. The latter study also raises the possibility of targeting the CaSRs in OBs and OCLs to enhance skeletal anabolism.

We have proposed to co-inject calcimimetics to enhance the anabolic effects of iPTH. Calcimimetics are non-ionic allosteric CaSR agonists that are being used clinically to treat HPT and hypercalcemia by potentiating extracellular Ca^{2+}-induced inhibition of PTH secretion and thereby suppressing the calciotropic actions in the kidney, intestine, and bone [203–206]. We theorize that transient activation of CaSR in PTCs with calcimimetics will dampen the secretion of endogenous PTH1–84, and therefore reduce its calciotropic activities in the kidney ($\circled{3}$) and gut ($\circled{4}$), therefore alleviating some of the adverse hypercalcemic effects in patients also receiving iPTH (Figure 1D). According to our working model, the injected PTH will continue to promote OB ($\circled{5}$) and then OCL activities ($\circled{6}$) in the BRUs. Although a smaller increase in sCa^{2+} level is anticipated to give a less increase in local $[Ca^{2+}]_e$ in the BRUs ($\circled{10}$), when compared to iPTH treatment alone, the injected calcimimetic is anticipated to enhance the Ca^{2+}-responsivenes of OB by shifting the Ca^{2+} set-point of the CaSR to the left and therefore promote the differentiation and functions of OBs ($\circled{8}$). The inhibitory actions of calcimimetics on OCL activities are anticipated to slow down bone resorption by inhibiting OCL recruitment, differentiation and survival ($\circled{9}$), therefore expanding the anabolic window. The actions of both agents together are expected to produce more robust anabolism with less or no hypercalcemia. Preliminary studies indeed showed that daily co-injections of a calcimimetic, (NPS-R568, 20 nmole/kg) with PTH1–34 (40–80 μg/kg) for 4–6 weeks in both adult (3 months old) male and aging (12 months old) female mice (i) completely prevented the development of hypercalcemia, (ii) produced anabolic effects on trabecular bone that was 2–3 fold more robust than that with iPTH treatment alone, and (iii) produced significant anabolic effects and increased bone strength at cortical sites, which were absent with iPTH treatment alone [207]. The ability of this combined PTH/calcimimetic treatment to address the issue of hypercalcemia may allow use of higher doses of PTH to build more bone mass perhaps over a shorter time-course to minimize possible risks of osteosarcoma and make treatment more effective and cost less. Cinacalcet, an orally active calcimimetic, is approved to treat hypercalcemia in patients with

primary and secondary HPT and has been in clinical use for several years. Translation of this novel combination drug strategy to human disease therapy could be facilitated.

12. Conclusion

In cartilage and bone, close complementary interactions between CaSR and PTH1R signaling are required for smooth progression of chondrocyte, OB, and OCL differentiation. Regimens with combined pharmaceutics concurrently targeting these two receptors have the propensity of producing more robust anabolic bone effects than treatments with individual compound. However, the dosing of the compounds and timing (concurrent vs sequential) for the drug deliveries remain to be optimized. Based on their cDNA sequences, the CaSRs expressed in OBs and chondrocytes are identical to that cloned from the PTGs [29, 122]. Immunoblotting analyses, however, showed distinct glycosylation patterns of the receptor in chondrocytes and OBs compared to that in PTGs and in HEK-293 cells expressing CaSR cDNA [29]. This difference in post-translational modification could produce different pharmacological profiles of the receptor at different anatomical sites (e.g., OCLs in the resorbing pits), but this concept has not been formally addressed. In addition, the CaSR forms heteromeric complexes with type B γ-aminobutyric acid receptor (GABA$_B$R1 and R2) in OBs and chondrocytes [[103], and unpublished data]. In GABA$_B$R1-deficient chondrocytes, the ability of Ca^{2+} to stimulate acute signaling responses was reduced significantly [103]. Since GABA$_B$R1 and R2 are co-localized with the CaSR in many tissues at various levels [103, 104], it is plausible that different stoichiometric interactions among these receptors and perhaps with other members of family C GPCRs could produce receptor complexes with distinct pharmacological files in a cell-specific manner. These differences in receptor processing and complex formation provide opportunities for designs of tissue-specific compounds to enhance skeletal anabolism.

Acknowledgments

Abbreviations

1,25D	1,25-dihydroxyvitamin D
Agg	aggregan
ALP	alkaline phosphatase
BRU	bone remodeling unit
CaSR	extracellular calcium-sensing receptor
[Ca^{2+}]$_e$	extracellular calcium concentration
CDM, Col(I)	type I collagen
α$_1$(I)	alpha 1 subunit of the type I collagen
Col(II)	type II collagen

$α_1(II)$	alpha 1 subunit of the type II collagen
DMP1	dentin matrix protieon-1
HPT	hyperparathyroidism
IGF1	insulin-like growth factor
Ihh	Indian hedgehog
KO	gene knockout
Gcm2	glial cells missing homolog 2
GP	growth plate
GPC	growth plate chondrocyte
iPTH	daily injection (or intermittent) PTH treatment
M-CSF	macrophage colony-stimulating factor
μCT	micro-computed tomography
OB	osteoblast
OCL	osteoclast
OCN	osteocalcin
ON	osteonectin
OPG	osteoprotegerin
OPN	osteopontin
PG	proteoglycan
PTC	parathyroid cell
PTG	parathyroid gland
PTH	parathyroid hormone
PTH1R	parathyroid hormone 1 receptor
PTHrP	parathyroid hormone-related protein
RANK	receptor activator of nuclear factor kappa-B
RANKL	RANK ligand
Floxed-CaSR	control mice carrying loxP sequences flanking the exon 7 of the *Casr* gene
^{Exon5}CaSR−/−	mice with insertion a neomycin gene cassette into the exon 5 of the *Casr* gene
^{Cart}CaSR $^{flox/ flox}$	mice with constitutive ablation of the exon 7 of the *Casr* gene targeted specifically to chondrocytes

Tam-Cart**CaSR** flox/ flox	mice with tamoxifen-induced ablation of the exon 7 of the *Casr* gene targeted specifically to chondrocytes
OB**CaSR** flox/ flox	mice with constitutive osteoclast-specific ablation of the exon 7 of the *Casr* gene
PTH–/–	mice with global deletion of *Pth* gene
Gcm2–/–	mice with global deletion of *Gcm2*

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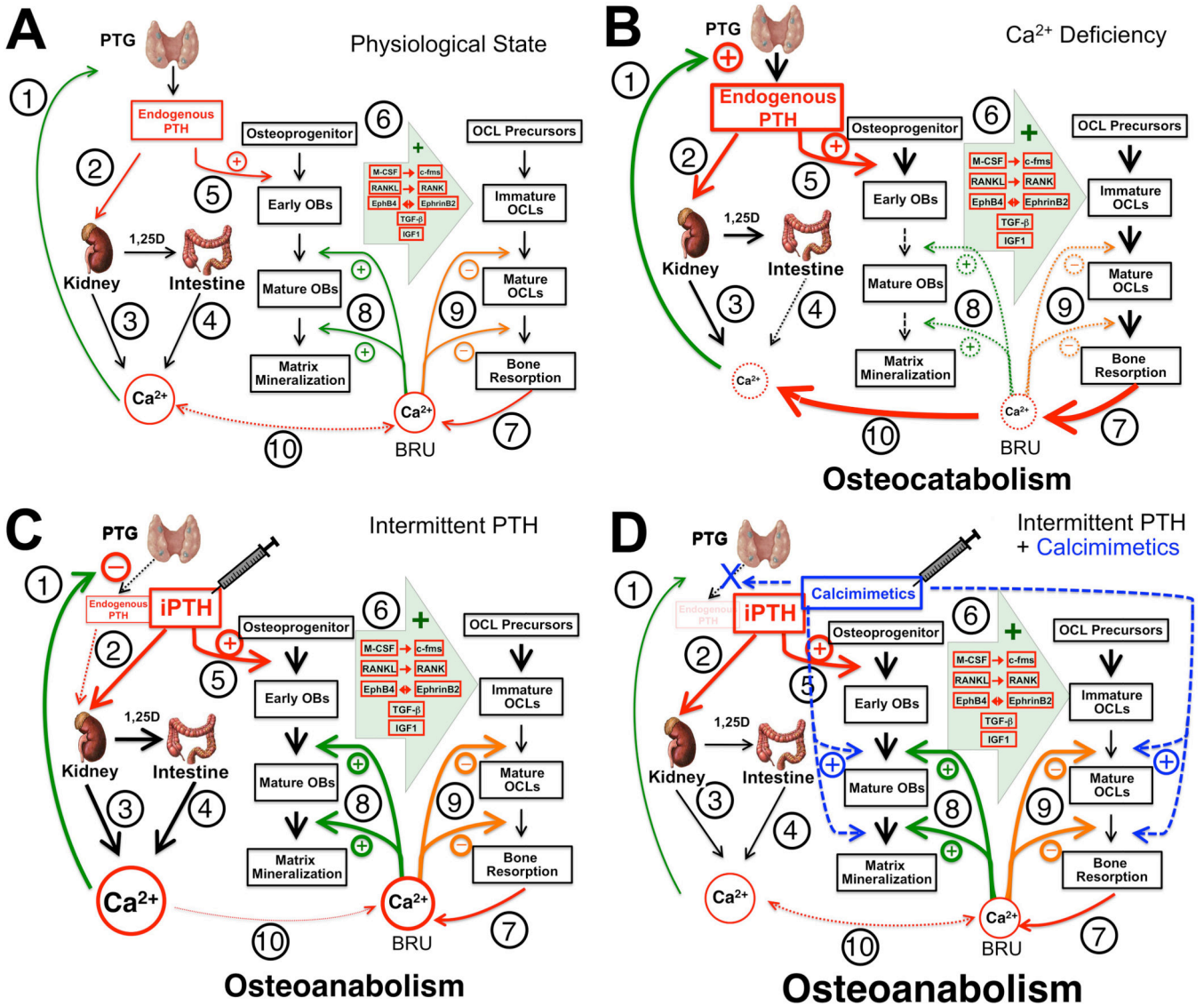
Figure 1.
Schemas for the actions of PTH/PTH1R and Ca^{2+}/CaSR signaling in the regulation of mineral and skeletal metabolism under (A) a physiological state and (B) Ca^{2+} deficiency and its responses to (C) iPTH or (D) combined iPTH and calcimimetics treatment. See the text for detailed descriptions.
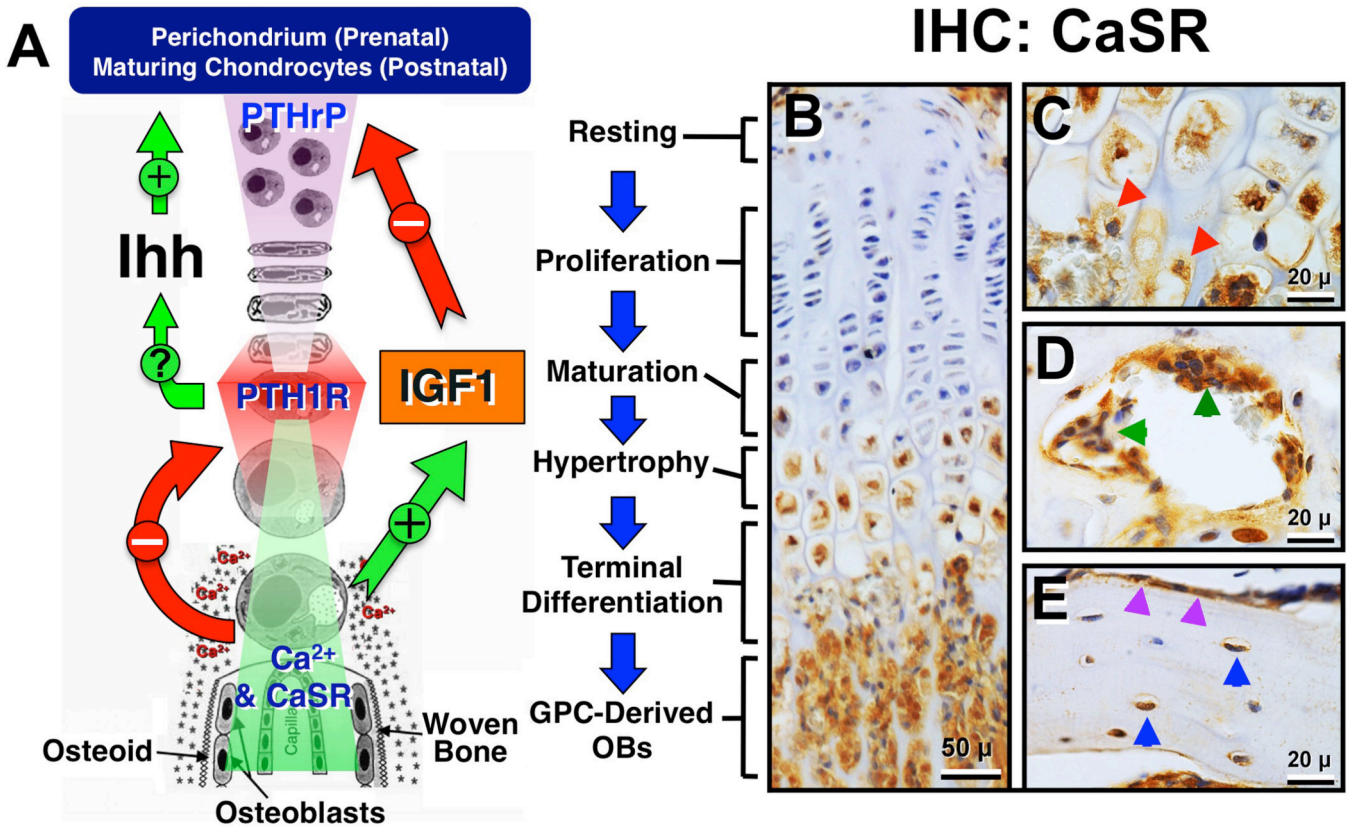
Figure 2.
(A) A schema for growth plate chondrocyte differentiation and its regulation by PTHrP/ PTH1R/Ihh, IGF1/IGF1R, and Ca^{2+}/CaSR signaling pathways. See the text for detailed descriptions. (B–E) Immunohistochemical (IHC) detection of CaSR protein in (B) mouse growth plate and primary spongiosa; (C) chondro-osseous junction; (D) resorbing pits in the secondary spongiosa, and (E) cortical bone of the tibia. Red, greed, purple, blue arrowheads depict terminally differentiated chondrocyte being released from cartilage matrix in (C), osteoclasts in (D), and bone-lining OBs and osteocytes in (E), respectively.
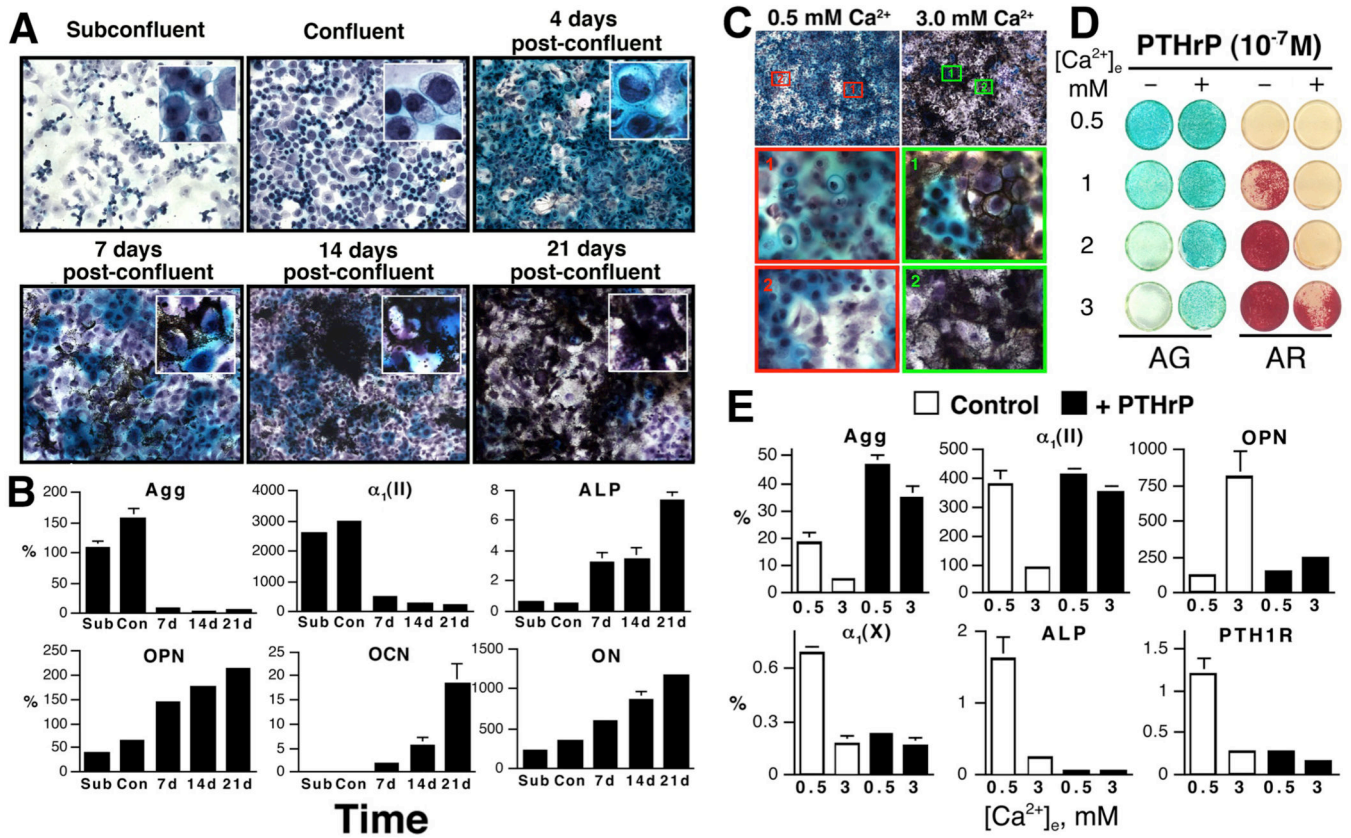
Figure 3.
(A) PG accumulation and mineral deposition by Alcian green and von Kossa staining, respectively, in mouse GPCs cultured for different times. Cultures were counterstained with hematoxylin. Insets: high-power (100×) views. (B) RNA levels, assessed by q-PCR, for Agg, and a$_1$(II), ALP, OPN, OCN, and ON, in mouse GPCs cultured for various times [subconfluent (Sub), confluent (Con), 7, 14, and 21 days post-confluence]. The level of gene is expressed as "%" of L19 expression. (C) Effects of different [Ca^{2+}]$_e$ on proteoglycans (PG) accumulation, mineralization in mouse GPCs cultured at 0.5 or 3.0 mM Ca^{2+} for 14 days after confluence and viewed at 20× and 100× in upper and 2 lower panels, respectively. (D) PG accumulation and mineral accumulation assessed by Alcian green and Alizarin red staining, respectively, and (E) RNA expression assessed by qPCR in mouse GPCs cultured at different [Ca^{2+}]$_e$ (0.5 to 3.0 mM) in the absence (−) or presence (+) of 10^{-7} M PTHrP for 14 days. RNA levels are presented as the percentage of ribosomal L19 gene expression.