Published in final edited form as:

Curr Pharm Biotechnol. 2009 April ; 10(3): 289–301.

The Role of the Calcium-Sensing Receptor in Bone Biology and Pathophysiology

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Abstract

Bone cells, particularly osteoblasts and osteoclasts, exhibit functional responses to calcium (Ca^{2+}). The identification of the calcium-sensing receptor (CaR) in parathyroid glands as the master regulator of parathyroid hormone (PTH) secretion proved that cells could specifically respond to changes in divalent cation concentration. Yet, after many years of study, it remains unclear whether this receptor, which has also been identified in bone, has functional import there. Various knockout and transgenic mouse models have been developed, but conclusions about skeletal phenotypes remain elusive. Complex endocrine feedback loops involving calcium, phosphorus, vitamin D, and PTH confound efforts to isolate the effects of a single mineral, hormone, or receptor and most models fail to account for other local factors such as parathyroid hormone related protein (PTHrP). We review the relevant mouse models and discuss the importance of CaR in chondrogenesis and osteogenesis. We present the evidence for a non-redundant role for CaR in skeletal mineralization, including our experience in patients with activating CaR mutations. Additionally, we review emerging research on the importance of the CaR to the regulation of serum calcium homeostasis independent of PTH, the role of the CaR in the hematopoietic stem cell niche with implications for bone marrow transplant, and early evidence that implies a role for the CaR as a factor in skeletal metastasis from breast and prostate cancer. We conclude with a discussion of drugs that target the CaR directly either as agonists (calcimimetics) or antagonists (calcilytics), and the consequences for bone physiology and pathology.

Keywords

calcium-sensing receptor; bone; metastasis; calcimimetic; calcilytic

Introduction

The calcium-sensing receptor (CaR), first cloned in 1993, is a G protein-coupled receptor that responds to multiple extracellular cations, with calcium as its major ligand [i]. The primary role of the CaR is to regulate parathyroid hormone (PTH) secretion and parathyroid hyperplasia in response to changes in extracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_e$). Since shortly after its discovery there has been a controversy as to whether the CaR is expressed and functions in the skeleton. This review will summarize the current knowledge regarding the role of the CaR in bone physiology and pathology.

CaR is also expressed in multiple human tissues not related to calcium homeostasis, such as the basal cell layer of human esophagus [ii], aortic smooth muscle cells [iii], oocytes [iv], and adipocytes [v]. In addition, CaR expression has been linked to numerous diverse functions including secretion of peptides, ion channel and transporter activity, proliferation, differentiation, apoptosis and chemotaxis [reviewed vi], and gastric acid secretion [vii].

Three human diseases have been linked to mutations in CaR. Inactivation of one allele results in familial hypocalciuric hypercalcemia (FHH) [viii], a relatively benign disease characterized by usually asymptomatic elevations in serum calcium (and, to a lesser extent, magnesium), relative hypocalciuria, and inappropriately normal or elevated PTH. While bone turnover tends to be marginally elevated, these patients have normal bone mineral density [ix,x] and are not at increased risk of fracture [xi]. Inactivation of both alleles, or expression of a dominant negative allele [xii], results in neonatal severe hyperparathyroidism (NSHPT) [viii]. This disorder presents in the very early postnatal period with failure to thrive, hypotonia and respiratory distress. Lab studies reveal severe hyperparathyroidism, hypercalcemia, hypophosphatemia, and an undermineralized skeleton with subperiosteal bone resorption. Furosemide, a calcium-wasting diuretic, and hydration are an effective short-term remedy, but without parathyroidectomy these patients usually die.

Activating mutations in the CaR cause a left-shift in the dose response curve of the parathyroid and renal tubule to both Ca^{2+} and Mg^{2+} , resulting in hypoparathyroidism with renal calcium and magnesium wasting and ensuing hypocalcemia, hyperphosphatemia, and hypomagnesemia. The familial form is autosomal dominant hypocalcemia (ADH) [xiii] but sporadic mutations also occur [xiv]. The skeletal phenotype in ADH is still being defined. Nephrocalcinosis and renal insufficiency can appear at a young age, particularly with over-replacement of calcium, and ectopic calcifications occur frequently. Autoantibodies that inhibit [xv,xvi] or activate [xvii] the CaR can cause acquired syndromes that mirror FHH and ADH, respectively.

CaR signaling

The CaR is a member of family C of G-protein coupled receptors (GPCRs) and bears both structural homology and some degree of ligand crossover with the metabotropic glutamate receptors and γ -aminobutyric acid receptors (GABA-R). The structure and signaling of CaR have been extensively reviewed [xviii,xix]. Briefly, based on amino acid sequence, the CaR is assumed to have a large “venus flytrap” extracellular domain, a characteristic seven transmembrane domain, and a smaller cytosolic domain. The parathyroid CaR couples through several G-proteins, including G_i , $G_{12/13}$ and G_q/G_{11} [xviii]. Ligand binding and activation of the receptor inhibits adenylate cyclase, decreasing cytosolic cAMP, and increasing production of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. These activate Akt (protein kinase B) and increase intracellular Ca^{2+} , and, through stimulation of phospholipase A_2 , C, and D, increase the production of arachidonic acid metabolites including cyclooxygenase-2 (COX-2) and prostaglandin E2. CaR activation is also linked to various mitogen activated protein kinases (MAPKs) including ERK 1 and 2, p38, and JNK. Protein kinase C exerts negative regulatory control over CaR signaling through active sites on the cytosolic domain. Of importance in bone, CaR can also transactivate the epidermal growth factor (EGF) receptor (EGFR) resulting in activation of Ras/MEK/ERK1/2 pathways and parathyroid hormone related protein (PTHrP) secretion [see for review xx].

Homo- and heterodimerization is common in family C GPCRs and is well established in CaR [xxi,xxii,xxiii]. Though the functional significance of heterodimerization is unknown, early data showing that a type B GABA-R, both physically and functionally, interacts with CaR in growth plate chondrocytes [xxiv]. This may be crucial for understanding the diverse array of

functions attributed to CaR in that tissue. To our knowledge, no bone-cell specific CaR signaling pathways have been reported.

There are two types of CaR agonists. Type I agonists, including calcium and other divalent (Mg^{2+}) and polyvalent cations (gadolinium, neomycin, polyamines), independently activate the receptor leading to the signaling cascades described above. In contrast, Type II agonists are positive allosteric modifiers that potentiate the response to type I ligands. These include aromatic amino acids and the calcimimetic drugs, only one of which (cinacalcet HCl) is currently FDA approved. Finally, calcilytics are a class of drugs being developed as small molecule inhibitors of the CaR.

Is CaR expressed in bone?

The cellular components of bone that express CaR are controversial, though CaR has been detected using various methodologies in osteoblasts, osteoclasts, and osteocytes. Initial studies found CaR mRNA expression in multiple bone marrow cells, including those in the osteoblast lineage [xxv], and in MC3T3-E1 cells, an osteoblast-like cell line [xxvi], and in culture of mature rabbit osteoclasts [xxvii]. CaR protein has been directly detected by immunocytochemistry, and immunoblotting in mouse, rat, and bovine osteoblasts, osteocytes, and chondrocytes as well as in various osteogenic cell lines (SaOS-2, UMR-106, ROS 17/2.8, MC3T3-E1) [xxviii]. Chang *et al* further demonstrated IP_3 and Ca^{2+} responses to increased $[Ca^{2+}]_e$ in all but one of these cell lines, similar to what is seen in “gold-standard” parathyroid cells [xxviii] (discussed in previous section).

Two groups have proposed the presence of a cation-sensing mechanism functionally similar to, but molecularly distinct from CaR in bone cells. Both detected at least partial responses to known CaR agonists, yet failed to detect CaR transcripts or protein in human and murine osteoblast cell lines [xxix,xxx], and a human osteoclast-like cell line [xxxi]. Extending their observation, Pi *et al* examined osteoblasts from wild type and $CaR^{-/-}$ mice and failed to detect CaR RNA in either cell type, yet documented normal functional responses to various CaR agonists (calcium, gadolinium, aluminum) in both [xxxii]. Another group found that primary cultures and cell lines of normal adult human osteoblastic and osteoclastic cells responded in a dose-dependent manner to calcium but not the calcimimetic cinacalcet HCl [xxxiii], providing further support for an alternative cation receptor on bone cells. Adding to the controversy, in a review of osteoclastic resorption, Zaidi *et al* described evidence for a ryanodine receptor that may function both as a calcium sensor and channel [xxxiv]. Also, Tu *et al* describe an intracellular calcium-binding protein, calcyclin, whose transfection confers calcium-sensing ability onto cells [xxxv].

Shoback and Chang, however, have suggested that the ability of $CaR^{-/-}$ mouse to respond to calcium may be due to the presence of CaR splice variants. It has been suggested, but never definitively demonstrated, that via alternative splicing exon 5 deficient $CaR^{-/-}$ mice have the ability to signal. Thus, it is possible that an alternatively spliced CaR transcript could explain the normal functional response of cells from knockout mice [xxxvi]. Indeed, there are data from $CaR^{-/-}$ mouse and human growth plate chondrocytes that show they not only express CaR splice variants but that these variants may mediate the cellular response to $[Ca^{2+}]_e$ [xxxvii]. A full mechanism has not been elucidated for any of the putative cation-sensing receptors and it has yet to be demonstrated that the CaR lacking exon 5 has any activity. Further study is needed.

Role of CaR in cartilage

Development and remodeling of cartilage is critical for endochondral bone formation, longitudinal growth, and craniofacial development [xxxviii]. Calcium is an important extracellular signal during chondrogenesis and, clinically, paucity of calcium results in soft,

demineralized, deformed growth plates, growth abnormalities, and rickets. Dietary calcium replacement cures rickets in calcium-deficient children and vitamin-D receptor knockout mice [xxxix]. CaR has been detected by *in situ* hybridization, immunocytochemistry, immunoblotting, and RT-PCR in articular and hypertrophic chondrocytes but was absent in proliferating and maturing chondrocytes [xxviii], possibly specifying regiospecific calcium signaling. Increasing $[Ca^{2+}]_e$ activates CaR and drives differentiation in cultured chondrocytes [xl,xli]. Calcimimetics stimulate chondrocyte proliferation and hypertrophy [xlii] while transfection of a CaR mutant with defective signaling inhibits functional responses to $[Ca^{2+}]_e$ [xli], showing that CaR mediates these functions in chondrogenesis (for review see [xlili]).

As in bone, chondrocytes from CaR^{-/-} mice elicit functional responses to increasing $[Ca^{2+}]_e$, indicating the presence of functional splice variants [xlili] or alternate receptors. Unfortunately, the homozygous knockout of cartilage-specific CaR is embryonic lethal in mice [xliv]. A tamoxifen-inducible chondrocyte-specific CaR knockout was recently reported [xlv]. Using the type II collagen α_1 promoter, Chang *et al* found that knockout mice had shorter skeletons, undermineralized growth plates, and fewer mature chondrocytes as demonstrated by decreased expression of type X collagen, RUNX2, and osteopontin, though markers of early chondrocyte differentiation (aggrecan, collagen II) were unaffected. It remains unclear why cartilage-specific CaR knockouts are embryonic lethal while CaR^{-/-} mice survive, and is one of the observations that has led some to propose that the knockout of CaR signaling is incomplete in that model.

Role of CaR in bone

Despite the lack of a detailed mechanism and disagreement over the identity of the cation receptor(s) on bone cells, there is general agreement that bone cells respond to calcium, and that high extracellular calcium stimulates proliferation in osteoblasts [xxvi] and inhibits osteoclastogenesis [xlvi]. Implications are potentially far-reaching: there are varying degrees of evidence suggesting a role for CaR in osteogenesis, the regulation of serum calcium homeostasis, homing of cancer metastases to bone, and as a factor that “couples” bone formation to resorption in bone remodeling [xlvi]. Bone remodeling is the carefully regulated process whereby osteoclasts resorb bone, followed closely by the deposition of new bone by osteoblasts. Though systemic factors such as PTH are important for regulating the overall balance of remodeling, there is substantial evidence that local autocrine and paracrine factors, mostly cytokines and growth factors, are involved in cell recruitment, chemotaxis, differentiation, and activation [xlvi]. The identification of the CaR makes two potentially substantial additions to the model: to support chemotaxis of osteoblast precursors to the site of resorption via calcium gradients, and to inhibit osteoclasts via elevated $[Ca^{2+}]_e$, thereby closing the negative feedback loop.

Development of a CaR knockout mouse quickly followed identification of the CaR gene and receptor [xlviii]. Mice with heterozygous (+/-) deletion of CaR mimic the phenotype of FHH with a modest elevation of serum calcium and relative hypocalciuria. Homozygous knockouts (-/-) mimic NSHPT with severe hypercalcemia, parathyroid hyperplasia, and early neonatal death. CaR^{+/-} mice had no skeletal abnormalities by whole body radiography while CaR^{-/-} mice showed marked reduction in radiodensity of all bones, kyphoscoliosis, and bowing of the long bones.

It was unclear at that time if the skeletal abnormalities in CaR^{-/-} mice were due to the gene knockout *per se* or were secondary to the severe metabolic abnormalities. Following the observation that parathyroidectomy is curative in humans and rapidly reverses the skeletal abnormalities [xlix,l], Kos *et al* attempted to untangle the confounding by crossing CaR^{-/-} mice

with $Pth^{-/-}$ mice [li]. The genetic ablation of *Pth* was sufficient to rescue the biochemical phenotype of $CaR^{-/-}$ mice, though mice were paradoxically rendered hypercalcemic with wide variation in serum and renal calcium handling (see standard deviations in Table 1). In light of more recent preliminary data showing that $Pth^{-/-}CaR^{-/-}$ mice are hypocalcemic (the second value given in the table) [lii], it is likely that over treatment with dietary vitamin D accounts for the hypercalcemia observed in the $CaR^{-/-} Pth^{-/-}$ double knockout mice. There were no reported differences in skeletal histology, mineral apposition rate (MAR), or bone formation rate (BFR) between $Pth^{-/-}CaR^{+/+}$ mice and $Pth^{-/-}CaR^{-/-}$ mice. Although bone mineral density (BMD) was increased in female double knockouts relative to sex-matched $Pth^{-/-}$ littermates, these data posit only a minor role, if any, for CaR in skeletal development. However, the utility of the $Pth^{-/-}CaR^{-/-}$ double knockout in delineating the role of the CaR in bone is limited due to the fact that they have a unique skeletal phenotype characterized by reduced resorption and increased cortical and trabecular bone volume [liii], findings also seen in $Pth^{-/-}$ mice and hypoparathyroid humans. This raises the possibility that in this model loss of PTH signaling may confound independent effects of CaR on the skeleton. Stimulation of a compensatory pathway *in utero* is another possibility [liv].

Similar findings were obtained by creating $Gcm2^{-/-}CaR^{-/-}$ double knockouts [lv]. *Gcm2*, the mouse homologue of the *Drosophila* glial cells missing gene, encodes a transcription factor specifically expressed in developing parathyroid glands. $Gcm2^{-/-}$ mice lack parathyroid glands but are only mildly hypoparathyroid due to ectopic thymic PTH production. Double knockouts show no major skeletal abnormalities, again offering little evidence of a non-redundant role for CaR in skeletal development. However, while some have claimed that these mice have normal skeletal growth and development [lv], they demonstrate reduced bone turnover and increased bone volume [lvi] like $Pth^{-/-}$ mice, again raising the possibility of confounding, similar to that seen in the $Pth^{-/-} CaR^{-/-}$ model.

Utilizing the $CaR^{-/-}$ mouse model, Garner *et al* performed a more sophisticated analysis of the $CaR^{-/-}$ skeletal phenotype and, surprisingly, reported that the predominant abnormality was rickets [lvii]. There were no differences between heterozygous and wild type mice, but $CaR^{-/-}$ mice demonstrated widened epiphyseal cartilage and rachitic changes, delayed endochondral bone formation, and osteomalacia characterized by excessive osteoid and impaired mineralization. While there is overlap between hyperparathyroid changes and those in the $CaR^{-/-}$ mice, osteomalacia is not a typical skeletal abnormality seen in primary hyperparathyroidism (or in NSHPT, the human disease analogue). Therefore, it is difficult to determine what the significance of these findings in this rodent model system.

Furthermore, mouse models of PTH over-activity have been developed and mirror many, but not all, of the skeletal features in $CaR^{-/-}$ mice. Mice with constitutively active PTH/PTHrP receptors have markedly delayed endochondral bone formation and decreased mineralization, similar to what is seen in $CaR^{-/-}$ mice, but have a skeletal phenotype with shortened, deformed limbs not seen in $CaR^{-/-}$ mice [lviii]. While $CaR^{-/-}$ mice showed only an enlarged hypertrophic zone of growth plate cartilage, transgenic mice that overexpress PTHrP in chondrocytes show increases in both proliferative and hypertrophic chondrocytes [lix]. Yet mice engineered with parathyroid specific knockout of two G-proteins involved in CaR signaling, essentially creating a parathyroid-specific CaR knockout (with resultant PTH excess but supposedly normal skeletal CaR), very closely resembled the phenotype seen in $CaR^{-/-}$ mice, with increased serum calcium and PTH, parathyroid gland hyperplasia and delayed bone formation [lx]. These PTH- $G\alpha_q/G\alpha_{11}$ -double knockouts were diffusely osteopenic and had rachitic changes similar to what was described by Garner *et al*, but had abnormal craniofacial development not seen in $CaR^{-/-}$ mice. More recently, Chang *et al* developed a novel CaR knockout targeting exon 7 [xlv], chosen to avoid any potential activity of the exon 5 splice variant as previously discussed. Using a Cre recombinase driven by the PTH promoter they created a “parathyroid-specific”

CaR knockout (PTH-Cre/CaR^{flox/flox}). Like other hyperparathyroid mice, this mouse also has a small, undermineralized skeleton and high neonatal mortality. Importantly, further molecular analysis showed an unexpected 70% reduction in CaR mRNA in the bones. To our knowledge, a hypercalcemia-induced reduction in CaR expression has never been observed, though increased PTH signaling or an idiopathic effect of the floxed CaR allele might have effects in tissues not targeted by the Cre recombinase. As in this and some other models, early perinatal mortality limits both biochemical and skeletal analysis to immature animals. More detailed analysis is needed to determine if osteomalacia is present in these models.

Seeing conflicting results using models of CaR inactivation, some investigators turned their attention to the other extreme of CaR activity. Using a mutagenic screen, Hough *et al* identified and characterized a mouse with an activating CaR mutation that mimics some of the findings in ADH [lxi]. Dubbed *Nuf* after the ocular “nuclear flecks” that were later identified as cataracts, the mouse is hypocalcemic, hyperphosphatemic, and has suppressed PTH. Dose-response to calcium is left-shifted, indicating greater receptor-ligand affinity. There are important departures from human ADH, however. Ectopic calcifications are ubiquitous in the mouse, unlike nephrocalcinosis, cataracts, and basal ganglia calcifications that are commonly seen in ADH. Furthermore, *Nuf* mice lack hypercalciuria and appear to be normomagnesemic. A skeletal phenotype was not described.

Using the osteocalcin promoter, Dvorak *et al* created a transgenic mouse with constitutively active CaR targeted to osteoblasts [lxii]. Following the observation that increased $[Ca^{2+}]_e$ increased osteoblast activity and inhibited osteoclastogenesis (discussed previously), we might expect that constitutive activity of the osteoblast CaR would lead to unopposed bone formation. Micro computed tomography instead revealed reduced cancellous bone volume and connectivity. Receptor activator of nuclear factor kappa-B (RANK) ligand (RANKL), the major stimulator of osteoclast activation and differentiation, was upregulated in osteoblasts cultured from mutant calvaria and femora, and histomorphometry confirmed an increase in osteoclasts. Mice had normal serum calcium and PTH, however, implying that local CaR signaling within bone can affect turnover absent systemic changes in calciotropic hormones.

These local changes may be effected not through PTH, but through a related hormone that shares its receptor, PTHrP. A recent review discusses evidence that CaR may effect local changes in PTHrP absent changes in systemic calcium or PTH concentration [xx]. Serum PTHrP levels are ordinarily low, although tissue distribution of PTHrP and its receptor is vast, implying an important role in local autocrine and paracrine signaling. Depending on the tissue type, CaR activation may lead to up- or downregulation of PTHrP. In bone, increasing extracellular calcium stimulates PTHrP production which upregulates RANKL and stimulates osteoclastogenesis. So while PTH and PTHrP share a receptor, independent regulation may allow either synergistic or antagonistic activity depending on *local* factors.

Returning to the transgenic mouse, it is unclear why the findings in the mouse model departed from the human phenotype. Patients with activating CaR mutations (ADH) present with nearly the opposite bone phenotype seen in the transgenic mouse described above. Children with ADH have normal BMD while adults have elevated BMD [lxiii]. Histomorphometry on a small number of children with ADH failed to show increased markers of osteoclast activation (eroded surface) [xiv]. The significance of the discrepancy is unknown but could reflect species differences.

Paradoxically, conditional ablation of CaR (by deletion of exon 7) in early osteoblasts using the collagen I promoter resulted in markedly reduced mineralization, bone volume, BMD and thickness of cancellous and cortical bone, as well as decreased cancellous connectivity and increased cortical porosity [lxiv]. As in the constitutively active transgenic mice, RANKL was

upregulated. Similar to observations in the parathyroid-specific knockout (PTH-Cre/CaR^{flox/flox}), genes important for bone development were markedly downregulated (Col I, alkaline phosphatase, DMP1, osteocalcin, sclerostin, and IGF-1, a gene important for overall growth as well as osteoblast differentiation) [xlv]. A second osteoblast-specific knockout using the osterix promoter produced a similar skeletal phenotype with delayed growth, abnormal cancellous and cortical bone, and decreased expression of genes encoding osteoblast markers [xlv]. Osterix is both more bone-specific than type 1 collagen and is expressed earlier in osteoblast development [lxv]. Conversely, ablating CaR in mature osteoblasts using the osteocalcin promoter resulted in no difference in bone volume, BMD, or growth compared to normal littermates [lxvi].

Though it is tempting to conclude the presence of an embryonic compensatory pathway, or that bone-specific CaR plays a role in skeletogenesis absent changes in systemic PTH, interpretation of these preliminary findings should be guarded. As in nearly all models under discussion, mechanistic determinations are impossible without side-by-side analysis of the systemic PTH-mineral axis and local PTHrP activity. PTHrP production has been demonstrated in both early and mature osteoblasts [lxvii], suggesting that it could play a role in one or both of these transgenics. Experiments which block local PTH/PTHrP production are needed to confirm if CaR mediates its bone specific effects through PTHrP signaling.

Despite years of work with a variety of knockout and transgenic mice, firm conclusions regarding a role for CaR in bone development remain elusive. CaR^{+/-}, CaR^{-/-} and *Nuf* mice very closely mimic the biochemical phenotype of their human disease counterparts FHH, NSHPT, and ADH respectively. Still, detailed skeletal analysis remains for the *Nuf* mouse and known differences in skeletal phenotype between CaR^{-/-} mice and NSHPT limit their use. The larger question, whether CaR is important for skeletogenesis, also remains unanswered. Various models designed to “rescue” the CaR phenotype and models of PTH excess or over-signaling through the PTH/PTHrP receptor suggest that most features of the CaR^{-/-} phenotype are secondary to PTH excess or altered mineral homeostasis. Though it remains unclear if CaR lacking exon 5 has functional activity, even the exon 7 CaR knockouts seem to affect the wider IGF-1 axis, making it difficult to attribute growth delay to calcium signaling. Furthermore, control mice from Chang *et al* were hypercalcemic relative to other studies [xlv], possibly suggesting that the floxed CaR allele itself has activity even without a Cre recombinase. Yet, important differences have also been observed and we are left to wonder why rescue is incomplete when CaR^{-/-} is crossed onto *Pth* null or *Gcm2* null backgrounds. The omission of crucial biochemical and histomorphometric data, even the use of different diets with varying amounts of vitamin D, in published reports also complicates interpretation. Few studies measured or controlled for local PTHrP production. None of the mice with rickets had reported FGF23 levels, a phosphatonin important in the pathogenesis of rickets and tumor-induced osteomalacia. The possibility of species differences or compensatory pathways, most obvious in the transgenic models targeting activating mutations to osteoblastic cells, is also a possibility, although data are still preliminary. Despite these unknowns, there is convincing data suggesting a role for CaR in other processes.

CaR and Mineralization

As alluded to above, CaR may regulate osteoblast and osteoclast function vis-à-vis bone mass and mineralization. High [Ca²⁺]_e increases matrix mineralization and blocks expression of early chondrocyte markers (proteoglycan, aggrecan) in favor of markers of terminal differentiation (osteopontin, osteonectin, osteocalcin) in maturing chondrocytes [xli]. Overexpression of a CaR with defective signaling blocked the response to high [Ca²⁺]_e, showing that CaR regulates chondrocyte matrix production and mineralization [xli]. In osteoblasts, high [Ca²⁺]_e stimulates differentiation and mineralized nodule formation, a

response that is blocked by the CaR inhibitor NPS 89636 [lxviii]. In agreement with these findings, expression of an antisense CaR oligonucleotide or dominant negative CaR vector suppressed Ca^{2+} -induced alkaline phosphatase activity, osteocalcin expression, and mineralization (by both Alizarin red and von Kossa staining) in osteoblast culture [liv]. One group, however, found that while high extracellular calcium increased osteocalcin and mineralization of human mesenchymal stem cells, the CaR agonist neomycin did not [lix]. The significance of this finding is uncertain but could reflect possible differences in ligand affinity for the CaR on hMSCs and more differentiated cell types.

We recently found low average bone mineral density distribution, as determined by quantitative backscattered electron imaging (qBEI), in three children with ADH that was not corrected with PTH replacement [xiv]. PTH replacement resulted in a dramatic increase in cancellous bone volume and trabecular number on histomorphometry. Bone mineral density lagged behind expected values given the increased bone mass, suggesting poor mineral content. Using qBEI we found low average bone mineralization at baseline in two adolescent girls with ADH. After one year of PTH treatment, average bone mineralization was even lower, presumably due to the formation of a significant amount of new bone of lower mineralization density. A shift toward lower mineralization with greater heterogeneity is also seen in osteoporotic patients treated with intermittent PTH for 18-36 months, although to a lesser degree [lxx]. Randomized studies in ovariectomized monkeys have demonstrated that intermittent administration of PTH increased bone mineral content at the spine, hip, and long bones [lxxi], but that bone had lower mineralization and reduced mineral crystallization by Fourier transform infrared imaging [lxxii].

Notably, though still “left-shifted” compared with normal adults, a bone sample from a 19-year old woman with ADH treated with PTH for 13 years had the same mineralization characteristics as the two hypoparathyroid patients mentioned above, *prior* to receiving PTH. This is suggestive that the underlying CaR mutation may contribute directly to abnormal mineralization and that the PTH-induced shift to lower mineralization may be overcome with time as more complete secondary mineralization ensues. We believe that altered serum mineral availability (hypocalcemia) likely does not contribute, as adults with hypoparathyroidism develop high bone mass despite hypocalcemia [lxxiii] and length of disease is positively correlated with BMD [lxiii]. Some have suggested that single nucleotide polymorphisms of the calcium-sensing receptor may influence BMD, although most studies find only a small, if any, contribution from CaR [lxxiv,lxxv].

In summary, there is conflicting evidence regarding the importance of CaR in skeletogenesis and mineralization. Future studies should address the presence of CaR splice variants and alternative cation receptors that may be present (and functioning) in $\text{CaR}^{-/-}$ mice. Though there is considerable overlap between each of three PTH “over-expressing” mouse models and the $\text{CaR}^{-/-}$ mouse, the differences suggest at least a local role for CaR in skeletogenesis and remodeling. In addition to controlling for serum phosphorus, systemic and local PTH/PTHrP signaling should be controlled for and evaluated independently as the effect of one likely conflates effects attributed to the other. A model of inducible CaR overactivity or deactivation could also be useful for investigating the role of CaR in skeletal development.

Does bone-specific CaR regulate plasma $[\text{Ca}^{2+}]$?

Plasma calcium concentration is maintained within a narrow range by regulating the flux of calcium in and out of the skeleton, intestine, and kidney. The current model involves a three-part negative feedback loop triggered when hypocalcemia elicits a rapid increase in PTH secretion from the parathyroid glands [lxxvi]. Increased PTH enhances distal renal tubular reabsorption of calcium, and upregulation of proximal tubule 1- α hydroxylase, the enzyme

which converts 25-(OH)D to its more active metabolite 1,25-(OH)₂D. Active vitamin D stimulates absorption of calcium and phosphate from the gut while, and in concert with PTH, reclaims stored calcium and phosphate from bone. These actions restore plasma [Ca²⁺] and close the feedback loop.

But some interesting observations seem to argue that those mechanisms, while important for setting the overall “calciostat” and maintaining long-term normocalcemia, are not responsible for restoring serum calcium after acute hypocalcemic stress. Contrary to common wisdom, a very rapid recovery from EGTA-induced hypocalcemia occurs in parathyroidectomized, thyroparathyroidectomized (TPTX), vitamin-D depleted, and nephrectomized rats [lxxvii,lxxviii,lxxix,lxxx]. While these models are not sufficient to implicate CaR directly in this process, some have argued that bone may sense acute changes in serum calcium and respond by altering calcium flux into or out of bone, independent of systemic hormones [lxxxi].

Bone stores the majority of body calcium but keeps the mineral in constant flux. Though calcium is liberated during remodeling, there is evidence of large-scale calcium flux at the quiescent surface unrelated to remodeling activity [lxxxii]. It is estimated that calcium flux across the quiescent bone surface is 12.5-100 mmol/day [lxxxiii,lxxxiv], vastly exceeding both the amount needed to correct induced hypocalcemia [lxxxv] and that associated with bone remodeling (10 mmol/day). Indeed, it has been shown that rapid calcium flux across the quiescent surface acutely buffers serum calcium in response to hyper- or hypocalcemic stress [lxxxiv] without changes in circulating PTH [lxxxvi]. These fluxes are probably cell-mediated and some have implicated the flat lining cells on trabecular and endocortical bone, which are derived from osteoblasts [lxxxiv], and therefore may express CaR.

Though the evidence remains circumstantial, we discussed previously that a high [Ca²⁺]_e stimulates osteoblast proliferation and inhibits osteoclastogenesis. The opposite also appears to be true. When osteoclasts are co-cultured with osteoblasts and bone marrow cells, exposure to a low-calcium environment increases osteoclast formation by inducing RANKL [lxxxvii]. Exposure to the CaR agonists gadolinium and neomycin reduced RANKL expression and osteoclast proliferation, implying that the CaR on osteoblasts may regulate bidirectional control on osteoblast/osteoclast behavior and potentially induce bone resorption in response to low [Ca²⁺]_e. Whether this activity is present in the lining cells is unknown.

In vivo observations also suggest that CaR participates in the regulation of [Ca²⁺]_e independent of PTH. PTH^{-/-}CaR^{-/-} double-knockout mice display wide variation in serum and urine [Ca²⁺] in contrast to normal serum calcium control in PTH^{-/-}CaR^{+/+} mice [li]. Possibly confounding their findings, animals in this study appeared to be over-treated with dietary vitamin D (serum calcium and phosphorus were paradoxically higher in the double knockout than in PTH^{-/-}CaR^{+/+} mice and *Pth*-null mice in this study had a mean serum calcium of 10.8 mg/dl vs. 6.8-7.5 mg/dl in other studies [lii,liii]). In a separate study Kantham *et al* showed that PTH^{-/-}CaR^{-/-} double-knockout mice become markedly hypercalcemic on a calcium-enriched diet, while PTH^{-/-}CaR^{+/+} mice remained normocalcemic [lii], again implying that, in the absence of PTH, the CaR is necessary for normal serum Ca²⁺ control. More convincingly, Huan *et al* used thyroparathyroidectomy to remove circulating PTH and calcitonin from rats followed by EGTA infusion to induce hypocalcemia. Rats were then treated with either of two CaR agonists (the type I agonist gentamycin or the calcimimetic R-568) or vehicle and the plasma [Ca²⁺] was allowed to recover. Both CaR agonists impaired recovery of plasma [Ca²⁺] [lxxxv], implying that CaR, at least in part, mediates acute (minutes to hours) recovery of [Ca²⁺]_e. These findings remain to be replicated. Again we emphasize that, as the CaR is upstream of other effectors such as PTHrP, other unmeasured local factors could also be

involved. Studies involving alternative CaR agonists, calcilytics, and other species may offer additional mechanistic insights.

CaR and the Hematopoietic Stem Cell Niche

Since 1978 when Shofield and colleagues proposed that hematopoietic stem cells (HSCs) reside in a highly specific niche that regulates the overall size of the pool and their development [lxxxviii], efforts to characterize that niche have increasingly acknowledged the role of bone. Adult hematopoietic stem cells (HSCs) reside within the bone marrow in close association with the endosteal surface of bone. Transplanted HSCs do not circulate randomly but instead lodge themselves at the same endosteal surface within hours of injection [lxxxix]. While new data confirms a role for CaR in stem cell engraftment, collected observations already established that mineralized bone is a prerequisite for normal HSC development.

During mammalian hematopoiesis, HSCs shift from the fetal liver to the bone marrow shortly after the onset of bone mineralization [xc]. Generating ectopic calcified tissue by injecting bone morphogenetic proteins induces hematopoiesis in that microenvironment [xci]. Transgenic mice for collagen X, a marker of hypertrophic cartilage beginning endochondral ossification, have deficient skeletogenesis and fail to develop mature hematopoiesis. Recent studies have also illuminated the importance of the osteoblastic cells to HSC localization, lodgment, and survival at the endosteal niche [xcii,xciii].

In a transgenic mouse model, conditional ablation of osteoblasts results in significant loss of bone and hematopoiesis [xciv]. Osteopontin, an acidic glycoprotein produced by osteoblasts (among other cell types), is highly enriched at the endosteal surface and was shown to be necessary for HSC lodgment [xcv] (see for review [xcvi]). Returning to the idea that the endosteal niche has uniquely enriched calcium content relative to serum, CaR expression has been demonstrated on HSCs [xxv], including the stem cell-enriched (lin⁻) Sca-1⁺ c-Kit⁺ (LSK) population [xcvii]. Finally, in a series of elegant experiments using CaR^{-/-} mice, Adams *et al* demonstrated that it is the mineral content of the niche dictates HSC localization via the CaR [xcvii].

Looking at antenatal CaR^{-/-} mice, it was found that primitive HSCs were abundant in the peripheral blood and spleen but sparse in the bone marrow, while HSCs from CaR^{+/+} mice were abundant in marrow. Furthermore, transplantation of CaR^{-/-} HSCs into lethally irradiated wild type mice revealed markedly fewer resident HSCs compared with HSCs from CaR^{+/+} mice, a lodgment failure that was not due to any measured host attachment factor. Lastly, CaR^{-/-} cells were shown to have a specific defect in attaching to collagen I, the most abundant bone ECM protein.

To our knowledge, there are no reports of a bone marrow phenotype associated with CaR mutations in humans. Still, drug therapies that target components of the stem cell niche are being studied for a potential effect on HSC mobilization and engraftment. Following the observation that osteoblast activation leads to enhanced stem cell growth, it was demonstrated in mice that PTH expands resident HSCs *in vivo*, increases HSC mobilization, enhances HSC engraftment following transplantation, and protects stem cells from cytotoxic chemotherapy [xcviii]. The subject was recently reviewed [xcix] and clinical trials of PTH to augment human bone marrow stem cell transplantation have begun. With CaR expression on both HSCs and surrounding stroma, strategies involving the use of calcimimetics or calcilytics have been suggested [c] but the US government clinical trials registry does not list any such effort at this time.

CaR and skeletal cancer metastasis

Stephen Paget formulated his now famous “seed and soil” hypothesis in 1889 after observing non-random patterns of tumor metastasis in the autopsies of 735 women who died with breast cancer [ci]. His surprising finding was the high incidence of tumor metastases in bone. While bone seems like an inhospitable environment for tumor growth, it is now well established that breast and prostate cancers, the second leading cause of cancer death in women and men, metastasize preferentially to bone. Recent work on CaR allows a reinterpretation of Paget's hypothesis: that CaR may impart a growth advantage on tumors that express it, and with respect to metastasis, may be the factor that signals to the seed (tumor) that it landed in soil (bone).

Successful skeletal metastasis requires chemotactic homing to the skeleton, extravasation within the bone, adherence to bone extracellular matrix (ECM), and finally growth and survival. Previous observations suggest that osteolysis, a characteristic feature of skeletal metastases, provides the driving force over these significant hurdles. For prostate cancer, skeletal metastases are most frequently found at sites of high bone turnover [cii], including the spine, pelvis, ribs, and the proximal metaphyses of long bones [ciii]. Even in classically osteoblastic lesions, such as prostate cancer, osteoclastic resorption is increased [civ]. Importantly, the process is mediated by osteoclasts themselves, not necessarily tumor cells [cv]. Stimulating osteoclasts with PTH increased metastatic seeding of bone [cii], while blocking osteoclast-mediated bone resorption with osteoprotegerin (OPG) [cvi] or bisphosphonates [cvii] reduced osteolytic lesions and bone metastatic burden, respectively.

In addition to carving out a geographic niche for tumor growth and invasion, it appears that osteolysis releases growth factors and calcium for autocrine signaling. Bone extracellular matrix (ECM) can be seen as a reservoir of growth factors, insulin-like growth factor (IGF)-I, IGF-II, fibroblast growth factor, platelet-derived growth factor, bone morphogenetic proteins, and transforming growth factor β (TGF- β) [cviii]. PTHrP is a product of breast and prostate cancer cells that functions to increase osteoclastic resorption of bone. Increased bone turnover releases growth factors that stimulate tumor progression and further PTHrP production, fueling a vicious cycle of tumor spread. In breast cancer, as expected, PTHrP expression is higher in osseous metastasis than in either soft tissue metastasis or the primary tumor [cix]. Reducing PTHrP activity using neutralizing antibodies [cx] or by knocking down gene expression [cxii] reduces osteolytic lesions and tumor burden.

CaR is expressed on normal mammary epithelium and responds to low Ca^{2+} by increasing production of PTHrP. Following classic negative feedback, PTHrP stimulates osteoclasts to resorb bone, releasing calcium, and signaling back through the CaR to reduce PTHrP production [cxii]. However, transformation to a malignant phenotype may involve subversion of the normal negative feedback that exists between CaR and PTHrP. Not only is the CaR overexpressed in breast and prostate cancer cell lines, but when cells were stimulated with increased calcium they *increased* PTHrP production [cxiii,cxiv], which may confer a growth advantage. Using an *in vivo* mouse model, one group found that induced expression of PTHrP converted a noninvasive prostate cancer cell line into one with skeletal progression [cxv].

To further investigate the role of CaR in tumor growth, Liao *et al* incubated three prostate cancer cell lines that have different tumorigenic and metastatic potential in low versus high calcium media. High calcium stimulated proliferation, but only in the two cell lines with high skeletal metastatic potential (PC-3, C4-2B) [cxvi]. Knockdown of the CaR via RNA interference decreased tumor proliferation *in vitro* as well as skeletal metastasis when injected into mice. This suggests CaR is responsible for the effect, however the investigators did not control for PTHrP production. It is well known that CaR activation (with Ca^{2+} and other polycations) stimulates PTHrP secretion in PC-3 prostate cancer cells [cxiv]. While Liao *et*

al cultured PC-3 cells in high versus low Ca^{2+} for six days, another study showed that PTHrP secretion was significantly higher in PC-3 cells using similar culture conditions after only 6 hours [cxvii]. Liao *et al* also observed that their LNCaP cells, which are androgen dependent, did not proliferate in response to increased calcium. LNCaP cells secrete PTHrP but, crucially, were shown not to respond to it in the absence of androgen [cxviii]. Liao *et al* did not add androgen to their media, possibly explaining the lack of response in that cell line. PTHrP stimulates prostate cancer proliferation and tumorigenesis *in vitro* [cxix], although its definitive role *in vivo* is still unknown. Therefore, while Liao *et al* mention the CaR/PTHrP axis, this analysis provides further evidence that PTHrP may actually mediate the proliferation and metastasis attributed to the CaR.

CaR may still be a robust target for intervention in cancer, however. Liao *et al* found that CaR activation (using both calcium and neomycin) increased attachment of PC-3 prostate cancer cells to extracellular matrix *in vitro*. Increased adherence was reduced by the addition of pertussis toxin, a CaR antagonist. PTHrP production may also respond to drugs targeting CaR pathways. CaR signals, in part, through MAP kinase to stimulate PTHrP. Blocking CaR-induced MAP kinase pathway elements inhibited calcium-induced PTHrP release [cxx]. TGF- β , one of the growth factors released from bone matrix, also acts synergistically with CaR agonists to stimulate PTHrP production [cxiii]. For further discussion of CaR and signal transduction in cancer see Kingsley *et al* [cviii].

Despite these mechanistic findings, clinical correlations remain elusive. PTHrP-positive primary breast tumors are associated with a *reduced* risk of osseous metastasis and better prognosis [cxxi]. This could be confounded by correlation between PTHrP status and progesterone receptor status [cxxii], which is independently associated with favorable prognosis and response to endocrine therapy [cxxiii]. It was also recently demonstrated that PTHrP has natural antiangiogenic properties [cxxiv]. However, overexpression of CaR in breast tumor samples is correlated with bony metastases but not visceral metastases [cxxv], as expected, although selection bias for advanced cases may contribute to this finding. Interestingly, PTH levels were negatively correlated with survival in a clinical study of a vitamin D analogue in advanced (androgen independent) prostate cancer [cxxvi]. Vitamin D both inhibits PTH secretion and has antiproliferative effects on prostate cancer cells, possibly confounding their result.

Clearly more work is needed to evaluate the role of CaR in skeletal metastasis. The existing data suggest that the CaR is upstream of multiple processes that support skeletal tumor progression including osteoclast-mediated osteolysis, PTHrP production, and tumor cell adhesion. Though a better understanding of CaR signaling is needed, the potential application of CaR-modifying therapies to block tumor metastasis or as adjuvant chemotherapy is exciting. CaR may also serve as a useful biomarker for predicting skeletal progression of tumors.

CaR, however, is just one of a number of receptors under investigation in skeletal metastasis. Prostate tumor cells express a number of “bone specific” markers including CaR, osteopontin, osteocalcin and bone sialoprotein [cxxvii] (see for review [cxxviii]). The chemokine CXCL12/SDF-1, which is highly expressed in bone marrow (among other tissues) may also be important. CaR^{-/-} HSCs successfully localized to the endosteal niche using a SDF-1 gradient, though they could not lodge, suggesting that CaR functions downstream of SDF-1 in HSC homing to bone [xcvii]. Breast cancer cells and their metastases express CXCR4, the SDF-1 ligand, and their interaction promotes chemotaxis and invasion [cxxix]. Blocking SDF-1 signaling reduced metastasis. Experiments using CaR^{-/-} mice may help to further elucidate the role of the CaR in directing tumor metastasis.

Drug therapy

Calcimimetics are small orally active agents that act as positive allosteric modifiers of the CaR. As type II agonists they do not activate the receptor alone but rather increase receptor sensitivity to the type I agonists calcium and polycations (e.g. neomycin, gadolinium). In practice, they shift the PTH-Ca²⁺ dose-response curve to the left, reducing serum PTH and [Ca²⁺]. Their use has been extensively reviewed [cxxx]. One agent, cinacalcet HCl (also known as AMG 073, NPS 1493, Sensipar, and KRN 1493 in Asia), received regulatory approval in March 2004 for the treatment of secondary HPT in chronic renal failure and parathyroid carcinoma. In two small studies of patients with chronic kidney disease, cinacalcet use reduced biomarkers of bone turnover [cxxx], reversed bone loss at the proximal femur, but did not improve BMD at the lumbar spine [cxxxii]. In a year-long placebo controlled trial in patients with primary hyperparathyroidism, cinacalcet administration increased biomarkers of bone turnover (though they remained in the normal range) despite a ~20% drop in serum PTH levels, but had no effect on BMD after one year compared to placebo [cxxxiii]. A meta-analysis of four double blind placebo controlled trials of cinacalcet in ESRD found that randomization to cinacalcet reduced the risk of fracture more than 2-fold [cxxxiv].

In summary, it seems that the use of calcimimetics in primary or secondary HPT is not harmful to bone, although long-term data and more detailed analysis by histomorphometry and quantitative backscattered electron microscopy are needed. While current studies do not rule out the possibility of a direct effect of cinacalcet on bone, the observed changes are likely secondary to changes in PTH.

Calcilytics are antagonists at the CaR that stimulate PTH secretion. They are being developed for the treatment of osteoporosis under the auspices of stimulating a short burst of endogenous PTH which, in theory, will have a similar anabolic effect as exogenous PTH (teriparatide, Eli Lilly) given intermittently. The development of calcilytics was recently reviewed [cxxxv]. Unfortunately, the published data have yet to show convincing proof of principle. Administration of the calcilytic NPS 2143 to ovariectomized rats caused an increase in PTH levels and biomarkers of bone turnover but no change in bone density or net gain of bone mass by dynamic histomorphometry [cxxxvi]. The lack of a beneficial skeletal effect of NPS 2143 (or other calcilytics) is likely due to the fact that the pharmacodynamic half life was too long. Instead of having the desired anabolic effect that intermittent PTH has, the drug was catabolic, as is seen in primary HPT. Furthermore, the drug appeared to have no direct effect on human or mouse osteoblasts or human osteoclasts *in vitro*. It is likely that calcilytics will be efficacious in treating ADH, which is due to activating mutations of the CaR. It has been shown *in vitro* that calcilytics can right-shift the dose response of disease causing CaR mutations towards normal (see for review [cxxxvii]). Other uses include capitalizing on the known interaction with PTHrP to block skeletal metastasis (discussed previously) or treat humeral hypercalcemia of malignancy.

PTH is currently in clinical trials as an adjuvant agent for stem cell mobilization and engraftment. Given the recent finding that stem cell homing and engraftment is specified by CaR [xcvii], there may be a role for drugs that target CaR directly in bone marrow transplant. At least *in vitro*, human osteoblasts and osteoclasts do not respond normally to either cinacalcet [xxxiii] or a calcilytic [cxxxvi], though not all functional responses have been tested. Other challenges include the CaR's ubiquity of expression, promiscuity for multiple ligands, and potential confounding by the presence of alternate cation receptors.

Strontium ranelate is under investigation to treat postmenopausal osteoporosis and has been shown to significantly reduce the risk of hip [cxxxviii] and vertebral fractures [cxxxix]. Strontium is also a divalent cation and was recently reported to be a type I agonist of CaR.

Chattopadhyay *et al* showed a dose-dependent, functional response to strontium in rat primary osteoblasts, indicating that strontium activates endogenous CaR in bone cells [cxl] and implying that the observed anabolic effect is secondary to CaR activation. Furthermore, their finding of a differential effect with calcium suggests that certain ligand combinations might exert novel effects and is worthy of further investigation.

Unanswered questions

One group has reported intracellular staining of the CaR in keratinocytes [cxli], osteoblasts, and chondrocytes [xxviii]. While the cytoplasmic variety of a CaR may be involved in sensing intracellular calcium stores, it is unknown to what extent intracellular localization of CaR participates in the actions attributed to its plasma membrane cousin. Recent work in keratinocytes provides some evidence that the intracellular CaR participates in regulating intracellular calcium stores and that inhibition of the intracellular CaR with antisense cDNA inhibited the cellular response to extracellular calcium and decreased Ca^{2+} -induced differentiation of cells [cxlii].

CaR has garnered interest in multiple areas of biology and as the pace of research continues to advance it is becoming clear that discoveries in areas unrelated to bone biology will have implications that resonate as ubiquitously as the CaR itself.

Acknowledgments

Funding sources: This research was supported in part by the Intramural Research Program of the NIH, NIDCR. Author TAT was supported by the Clinical Research Training Program, a public-private partnership supported jointly by the NIH and Pfizer Inc (via a grant to the Foundation for NIH from Pfizer Inc).

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Table 1

Genotype	Description	Serum Ca ²⁺ (mg/dl)	Phosphorus (mg/dl)	PTH (pg/ml)	Urine Ca ²⁺ (mg/dl)	Urine Ca/Cr	Skeletal phenotype	Cartilage phenotype	Rickets/osteomalacia	Mineralization or Bone ash % dry wt	Histomorphometry parameters	Parathyroid hyperplasia	High neonatal mortality	Comments	Ref.
Background															
CaR ^{+/+}	Wild type	9.6 ± 0.3	8.2 ± 0.3 ^a	25 ± 6.4 ^c	20.2 ± 9.6	0.6 ± 0.05 ^a	Normal	Normal	-	33 ± 2.0 ^b 59.6 ± 0.3 ^a	Normal	-	-		48,54,56
CaR ^{+/-}		↑ 10.4 ± 0.6	↔ 8.2 ± 0.3 ^a	↔↗ 39.1 ± 14.7 ^c	↓ 11.7 ± 9.3	0.5 ± 0.08 ^a	Normal	Normal physis	-	33.0 ± 1.0 ^b 59.3 ± 0.2 ^a		-	-		48,54,56
Pth ^{-/-}	Complete hypoparathyroid	↓ (6.8-7.5)	↑↑ (>13)	0			Low turnover; ↑ bone mass, ? normal adults	Normal	-	↑ Von Kossa staining	1.8-fold ↑ Cn BV/TV and Ct.Th, ↓↓ MAR, ↑↑ Cn BV/TV, ↑ Tb.No, ↑ Tb.Th, ↔ OV/BV	+			51,52
Gcm2 ^{-/-}	Mild hypoparathyroid	↓↓ 6.3 ± 0.4 ^a	↑↑ 13.2 ± 1.0 ^a	↔↘ 17 ± 2.8 ^a		↔↗ 0.8 ± 0.1 ^a	Low turnover, ↑ bone mass	Normal	-	33.9 ± 3.0 ^b 60.2 ± 0.4 ^a	↓ osteoblast & osteoclast surface	-	+	Bone phenotype normalizes with PTH tx	54,55
CaR knockout															
CaR ^{-/-}	Single KO (exon 5)	↑↑ 14.8 ± 1.0	↓ 5.5 ± 0.5	↑↑ 129.1 ± 90	↓↓ 10.5 ± 7.7		osteopenia, kyphoscoliosis, rachitic changes, delayed endochondral formation	↑ hypertrophic zone	+	↓↓ 21 ± 2.0 ^d	↑ Cn BV/TV, Ob/BS, ↑↑ OV/BV, O.Th; ↓↓ MAR, BFR	+	+		48,56
CaR ^{-/-} Gcm2 ^{+/+} or Gcm2 ^{+/-}	Single KO (exon 5)	↑↑ 14.5 ± 0.9 ^(b)	↓ 4.9 ± 0.5 ^b	↑↑ 361 ± 54 ^b		↓ 0.35 ± 0.03 ^b	osteopenia, wide physes, delayed endochondral formation	↑ hypertrophic zone	+	↓↓ 23.8 ± 1.6 ^b	↑ osteoid	+	+	CaR ^{-/-} on different background is consistent with other CaR ^{-/-} KO	54
Col I-Cre/CaR ^{fllox/fllox}	Early osteoblast CaR KO (exon 7)						Fractures; growth delay, under-mineralized skeleton, ↓↓ BMD				↓↓ Tb.BV/TV, Tb. Conn. ↓ Tb.Th, Cn BV, Ct.Th ↑ osteoid		+		63,65,45
OC-Cre/CaR ^{fllox/fllox}	Mature osteoblast CaR KO (exon 7)						Normal growth, normal BMD		-		Normal Cn.BV/TV, Ct.Th			Preliminary data	63,65
OSX-Cre/CaR ^{fllox/fllox}	Embryonic osteoblast CaR KO (exon 7)						Delayed growth, undermineralized skeleton				↓↓ Tb.BV/TV, Tb. Conn. ↓ Tb.Th, Tb. N, Cn BV			OSX is expressed before <i>Col(I)</i>	45
Tam-Col II-Cre/CaR ^{fllox/fllox}	Tamoxifen-inducible Chondrocyte CaR KO (exon 7)						Shorter long bones	↑ hypertrophic zone, decreased mineral deposition						Pure KO is embryonic lethal	45
CaR “rescue”															
Gcm2 ^{-/-} CaR ^{-/-}	Double KO	↓↓ 6.0 ± 0.3 ^a	↑↑ 14.8 ± 0.9 ^a	↔↘ 12 ± 1.3 ^a		↓↓ 0.4 ± 0.07 ^a	Normal histology	Normal	-	↔ 32.0 ± 1.9 ^b 59.0 ± 0.5 ^a	Normal osteoid volume	-			54
CaR ^{-/-} PTH ^{-/-}	Double KO	↓↑ 13.2 ± 4.0 [*] (alt 6.2)	* ↑	* ↑		↑ 1.59 ± 1.17	Normal histology, ↑ vertebral BMD in females only		-		↔ MAR, BFR	+	-	Probably over-treated with vit. D (see text)	51,85
Activating CaR mutations															
<i>Nuf</i>	Activating CaR mutant	↓↓ 5.92 ± 0.5 ^e	↑ 12.2 ± 3.3 ^e	↓↔ ↓↔ ^{ff}		↓↔ 0.2 ± 0.08 ^e						-			60

Genotype	Description	Serum Ca2+ (mg/dl)	Phosphorus (mg/dl)	PTH (pg/ml)	Urine Ca2+ (mg/dl)	Urine Ca/Cr	Skeletal phenotype	Cartilage phenotype	Rickets/osteomalacia	Mineralization or Bone ash % dry wt	Histomorphometry parameters	Parathyroid hyperplasia	High neonatal mortality	Comments	Ref.
Act-CaR Oblast	Osteoblast-specific activating CaR mutant	10.5 ± 0.7 <i>f</i>	$\leftrightarrow$	34.8 ± 11 <i>f</i>	$\leftrightarrow$		Cancellous osteopenia		-		$\downarrow$ Tb.N., Tb Conn, and MAR $\uparrow$ osteoclasts, $\uparrow$ eroded surface, $\downarrow\downarrow$ Ct BV/TV, norm Ct.Th, O.Th, OV/BV, BFR		-		61
Models of PTH excess															
PTH-Gα _q /Gα ₁₁	Parathyroid gland-specific CaR KO	$\uparrow\uparrow$ (~ 16)	normal $\S$	$\uparrow\uparrow$ (>250)	$\leftrightarrow$ (~ 22)		Osteopenia, rachitic changes, craniofacial dysmorphism, delayed endochondral formation					+	+	Serum data is from minority of mice with longer survival	59
PTH-Cre/CaR ^{fllox/fllox}	Parathyroid-specific CaR KO (exon 7)	$\uparrow\uparrow$ 16.4 ± 0.5		$\uparrow\uparrow$ 959 ± 122	13.4 ± 2.9 (<i>g</i>)		Fractures; small, undermineralized skeleton impaired mineralization, limb deformity, no tooth eruption, delayed endochondral formation	Abnormal proliferating and hypertrophic chondrocytes			$\downarrow$ Tb.BV/TV, Tb.Th., Tb.N, Ct.Th; Ct. BV	+	+		45
Act-Col2A1-PPR	Activating PPR mutant targeted to growth plate												+		57
Act-Col1A1-PPR	Activating PPR mutant targeted to osteogenic cell													$\uparrow$ Osteoblasts $\uparrow$ Osteoclasts	143-145

* animals were probably over-treated with vitamin D

$\S$ preliminary results (Nina Wettschureck, personal communication)

$\P$ value was identical to control mice within study

a data from 6-week old mice

b data from 1-week old mice

c data from 61-73 day old mice

d data from 5-day old mice

e data from ~120 day old males (see ref. for females)

f data from 12 week old males (see ref. for females)

g represents a 380% increased urinary calcium excretion over controls (see ref)

Col I, collagen type I; OC, osteocalcin; OSX, osterix; Act, activating; BMD, bone mineral density; CaR, calcium-sensing receptor; PTH, parathyroid hormone; PPR, PTH/PTHrP receptor; KO, knockout; +, trait present; -, trait absent; tx, treatment. Histomorphometry parameters: Cn, cancellous; Ct, cortical; Tb, trabecular; BV/TV, bone volume; Th, thickness; OTh, osteoid thickness; OV/BV, osteoid volume; Ob/BS, osteoblast perimeter; MAR, mineral apposition rate; BFR, bone formation rate;