Rescue of the skeletal phenotype in CasR-deficient mice by transfer onto the Gcm2 null background

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To understand the role of the calcium-sensing receptor (CasR) in the skeleton, we used a genetic approach to ablate parathyroid glands and remove the confounding effects of elevated parathyroid hormone (PTH) in CasR-deficient mice. CasR deficiency was transferred onto the glial cells missing 2–deficient (Gcm2-deficient) background by intercrossing CasR- and Gcm2-deficient mice. Superimposed Gcm2 deficiency rescued the perinatal lethality in CasR-deficient mice in association with ablation of the parathyroid glands and correction of the severe hyperparathyroidism. In addition, the double homozygous CasR- and Gcm2-deficient mice demonstrated healing of the abnormal mineralization of cartilage and bone associated with CasR deficiency, indicating that rickets and osteomalacia in CasR-deficient mice are not due to an independent function of CasR in bone and cartilage but to the effect of severe hyperparathyroidism in the neonate. Analysis of the skeleton of 6-week-old homozygous CasR- and Gcm2-deficient mice also failed to identify any essential, nonredundant role for CasR in regulating chondrogenesis or osteogenesis, but further studies are needed to establish the function of CasR in the skeleton. In contrast, concomitant Gcm2 and CasR deficiency failed to rescue the hypocalciuria in CasR-deficient mice, consistent with direct regulation of urinary calcium excretion by CasR in the kidney. Double Gcm2- and CasR-deficient mice provide an important model for evaluating the extraparathyroid functions of CasR.


Introduction

The heptahelical extracellular calcium-sensing receptor (CasR) plays a central role in controlling systemic calcium homeostasis, predominately through its effects on regulation of parathyroid hormone (PTH) secretion by the parathyroid glands and urinary calcium excretion by the kidney (1–4). CasR also is widely expressed in many other tissues, including bone and cartilage, where its biological function has not been established (1, 5–7). Whether CasR has a direct role in regulating bone and cartilage function is a subject of much debate. In vitro studies and indirect in vivo evidence implicate CasR or a related receptor in the regulation of important steps in osteoblast-mediated bone formation, as well as in the differentiation and mineralization of cartilage (5–10). Consistent with the role of CasR in mineralization of bone and cartilage, rickets and osteomalacia were the major skeletal abnormalities found in CasR-deficient mice (11). The hyperparathyroidism, hypercalcemia, and hypophosphatemia that accompany deletion of CasR in the parathyroid glands, however, confound the independent assessment of CasR effects on bone and cartilage. The neonatal mortality in CasR-deficient mice also prevents assessment of the role of CasR in the adult skeleton (11). In addition, CasR transcripts have been inconsistently detected in mineralized bone containing mature osteoblasts and osteocytes after removal of marrow elements (11). Moreover, the putative osteoblastic cation-sensing receptor is characterized by different cation specificity from CasR, namely failing to respond to calcimimetics or magnesium (12–15), and osteoblasts derived from CasR-deficient mice retain their ability to respond to extracellular calcium through a G protein–coupled mechanism, suggesting that the osteoblastic receptor is distinct from CasR (14).

Regardless, a full understanding of the direct effect of CasR on bone and cartilage function requires correction of the hyperparathyroidism that accompanies CasR deficiency. Surgical parathyroidectomy is not technically feasible in neonatal mice. Although transfer of CasR deficiency onto PTH- or PTH receptor–deficient mice provides a means to remove the effects of excess PTH (16), the complete loss of PTH signaling leads to dysmorphic skeletons that might mask potential independent effects of CasR (17). Deletion of glial cells missing 2

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Nonstandard abbreviations used: calcium-sensing receptor (CasR); parathyroid hormone (PTH); glial cells missing 2 (Gcm2); deoxypyridinoline (Dpd); bone mineral density (BMD); dual-energy X-ray absorptiometry (DEXA); neonatal severe hyperparathyroidism (NSHPT).
(Gcm2), a mouse homologue of Drosophila Gcm, is a master regulatory gene of parathyroid gland development and provides an alternative strategy for achieving a molecular parathyroidectomy (18). Gcm2-deficient mice lack parathyroid glands but exhibit mild hypoparathyroidism due to the auxiliary production of PTH as well as normal skeletal growth and development (19).

In the current study, we transferred CasR deficiency onto the Gcm2-deficient background to create a model to assess the independent function of CasR in bone and cartilage and the importance of CasR beyond the early postnatal period. Molecular ablation of parathyroid glands and rescue of the perinatal lethality was achieved in double homozygous CasR- and Gcm2-deficient mice. Correction of the severe hyperparathyroidism prevented the rickets and osteomalacia in CasR-deficient mice but did not rescue the hypocalciuria. In addition, analysis of the skeleton of homozygous CasR- and Gcm2-deficient mice failed to identify any essential, nonredundant role for CasR in regulating growth-plate and bone mineralization. These studies indicate that defective mineralization of bone and cartilage is due to metabolic abnormalities associated with hyperparathyroidism rather than the absence of skeletal CasR and establish that the low urinary calcium is mediated by the absence of CasR in the kidney.

### Methods

**Creation of double CasR- and Gcm2-deficient mice.** We obtained heterozygous mice (CasR+/−) with targeted disruptions of exon 5 of the CasR gene from the laboratory of David Conner (Harvard University, Boston, Massachusetts, USA) (20). These mice were bred and maintained on a Black Swiss/129SvEv background. CasR-deficient mice express nonfunctional CasR splice variants lacking exon 5, thereby creating a functional CasR knockout (21). We obtained heterozygous Gcm2−/− mice, which have the targeted replacement of all four exons of the Gm2 gene, from the Baylor College of Medicine (19). Gm2−/− mice had been bred in a C57BL/6J/129SvEv background. Mice were maintained and used in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Duke University.

We created double heterozygous CasR- and Gcm2-deficient mice on a mixed genetic background by inbreeding CasR+/− and Gm2+/− mice for two generations. Crosses between CasR+/− and Gm2+/− mice produced double heterozygous CasR+/−/Gm2+/− mice. Male and female F2 CasR+/−/Gm2+/− mice were mated to generate nine distinct genotypes: CasR+/−/Gm2+/−, CasR+/−/Gm2−/−, CasR+/−/Gm2−/−, CasR+/−/Gm2−/−, CasR+/−/Gm2+/−, CasR+/−/Gm2−/−, and CasR+/−/Gm2−/−. This report focuses on results obtained

### Table 1

Data from 1-week-old mice

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<td>Bone ash (% dry wt)</td>
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<td>23.8 ± 1.6a</td>
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Values represent mean ± SEM of the number of animals per group as indicated in parentheses. Dpd, deoxypyridinoline; PTH, parathyroid hormone. a,b,c indicates significant differences between groups for the indicated parameter. Values sharing the same superscript in a given category are not significantly different, whereas values with a different superscript are significantly different at indicated P values at the end of the row. Group I, wild-type and heterozygous CasR+/−/Gcm2+/−; Group II, homozygous CasR+/− and mixed homozygous CasR+/−/heterozygous Gcm2+/−; Group III, homozygous Gm2−/−; Group IV, double homozygous mutant CasR+/−/Gm2−/−; Group V, heterozygous CasR+/− and double heterozygous CasR+/−/Gm2+/−; Group VI, heterozygous CasR+/− and homozygous Gm2−/−.
from the analysis of F3 generation littermates. We found that heterozygous Gcm2−/+ mice were identical to wild-type mice in all parameters tested. In addition, the absence of one Gcm2 allele did not influence the phenotype of either heterozygous CasR+/− or homozygous CasR−/− mice. This allowed the Gcm2−/+ mice to be combined with their wild-type counterparts to create six groups with distinct phenotypes, as defined in Table 1.

Genotyping. Genomic DNA was extracted and purified from the tail of each mouse using a QIAGEN DNeasy Tissue kit (QIAGEN Inc., Valencia, California, USA). To detect the presence of CasR+/−, we performed PCR using the reverse primerCasR2144.R (5′-TGAAAGCACCTACGGCACCCTG-3′), specific for the native mouse CasR gene sequence, in combination with a primer designed for upstream elements in exon 5, CasR1956.F (5′-TGTGAGAGCTCTTTCGG-3′), or primer KCM-F (5′-TTTCAGATCCCTTGGTGGTCG-3′) for the inserted neomycin gene sequence used for targeted disruption of CasR. The amplified band (1.4 kb) was accurately excised from agarose gel and visualized by ethidium bromide staining. Three groups with distinct phenotypes, as defined in Table 1.

Results

CasR deficiency causes hypocalciuric hyperparathyroidism, perinatal lethality, and rickets/osteomalacia. Compared with group I control mice, group II CasR-deficient mice had high perinatal mortality (Figure 1) in association with severe hyperparathyroidism, growth retardation, and abnormal growth-plate and bone histology (Figures 2–4 and Table 1). Most of the group II homozygous CasR-deficient mice died by the end of the first week after birth, with none of the mice surviving for more than 6 weeks (Figure 1). We observed a 28-fold increase in serum PTH in 1-week-old group II mice (Table 1) and marked enlargement of their parathyroid glands (data not shown) as compared with normal group I controls. The elevated PTH resulted in increases in serum calcium

Figure 1
Survival of CasR- and Gcm2-deficient mice. The survival rate of group I, which combines data from heterozygous Gcm2+/− and wild-type mice (n = 38), was 100%, whereas no group II homozygous CasR-deficient mice (n = 29) survived beyond 3 weeks. Group III homozygous Gcm2-deficient mice (n = 13) had a survival rate of 69%. The survival rates of group IV double homozygous CasR- and Gcm2-deficient mice (71%, n = 14) and group VI mixed heterozygous CasR- and homozygous Gcm2-deficient mice (74%, n = 23) are not different from that of group III Gcm2−/− mice. Group V, which combines heterozygous CasR+/− mice and the double heterozygous Gcm2+/−/CasR−/− mice (n = 69), had a survival rate of 97%.
and hypophosphatemia in group II CasR-deficient mice (Table 1). CasR deficiency also resulted in a twofold reduction in urinary calcium excretion, due to the absence of CasR in the kidney (Table 1).

Group II CasR-deficient mice also weighed significantly less than group I control mice and showed an obvious reduction in body size as compared with group I controls at 1 week of age (Figure 2, d–f versus a–c, and Table 1). Comparison of skeletal radiographs (Figure 2, b versus e), and alizarin red S/Alcian blue–stained whole skeletons (c, f, i, l, o, and r) of 1-week-old mice are shown. The marked reduction in body size, diffuse osteopenia, growth-plate widening (indicated by arrows), and delayed endochondral mineralization in group II homozygous CasR-deficient mice are rescued by superimposed Gcm2 deficiency in group IV double knockout mice. The growth appearance and skeletal phenotype of groups I (normal control), III (homozygous Gcm2-deficient), IV (double homozygous CasR- and Gcm2-deficient), V (double heterozygous CasR- and Gcm2-deficient), and VI (mixed heterozygous CasR- and homozygous Gcm2-deficient mouse) are indistinguishable.

Figure 2
Skeletal phenotype of CasR- and Gcm2-deficient mice. Gross appearance (a, d, g, m, and p), skeletal radiographs (b, e, h, k, n, and q), and alizarin red S/Alcian blue–stained whole skeletons (c, f, i, l, o, and r) of 1-week-old mice are shown. The marked reduction in body size, diffuse osteopenia, growth-plate widening (indicated by arrows), and delayed endochondral mineralization in group II homozygous CasR-deficient mice are rescued by superimposed Gcm2 deficiency in group IV double knockout mice. The growth appearance and skeletal phenotype of groups I (normal control), III (homozygous Gcm2-deficient), IV (double homozygous CasR- and Gcm2-deficient), V (double heterozygous CasR- and Gcm2-deficient), and VI (mixed heterozygous CasR- and homozygous Gcm2-deficient mouse) are indistinguishable.

The growth plate was enlarged in group II CasR-deficient mice due to the increased height of the hypertrophic zone (Figure 4, e and f) as compared with that of 1-week-old group I control mice (Figure 4, a and b). In addition, excessive amounts of osteoid were present covering trabecular bone in group II CasR-deficient mice (Figure 4g). Examination of the zone of calcified cartilage and primary spongiosa under fluorescent light in mice labeled with tetracycline and calcein demonstrated a normal pattern of mineralization in group I mice (Figure 4d) but a significant reduction in mineralized cartilage and bone in CasR–/– mice (Figure 4h). Consistent with the marked hyperparathyroidism, group II CasR-deficient mice had elevated serum osteocalcin, which is a marker of osteoblast activity, and increments in urinary excretion of Dpd, which is a marker of osteoclast activity (23) (Table 1).

Gcm2 deficiency results in the molecular ablation of parathyroid glands, mild hypoparathyroidism, and a normal-appearing skeleton. Approximately 30% of the homozygous Gcm2-deficient mice (group III) died soon after birth (Figure 1). The surviving homozygous Gcm2-deficient mice were viable and developed like group I control littermates. Gcm2-deficient mice had a trend toward lower circulating PTH concentrations (group III) than control littermates (group I) at both 1 (Table 1) and 6 weeks of age (Table 2), but although the difference did not achieve statistical significance (Table 1), it was
inappropriately low for the degree of hypocalcemia. Homozygous Gcm2-deficient mice had significantly lower serum calcium and higher serum phosphorus than group I controls (Tables 1 and 2), consistent with a functional state of PTH deficiency. Parathyroid glands could not be identified in Gcm2-deficient mice (data not shown).

Neither serum osteocalcin nor urinary Dpd levels were suppressed in group III Gcm2-deficient mice at either 1 or 6 weeks of age (Tables 1 and 2). Also, we did not identify any abnormalities of cartilage (Figure 2i; Figure 3, g–i; Figure 4, i and j; and Figure 5, e and f). The size and gross appearance of the skeletons of group III Gcm2-deficient mice at both 1 and 6 weeks of age were indistinguishable from those of group I normal controls (Tables 1 and 2 and Figure 2, g and h versus a and b, and Figure 3, g–i versus a–c). Gcm2-deficient mice had normal bone length, cartilaginous growth plates and centers of secondary ossification, and mineralization of caudal vertebrae as well as normal BMD. Dry ashed weight (Tables 1 and 2) and BMD by DEXA were also normal in group III mice (Table 2). Histologic evaluation revealed a normal fluorescent labeling of bone with tetracycline and calcine and osteoid-covered surfaces (Figure 4l and Figure 5, f and h).

Rescue of perinatal lethality, hyperparathyroidism, and rickets/osteomalacia but not hypocalciuria in double CasR- and Gcm2-deficient mice. In group IV mice double homozygous for CasR and Gcm2 deficiency, 71% of the pups were alive at 6 weeks (Figure 1). Their survival curve overlapped that of group III Gcm2-deficient mice (Figure 1), indicating that CasR deficiency was no longer lethal when transferred onto the Gcm2-deficient background. Associated with the improved survival, group IV double CasR- and Gcm2-deficient mice no longer displayed evidence of hyperparathyroidism, having serum PTH, calcium, and phosphate levels not statistically different from those of group III Gcm2-deficient littermates (Tables 1 and 2). Similar to homozygous Gcm2-deficient mice, double homozygous CasR- and Gcm2-deficient mice had no detectable parathyroid glands (data not shown).

In contrast to the improved survival associated with prevention of neonatal severe hyperparathyroidism (NSHPT), transfer of CasR deficiency onto the Gcm2

Figure 3
Alizarin red S/Alcian blue–stained skeletal regions of CasR- and Gcm2-deficient mice. (a–c) Heterozygous Gcm2+/– and wild-type mice. (d–f) Homozygous CasR-deficient mice. (g–i) Homozygous Gcm2-deficient mice. (j–l) Double homozygous CasR- and Gcm2-deficient mice. (m–o) Heterozygous CasR+/– and double heterozygous Gcm2+/–/CasR+/– mice. (p–r) Heterozygous CasR+/– and homozygous Gcm2-deficient mice. Normal appearance of the wrist from a group I control mouse (a) is compared to the widening of the growth plate of the wrist in a 1-week-old group II homozygous CasR-deficient littermate (d). These abnormalities are rescued in group IV double knockout mice (j) and are not present in the other groups (g, m, and p). Mineralization in the epiphysis cartilage of the femur is present in group I control mice (b) but not in the CasR+/– mice (e). Secondary centers of ossification are restored in group IV homozygous CasR- and Gcm2-deficient mice (k). No abnormalities of ossification centers were observed in the other groups (h, n, and q). Endochondral mineralization, as assessed by the number of alizarin red–positive mineralized caudal vertebrae, is reduced in 1-week-old group II CasR+/– mice (f) as compared with group I controls (c), whereas group IV homozygous CasR- and Gcm2-deficient mice (l) as well as the other groups (i, o, and r) show the same number of alizarin red–positive mineralized caudal vertebrae, consistent with a normal temporal and spatial pattern of endochondral bone formation.
null background did not rescue the hypocalciuria, which defines the kidney phenotype of CasR deficiency in the renal tubules. In this regard, the urinary calcium/creatinine ratio, which was normal in group III Gcm2-deficient mice and suppressed in group II CasR-deficient mice, remained diminished in group IV double Gcm2- and CasR-deficient mice at both 1 and 6 weeks of age (Tables 1 and 2).

Transfer of CasR deficiency onto the Gcm2 null background, however, rescued the rickets and osteomalacia associated with CasR deficiency. The size and gross appearance of group IV mice double homozygous for CasR and Gcm2 deficiency at both 1 and 6 weeks of age were indistinguishable from those of group I control and group III Gcm2-deficient mice (Figure 2 and Tables 1 and 2), indicating that the effect of CasR deficiency on the gross appearance of the skeleton was corrected on the Gcm2-deficient background. In spite of the absence of CasR, group IV double knockout mice also had normal bone density and bone length (Table 2). The rachitic changes disappeared in double knockout mice as evidenced by the normal growth plates, centers of secondary ossification, and mineralization of caudal vertebrae (Figure 2, j–l, and Figure 3, j–l). Histologic examination of cartilage confirmed the correction of the widened zone of hypertrophic chondrocytes and the normalization of calcification in cartilage and primary spongiosa that was observed in CasR-deficient mice (Figure 4, m and n, and Figure 5, i and j). The impaired mineralization of trabecular and cortical bone was also corrected, as evidenced by the absence of excess osteoid and the presence of fluorescence-labeled bone surfaces (Figure 4, o and p, and Figure 5, n–p). Osteocalcin and urinary Dpd levels were similar in Group III and IV mice and significantly lower than in 1-week-old group II CasR-deficient mice (Table 1).

Gcm2 deficiency predominates over effects of heterozygous CasR deficiency. Heterozygous group V CasR-deficient mice had a less severe phenotype that took longer to manifest. Survival was normal in group V heterozygous CasR-deficient mice (Figure 1). Group V mice were indistinguishable from group 1 control mice, except for mild hyperparathyroidism at 6 weeks of age and hypocalciuria at both 1 and 6 weeks of age (Tables 1 and 2). Serum calcium and phosphorus in group V mice did not differ from values in group I controls in spite of a twofold increase in PTH levels at 6 weeks of age. Serum osteocalcin but not DPD levels were increased in 6-week-old group V mice (Table 2), consistent with the effect of increased PTH on bone. The skeletons of 1- and 6-week-old heterozygous CasR-deficient mice, however, were indistinguishable from those of controls as assessed by skeletal radiography (Figure 2, n versus b), whole skeletal mounts (Figure 2, o versus c, and Figure 3, m–o versus a–c), bone ashed weight and BMD (Table 2), and bone histology (Figure 5, o and p versus c and d). In group VI heterozygous CasR- and Gcm2-deficient mice, superimposed Gcm2 deficiency predominated over that of heterozygous CasR deficiency, leading to a reduced survival (Figure 1), low PTH, and hypocalcemia (Tables 1 and 2). Consistent with the similar biochemical profiles, the growth plate and calcified cartilage in group V heterozygous CasR-deficient mice were also indistinguishable from those of group I normal mice at 6 weeks of age (Figure 5, q–t).

Discussion
In the current study, we created a molecular parathyroidectomy in CasR-deficient mice by transferring CasR deficiency onto the Gcm2 null background in order to...
assess the independent role that CasR deficiency plays in the abnormalities of cartilage and bone. We found that Gem2 deficiency exerted a dominant effect to prevent the development of parathyroid glands in CasR-deficient mice, thereby replacing severe hyperparathyroidism with a state of mild hypoparathyroidism (Tables 1 and 2) due to the accessory production of PTH (19). Correction of the metabolic abnormalities was sufficient to rescue the perinatal lethality of CasR deficiency (Tables 1 and 2). In this regard, double heterozygous CasR- and Gem2-deficient mice, rather than dying in the early neonatal period as occurs with CasR deficiency, had survival rates indistinguishable from those of Gem2-deficient littermates (Figure 1).

The survival of double CasR- and Gem2-deficient mice permitted the role of CasR to be assessed in the adult mouse skeleton and in other tissues where the function of CasR remains uncertain. Analysis of 1- and 6-week-old mice demonstrated that transfer of CasR deficiency onto the Gem2 null background also corrected growth retardation and skeletal abnormalities associated with CasR deficiency (Figures 2–5). The bone length, width of the growth plates, centers of ossification, and bone mineral density were indistinguishable among Gem2-deficient mice, CasR- and Gem2-deficient mice, and normal mice. Histologic analysis of nondecalcified cartilage and bone also demonstrated that the increased zone of hypertrophic cartilage, hyperostosis, and mineralization abnormalities had resolved (Figures 4 and 5). These results are consistent with the observation that parathyroidectomy in humans with NSHPT apparently cures the skeletal abnormalities (24, 25). In contrast to the rescue of the bone and cartilage phenotype of CasR-deficient mice, hypocalciuria remained in double CasR- and Gem2-null mice (Tables 1 and 2), indicating that the manifestation of CasR loss on renal tubular excretion of calcium was not rescued (1). The fact that the effects of CasR deficiency remain at sites where the receptor plays a physiological role adds further support to the conclusion that the absence of skeletal CasR is not responsible for rickets and osteomalacia in CasR-deficient mice.

The rescue of the skeletal phenotype by ablation of the parathyroid glands in double CasR- and Gem2-deficient mice indicates that excessive PTH, when combined with rapid skeletal growth during the neonatal period, is predominantly responsible for rickets and osteomalacia. The ability of parathyroid gland ablation to rescue the rickets and osteomalacia in CasR-deficient mice is surprising, since excess PTH typically leads to increased

### Table 2
Data from 6-week old mice

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</table>

Values represent mean ± SEM of the number of animals per group as indicated in parentheses. None of the Group II animals survived 6 weeks. a,b,cIndicates significant differences. Values sharing the same superscript or lacking superscripts in a given category are not significantly different, whereas those having a different superscript are significantly different at indicated P values at the end of the row. Groups are the same as described in Table 1. BMD, bone mineral density. Other abbreviations are the same as in Table 1.
bone turnover and accelerated mineralization in adults (11). However, the skeletal phenotype associated with gain-of-function mutations of PTH/PTH related peptide targeted to cartilage has some similarities to the skeletal phenotype of CasR deficiency (26). The intensity and persistence of activation of the PTH signal-transduction pathway might explain these differences in the skeletal phenotype induced by increased PTH secretion in CasR-deficient mice, which could lead to matrix biosynthesis in cartilage and bone in excess of the capacity to mineralize or production of mineralization inhibitors, such as matrix γ-carboxyglutamic acid protein from osteoblasts, as demonstrated in vitro (27).

Indeed, the current findings add further support to an important role of hyperparathyroidism in the development of rickets and osteomalacia in vitamin D–deficient rats and vitamin D receptor–deficient mice (28, 29). Hypophosphatemia also does not seem to be sufficient to account for the skeletal phenotype in CasR-deficient mice. In this regard, Npt2 null mice, which are hypophosphatemic due to the absence of the sodium-phosphate transporter in the proximal tubule, exhibit only mild retardation of secondary ossification and minimal growth-plate abnormalities (30, 31) and do not manifest overt signs of rickets and osteomalacia.

Although the current studies fail to support an essential and nonredundant role of CasR in normal cartilage and bone development, they do not prove the lack of function of CasR in bone/cartilage formation. Since in vitro data are consistent with a functional role for CasR or a related extracellular calcium-sensing mechanism in chondrocytes and bone marrow (5), it is possible that low calcium and/or hypoparathyroidism could have masked the effects of CasR to regulate cartilage and bone function. Further studies are needed to compare the skeletal phenotypes of Gcm2-deficient and double homozygous CasR- and Gcm2-deficient mice after normalization of calcium and PTH. In addition, long-term observation of double homozygous CasR- and Gcm2-deficient mice and provocative studies with calcimimetics may be needed to uncover effects of CasR on cartilage and bone function. The presence of redundant calcium-sensing mechanisms due to other calcium-sensing receptors and/or membrane channels provides another explanation for the lack of apparent in vivo CasR function in the skeleton (14). Indeed, there is an ongoing controversy as to whether all of the functional responses to cations in osteoblasts and osteoclasts are mediated solely by CasR or whether another CasR-like receptor is present that differs from CasR with regard to cation specificity, response to calcimimetics, and...

Figure 5
Histologic analysis of the growth plate and bone of 6-week-old homozygous and heterozygous CasR- and Gcm2-deficient mice. Shown are group I normal controls (a–d), group III homozygous Gcm2-deficient mice (e–h), group IV double homozygous CasR- and Gcm2-deficient mice (i–l), group V heterozygous Gcm2-deficient mice (m–p), and group VI heterozygous CasR- and Gcm2-deficient mice (q–t). Goldner-stained sections (×250 in a, e, i, m, and q) show normal growth plates in all groups. Villanueva-stained sections (×125 in b, f, j, n, and r) of the growth plate and adjacent metaphysis show normal mineralization of primary and secondary spongiosa in all groups. Goldner-stained sections (×500 in c, g, k, o, and s) show normal amounts of osteoid in all groups. A highly magnified view (×500 in d, h, l, p, and t) of trabecular bone under fluorescent light reveals normal mineralization in all groups as evidenced by dual calcein and tetracycline labels on the bone surfaces.
signaling pathways (11, 13). It is possible that the severe hypercalcemia acting through another calcium-sensing receptor or mechanism might contribute to skeletal changes in CasR-deficient mice. In this regard, aluminum, which stimulates the putative novel cation-sensing receptor in osteoblasts but not CasR (14), causes osteomalacia in both humans and animal models (15, 32). Finally, since the genetic background can influence the severity of any given phenotype, it is possible that the mixed background of CasR- and Gcm-deficient mice might have obscured the effect of CasR deficiency on bone and cartilage. Although we did not observe major strain-specific variations in cartilage and bone formation in this study, it may be important to evaluate the skeletal effect of CasR deficiency in different genetic backgrounds.

The current studies provide additional insights into the role of CasR and age in the phenotype of Gcm2-deficient mice. Consistent with previous reports of maximal stimulation of PTH secretion by CasR at extraparathyroid sites (19), we failed to see further compensatory increases in PTH levels with superimposed CasR deficiency. Unlike previously reported data from 6-month-old mice (19), we found no demonstrable effects of Gcm2 deficiency on biochemical markers of bone turnover, bone ashed weight, BMD, or bone histology in 6-week-old mice. The failure to observe these skeletal abnormalities in 6-week old mice might be due to compensatory factors associated with skeletal growth in young mice.

In conclusion, correction of severe hyperparathyroidism and the accompanying hypercalcemia and hypophosphatemia is sufficient to correct the abnormalities of mineralization of the extracellular matrix in CasR-deficient mice. The rescue of the skeletal phenotype fails to support an essential role for CasR in cartilage and bone, but long-term studies and additional in vivo and in vitro experiments are needed to define the role of CasR in bone and cartilage as well as at other sites. The rescue of the perinatal lethality of CasR null mice by backcrossing onto the Gcm2-deficient background should provide a useful model for evaluation of the role CasR in regulating the physiological function of other organs and tissues where it is expressed, without the confounding effects of excess PTH.

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