Alterations in phosphorus, calcium and PTHrP contribute to defects in dental and dental alveolar bone formation in calcium-sensing receptor-deficient mice

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SUMMARY
To determine whether the calcium-sensing receptor (CaR) participates in tooth formation and dental alveolar bone development in mandibles in vivo, we examined these processes, as well as mineralization, in 2-week-old CaR-knockout (CaR\(^{-/-}\)) mice. We also attempted to rescue the phenotype of CaR\(^{-/-}\) mice by genetic means, in mice doubly homozygous for CaR and 25-hydroxyvitamin D 1α-hydroxylase [1α(OH)ase] or parathyroid hormone (Pth). In CaR\(^{-/-}\) mice, which exhibited hypercalcemia, hypophosphatemia and increased serum PTH, the volumes of teeth and of dental alveolar bone were decreased dramatically, whereas the ratio of the area of predentin to total dentin and the number and surface of osteoblasts in dental alveolar bone were increased significantly, as compared with wild-type littermates. The normocalcemia present in CaR\(^{+/+}\); 1α(OH)ase\(^{+/+}\) mice only slightly improved the defects in dental and alveolar bone formation observed in the hypercalcemic CaR\(^{-/-}\) mice. However, these defects were completely rescued by the additional elimination of hypophosphatemia and by an increase in parathyroid hormone-related protein (PTHrP) expression in the apical pulp, Hertwig’s epithelial root sheath and mandibular tissue in CaR\(^{-/-}\); Pth\(^{+/+}\) mice. Therefore, alterations in calcium, phosphorus and PTHrP contribute to defects in the formation of teeth and alveolar bone in CaR-deficient mice. This study indicates that CaR participates in the formation of teeth and in the development of dental alveolar bone in mandibles in vivo, although it appears to do so largely indirectly.

KEY WORDS: Calcium-sensing receptor (CaR; CASR), 25-hydroxyvitamin D 1α-hydroxylase [1α(OH)ase; CYP27B1], Parathyroid hormone (PTH), Parathyroid hormone-related protein (PTHrP; PTHLH), Teeth, Mandible, Mouse

INTRODUCTION
The calcium-sensing receptor (CaR; CASR – Mouse Genome Informatics) is a plasma membrane G-protein-coupled receptor that is activated by extracellular calcium. CaR plays a central role in controlling systemic calcium homeostasis, predominately through its effects on the regulation of parathyroid hormone (PTH) secretion by the parathyroid glands and on urinary calcium excretion by the kidney (Brown et al., 1993; Brown and MacLeod, 2001). Evidence for its crucial role in parathyroid function came from the identification of inactivating mutations in CaR in familial hypocalciuric hypercalcaemia (FHH) and neonatal severe hyperparathyroidism (NSHPT) (Pollak et al., 1993). Patients with FHH are heterozygous for inactivating mutations in CaR and exhibit mild to moderate hypercalcaemia, normal or mildly increased circulating PTH levels, and parathyroid histology that ranges from normal to mild hyperplasia. Patients with NSHPT are homozygous for inactivating mutations in CaR and exhibit severe abnormalities of CaR impacts tooth formation and dental alveolar bone development. CaR is expressed in the mandible and developing tooth, and might provide a mechanism for sensing and responding to the alterations in extracellular calcium concentrations that take place during the formation of the mandibles and teeth (Dvorak et al., 2004; Mathias et al., 2001). Calcium is, of course, a key component of teeth. It is found in the enamel, dentin and the surrounding extracellular matrix. Moreover, the teeth and dental alveolar bone are highly active tissues that constantly undergo remodeling throughout the life cycle (Marks and Schroeder, 1996; Roberts, 1999). Thus, CaR could potentially play important roles in the formation and development of the teeth and dental alveolar bone.

The vitamin D-PTH axis plays a central role in calcium and phosphorus homeostasis and is essential for skeletal development and mineralization. PTH and 1,25-dihydroxyvitamin D\(_3\) [1,25(OH)\(_2\)D\(_3\)] directly affect calcium homeostasis and each exerts important regulatory effects on the other. PTH stimulates the production of 1,25(OH)\(_2\)D\(_3\) by activating the renal 25-
hydroxyvitamin D 1α-hydroxylase [1α(OH)ase; CYP27B1 – Mouse Genome Informatics] (Brenza et al., 1998; Murayama et al., 1998), and increased 1,25(OH)2D3 in turn suppresses the production of PTH (Cantley et al., 1985; Chan et al., 1986) and controls parathyroid cell growth (Szabo et al., 1989). Suppression of PTH synthesis by 1,25(OH)2D3 occurs through negative regulation of Pth transcription by a 1,25(OH)2D3-vitamin D receptor (VDR)-retinoid X receptor (RXR) complex (Liu et al., 1996) in parathyroid cells (Beckerman and Silver, 1999).

We have previously reported the creation of a mouse model that is deficient in PTH by targeting the Pth gene in embryonic stem cells. Although adult Pth-null mice develop hypocalcemia, hyperphosphatemia and low circulating 1,25(OH)2D3 levels consistent with primary hyperparathyroidism (Miao et al., 2004a), this phenotype is not lethal. We (Panda et al., 2001) and others (Dardenne et al., 2001) have also previously reported the generation of a mouse model that is deficient in 1,25(OH)2D3 as a result of targeted ablation of the 1α(OH)ase gene. After weaning, 1α(OH)ase–/– mice that are fed a diet of regular mouse chow developed secondary hyperparathyroidism, retarded growth and the skeletal abnormalities characteristic of rickets. These abnormalities mimic those described in the human disease vitamin D-dependent rickets type I (Fraser et al., 1973). By comparing Pth–/– or 1α(OH)ase–/– mice with Pth+/–; 1α(OH)ase–/– double-null mice, we found that PTH plays a predominant role in appositional bone growth, whereas 1,25(OH)2D3 acts predominantly on endochondral bone formation, and both play collaborative roles in modulating skeletal and calcium homeostasis (Xue et al., 2005). However, the relative contributions of calcium, phosphorus, 1,25(OH)2D3 and PTH to dental formation and intramembranous bone formation remain unknown. We therefore used CaR–/– mice, CaR+/–; 1α(OH)ase–/– and CaR+/–; Pth–/– double-homozygous mice to dissect the individual contributions of calcium (acting via CaR), phosphorus, 1,25(OH)2D3 and PTH to the formation of teeth and dental alveolar bone.

Genotyping of mice
Tail fragment genomic DNA was isolated by standard phenol-chloroform extraction and isopropanol precipitation. To determine the genotype at the CaR, Pth and 1α(OH)ase loci, six PCR amplification reactions were conducted. To assay the presence of the wild-type CaR allele, samples were amplified with CaR forward primer (5′-TCTGTCTTCTTTAGGTCCCTGAAAACA-3′) and CaR reverse primer (5′-TCATTGTGAAACAGTCTTCTCCCT-3′). To detect the presence of the null CaR allele, the Neo forward primer r-Neo-2 (5′-TCTGATTCCTACATTGTTGTCCTA-3′) was used with the CaR reverse primer. The presence of the wild-type Pth allele was detected using the PTH forward primer (5′-GAGTGTGCGAAAACCGTTGGCTAAA-3′) and PTH reverse primer (5′-TCCAAGGTTCATTACATAGAAG-3′). The null Pth allele was detected using the Neo forward primer r-Neo-2 and the PTH reverse primer (Kos et al., 2003). For the wild-type 1α(OH)ase allele, forward primer (5′-AGACTGCACTCCACTCTGAG-3′) and reverse primer (5′-GTTTCCTACACGGATGTCTC-3′) were used.

Biochemical and hormone analyses
Serum calcium and phosphorus levels were determined using an autoanalyzer (Synchrone 67, Beckman Instruments). Serum intact PTH was measured by a two-site immunoradiometric assay (ImmunoTech, San Clemente, CA, USA).

Materials and Methods
Derivation of CaR+/–, CaR–/–, 1α(OH)ase–/– and CaR+/–; Pth–/– mice
The derivation of the three parental strains of CaR+/–, 1α(OH)ase–/– and Pth–/– mice has been described (Ho et al., 1995; Panda et al., 2001; Miao et al., 2002). Briefly, a neomycin resistance gene was inserted into exon 5 of the mouse CaR gene. Western blot analysis of kidney protein membrane extracts from CaR+/– mice confirmed that no detectable protein is expressed from this allele in this tissue (Ho et al., 1995). A neomycin resistance gene replaced exons 6-8 of the mouse 1α(OH)ase gene, removing both the ligand-binding and the heme-binding domains (Panda et al., 2001). RT-PCR of renal RNA from 1α(OH)ase–/– mice confirmed the lack of 1α(OH)ase expression. A neomycin resistance gene was inserted into exon 3 of the mouse Pth gene, resulting in replacement of the entire coding sequence of mature PTH. Lack of PTH expression in parathyroid glands was confirmed by immunostaining (Miao et al., 2002). CaR+/– mice and 1α(OH)ase–/– mice were fertile and were mated to produce offspring heterozygous at both loci, which were then mated to generate CaR+/–; 1α(OH)ase–/– pups. Lines were maintained by mating CaR+/–; 1α(OH)ase–/– mice and females on a mixed genetic background with contributions from 129/SvJ and BALB/c strains. CaR+/– mice and Pth–/– mice were fertile and were mated to produce offspring heterozygous at both loci, which were then mated to generate CaR+/–; Pth–/– pups. These mice were maintained on a mixed genetic background with contributions from 129/SvJ and C57BL/6J strains. Mutant mice and control littermates were maintained in a virus- and parasite-free barrier facility and exposed to a 12-hour light/12-hour dark cycle. In the current study, 2-week-old wild-type, CaR+/–, CaR–/–, 1α(OH)ase–/– and CaR+/–; Pth–/– mice were used. All animal experiments were carried out in compliance with, and approval by, the Institutional Animal Care and Use Committee.
Enzyme histochemistry for ALP activity was performed as described (Miao and Scott, 2002b). Briefly, following preincubation overnight in 100 mM MgCl₂ in 100 mM Tris-maleate buffer (pH 9.2), dewaxed sections were incubated for 2 hours at room temperature in 100 mM Tris-maleate buffer containing naphthol AS-MX phosphate (0.2 mg/ml, dissolved in ethylene glycol monomethyl ether, both Sigma) as substrate and Fast Red TR (0.4 mg/ml, Sigma) as a stain for the reaction product. After washing with distilled water, the sections were counterstained with Vector Methyl Green nuclear counterstain (Vector Laboratories, Burlington, Ontario, Canada) and mounted with Kaiser’s glycerol jelly.

Enzyme histochemistry for TRAP was performed using a modification of a previously described protocol (Miao and Scott, 2002b). Dewaxed sections were preincubated for 20 minutes in buffer containing 50 mM sodium acetate and 40 mM sodium tartrate (pH 5.0). Sections were then incubated for 15 minutes at room temperature in the same buffer containing 2.5 mg/ml naphthol AS-MX phosphate in dimethylformamide as substrate and 0.5 mg/ml Fast Garnet GBC (Sigma) as a color indicator for the reaction product. After washing with distilled water, the sections were counterstained with Methyl Green and mounted in Kaiser’s glycerol jelly.

Immunohistochemical staining

Immunohistochemical staining was carried out for biglycan, dentin sialoprotein (DSP), proliferating cell nuclear antigen (PCNA) and parathyroid hormone-related protein (PTHrP) using the avidin-biotin-peroxidase complex technique with affinity-purified rabbit anti-mouse biglycan (LF-106) antibody (courtesy of Dr L. W. Fisher, NIDCR, NIH, Bethesda, MD, USA), affinity-purified rabbit anti-mouse dentin sialoprotein (Santa Cruz, CA, USA), mouse anti-PCNA monoclonal antibody (Medicorp, Montreal, Canada), and rabbit antiserum against PTHrP[1-34] as described previously (Bai et al., 2007). Briefly, dewaxed and rehydrated paraffin-embedded sections were incubated with methanol/hydrogen peroxide (1:10) to block endogenous peroxidase activity and then washed in Tris-buffered saline (pH 7.6). The slides were then incubated with the primary antibodies overnight at room temperature. After rinsing with Tris-buffered saline for 15 minutes, tissues were incubated with secondary antibody (biotinylated goat anti-rabbit or anti-mouse IgG, Sigma). Sections were then washed and incubated with the Vectastain Elite ABC reagent (Vector Laboratories) for 45 minutes. Staining was developed using 3,3-diaminobenzidine (2.5 mg/ml) followed by counterstaining with Mayer’s Hematoxylin.

Quantitative real-time PCR

RNA was isolated from mouse mandible bodies using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. Reverse transcription reactions were performed using the SuperScript First-Strand Synthesis System (Invitrogen) as described (Xue et al., 2005). To determine the number of cDNA molecules in the reverse-transcribed samples, real-time PCR analyses were performed using the LightCycler system (Roche, Indianapolis, IN, USA). PCR was performed using 2 µL LightCycler DNA Master SYBR Green I (Roche), each 5' and 3' primer at 0.25 µM, and 2 µl of samples or water to a final volume of 20 µl. The MgCl₂ concentration was adjusted to 3 mM. Samples were denatured at 95°C for 10 seconds, with a temperature transition rate of 20°C/second. Amplification and fluorescence determination were carried out in four steps: denaturation at 95°C for 10 seconds, with a temperature transition rate of 20°C/second; annealing for 5 seconds, with a temperature transition rate of 8°C/second; extension at 72°C for 20 seconds, with a temperature transition rate of 4°C/second; and detection of SYBR Green fluorescence, which reflects the amount of double-stranded DNA, at 86°C for 3 seconds. Thirty-five amplification cycles were performed. To discriminate specific from non-specific cDNA products, a melting curve was obtained at the end of each run: products were denatured at 95°C for 3 seconds, and the temperature was then decreased to 58°C for 15 seconds and raised slowly from 58 to 95°C using a temperature transition rate of 0.1°C/second. To determine the number of copies of the targeted DNA in the samples, purified PCR fragments of known concentration were serially diluted to serve as external standards in each experiment. Data were normalized to Gapdh levels in the samples. The primer sequences used for the real-time PCR were as described (Miao et al., 2004b; Xue et al., 2005).

Western blot analysis

Proteins were extracted from mandibular bones and quantitated using a protein assay kit (Bio-Rad, Mississauga, Ontario, Canada). Protein samples (30 µg) were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was carried out as described (Xue et al., 2005) using antibodies against PTHrP[1-34] (Upstate, NY, USA) and β-tubulin (Santa Cruz). Bands were visualized using ECL chemiluminescence (Amersham) and quantitated by Scion Image Beta 4.02 (Scion Corporation, NIH).

Computer-assisted image analysis

After HE staining or histochemical or immunohistochemical staining of sections from six mice of each genotype, images of fields were photographed with a Sony digital camera. Images of micrographs from single sections were digitally recorded using a rectangular template, and recordings were processed and analyzed using Northern Eclipse image analysis software as described (Miao et al., 2001; Miao et al., 2002).

Statistical analysis

Data from image analysis are presented as mean ± s.e.m. Statistical comparisons were made using a two-way ANOVA, with P<0.05 considered significant.

RESULTS

Changes in serum biochemistry in wild-type and mutant mice

First, we compared the changes in serum calcium, phosphorus and PTH in mutant and wild-type mice. At 2 weeks of age, the CaR–/– mice displayed hypercalcemia, hypophosphatemia and increased serum PTH. The CaR–/–; Iα(OH)ase–/– mice displayed norocalcemia, hypophosphatemia and more severe PTH elevations, whereas the CaR–/–; Pth–/– mice exhibited norocalcemia, hyperphosphatemia and undetectable serum PTH (Fig. 1A-C).

Imaging changes in wild-type and mutant mice

We next examined the phenotypes of teeth and mandibles in 2-week-old wild-type, CaR–/–, CaR–/–; Iα(OH)ase–/– and CaR–/–; Pth–/– mice by radiography and micro-CT scanning. The teeth and mandibles were smaller in CaR–/– than in wild-type mice. Radiolucency was increased in all teeth, including molars and incisors, and in the mandibles of CaR–/– mice compared with their wild-type littermates. The teeth and mandible were enlarged and their mineral density increased in CaR–/–; Iα(OH)ase–/– mice compared with CaR–/– littermates, but these parameters were still reduced significantly compared with wild-type littermates. These parameters were, however, normalized in CaR–/–; Pth–/– mice (Fig. 1D).

Four micro-CT-scanned sections through the incisors in front of the first molar, and through the first, second and third molars in mandibles, were compared between wild-type mice and the three mutant models. The mineralized tooth volume in incisor and molars and the mineralized cortical and alveolar bone volume in mandibles were decreased in CaR–/– mice compared with wild-type littermates. The mineralization defects in teeth and alveolar bone were slightly improved in CaR–/–; Iα(OH)ase–/– mice, but were almost completely rescued in CaR–/–; Pth–/– mice as compared with CaR–/– littermates (Fig. 1E).

Tooth volume and dental alveolar bone volume in mandibles

We next examined the tooth volume and dental alveolar bone volume in mandibles, using paraffin-embedded sections through the first molars, which were stained with HE (Fig. 2A) and histochemically for total collagen (Fig. 2B). The dental volume (Fig.
and immuno-histochemistry, respectively. The ratio of the areas of predentin to dentin in sections stained with HE (Fig. 3A-E) and the areas of biglycan-immunopositive dentin (Fig. 3B,F) were increased significantly, whereas the areas of mineralized dentin (Fig. 3C,G) and DSP immunoreactivity (Fig. 3D,E) were decreased dramatically, in the first molars of CaR–/– mice compared with wild-type littermates. The ratio of predentin to dentin (Fig. 3A,E) and the biglycan-immunopositive dentin areas (Fig. 3B,F) were not significantly different between CaR–/– and CaR+/+; 1α(OH)ase–/– mice. The areas of mineralized dentin and of DSP immunoreactivity in the first molar were, however, increased in CaR–/–; 1α(OH)ase–/– mice compared with CaR–/– littermates, but were still reduced significantly compared with wild-type littermates (Fig. 3C,D,G,H). In CaR–/–; Pth–/– mice, these parameters were all normalized (Fig. 3A-H).

Cell proliferation and PTHrP expression in the apical pulp and Hertwig’s epithelial root sheath

We immunostained for proliferating cell nuclear antigen (PCNA) to assess alterations in cell proliferation, and also for changes in PTHrP expression in the apical pulp and Hertwig’s epithelial root sheath (HERs). In wild-type mice, immunoreactivity of both antigens was observed in the apical pulp, and in the nuclei of cells of Hertwig’s epithelial root sheath (HERs). In CaR–/– mice, cells positive for both PCNA (Fig. 4A,C) and PTHrP (Fig. 4B,D) were clearly decreased. The reductions in PCNA- and PTHrP-positive cells in the apical pulp and HERs were improved in CaR–/–; 1α(OH)ase–/– as compared with CaR–/– mice (Fig. 4A-D). There was no significant difference in the numbers of PCNA-positive cells in the CaR–/–; Pth–/– and wild-type mice (Fig. 4E,F), whereas the number of PTHrP-positive cells was increased markedly in CaR–/–; Pth–/– mice as compared with wild-type littermates (Fig. 4B,D). The alterations in PTHrP gene and protein

2C,D) and dental alveolar bone volume (Fig. 2E) were decreased dramatically in CaR–/– mice compared with wild-type littermates. Both parameters were increased in CaR+/+; 1α(OH)ase–/– mice compared with CaR–/– littermates, but were still reduced significantly compared with wild-type littermates (Fig. 2A-E). Ablation of Pth in CaR–/– mice was sufficient to rescue the CaR–/– phenotypes as the dental volume and alveolar bone volume were normalized (Fig. 2A-E).

Predentin maturation and dentin formation

We then determined predentin maturation and dentin formation in the mutant and wild-type mice. The thickness of the predentin and mineralized dentin in the first molars and the immunoreactivity of biglycan and dentin sialoprotein (DSP) were assessed by histology

and imaging of teeth and mandibles. (A,B) Decalcified sections through the first molars and the incisors from 2-week-old wild-type, CaR–/–; 1α(OH)ase–/– and CaR+/+; Pth–/– mice that were stained with Hematoxylin and Eosin (HE) (A) or Sirius Red for total collagen (B). Magnification, 50×. (C-E) Dental volume of incisors (C) and of the first molars (D), and dental alveolar bone volume of the mandibles (E), presented as mean ± s.e.m. of determinations in six animals of each group. *, P<0.05; **, P<0.01; ###, P<0.001, compared with wild-type mice; #, P>0.05, compared with CaR–/– mice.
expression in mandibular extracts, as demonstrated by real-time RT-PCR (Fig. 4E) and western blots (Fig. 4F,G), were consistent with those observed by immunohistochemistry.

Osteoblastic dental alveolar bone formation
We next determined osteoblastic dental alveolar bone formation in paraffin-embedded sections that were stained with HE and then histochemically stained for biglycan (B) or dentin sialoprotein (DSP) (D). (C) Non-decalcified sections through the first molars and incisors stained by the von Kossa procedure. Magnification, 400×. (E-H) The quantitative ratio of predentin to dentin (E), the biglycan-positive area (F), the mineralized dentin area (G) and the DSP-positive area (H) in the first molars were determined by image analysis, and the percentages are presented as the mean ± s.e.m. of determinations in six animals of each group. *, P<0.05; **, P<0.01; ###, P<0.001, compared with wild-type mice. #, P<0.05; ##, P<0.01; ###, P<0.001, compared with CaR−/− mice.

Osteoclastic dental alveolar bone resorption
Osteoclastic bone resorption in dental alveolar bone was examined in paraffin-embedded sections stained histochemically for TRAP. The number of TRAP-positive osteoclasts was increased in the dental alveolar bone in CaR−/− and CaR−/−;1α(OH)ase−/− mice compared with their wild-type littermates, but there was no significant difference in the TRAP-positive osteoclast surface (Fig. 6A-C). TRAP-positive osteoclast number and surface area were not

Fig. 3. Predentin maturation and dentin formation in teeth.
(A-D) Sections through the root wall of the first molars and incisors from 2-week-old wild-type, CaR−/−;1α(OH)ase−/− and CaR−/−;Pth−/− mice stained with HE (A), or immunohistochemically stained for biglycan (B) or dentin sialoprotein (DSP) (D). (C) Non-decalcified sections through the first molars and incisors stained by the von Kossa procedure. Magnification, 400×. (E-H) The quantitative ratio of predentin to dentin (E), the biglycan-positive area (F), the mineralized dentin area (G) and the DSP-positive area (H) in the first molars were determined by image analysis, and the percentages are presented as the mean ± s.e.m. of determinations in six animals of each group. *, P<0.05; **, P<0.01; ###, P<0.001, compared with wild-type mice; #, P<0.05; ##, P<0.01; ###, P<0.001, compared with CaR−/− mice.

Fig. 4. Cell proliferation and PTHrP expression in the apical pulp, Hertwig's epithelial root sheath (HERs) and mandibular tissue.
(A, B) Sections through the first molars and incisors, showing the apical pulp and HERs, from 2-week-old wild-type, CaR−/−;1α(OH)ase−/− and CaR−/−;Pth−/− mice stained immunohistochemically for PCNA (A) and PTHrP (B). Magnification, 400×. (C, D) Numbers of PCNA-positive (C) or PTHrP-positive (D) cells per field were determined by image analysis, and the percentages of immunopositive cells relative to total cells are presented as the mean ± s.e.m. of determinations in six animals of each group. (E) Comparison of Pthrp gene expression levels in mandibular tissue of wild-type, CaR−/−, CaR−/−;1α(OH)ase−/− and CaR−/−;Pth−/− mice as quantified by real-time RT-PCR. mRNA expression, normalized to that of Gapdh, is shown relative to levels in wild-type mice. (F) Western blots of mandibular extracts were carried out for expression of PTHrP, with β-tubulin as a loading control. (G) PTHrP protein levels relative to those of β-tubulin were assessed by densitometric analysis and are expressed relative to levels in wild-type mice. *, P<0.05; **, P<0.01; ***, P<0.001, compared with wild-type mice; #, P<0.05; ##, P<0.01; ###, P<0.001, compared with CaR−/− mice.
altered significantly in CaR<sup>−/−</sup>; Pth<sup>−/−</sup> mice (Fig. 6A-C). The ratio of RANKL/OPG (Tnfsf11/Tnfrsf11b – Mouse Genome Informatics) mRNA levels showed no significant differences among the three knockout models and wild-type mice, as demonstrated by real-time RT-PCR (Fig. 6D).

**DISCUSSION**

We used CaR<sup>−/−</sup> mice, and CaR<sup>−/−</sup>; 1α(OH)ase<sup>−/−</sup> and CaR<sup>−/−</sup>; Pth<sup>−/−</sup> double-mutant mice and analyzed them biochemically, by radiography, micro-CT imaging, histology, immunohistochemistry, real-time RT-PCR and western blotting, to gain insight into the role of CaR and of calcium, phosphorus, 1,25(OH)<sub>2</sub>D, PTH and PTHrP in the formation of teeth and the development of dental alveolar bone.

We found that in CaR<sup>−/−</sup> mice, the area of biglycan-immunopositive predentin was increased, but the dental volume and DSP level were reduced. We also found that osteoid volume in dental alveolar bone was increased, but that dental alveolar bone volume was reduced dramatically, although the number of ALP-positive osteoblasts was increased. These results demonstrate that CaR deficiency produces defects in dental and dental alveolar bone mineralization and formation.

CaR<sup>−/−</sup> mice develop primary hyperparathyroidism and are hypercalcemic. To determine whether the hypercalcemia in CaR<sup>−/−</sup> mice contributed to the defects in dental and dental alveolar bone development in these animals, we bred them with mice in which Tnfrsf11b<sup>−/−</sup>; Pth<sup>−/−</sup> mice had been deleted. We recently examined 1α(OH)ase<sup>−/−</sup> mice, which are hypocalcemic and display secondary hyperparathyroidism and resultant hypophosphatemia, to assess the effects of 1,25(OH)<sub>2</sub>D deficiency on dental and dental alveolar bone formation and mineralization in the mandibles. These studies revealed defects in dental and dental alveolar bone formation and mineralization (Liu et al., 2009). These defects were, however, less severe than in CaR<sup>−/−</sup> mice. In the present studies, after deletion of 1α(OH)ase in CaR<sup>−/−</sup> mice in order to correct the hypercalcemia, a mild improvement in dental and dental alveolar bone formation was observed. This included slight increases in dental volume, DSP production and dental alveolar bone volume. By contrast, mineralization of the teeth and dental alveolar bone was not improved. These results therefore suggest that correction of the hypercalcemia partly corrects dental and dental alveolar bone formation, but does not correct the abnormal mineralization. In view of the failure to completely rescue the defects of dental and dental alveolar bone formation and mineralization in CaR<sup>−/−</sup> mice by concomitant deletion of 1α(OH)ase, we sought to determine whether these abnormalities were related to the more marked hyperparathyroidism and the presence of hypophosphatemia in the CaR<sup>−/−</sup>; 1α(OH)ase<sup>−/−</sup> mice, by deleting Pth from CaR<sup>−/−</sup> mice.

Ablation of Pth in CaR<sup>−/−</sup> mice has previously been reported to reverse the hyperparathyroidism and the associated hypercalcemia and hypophosphatemia (Kos et al., 2003; Tu et al., 2003), and to rescue the rachitic skeletal phenotype (Kos et al., 2003; Tu et al., 2003). In the present study, ablation of Pth in CaR<sup>−/−</sup> mice
ameliorated the defects in dental and dental alveolar bone formation and mineralization. Our results therefore suggest that hyperparathyroidism is the major factor contributing to defects in the dental and dental alveolar bone development caused by CaR deficiency, at least in part via the accompanying hypophosphatemia.

PTHrP is a ubiquitously produced local paracrine/autocrine/intracrine factor, the role of which is to regulate cellular proliferation, differentiation and differentiated function, as well as cell death, both during development and in adult life (Bisello et al., 2000; Fiaschi-Taesch and Stewart, 2003; Miao et al., 2008). Because PTHrP can modulate bone development both by binding to the type I PTH/PTHrP receptor (PTH1R – Mouse Genome Informatics) (Juppner et al., 1991) and by an intracrine mode of action (Miao et al., 2008), we assessed whether defects in teeth and mandible development are associated with any alteration of PTHrP expression in teeth and mandibles. Our results showed that PTHrP-immunopositive cells in the apical pulp and HERs, as well as PTHrP mRNA and protein levels in mandibular tissues, including teeth, were clearly decreased in CaR–/– mice. These parameters were only slightly increased in CaR+/-; 1α(OH)ase–/– relative to CaR–/– mice, but were increased markedly in CaR+/-; Pth–/– mice compared with their wild-type littermates. Previous studies have demonstrated that elevated extracellular calcium acting via CaR can increase PTHrP release (Ahlstrom et al., 2008; Tfelt-Hansen et al., 2003). Our results support the possibility that extracellular calcium stimulates PTHrP production via CaR and that CaR deficiency contributed to the low PTHrP expression in the CaR–/– mice. The secosteroid 1,25(OH)2D3 has been reported to downregulate PTHrP (Kremen et al., 1996; Tovar Sepulveda and Falzon, 2002), and we found that deletion of 1α(OH)ase in CaR-deficient mice upregulated PTHrP gene and protein expression in teeth and mandibles. This result suggests that 1,25(OH)2D3 modulates PTHrP production in a CaR-independent fashion. Although 1,25(OH)2D3 levels are reduced in CaR–/–; Pth–/– mice, the levels are still higher than in CaR+/-; 1α(OH)ase–/– mice, yet PTHrP gene and protein expression were upregulated to a greater degree in CaR–/–; Pth–/– than in CaR+/-; 1α(OH)ase–/– mice. Reduced 1,25(OH)2D might, therefore, mediate increased PTHrP expression in these PTH-deficient mice, but other mechanisms might be operative as well. In CaR–/– mice, the downregulation of PTHrP expression in mandibular tissues, including teeth, was associated with the defects in teeth and mandible caused by CaR deficiency. Conversely, upregulation of PTHrP expression was associated with either improvement of the teeth and mandible phenotypes in CaR+/-; 1α(OH)ase–/– mice or rescue of these phenotypes in CaR–/–; 1α(OH)ase–/– mice. We previously reported that PTHrP is required for increased trabecular bone volume in Pth–/– mice (Miao et al., 2004b). Our present studies suggest that PTHrP might play a local anabolic role in teeth and mandibular tissues, as well as in bone.

Our studies and the studies of others have shown that osteoblast numbers and trabecular bone volume in long bones are increased significantly in 1α(OH)ase–/– mice and Vdr–/– mice on a normal or even on a high calcium intake (Panda et al., 2004). This increase disappeared when circulating PTH was normalized and is, therefore, attributable to the anabolic effect of PTH. In the present study, however, circulating PTH levels, osteoblast numbers in the mandibles and the expression levels of several osteoblast-related genes were all increased in CaR–/– and CaR+/-; 1α(OH)ase–/– mice, but dental alveolar bone volume was not increased. Because alveolar bone volume was restored in CaR+/-; Pth–/– mice, the linkage of osteoblast activity to alveolar bone volume did not appear to be related directly to CaR deficiency. Consequently, hypophosphatemia per se, low PTHrP, or other factors yet to be determined, might play a role in preventing the coupling of the enhanced osteoblast activity to the development of increased alveolar bone volume in CaR+/- and CaR–/–; 1α(OH)ase–/– mice.

In summary, our data suggest that CaR is crucial for the development of teeth and alveolar bone postnatally. It is uncertain, however, what direct role, if any, CaR has on dental and alveolar bone formation. A recent study demonstrated that deletion of CaR in osteoblasts resulted in profound bone defects (Chang et al., 2008), however, the mechanism underlying any putative direct action of CaR on skeletal development remains unclear. The role of CaR in teeth and alveolar bone formation may therefore be, at least in part, to directly regulate the levels of ambient calcium (Brown et al., 1993; Brown and MacLeod, 2001) and PTHrP (Ahlstrom et al., 2008; Tfelt-Hansen et al., 2003) and to indirectly regulate phosphorus levels by altering PTH levels (Brown et al., 1993; Brown and MacLeod, 2001). These analyses, per se, may then modulate the postnatal development of teeth and alveolar bone.

Acknowledgements
This work was supported by Key Project grants from the National Natural Science Foundation of China (No. 30830103) and by the Program for Changjiang Scholars and Innovative Research Team in University (to D.M.), a grant from the Canadian Institutes for Health Research (to D.G.), and a grant from the National Institutes of Health (DK7083310E and DK70756 to M.R.P.). Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

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