Involvement of calcium-sensing receptor in osteoblastic differentiation of mouse MC3T3-E1 cells

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Yamauchi, Mika, Toru Yamaguchi, Hiroshi Kaji, Toshitsugu Sugimoto, and Kazuo Chihara. Involvement of calcium-sensing receptor in osteoblastic differentiation of mouse MC3T3-E1 cells. Am J Physiol Endocrinol Metab 288: E608–E616, 2005. First published November 16, 2004; doi:10.1152/ajpendo.00229.2004.—We have previously shown that the extracellular calcium-sensing receptor (CaR) is expressed in various bone marrow-derived cell lines and plays an important role in stimulating their proliferation and chemotaxis. It has also been reported that the CaR modulates matrix production and mineralization in chondrogenic cells. However, it remains unclear whether the CaR plays any role in regulating osteoblast differentiation. In this study, we found that mineralization of the mouse osteoblastic MC3T3-E1 cells was increased when the cells were exposed to high calcium (2.8 and 3.8 mM) or a specific CaR activator, NPS-R467 (1 and 3 μM). Next, we stably transfected MC3T3-E1 cells with either a CaR antisense vector (AS clone) or a vector containing the inactivating R185Q variant of the CaR (DN clone) that has previously been shown to exert a dominant negative action. Alkaline phosphatase activities were decreased compared with controls in both the AS and DN clones. However, the levels of type I procollagen and osteopontin mRNA in the AS clone, as detected by Northern blotting, were almost the same as in the controls. On the other hand, the expression of osteocalcin, which is expressed at a later stage of osteoblastic differentiation, was significantly reduced in both the AS and DN clones. Mineralization was also decreased in both clones. In conclusion, this study showed that the abolition of CaR function results in diminishing alkaline phosphatase activity, osteocalcin expression, and mineralization in mouse osteoblastic cells. This suggests that the CaR may be involved in osteoblastic differentiation.

osteoblast; mineralization; osteocalcin

THE CALCIUM-SENSING RECEPTOR (CaR) plays an important role in calcium homeostasis, predominantly through its effects on the regulation of parathyroid hormone (PTH) secretion by parathyroid glands and on urinary calcium excretion by the kidney (1, 5). We have previously shown that the CaR is expressed in the osteoblastic cell lines UMR-106 and SAOS-2 (37) and in the MC3T3-E1 cell line (36). It has been reported that the CaR is expressed in mouse, rat, and bovine cartilage (in articular chondrocytes and in hypertrophic chondrocytes of the epiphyseal growth plate) and bone (in osteoblasts and in osteocytes) (3). The CaR may play a key role in regulating bone turnover by stimulating the proliferation and chemotaxis of osteoblasts (36). Overexpression of signal-deficient CaR suppressed the expression of early differentiation markers and increased the expression of terminal differentiation markers in chondrogenic RCJ3.1C5.8 cells (4), suggesting that the CaR modulates matrix production and mineralization in chondrogenic cells. The local changes in extracellular calcium (Ca 2+) acting through the CaR may serve a physiological role in regulating the differentiation and function of osteoblasts and chondrocytes and therefore may have a direct effect on bone function. In addition, Garner et al. (10) have recently reported that CaR-deficient mice show the abnormalities of mineralization, similar to those seen in rickets and osteomalacia, despite having hypercalcemia caused by PTH excess. These studies suggest that the CaR might be indispensable for mineralization of bone. It is of interest, however, that the mineralized abnormalities of CaR-deficient mice could be rescued by abolishing hyperparathyroidism through the genetic ablation of the PTH gene or by the transfer of CaR deficiency onto the glial cell missing 2 (Gcm2)-deficient background (33). In addition, no model showed any obvious skeletal changes associated with CaR deficiency, except for elevated bone mineral density in the spine of female Pth −/−-CaR −/− mice (18). These two aforementioned studies using double-deficient mice, Pth −/−-CaR −/− or Gcm2 −/−-CaR −/−, failed to support an essential and nonredundant role of CaR in bone and cartilage metabolism. However, because embryonic knockout may produce compensatory pathways on occasion, the results of embryonic ablation may not be conclusive. Additional in vivo and in vitro experiments and long-term studies are needed to define the role of CaR in these organs. In this study, to clarify the roles of CaR in bone mineralization, we transfected mouse osteoblastic MC3T3-E1 cells with antisense (AS) and dominant negative (DN) vectors of the CaR to suppress endogenous CaR expression and functions. We then examined the effects of CaR suppression on osteoblastic differentiation and, in particular, the effects on mineralization and the production of osteoblast-specific substances.

MATERIALS AND METHODS

Materials. All routine cell culture media were obtained from Gibco-BRL (Rockville, MD). Anhydrous calcium chloride (CaCl2) was purchased from Wako Industries (Osaka, Japan). NPS-R467 was a generous gift from Dr. Edward F. Nemeth, NPS Pharmaceuticals (Salt Lake City, UT). A CaR-specific polyclonal antiserum (4637) was generously provided by Drs. Forrest Fuller and Karen Krapcho of NPS Pharmaceuticals. This antiserum was raised in rabbit against a peptide (FF-7) corresponding to amino acids 345–359 of bovine CaR that resides within the predicted amino-terminal extracellular domain of the CaR.

Cell culture. MC3T3-E1 cells, an osteoblastic cell line established from normal mouse calvaria, were kindly provided by Dr. H. Kodama. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Stable transfection. MC3T3-E1 cells were stably transfected with a CaR AS and the CaR bearing an inactivating mutation (R185Q), previously shown to exert a DN action on the wild-type receptor. Plasmid pCRII (Invitrogen, San Diego, CA) and mammalian expression vector pcDNA3 (Invitrogen) were used to construct an AS CaR expression vector. A 324-bp CaR cDNA containing 5 bp of upstream DNA from the open reading region was cloned into plasmid pCRII (pCR-CaR). pCR-CaR was digested with EcoRI, and a 324-bp cDNA fragment from the complementary 5′-CaR coding region was subcloned into the cloning sites of pcDNA3. The orientation of the inserts was determined by restriction mapping and confirmed by sequencing. A DN-CaR, R185Q, which was cloned in pcDNA3 and tagged with the Flag epitope tag, as well as the empty vector were prepared using the Midi plasmid kit (Qiagen). AS, DN, and empty vectors (each 2 μg) were transfected into MC3T3-E1 cells with lipofectamine (GIBCO-BRL). After 48 h, cells were passaged and clones were selected in α-MEM supplemented with G418 (0.3 mg/ml; GIBCO-BRL) and 10% FBS. Cells that exhibited Flag-positive immunoreactivity were used as DN-transfected cells. Empty vector-transfected cells similarly selected with G418 were used as negative controls.

Selection of AS clones by Western blot analysis. Western blot analysis was performed to select the AS clones, as previously described (36). In brief, cells were lysed with a lysis solution (1% SDS, 10 mM Tris·HCl, pH 7.4) and heated to 65°C. The cells were scraped from the dishes, transferred to microcentrifuge tubes, and heated for an additional 5 min at 65°C. The viscosity of the sample was reduced by brief sonication, and insoluble material was removed by centrifugation for 5 min. The resultant whole cell lysate in the supernatant was stored at −80°C until Western blot analysis was carried out. Protein quantitation was performed with bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL). Aliquots of 150 μg of protein were dissolved in SDS-Laemmli gel loading buffer containing 100 mM dithiothreitol, incubated at 37°C for 15 min, and resolved electrophoretically on 6% SDS-polyacrylamide gels. Proteins were transferred in 25 mM Tris, 192 mM glycine, and 20% methanol to polyvinylidene difluoride blots. The blots were blocked for 2 h with Tris-buffered saline [TBS; 20 mM Tris·HCl (pH 7.5) and 137 mM NaCl] plus 0.1% Tween 20 containing 3% dried milk powder, and then incubated overnight at 4°C with the affinity-purified antiseraum (4637). The blots were further incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated, goat anti-rabbit IgG (Sigma Chemical) for 1 h at room temperature. The specific protein bands were detected with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Arlington Heights, IL).

Selection of DN clones by immunocytochemistry. To select DN clones, we performed immunocytochemistry. Each coverslip with DN-transfected cells was fixed with 4% formaldehyde in PBS for 5 min. Fixed DN-transfected cells were treated with peroxidase-block reagent for 10 min to inhibit endogenous peroxidases, followed by treatment with protein block serum-free solution for 1 h, and then incubated overnight at 4°C with peroxidase-coupled anti-Flag antibody. The color reaction was developed for 10 min using the DAKO AEC substrate system (DAKO, Carpenteria, CA).

RNA extraction and Northern blot analysis. The levels of type I procollagen (COL I), mouse osteocalcin (OCN), and mouse osteopontin (OPN) mRNA were detected by Northern blot analysis. Total RNA was prepared from MC3T3-E1 cells using the acid guanidinium thiocyanate-phenol-chloroform extraction method (8). Twenty micrograms of total RNA were denatured, electrophoresed on a 1% agarose gel containing 2% formaldehyde, and then transferred to a nitrocellulose membrane and fixed with UV light (FUNA-UV-LINKER; Funakoshi, Tokyo, Japan). The membranes were hybridized to a 32P-labeled DNA probe. The hybridization probes used were the 2.8-kb fragment of the gene of COL I (a gift from Dr. H. Kodama, Ohu Dental College) as previously described (29). After hybridization, the filter was washed twice with 2× SSC containing 0.5% SDS and subsequently washed twice with 0.1× SSC containing 0.5% SDS at 58°C for 1 h. The filter was exposed to X-ray film using an intensifying screen at −80°C. All values were normalized for RNA loading by probing the blot with human β-actin cDNA (Wako Industries).

Semiquantitative RT-PCR. Reverse transcription of 5 μg of cultured cell total RNA was carried out for 50 min at 42°C and then 15 min at 70°C, using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen), which contained RT buffer, oligo(dt)12–18, 5× first-strand solution, 10 mM dNTP, 0.1 M dithiothreitol, SuperScript II (RT-enzyme), and RNase H (ribonuclease inhibitor). mRNA levels of OCN were detected by RT-PCR. With the use of primers unique to mouse OCN cDNA sequences, RT-PCR was carried out in a volume of 10 μl of reaction mixture for PCR (as supplied by TaKaRa, Otsu, Japan) supplemented with 2.5 U of TaKaRa Taq, 1.5 mM each dNTP (TaKaRa), and PCR buffer (10×) that contained 100 mM Tris·HCl (pH 8.3), 500 mM KCl, and 15 mM MgCl2. An amount of 25 ng of each primer and 1 μg of template (from a 50-μl RT reaction) were used. Thermal cycling conditions and primer sequences are described as follows: 1) initial denaturation at 96°C for 2 min, 2) cycling for 30cycles [99°C for 1 min, cDNA-specific annealing temperature for 2 min, and 72°C for 2 min], and 3) final extension at 72°C for 5 min. Primer sequences, annealing temperature, and cycle numbers were as follows: OCN, 5′-CTGGTGCCTTCTGACCTCTACAG-3′ and 5′-GGAGCTGCTGTGACATCCATAC-3′ (53°C, 28 cycles); GAPDH, 5′-ATCCATCACCTTCCGAGG-3′ and 5′-CTGCTTACCACCTTCCAGG-3′ (47°C, 24 cycles). For semiquantitative RT-PCR, the number of cycles was chosen so that amplification was stable within the linear range, as assessed by densitometry (NIH Image J, v. 1.08i, Public Domain Program). PCR products were fractionated on 1% agarose gels. OCN gene expression was normalized to GAPDH expression in each sample.

Assay of alkaline phosphatase activity and alkaline phosphatase staining. After reaching confluence, cells in 24-well plates were rinsed three times with PBS, and 600 μl of distilled water were added to each well and sonicated. The protein assay was performed with the BCA protein assay reagent. Alkaline phosphatase (ALP) activity was assayed by a method modified from that of Lowry et al. (20). In brief, the assay mixtures contained 0.1 M 2-amino-2-methyl-1-propanol, 1 mM MgCl2, 8 mM p-nitrophenylphosphate disodium (Sigma), and cell homogenates. After 3 min of incubation, the reaction was stopped with 0.1 N NaOH, and the absorbance was read at 405 nm. A standard curve was prepared with p-nitrophenol (Sigma). Each value was normalized to the protein content value.

ALP staining was performed by a standard protocol. In brief, cultured cells were rinsed with PBS, fixed in 100% methanol, rinsed with PBS, and then overlaid with 1.5 ml of 0.15 ml of 5-bromo-4-chloro-3-indolylphosphate (GIBCO-BRL) plus 0.3 mg/ml nitroblue tetrazolium chloride (GIBCO-BRL) in 0.1 M Tris·HCl, pH 9.5, 0.01 N NaOH, and 0.05 mM MgCl2, followed by incubation at room temperature for 2 h in the dark.

Transient transfection. MC3T3-E1 cells were transiently transfected with 1–3 μg of AS, 0.3–1 μg of DN, or empty vector using lipofectamine. The total amounts of vector were the same (10 μg each). ALP activity was measured 48 h after transfection.

Assay of mineralization. The mineralization of MC3T3-E1 cells was determined in 6-well and 12-well plates using von Kossa staining and Alizarin red (Sigma) staining, respectively. After reaching confluence, cells were grown in α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 10 mM β-glycerophosphate for 2 wk.
After confluence, nontransfected MC3T3-E1 cells were grown in the same medium as well as in various concentrations of calcium and CaR agonist (2.8 and 3.8 mM CaCl₂ or 1 and 3 μM NPS-R467). The cells were then fixed with 95% ethanol and stained with AgNO₃ by the von Kossa method to detect phosphate deposits in bone nodules (7). At the same time, the other plates were fixed with ice-cold 70% ethanol and stained with Alizarin red to detect calcification. For quantitation, cells stained with Alizarin red were stained with ethylpyridinium chloride (Wako Industries), then the extracted stain was transferred to a 96-well plate, and the absorbance at 562 nm was measured using a microplate reader, as previously described (27).

Statistics. Results are expressed as means ± SE. Statistical evaluation for differences between groups was carried out with one-way ANOVA, followed by Fisher’s protected least significant difference. For all statistical tests, a value of \( P < 0.05 \) was considered to indicate a statistically significant difference.

RESULTS

Effects of high \( \text{Ca}^{2+} \) or CaR agonist on mineralization in MC3T3-E1 cells. To investigate the effects of high \( \text{Ca}^{2+} \) or CaR agonist on mineralization in MC3T3-E1 cells, we performed Alizarin red staining and von Kossa staining. The mineralization of the mouse osteoblastic MC3T3-E1 cells was increased when the cells were exposed to high calcium (2.8 and 3.8 mM) or a specific CaR activator, NPS-R467 (1 and 3 μM), for 10 days after reaching confluence. We also found that treatment of MC3T3-E1 cells with increasing levels of \( \text{Ca}^{2+} \) up to 3.8 mM resulted in a dose-dependent stimulation of mineralization compared with the control-level treatment (1.8 mM \( \text{Ca}^{2+} \); \( P < 0.01 \); Fig. 1). A specific CaR activator, NPS-R467 (1 and 3 μM), also induced significant stimulation of mineralization compared with the control-level treatment (\( P < 0.01 \)).

Stable transfection. We identified 10 and 20 clones for AS and DN clones, respectively. Several of the AS clones were selected for Western blotting with the anti-CaR antibody. As shown in Fig. 2, CaR signals were decreased in the AS3 and AS4 clones, although the signals in the vector (V) and AS1 clones were almost the same as those in the wild cells. We selected the AS3 and AS4 clones for further examination. The DN1 and DN7 clones, in which over 80 and 90% of the cells, respectively, exhibited Flag-positive immunoreactivity, were selected (Fig. 3).

Effects of the CaR on ALP activities of osteoblastic cells. Measurement of ALP activity is important to assess the process of bone formation as well as mineralization. Therefore, we examined whether a reduction in the CaR expression would affect ALP activity. ALP activity was evaluated both histochromically and biochemically. ALP staining and activity were decreased in the AS and DN clones compared with the V clone (Fig. 4, A and B). Transient transfection of the cells with the AS or DN vectors also resulted in decreases in ALP activity in a manner dependent on vector dose (Fig. 5).

Effects of the CaR on mineralization of osteoblastic cells. Mineralization is the most important phenotype in osteoblast differentiation. Mineralization was examined by Alizarin red staining and von Kossa staining as well as by a quantitative assay of mineralization based on Alizarin red staining. The mineralization of the AS and DN clones was decreased compared with that of the empty V clones (Fig. 6, A and B). The quantitative assay revealed that the differences in mineralization between the AS or DN clones and the V clones were significant (\( P < 0.05 \)).

Effects of the CaR on the expression of COL I, OPN, and OCN of osteoblastic cells. The production of bone matrix protein is also important for osteoblast differentiation. Therefore, we examined whether the reduction in the CaR expression would affect the expression of bone matrix proteins in MC3T3-E1 cells. In the AS clones, the levels of COL I and OPN mRNA in Northern blotting were similar to those in the V clones (Fig. 7A). The expression of COL I and OPN in the DN1 and DN7 clones was slightly decreased compared with that in the V clone. On the other hand, the expression of OCN, which appeared at a later stage of osteoblastic differentiation, was remarkably reduced in both the AS (Fig. 8A) and the DN clones (Fig. 8B), as determined by Northern blotting and semiquantitative RT-PCR.
CAR ENHANCES OSTEOBLASTIC DIFFERENTIATION

DISCUSSION

Osteoblasts are known to play a crucial role in the formation phase of bone remodeling by laying down the structural components of bone (matrix and mineral) and secreting various cytokines and growth factors that influence both bone formation and resorption. Bone formation is initiated by the migration of preosteoblasts into resorption pits at the end of osteoclastic bone resorption. Substantial amounts of Ca\(^{2+}\) are released from the mineralized bone matrix during osteoclastic resorption, raising the level of Ca\(^{2+}\) in the vicinity of resorption sites. It is possible that the CaR senses these high levels of Ca\(^{2+}\), thereby providing a signal for preosteoblasts that induces their migration into sites where new bone formation is required. In fact, we previously showed that high Ca\(^{2+}\) induced both a chemotaxis and proliferation of MC3T3-E1 cells (31). The CaR is expressed in mouse, rat, and bovine cartilage and bone (3), bone marrow cells (13), chondrogenic cells (2), and osteoclastic cell lines (15, 16) as well as in various osteoblastic cell lines (35–37). In vitro studies and indirect in vivo evidence implicate the CaR as having an important role in the regulation of essential steps in bone formation and resorption. We previously reported that CaR agonists stimulated both chemotaxis and proliferation of MC3T3-E1 cells (36) and proposed that the CaR may play a key role in regulating bone turnover by sensing increases in Ca\(^{2+}\). Chattopadhyay et al. (6) demonstrated expression of CaR in rat calvarial osteoblasts, which promotes mitogenesis in these cells (6).

Cells of the chondrocyte lineage produce matrix proteins, and mineralization corresponds to the developmental state of the cells. High Ca\(^{2+}\) inhibits the expression of early chondrogenic markers and enhances the expression of terminal differentiation markers and the mineral deposition in the chondrogenic RCJ3.1C5.18 cells (2). The CaR is also expressed in chondrogenic cells and modulates matrix production and mineralization. Overexpression of signal-deficient CaR suppresses the expression of early differentiation markers and increases the expression of terminal differentiation markers in the cells (4). These results suggest that the high Ca\(^{2+}\) regulates chondrocyte differentiation potentially via activation of the CaR.

In osteoclasts, the high concentration of Ca\(^{2+}\) that is sensed through the CaR can inhibit osteoclast activity (15). We previously reported that the CaR in osteoclast precursor cells could play a key role in regulating osteoclast formation by sensing local changes in Ca\(^{2+}\) at the resorptive sites (16). Taken together, these data demonstrate that the CaR may have important functions at various steps in bone remodeling. Recently, Dvorak et al. (9) reported that mRNA expression of the osteoblast differentiation markers COL I, OPN, and OCN mRNAs was increased by high Ca\(^{2+}\), as was mineralized nodule formation. Moreover, the high Ca\(^{2+}\)-induced mineralization was inhibited by the CaR inhibitor NPS-89636. This suggests that the CaR participates in the differentiation of osteoblasts.

MC3T3-E1 cells are known to provide an in vitro model for studying the developmental process from preosteoblast to mature osteoblast (26, 38). We previously showed that MC3T3-E1 cells expressed the CaR over the entire range of culture periods (36). In the present study, we found that both high Ca\(^{2+}\) and the CaR-specific agonist increased the mineralization of MC3T3-E1 cells. To further analyze the function of the CaR in sensing high Ca\(^{2+}\) in osteoblasts, the activity of endogenous CaR in MC3T3-E1 cells was suppressed by transfecting the cells with a vector containing either an AS or DN form of the CaR.
receptor. The stably transfected AS and DN clones showed a reduction in both ALP activities and mineralization. The transiently transfected cells also revealed a reduction in ALP activities in a manner dependent on dose of vector. The OCN gene transcripts were significantly downregulated in both the AS and the DN clones compared with the control clones. It is well known that ALP activity is essential for both mineralization and the production of bone matrix proteins, and that OCN is considered as a highly predictive marker for the osteoblast phenotype. Mineralization of newly formed matrix takes place after the expression of OCN. Thus, together, these results suggest that the reduction and dysfunction of the CaR in MC3T3-E1 cells may lead to an impediment in the differentiation of the osteoblastic phenotype at a later stage.

This study also showed that the expression of COL I and OPN in the DN clones was slightly decreased compared with that in the vector clone. Although their expression was not different in the AS clones, our results seem in partial accordance with the findings by Dvorak et al. (9) that high Ca\(^{2+}\) increased the expression of COL I and OPN in osteoblasts (9).
There have been only a few other studies of the relationship between the CaR function and cell differentiation. In keratinocytes, the expression of CaR is upregulated in a differentiation-dependent manner, and high Ca\textsuperscript{2+} induces differentiation markers in the cells (32). Regulated expression of the CaR was also evident in CFK2 chondrogenic cells at a later stage of differentiation and could participate in changes in the transport of calcium by Ca\textsuperscript{2+} (34).

CaR agonists or antagonists have been under the consideration of clinical use for treatment of osteoporosis, idiopathic hypoparathyroidism, and primary and secondary hyperparathyroidism. It has been reported that the CaR agonist NPS-R568...
caused a decrease in the circulating levels of PTH and an increase in bone volume in animal models of secondary hyperparathyroidism produced by chronic renal failure (14). The anabolic effect of the CaR agonist on bone seemed to depend on the oscillation of PTH levels. On the other hand, our results indicate that activation of the CaR in osteoblasts could directly evoke anabolic effects on bone. Thus CaR agonists might have a therapeutic benefit on bone mineralization via two different mechanisms.

The CaR-deficient mice show a skeletal phenotype of rickets, suggesting that CaR might be essential for normal mineralization (10, 12). However, hyperparathyroidism caused by CaR deletion confounded the independent assessment of CaR effects on bone and cartilage. Moreover, the neonatal mortality in CaR-deficient mice also prevented assessment of the role of the CaR in the adult skeleton. To fully understand the direct effect of the CaR on bone and cartilage function, correction of hyperparathyroidism was required. Recently, two research groups have reported double knockout models. Tu et al. (33) transferred CaR deficiency onto the Gcm2-deficient background to correct the severe hyperparathyroidism accompanied by hypercalcemia and hypophosphatemia observed in the CaR-deficient mice. Gcm2 is a master regulatory gene of parathyroid gland development (17), and Gcm2 knockout mice lack parathyroid glands (11). Kos et al. (18), on the other hand, succeeded in achieving genetic ablation of the PTH gene in...
CaR-deficient mice (18). In both studies, the results were concordant that elimination of hyperparathyroidism rescued the increased neonatal mortality as well as rickets-like skeletal abnormality in these mice. The findings in these double knock-out mice suggest that the CaR may not be indispensable for bone differentiation and remodeling. In agreement with this possibility, immortalized osteoblasts derived from CaR-deficient mice were shown to retain their ability to respond to Ca\(^{2+}\) through a G protein-coupled mechanism and to reveal a pattern of mineralization similar to osteoblasts derived from wild-type mice (22). In addition, patients with neonatal severe hyperparathyroidism, which is often caused by homozygous CaR gene mutations (24), do not develop rickets.

Some reports have suggested the existence of another cation receptor, the functional properties of which differ from the CaR in several ways (21, 25, 30). These studies suggested that novel cation receptors may play a more important role than the CaR in bone mineralization (22, 23, 25). Because calcium regulation is so fundamental to the maintenance of normal cell function, it is plausible that cells have some other pathways that are compensatory for the embryonic defects in the CaR. Glutamate receptors belonging to the same family as the CaR may be such candidates. These receptors are present in bone and respond to the changes in Ca\(^{2+}\) (19) as well as to mechanical force (28).

In conclusion, we showed in this study that the abolishment of the CaR function in mouse osteoblastic cells results in diminishing ALP activity, OCN expression, and mineralization. Our findings support the hypothesis that the CaR plays a role in osteoblastic differentiation.

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