

Cinacalcet HCl Suppresses *Cyclin D1* Oncogene-Derived Parathyroid Cell Proliferation in a Murine Model for Primary Hyperparathyroidism

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Abstract Cinacalcet HCl (cinacalcet) is a calcimimetic compound, which suppresses parathyroid (PTH) hormone secretion from parathyroid glands in both primary hyperparathyroidism (PHPT) and secondary hyperparathyroidism (SHPT). We previously reported the suppressive effect of cinacalcet on PTH secretion in vivo in a PHPT model mouse, in which parathyroid-targeted overexpression of the *cyclin D1* oncogene caused chronic biochemical hyperparathyroidism and parathyroid cell hyperplasia. Although cinacalcet suppressed parathyroid cell proliferation in SHPT in 5/6-nephrectomized uremic rats, its effect on PHPT has not yet been determined. In this study, the effect of cinacalcet on parathyroid cell proliferation was analyzed in PHPT mice. Cinacalcet (1 mg/g) was mixed into the

rodent diet and orally administrated to 80-week-old PHPT mice for 10 days before death. 5-Bromo-2'-deoxyuridine (BrdU, 6 mg/day) was infused by an osmotic pump for 5 days before death, followed by immunostaining of the thyroid–parathyroid complex using an anti-BrdU antibody to estimate parathyroid cell proliferation. Compared to untreated PHPT mice, cinacalcet significantly suppressed both serum calcium and PTH. The proportion of BrdU-positive cells to the total cell number in the parathyroid glands increased considerably in untreated PHPT mice ($9.5 \pm 3.1\%$) compared to wild-type mice ($0.7 \pm 0.1\%$) and was significantly suppressed by cinacalcet ($1.2 \pm 0.2\%$). Cinacalcet did not affect apoptosis in the parathyroid cells of PHPT mice. These data suggest that cinacalcet suppressed both serum PTH levels and parathyroid cell proliferation in vivo in PHPT.

The authors have stated that they have no conflict of interest.

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Primary hyperparathyroidism (PHPT), frequently caused by sporadic parathyroid adenomas, is characterized by hyperfunctioning parathyroid tissue that responds with decreased sensitivity to extracellular calcium (Ca) concentration. Two specific genes have been identified as participating in the tumorigenesis of parathyroid adenomas: the *cyclin D1/PRAD1* oncogene [1] and the multiple endocrine neoplasia type 1 (*MEN1*) tumor-suppressor gene [2, 3]. Parathyroid-targeted overexpression of the *cyclin D1* oncogene resulted in the development not only of abnormal parathyroid cell proliferation in mice but also of chronic biochemical hyperparathyroidism with characteristic abnormalities in bone [4].

An increase in the secretory set point of the PTH–Ca sigmoidal curve was associated with reduced calcium-sensing receptor (CaR) expression in parathyroid glands *in vivo* in CaR-knockout mice [5] and PHPT model mice [4, 6] and resulted in elevated PTH with hypercalcemia. Reduced CaR expression in parathyroid glands has been observed in PHPT and secondary hyperparathyroidism of uremia (SHPT) [7–9]; thus, CaR is a therapeutic target in hyperfunctioning parathyroid diseases.

Cinacalcet HCl, or cinacalcet, an allosteric modulator of CaR, increases the sensitivity of the receptor to activation by extracellular Ca, which leads to the suppression of PTH release from parathyroid cells [10]. Cinacalcet has met with success as a novel therapeutic agent in the treatment of SHPT [11–13] and, to some extent, of PHPT [14] in clinical studies. In 5/6-nephrectomized uremic rats, calcimimetics, such as R-568 [15] and cinacalcet [16], successfully suppress parathyroid cell proliferation as well as PTH secretion. Although cinacalcet HCl demonstrated potency at suppressing PTH in spite of any presumed endogenous CaR activation by hypercalcemia and hypoexpression of CaR in the parathyroid glands in PHPT model mice [17], it is unknown whether the calcimimetics can also suppress parathyroid cell proliferation when induced by an oncogene.

The aim of this study was to evaluate the effects of cinacalcet on the suppression of parathyroid cell proliferation caused by the *cyclin D1* oncogene and on the suppression of serum PTH and Ca levels, *in vivo*, in a murine model of PHPT and to investigate its molecular mechanism. Finally, we aimed to learn more about the effect of cinacalcet on parathyroid glands with reduced CaR expression.

Materials and Methods

Materials

Cinacalcet HCl— α R-(–)- α -methyl-N-[3-[3-trifluoromethylphenyl]propyl]-1-naphthalenemethanamine hydrochloride—was generated by Kyowa Hakko Kirin. (Takasaki, Japan). Methylcellulose was obtained from Sigma (St. Louis, MO). NPS Pharmaceutical (Salt Lake City, UT) kindly provided the anti-CaR antibody (4638 polyclonal antibody).

Animals

FVB/N background PTH–*cyclin D1* transgenic mice (PC2), which exhibit parathyroid-targeted overexpression of the human *cyclin D1* oncogene, were used in this study as a model for PHPT [4]. Offspring were genotyped by

Southern blotting using h-*cyclin D1* cDNA as a probe [4] to select mice bearing the transgene. All mice were provided with the commercially available rodent diet CE-2 (Clea Japan, Tokyo, Japan), containing 1.03% Ca and 0.97% phosphate (P), and water ad libitum. Studies were approved by the appropriate institutional animal care committees at Osaka City University Medical School.

Experimental Protocols

Seventy PC2 and wild-type (WT) mice, 7–84 weeks old, were used in this study because established PHPT was observed in all PC2 mice at these ages [6]. PC2 mice were randomly assigned to cinacalcet-treatment and nontreatment groups. Once assigned to their groups, all mice were fed a CE-2 powder diet for 10 days, apart from mice in the cinacalcet-treatment group, which were fed a CE-2 powder diet containing 0.1% cinacalcet HCl (100 mg/100 g chow). All mice were fed the experimental diet for 10 days and then killed. All mice received 6 µg/day of subcutaneous 5-bromo-2'-deoxyuridine (BrdU) continuously via Alzet osmotic pumps, implanted subcutaneously (model 2001; Alza, Mountain View, CA), for the last 5 days before death. Mice were killed under ether anesthesia and the thyroid–parathyroid complexes immediately removed.

Measurement of Biochemical Parameters

Serum PTH was measured using rat PTH IRMA kits (Immutopics, San Clemente, CA). Serum biochemistry profiles, including Ca, inorganic phosphorus (IP), and serum urea nitrogen (SUN), were determined using a series of TESTWAKO kits (Wako Chemical, Osaka, Japan). Serum 1.25(OH)₂D concentrations were measured using radioimmunoassay kits (Immunodiagnostic Systems, Boldon, UK). Blood samples were collected from orbital cavities pre- and post-cinacalcet treatment for the measurement of serum Ca, IP, and PTH. Blood samples were also collected by cardiac puncture at the time of death and used for the measurement of SUN and serum 1.25(OH)₂D levels.

Parathyroid Histology

CaR expression in the parathyroid glands was determined as previously described [17]. Resected parathyroid glands were immediately fixed in 10% formalin for 2 days and embedded in paraffin. Total parathyroid glands were sliced into 3-µm-thick sections, and the number of slides that contained parathyroid tissue was counted. Every quarter section defined by slide number was selected for analysis as the indicator of the parathyroid gland size. Immunohistochemical tests were performed, followed by inactivation of intrinsic peroxidase by incubation in 3% hydrogen

peroxide diluted in methanol. Sections were then treated with skim milk to prevent background staining and incubated with anti-CaR (0.8 µg/ml) or anti-PTH (dil 1:1000) antibody overnight at 4°C in a humidified chamber. After rinsing, sections were incubated with the biotinylated secondary antibody and peroxidase-conjugated streptavidin, followed by visualization with alkaline phosphatase and counterstaining with hematoxylin.

The outside of the parathyroid gland was carefully traced in each slide, and a person who was blinded to tissue samples calculated the area using Adobe Photoshop 5.5 software (Adobe Systems, San Jose, CA). The size given in pixels was changed to millimeters squared using NIH image 1.62 (National Institutes of Health, Bethesda, MD) and adjusted by kilograms of body weight. The size of each gland was represented by the mean value of three slides. Unstained area was defined by the area apparently stained fainter than the area of clearly stained normal parathyroid gland. The ratio of CaR reduced area size to whole area size in a parathyroid section—CaR(−)/whole area—represented CaR expression in the parathyroid glands.

To identify proliferating cells, BrdU-incorporated cells were detected using a BrdU immunostaining kit (Zymed Labs, San Francisco, CA) [15]. BrdU-positive cells were counted under 400× magnification, and the number was expressed as a percentage of the total cell number. The percentage was determined by counting a minimum of 1,000 cells per sample.

To identify apoptosis, nuclear DNA fragmentation was detected *in situ* by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using the ApopTag Kit (Chemicon International, Temecula, CA) [15].

Statistical Analyses

Intergroup comparisons were made using the Tukey-Kramer multiple comparison test. Differences in serum parameter levels between pre- and postadministration of cinacalcet were analyzed by the paired *t*-test. Correlations between two variables were examined by linear regression analysis. All data are presented as the mean ± SE. $P < 0.05$ was taken to indicate statistical significance.

Results

Effects of Cinacalcet on Serum Parameters

PC2 mice exhibited significant elevation of serum PTH and Ca levels and a reduction of serum phosphate levels compared to WT before the administration of cinacalcet (Fig. 1). Daily dietary administration of cinacalcet

significantly suppressed serum PTH and Ca levels to the levels found in WT mice and partially increased serum phosphate levels. PC2 mice exhibited significant elevation of serum 1,25(OH)₂D compared to WT mice, which decreased in response to the administration of cinacalcet (Table 1). No significant differences were observed in SUN and body weight among the groups at the time of death.

Effects of Cinacalcet on CaR Expression in Parathyroid Glands

Focal regions of reduced CaR expression were observed in some PC2 mice by immunohistochemical analysis (Fig. 2b), while other PC2 mice exhibited a general reduction in CaR expression in the parathyroid glands (Fig. 2c). This hypoexpression of CaR or CaR(−)/whole area ratio in parathyroid glands was much greater in PC2 mice compared to WT mice (Table 1). The CaR(−)/whole area ratio in parathyroid glands showed a significant positive correlation with serum Ca ($r = 0.636$, $P = 0.0038$) and PTH ($r = 0.442$, $P = 0.0071$) before the administration of cinacalcet (Fig. 3). These correlations were diminished when the WT mice data were excluded. No significant difference in CaR expression was observed following the administration of cinacalcet in PC2 mice (Table 1).

Effects of Cinacalcet on the Proliferation of Parathyroid Cells

The parathyroid cell proliferation rate showed a significant increase (by a factor of 10) in PC2 mice compared to WT mice, as measured by BrdU uptake (Fig. 4). The parathyroid glands with focal regions of reduced CaR expression in PC2 mice also exhibited focal regions of BrdU uptake (Fig. 2b, f) compared to those in WT mice (Fig. 2a, e). The PC2 mice exhibiting a general reduction in CaR expression in the parathyroid glands also exhibited a general uptake of BrdU (Fig. 2c, g). The CaR(−)/whole area ratio in parathyroid glands showed significant positive correlation with BrdU-positive cells ($r = 0.515$, $P = 0.0013$) among cinacalcet-untreated mice (WT and PC2-untreated) (Fig. 5).

A significant reduction in BrdU uptake, similar to that observed in WT mice, was seen in PC2 mice following the dietary administration of cinacalcet (Fig. 4). Although the expression of CaR, the target molecule of cinacalcet, was reduced, BrdU uptake was strongly suppressed by cinacalcet (Fig. 2d, h). No significant correlation was observed between the CaR(−)/whole area ratio and BrdU-positive cells in WT and PC2 cinacalcet mice ($r = 0.256$, $P = 0.3209$).

Fig. 1 Serum biochemistry for WT, PC2-untreated, and PC2 cinacalcet-treated mice. *Ca* calcium; *PTH* parathyroid hormone; *IP* inorganic phosphate. Values represent means \pm SE. ^a*P* < 0.05 versus WT, ^b*P* < 0.05 versus PC2-untreated, ^t*P* < 0.05 versus preadministration

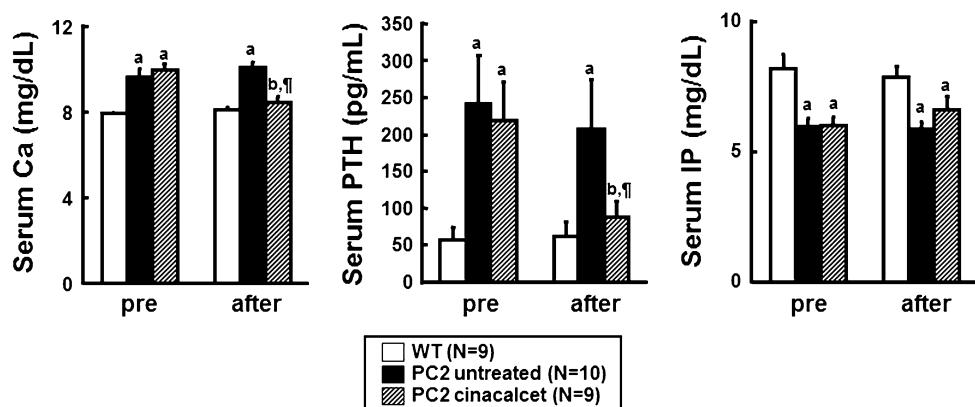


Table 1 Profiles for experimental mice at time of death

	<i>n</i>	Body weight (g)	SUN (mg/dl)	1,25(OH) ₂ D (pmol/l)	CaR(−)/whole area (%)
WT	9	41.4 \pm 0.8	24.9 \pm 0.4	241 \pm 23	15.5 \pm 0.8
PC2-untreated	10	41.7 \pm 1.4	24.8 \pm 0.6	439 \pm 35*	64.2 \pm 4.7*
PC2 cinacalcet	9	43.3 \pm 3.8	24.3 \pm 0.4	352 \pm 33	63.0 \pm 5.0*

Values represent mean \pm SE

SUN serum urea nitrogen; CaR(−)/whole area, ratio of CaR reduced area size against whole area size in a parathyroid section

**P* < 0.05 versus WT

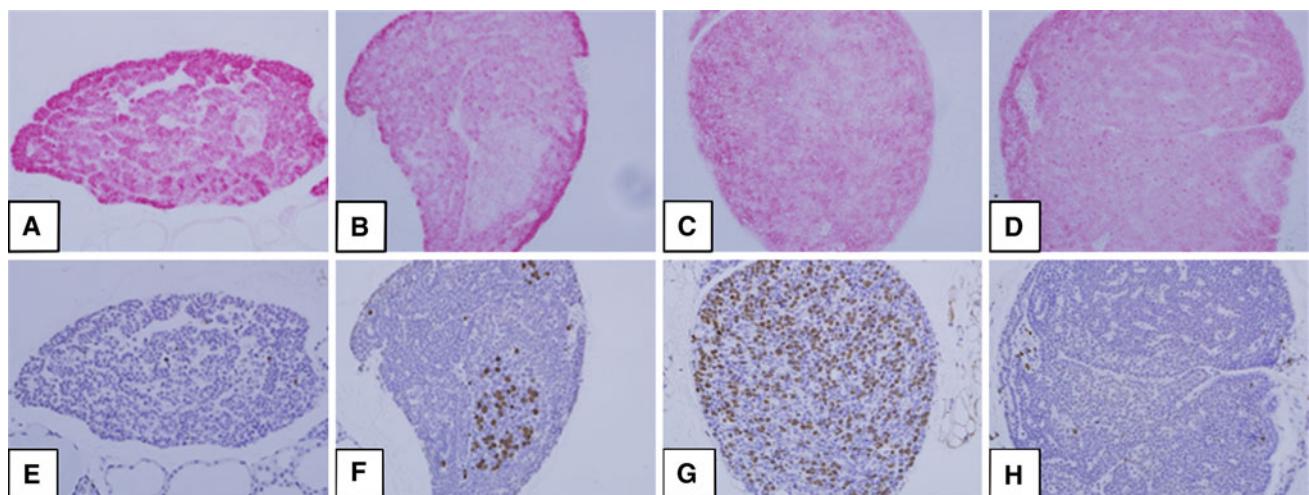


Fig. 2 Immunohistochemical analyses of CaR expression and BrdU incorporation in parathyroid glands. Parathyroid glands obtained from WT (a, e), PC2-untreated (b, c, f, g), and PC2 cinacalcet-treated (d, h) mice. Parathyroid glands were stained with an anti-CaR antibody (a–d) and an anti-BrdU antibody (e–h). Some PC2 mice exhibited focal regions of CaR hypoexpression (b) and focal

regions of BrdU incorporation (f). Other parathyroid glands exhibited global CaR hypoexpression (c) and global incorporation of BrdU. BrdU uptake was strongly suppressed by cinacalcet (h), and these glands also showed reduced CaR expression (d). Magnification is 200 \times

Effects of Cinacalcet on Apoptosis in Parathyroid Glands

Almost no apoptosis was observed in WT and PC2 mice by TUNEL staining (data not shown). Cinacalcet administration did not affect apoptosis in PC2 parathyroid glands.

Discussion

Cinacalcet, an allosteric modulator of CaR which acts as a calcimimetic, suppresses PTH secretion and has been approved for the treatment of SHPT patients on maintenance dialysis. Although calcimimetics, such as R-568 [15]

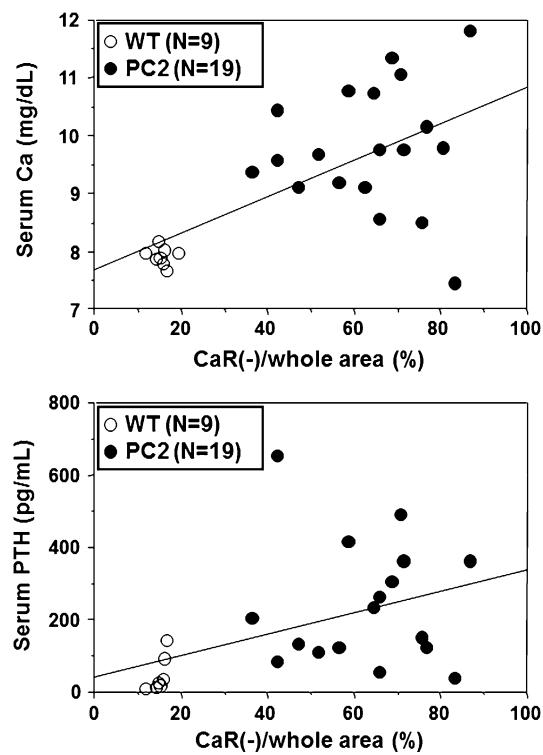


Fig. 3 The effects of CaR hypoexpression on serum parameters in parathyroid glands. The CaR(-)/whole area ratio in parathyroid glands showed a significant positive correlation with serum Ca ($r = 0.636, P = 0.0038$) and PTH ($r = 0.442, P = 0.0071$) before the administration of cinacalcet

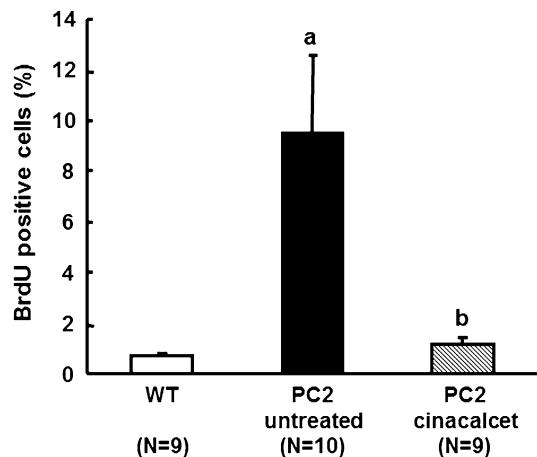


Fig. 4 Effects of cinacalcet on the proliferation of parathyroid cells. The proliferation rate is expressed as the ratio of the number of BrdU-positive cells to the total cell number. Results are shown as means \pm SEM. ^a $P < 0.05$ versus WT, ^b $P < 0.05$ versus PC2 vehicle

and cinacalcet [16], successfully suppress parathyroid cell proliferation, as well as PTH secretion, induced by 5/6-nephrectomy in uremic rat parathyroid glands, it is unknown whether these compounds can also suppress parathyroid cell proliferation when induced by an oncogene. To examine the suppressive effects of cinacalcet on

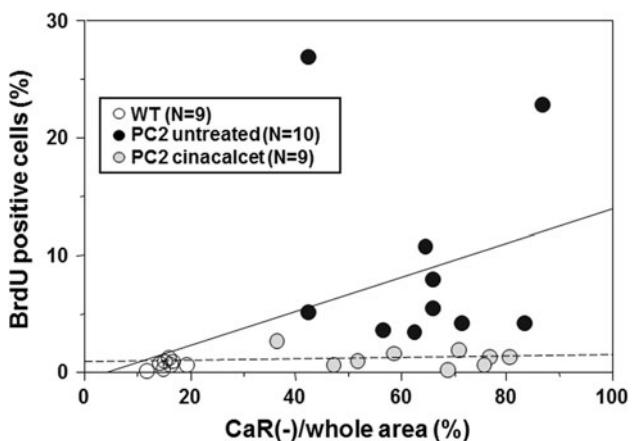


Fig. 5 The effects of CaR hypoexpression on parathyroid cell proliferation in parathyroid glands. The CaR(-)/whole area ratio in parathyroid glands showed a significant positive correlation with BrdU-positive cells ($r = 0.515, P = 0.0013$, solid line) among cinacalcet-untreated mice (WT and PC2-untreated), whereas no significant correlation was observed in WT and PC2 cinacalcet-treated mice ($r = 0.256, P = 0.3209$, dotted line). Administration of cinacalcet suppressed parathyroid cell proliferation regardless of CaR expression (PC2 cinacalcet)

parathyroid cell proliferation, cinacalcet was administered to PC2 mice, which developed parathyroid cell hyperplasia followed by monoclonal expansion, a phenotype observed in refractory SHPT patients [6]. Cinacalcet suppressed parathyroid cell proliferation as well as biochemical hyperparathyroidism in PC2 mice, although CaR expression in the parathyroid glands was reduced. Cinacalcet did not affect apoptosis in PC2 parathyroid glands, although uremic rat parathyroid glands exhibited apoptosis in response to treatment with R-568 [18].

The reduction in CaR expression in PC2 mouse parathyroid glands occurred as previously described [17]. The hypoexpression of CaR or CaR(-)/whole area ratio in parathyroid glands correlated positively with serum Ca and PTH levels, suggesting that an attenuated feedback signal from serum Ca apparently fails to suppress PTH secretion in PC2 mice [19].

Parathyroid cell growth was accelerated in PC2 mice compared to WT mice. Interestingly, the parathyroid glands with focal regions of reduced CaR expression also exhibited focal regions of BrdU uptake in which CaR expression was reduced, while the glands exhibiting a general reduction in CaR expression revealed a general uptake of BrdU in PC2 mice. This hypoexpression of CaR in parathyroid glands also correlated with parathyroid cell proliferation, suggesting a relationship between CaR expression and proliferation in parathyroid cells. Reduced CaR signaling in parathyroid cells enhances parathyroid cell proliferation not only in total CaR knockout mice [5] but also in parathyroid-specific heterotrimeric G protein-knockout mice that were created to investigate the role of

heterotrimeric G proteins in CaR signaling [20]. CaR signaling, in the absence of signaling through the vitamin D receptor, was also shown to have regulatory roles in PTH secretion and parathyroid cell proliferation [21]. The current study also supported the impact of CaR signaling in parathyroid glands in both PTH secretion and parathyroid cell proliferation.

Daily dietary administration of cinacalcet significantly suppressed serum PTH and Ca levels, as did a single oral administration of the drug in PC2 mice [17]. Cinacalcet also suppressed parathyroid cell proliferation in these mice, although the CaR expression in their parathyroid glands was reduced. In addition, it was shown that the calcimimetic compound R-568 suppressed parathyroid cell proliferation in uremic rat parathyroid glands [15]. Cinacalcet successfully suppressed parathyroid cell proliferation independently of its effect on CaR expression in this experiment. The pathological parathyroid cells were still sensitive to the oral administration of cinacalcet, despite the decrease in parathyroid CaR expression and putative ongoing CaR-mediated signaling due to hypercalcemia in these mice. These data indicated that cinacalcet could be an effective medication for the prevention of parathyroid cell proliferation in PHPT, even under hypercalcemic conditions.

R-568 and other calcimimetics, such as AMG641, upregulate CaR expression in uremic rat parathyroid glands [22, 23]; however, cinacalcet failed to upregulate CaR expression in PC2 mice in the current study. In uremic rats, a high concentration of R-568 accelerated apoptosis in parathyroid cells ex vivo [18], while oral administration of cinacalcet failed to induce apoptosis [16]. Additionally, apoptosis was not observed in untreated PC2 mice in spite of the fact that some TUNEL-positive cells were observed in uremic rats [18]. Cinacalcet did not induce apoptosis in PC2 parathyroid cells in the current study, and it is possible that parathyroid cells in PC2 mice might be resistant to apoptosis due to *cyclin D1* overexpression. It is clear from the accumulated data that the optimization of the administration of calcimimetics is necessary to obtain beneficial effects such as the upregulation of CaR expression and apoptosis in the parathyroid glands.

In conclusion, CaR is a potentially useful target for a therapeutic agent such as cinacalcet, not only for the suppression of PTH secretion but also for the suppression of parathyroid cell proliferation, even though CaR expression is reduced in parathyroid glands, as observed in advanced PHPT and SHPT. Cinacalcet could be beneficial at preventing the progression of hyperfunctioning parathyroid diseases by suppressing parathyroid cell proliferation.

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