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See related article, "*In Vivo* Maturation of Functional Renal Organoids Formed from Embryonic Cell Suspensions," on pages 1857–1868.

Parathyroid Hormone–Independent Role for the Calcium-Sensing Receptor in the Control of Urinary Calcium Excretion

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Owing to the involvement of calcium ions (Ca^{2+}_o) in many vital functions, the ability to detect and respond to changes in their concentration in extracellular fluid is of paramount importance for all living organisms. The molecular identification of the extracellular calcium-sensing receptor (CaSR) from parathyroid glands[1] constituted a major breakthrough in our understanding of how the body ensures Ca^{2+}_o homeostasis through regulation of parathyroid hormone (PTH) secretion. Studies in human participants and isolated parathyroid glands show the existence of a tight relationship between Ca^{2+}_o, CaSR, and PTH release, with deviations in Ca^{2+}_o concentrations of even $<$10% within the physiologic range (1.1–1.3 mM) capable of evoking rapid and substantial changes in PTH release.

In humans, activating mutations in the CaSR gene (*Casr*) are associated with hypocalcemia with hypercalciuria[2] and, in certain circumstances, a Bartter-like phenotype,[3] with renal salt wasting, alkalosis, and an increase in plasma renin and aldosterone levels. Conversely, heterozygous inactivating mutations in the *Casr* lead to familial hypocalciuric hypercalcemia (FHH), a relatively benign and asymptomatic condition. However, if the mutation is present on both alleles, it leads to neonatal severe hyperparathyroidism, with profoundly symptomatic hypercalcemia, hypermagnesemia, and hyperparathyroidemia, and can be life-threatening unless the parathyroid glands are removed early in life.[4] Although FHH could largely be accounted for by a reset of the parathyroid calciostat, studies by Attie *et al.* demonstrated that unusually low calcium clearance seen in FHH patients was caused by abnormal calcium sensing by the ascending limb of Henle's loop, in a fashion that was independent of systemic changes in PTH.[5] Subsequent to the cloning of the CaSR from parathyroid glands, the receptor was also identified from the rat kidney,[6] with the highest expression at the basolateral membrane of thick ascending limb cells and, to a lesser extent, in proximal convoluted tubules and collecting ducts.[7] Functional studies in isolated nephron segments and kidney-derived cell lines suggest that the renal CaSR is involved in mineral ion homeostasis, regulation of urine acidification, concentration, and of renin secretion (reviewed by Riccardi and Brown[8]). Yet, direct roles for the CaSR outside the parathyroid glands have been difficult to investigate because they cannot be dissociated easily from systemic changes in Ca^{2+} and PTH levels. Therefore, the availability of suitable experimental models of targeted *Casr* deletion *in vivo* became a necessity.

Ho *et al.* generated the first mouse model in 1995 by deleting exon 5 of the *Casr*, which encodes a 77 amino acid region in the amino terminal domain of the CaSR.[9] The exon 5-less mouse recapitulated many of the phenotypic features of neonatal severe hyperparathyroidism, including parathyroid gland hyperplasia, high serum PTH levels, hypercalcemia, and high perinatal mortality.[10] To investigate the role of the CaSR in the kidney, two groups rescued the hyperparathyroidism and hypercalcemia of the exon 5-less *Casr* murine model by breeding these mice with either glial cell missing 2 (*Gcm2*)–deficient mice (which are born without parathyroid glands[11]), or with PTH-deficient mice.[12] Both of these studies highlighted the primary role of the CaSR in the fine-tuning of urinary calcium excretion, independently of PTH-mediated effects. Although the two different models reached very similar conclusions, the effect of these studies was somewhat diminished by the identification of a splice variant in the exon 5-less mouse in some tissues (including the kidney), which was shown to be functionally active,[13] and that could partially compensate for the constitutive CaSR ablation.

A significant leap forward was made when Chang *et al.* produced conditional CaSR ablation by flanking LoxP sites to exon 7,[14] whose excision leads to the removal of the whole of the transmembrane domain and carboxy terminus of the CaSR. In a tour-de-force study, the authors used the $Casr^{fl/fl}$ mice to produce five cell-specific *Casr* deletions from the

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parathyroid, osteoblast cell lineages, and chondrocytes. Phenotypic characterization of these mice confirmed the existence of a complex interplay between the parathyroid and the bone. Crucially, and for the first time, the studies by Chang *et al.* demonstrated the critical role of the CaSR in the regulation of skeletal development, which was independent of calciotropic hormones.[14] To date, kidney-specific ablation of the CaSR using this mouse model is yet to be produced. Furthermore, exon 7-less mice still express exon 1–6, which encodes the whole of the amino terminus of the CaSR. Albeit the truncated protein is functionally inert when expressed in recombinant systems and it does not affect either the function or the expression of the full-length receptor, it does not constitute a model of complete CaSR deficiency.

A further development of conditional *Casr* deletion is described in this issue of *JASN*. Toka *et al.* generated a novel floxed mouse by flanking exon 3 of the *Casr* with LoxP sites.[15] Exon 3 encodes a peptide in the putative extracellular domain. Exon 3 excision causes a frameshift in the open reading frame that results in a protein lacking all of the putative $Ca^{2+}{}_o$ binding sites and the transmembrane region. Arguably, this is the most complete model of CaSR ablation available thus far. Transgenic mice with germline deletion of exon 3 develop NSHPT-like phenotype and die within 10 days of birth, therefore confirming the validity of the floxed model. Kidney tubular epithelial cell-specific deletion of the *Casr* was achieved by breeding $Casr^{fl/fl}$ animals with mice in which the enzyme Cre recombinase is under the control of the Sine oculis homeobox homolog 2 (Six2) gene promoter. Six2-Cre *Casr*-floxed mice showed no differences in serum biochemistries, even when the animals were challenged by a high Ca^{2+} diet (1.5% administered in drinking water). However, the authors observed a significant decrease in the urinary Ca^{2+}/creatinine ratio in *Six2-Cre;Casr-floxed* mice, compared with control mice, which augmented subsequently to increased dietary Ca^{2+} load. Hypocalciuria was rectified by treatment with the loop diuretic furosemide, suggestive of an involvement of the thick ascending limb-specific isoform of Na^+-K^+-$2Cl^-$ cotransporter, NKCC2, in this process. Although no changes in the total, nonphosphorylated NKCC2 protein expression levels were observed, *Six2-Cre;Casr-floxed* mice exhibited an increase in phosphorylated (active) NKCC2. Increased NKCC2 activity would be expected to lead to changes in BP, urinary concentrating ability, and/or formation of edema. Although the authors did not measure BP or vasopressin response in both experimental groups, no obvious signs of edema were observed in the kidneys of *Six2-Cre;Casr-floxed* mice. On the basis of these results, the authors hypothesized that loss of the CaSR leads to NKCC2 activation, resulting in an increase in basolateral Cl^- exit. The attendant increase in the lumen-positive transepithelial potential difference would increase the driving force for paracellular Ca^{2+} transport in this nephron segment.[16] Consistent with this hypothesis that the CaSR regulates paracellular Ca^{2+} transport, Western analysis performed to assess the expression levels of the tight junctional proteins involved in the regulation of the cation-selective paracellular pathway showed a decrease in the negative regulator, paracellin 14, and a small, albeit significant, increase of the positive regulator, claudin 16, in *Six2-Cre;Casr-floxed* mice. These observations are in line with results obtained by Loupy *et al.* in thyroparathyroidectomized, PTH-supplemented rats, which demonstrate the importance of CaSR-mediated signaling in the regulation of the paracellular pathway specifically in the thick ascending limb.[17]

A surprising finding of Toka *et al.*[15] is that serum or urine Mg^{2+} levels were not differentially affected, even after dietary calcium challenge, in *Six2-Cre;Casr-floxed* mice. Yet, FHH patients exhibit hypermagnesemia. The authors further investigated the possibility that compensative upregulation of genes involved in renal Mg^{2+} transport might take place distally to the thick ascending limb, and measured the expression levels of TRPV5, TRPM6, and the thiazide-sensitive Na^+-Cl^- cotransporter, NCC. None of these were differentially expressed in *Six2-Cre;Casr-floxed* mice compared with control animals.

An additional intriguing finding is that although serum Ca^{2+} levels were unchanged between the two experimental groups, there was a trend toward an increase in levels of 1,25 $(OH)_2D_3$ levels and 1-hydroxylase (*CYP27B1)* in *Six2-Cre; Casr-floxed* animals. Together with the reduced urinary Ca^{2+} excretion measured in these animals fed a high Ca^{2+} diet, these observations suggest that mice lacking the kidney CaSR ought to be in positive calcium balance. Toka *et al.* propose to carry out observations aimed at assessing the effects of long-term dietary Ca^{2+} supplementation on the skeleton of *Six2-Cre;Casr-floxed* animals.

The results presented in this study suggest that the renal CaSR prevents the development of hypercalcemia. If these observations were extended to humans, they suggest the possibility of using pharmacologic manipulation of the renal CaSR to rectify hypercalciuric diseases due to either gain-of-function Casr mutations, or hypercalciuric kidney stones.

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DISCLOSURES

None.

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