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Review

Calcium Homeostasis and Regulation of Calbindin-D_{9k} by Glucocorticoids and Vitamin D as Bioactive Molecules

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Abstract – Calbindin-D_{9k} (CaBP-9k), a cytosolic calcium-binding protein, is expressed in a variety of tissues, i.e., the duodenum, uterus, placenta, kidney and pituitary gland. Duodenal CaBP-9k is involved in intestinal calcium absorption, and is regulated at transcriptional and post-transcriptional levels by 1,25-dihydroxyvitamin D3, the hormonal form of vitamin D, and glucocorticoids (GCs). Uterine CaBP-9k has been implicated in the regulation of myometrial action(s) through modulation of intracellular calcium, and steroid hormones appear to be the main regulators in its uterine and placental regulation. Because phenotypes of CaBP-9k-null mice appear to be normal, other calcium-transporter genes may compensate for its gene deletion and physiological function in knockout mice. Previous studies indicate that *CaBP-9k* may be controlled in a tissue-specific fashion. In this review, we summarize the current information on calcium homeostasis related to CaBP-9k gene regulation by GCs, vitamin D and its receptors, and its molecular regulatory mechanism. In addition, we present related data from our current research.

Keywords: Calcium-binding proteins, Calbindin-D9k and vitamin D

CALCIUM HOMEOSTASIS AND ITS REGULATING PROTEINS

Calcium homeostasis is maintained via regulation of calcium absorption in various tissues, i.e., the small intestine, skeleton and kidney, and it has been shown to be regulated by various molecules, including 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), parathyroid hormone (PTH), calcitonin, and sex hormones (Hurwitz, 1996; Fukugawa and Kurokawa, 2002; Frick and Bushinsky, 2003). In the intestine and kidney, calcium can be transferred from the intestinal and renal lumen to the circulatory system through paracellular and transcellular pathways (Bindels, 1993; Wasserman and Fullmer, 1995). The active absorption of duodenal and renal calcium is controlled by calcium transporter proteins. These calcium transporters include calcium entry-channel proteins in the plasma membrane, cytosolic buffering or transfer proteins, and excretory pump proteins. The movement of luminal calcium across microvillar membrane into enterocytes appears to be regulated by two epithelial calcium channels, transient receptor potential vanilloid 5 and 6 (TRPV6 and TRPV5) (Kim *et al.,* 2006; Kim *et al.,* 2009).

Calbindin-D_{9k} (CaBP-9k), a calcium-binding protein, has been implicated in active calcium absorption in various tissues as an intracellular calcium ion-binding protein (Choi *et al.,* 2005; Lambers *et al.,* 2006; Choi and Jeung 2008). Apical calcium influx is rapidly buffered by this protein, which then shuttles calcium ions from the apical to the basolateral membrane, where Na^+/Ca^{2+} exchanger (NCX1) and plasma membrane Ca^{2+}-ATPase 1b (PMCA1b) account for calcium ion extrusion as seen in Fig. 1 (Wasserman and Fullmer, 1995; Hoenderop *et al.,* 2002; Peng *et al.,* 2003; van Abel *et al.,* 2005; Lee *et al.,* 2007). *CaBP-9k* knockout (KO) mice normally survive without any abnormality, apparently supported by calcium processing genes acting in a compensatory manner (Lee *et al.,* 2007). In the previous studies, duodenal CaBP-9k was up-regulated by 1,25-dihydroxyvitamin D_3, and down-regulated by gluco-

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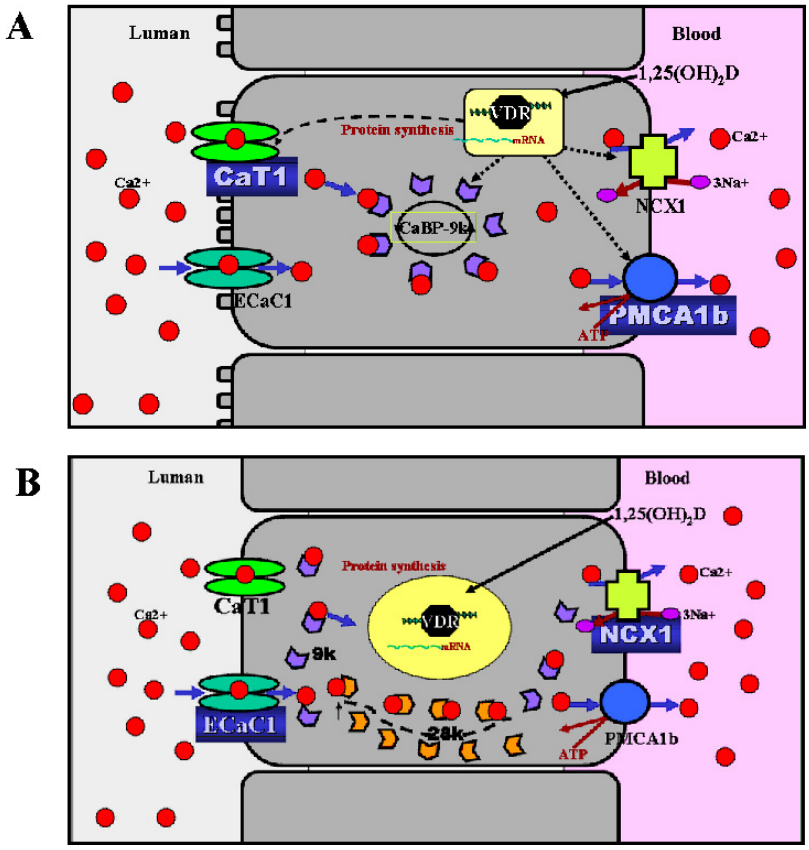

Fig. 1. Calcium transport system in the duodenum and kidney. The dynamic calcium transport pathways may be differentially controlled by various calcium transporter genes in the duodenum (A) and kidney (B). TRPV6 is known as epithelial calcium channel 2 (EcaC2) and calcium transporter 1 (CaT1), while TRPV5 is also called EcaC1 and CaT2.

corticoids (GCs) (Darwish and DeLuca 1992; Barley *et al.,* 1999; Lee *et al.,* 2006), indicating that vitamin D and GCs are potential regulators in the control of the *CaBP-9k* gene in the small intestine. The KO mice of *CaBP-9k* gene showed normal serum concentrations of calcium and exhibited no abnormal phenotype (Gkika *et al.,* 2006; Akhter *et al.,* 2007; Lee *et al.,* 2007), suggesting that the function(s) of this gene in the duodenum and kidney may be compensated by the increased expression of other active calcium transport proteins (Kutuzova *et al.,* 2006; Lee *et al.,* 2007).

GLUCOCORTICOIDS AND THEIR RECEPTORS IN THE REGULATION OF CaBP-9k

GCs have been used in the treatment of various diseases, e.g., asthma, rheumatoid arthritis and atopic dermatitis. The favorable efficacy of GCs as an anti-inflammatory agent is offset by the occurrence of side effects, including osteoporosis, hypothalamus-pituitary-adrenal axis suppression, cataract formation, skin thinning, growth retardation and bruising (McLaughlin *et al.,* 2002). Of these side effects, potentially the most serious and debilitating is the development of glucocorticoid-induced osteoporosis (GIO) (Patschan *et al.,* 2001). Potential mechanism(s) in GC-induced GIO have been suggested (Schacke *et al.,* 2002). For instance, GCs inhibit bone formation by suppressing the proliferation and activity of osteoblasts and osteocytes, and reduce the population of bone cells by increasing apoptosis (Silvestrini *et al.,* 2000). GCs decrease absorption of gastrointestinal calcium ions and increase excretion of urinary calcium ions. A number of critical mediators in bone homeostasis, i.e., sex hormones, growth hormone, insulin-like factor-1, and TGF-beta, seem to be also suppressed by GCs.

Although studies of GC-induced effects on bone cells have been carried out over the last several decades, the exact GC-effects on intestinal calcium transport and renal calcium elimination have not clearly been shown. Intestinal calcium absorption decreases in the presence of long-term and high doses of GCs in humans and rats (Lukert and Raisz, 1990). Decreases in active transcellular transport and in normal brush-border vesicle uptake of calcium and reduced calcium-binding proteins in the intestine are considered as cellular mechanisms resulting in diminished intestinal calcium absorption (Shultz *et al.,* 1982). In parallel, GCs have been demonstrated to increase renal calcium elimination and epithelial calcium transport capacity

(Nijenhuis *et al.,* 2004). Long-term treatment with GCs resulted in marked hypercalciuria and fasting urine calcium excretion (Cosman *et al.,* 1994). This GC-induced imbalance of calcium promotes secondary hyperparathyroidism, and the increased parathyroid hormones (PTHs) cause bone re-absorption due to an increase in osteoclast activity (Ziegler and Kasperk, 1998), which eventually results in osteoporosis.

In our previous study, we showed that duodenal *CaBP-9k* mRNA and protein levels were significantly decreased by dexamethasone (Dex) treatment, whereas renal *CaBP-9k* mRNA and protein levels were unchanged (Lee *et al.,* 2006). Huybers *et al.* reported that daily prednisolone treatment for 7 days reduced duodenal *TRPV6* (Huybers *et al.,* 2007). The expression of duodenal *TRPV6* declined in Dex-treated mice, and its level in CaBP-9k and CaBP-28k KO mice was up-regulated when compared to that of WT mice (Lee *et al.,* 2006; Lee *et al.,* 2007; Kim *et al.,* 2009). With regard to duodenal gene regulation by Dex, these results imply that the decrease in duodenal *CaBP-9k* and *TRPV6* gene expression may contribute to the pathogenesis of GIO (Kim *et al.,* 2009). In addition, our results suggest that a compensatory mechanism of *CaBP-9k* and *CaBP-28k* deficiency appears to function in the KO mice; but GCs down-regulate duodenal *CaBP-9k* and *TRPV6* transcription in parallel with the previous studies (Lee *et al.,* 2006; Huybers *et al.,* 2007). Because duodenal active calcium-processing genes were considered to be the major target for GC-induced gene suppression, we further clarified any alteration in the duodenal protein levels and their spatial expression. However, there were no differences in the expression patterns of duodenal *TRPV6, CaBP-9k, NCX1* or *PMCA1b* after Dex treatment (Kim *et al.,* 2009). Taken together, these results suggest that duodenal CaBP-9k and TRPV6 in the active calcium transport system may play key roles in the disruption of calcium homeostasis by chronic GC treatment, and the absence of *CaBP-9k* and *CaBP-28k* can be compensated by other active calcium transport components.

GCs and the glucocorticoid receptor (GR) are known to be essential factors in cellular differentiation and maintenance of mineral absorption (Beaulieu and Calvert, 1985; Sheppard *et al.,* 1999). The previous reports described GR expression and distribution in the duodenum and kidney (Merot *et al.,* 1989; Farman *et al.,* 1991; Lee *et al.,* 2006). In our previous study, we further employed Dex to elucidate the role of GR in the regulation of calcium-processing genes, and its differential expression in *CaBP-9k* KO mice (Kim *et al.,* 2009). Interestingly, the expression of duodenal and renal GR was significantly down-regulated by Dex in wild-type (WT) mice. However, *GR* mRNA levels in the duodenum and kidney were unaltered in CaBP-9k or CaBP-28k KO mice. Therefore, it can be concluded that the number of GRs may not cause the induction of compensatory genes in these KO mice (Lee *et al.,* 2006; Lee *et al.,* 2007; Kim *et al.,* 2009). Serum corticosterone levels were examined as a potential candidate for the compensatory gene induction and differential effect of Dex treatment on the expression of calcium processing genes in the KO mice. The serum corticosterone levels in these mouse models revealed surprising information, indicating that the corticosterone levels in CaBP-9k and -28k KO mice were significantly lower than those of WT mice (Kim *et al.,* 2009). The gene induction in CaBP-9k and CaBP-28k KO mice may be the result of low serum corticosterone levels. This conclusion is supported by previous reports demonstrating that synthetic GCs down-regulated duodenal CaBP-9k and TRPV6 expression (Lee *et al.,* 2006; Huybers *et al.,* 2007). Serum corticosterones in all animals were significantly reduced after Dex treatment. Dex provides negative feedback to the pituitary to suppress the secretion of adrenocorticotropic hormone, resulting in the suppression of serum corticosterone levels. Therefore, these results imply that these mice may be affected by exogenous Dex treatment (Cole *et al.,* 2000; McGowan *et al.,* 2000).

VITAMIN D AND ITS RECEPTORS IN THE REGULATION OF CaBP-9k

Intestinal CaBP-9k is involved in intestinal calcium absorption and is regulated at the transcriptional and post-transcriptional level by 1,25-dihydroxyvitamin D3 in rodents (Roche *et al.,* 1986; Wasserman and Fullmer, 1989; Darwish and DeLuca, 1992). In addition, the expression of duodenal CaBP-9k has been shown to be controlled by 1,25-dihydroxycholecalciferol in humans (Walters *et al.,* 1999). Furthermore, vitamin D regulation of this gene may also be important in reproductive tissues (Kwiecinksi *et al.,* 1989; Uhland *et al.,* 1992). Although the intestinal level of CaBP-9k is clearly vitamin D-controlled, its placental and uterine levels do not respond to vitamin D depletion. Therefore, CaBP-9k does not seem to be under the control of vitamin D despite the presence of vitamin D receptors in the uterus. Rather, uterine CaBP-9k appears to be controlled by sex hormones (Darwish *et al.,* 1991; L'Horset *et al.,* 1994). Renal CaBP-9k is expressed at distal convoluted tubule and appears to facilitate calcium re-absorption in this tissue (Peng *et al.,* 2000).

The biological actions of the active form of vitamin D_3

and its synthetic analogs are mediated by the nuclear vitamin D receptor (VDR) (Panda *et al.,* 2004; Nagpal *et al.,* 2005). VDR is a ligand-dependent transcription factor belonging to the superfamily of steroid/thyroid hormone receptors, and has been associated with calcemic activities, including calcium and phosphorus homeostasis, and maintenance of bone content (Nagpal *et al.,* 2005). In *VDR* KO mice, the expression levels of *CaBP-9k, TRPV6* and *TRPV5* were considerably reduced as compared to those in WT littermates, whereas the expression levels of *CaBP-28k, NCX1* and *PMCA1b* were not altered (Li and Christakos, 1991; Chailley- Heu *et al.,* 2001; Li *et al.,* 2001; Van Cromphaut *et al.,* 2001). Previously, we showed that Dex decreased duodenal *VDR* mRNA levels, suggesting that diminished levels of *VDR* mediate the down-regulation of duodenal *CaBP-9k* and *TRPV6* (Kim *et al.,* 2009). Furthermore, *VDR* expression increased in *CaBP-9k* or CaBP-28k KO mice. Conversely, renal *VDR* mRNA levels remained unchanged in all groups, with the exception of Dex-treated *CaBP-9k* KO mice. This result may suggest that renal VDR sensitivity in *CaBP-9k* KO mice can be increased by GCs (Kim *et al.,* 2009). Previously, it was shown that a 1-day treatment with Dex significantly increased duodenal and renal *VDR* mRNA levels, while 5-day Dex treatment diminished duodenal *VDR* and restored renal *VDR* expression to the level observed in vehicle only treated animals (Unpublished data). Taken together, these results imply that Dex regulates *VDR* transcription, and that this may directly regulate the expression of duodenal *CaBP-9k* and *TRPV6* through a GC-mediated pathway (Kim *et al.,* 2009).

The regulation of CaBP-9k in gastrointestinal tissues is not completely understood. The vitamin D-responsive DNA element is found in the CaBP-9k gene promoter region, which has been shown to regulate the expression of CaBP-9k in the intestine (Delorme *et al.,* 1983). CaBP-9k is actively expressed in enterocytes, which are the dominant epithelial cells in the duodenal mucosa (Walters *et al.,* 1999). CaBP-9k expression level decreases downstream from the duodenum and its levels are barely detectable in the distal ileum and large intestine. CaBP-9k is considered to be an important factor in calcium absorption and metabolism in the intestine. Although several studies examined the molecular regulatory mechanisms of active calcium-transport genes in regards to hypocalcemia, rickets and osteomalacia in VDR-null mice and 1α-hydroxylase-deficient mice, the exact mechanism remains unclear (Van Cromphaut *et al.,* 2001; Hoenderop *et al.,* 2002; Zheng *et al.,* 2004). In our previous study, CaBP-9k and other calcium-transport proteins were thought to be the rate-limiting factors in active calcium transport; however, our *CaBP-9k* KO mice exhibit no abnormalities (Lee *et al.,* 2007). It is known that some KO animals present normal phenotypes due to compensatory gene induction (Wertheimer *et al.,* 2001; Xu *et al.,* 2004; Tanabe *et al.,* 2005). Thus, we hypothesized that these KO animals might have compensatory gene induction that can replace CaBP-9k, and that this compensatory regulation might help to elucidate the mechanisms of active calcium transport genes.

As mentioned above, hypocalcemia, rickets and osteomalacia of *VDR*-KO mice may be a result of reduced *VDR* gene expression. Li *et al.* demonstrated that renal CaBP-9k may take part in renal calcium re-absorption, and Van Chromphaut *et al.* suggested that TRPV6 and CaBP-9k appear to be involved in duodenal calcium absorption (Li *et al.,* 2001; Van Cromphaut *et al.,* 2001). To understand the exact role(s) of CaBP-9k, we generated CaBP-9k-null mice. However, no abnormal phenotypes were observed, as was the case in the *VDR*-KO mice. In addition, *CaBP-9k*-KO mice were phenotypically normal at birth and survived normally for at least one year.

PTH is secreted by the parathyroid gland, and acts principally to regulate calcium and phosphate metabolism by binding to its receptors (PTHRs), which are expressed in the kidney and bone (Juppner *et al.,* 1991; Pausova *et al.,* 1994). PTHR is also widely expressed in the bone cells of osteoblast lineage, and in the kidney it is expressed in the glomerulus and at several sites along the nephron (Rouleau *et al.,* 1988; McCuaig *et al.,* 1995). PTH regulates the conversion of 25-hydroxyvitamin D to the active metabolite $1,25(OH)_2D_3$. PTH also activates dihydropyridine-sensitive channels, which mediate calcium entry (Bacskai and Friedman, 1990). Microtubule-dependent exocytosis stimulated by PTH is required for the activation of calcium channels and calcium influx. In our study, we investigated whether there is cross-talk between PTHR and VDR in GC-mediated regulation of calcium-processing genes in *CaBP* KO mice. There was no distinct alteration in renal *PTHR* mRNA levels in Dex-treated animals compared to control, suggesting that *CaBP* gene deficiency and Dex treatment may not to be related to the transcription of *PTHR* (Kim *et al.,* 2009).

CaBP-9k AND ITS COMPENSATORY MECHANISM IN THE KNOCKOUT MODELS

In spite of CaBP-9k depletion, CaBP-9k-null mice maintained normal serum calcium concentrations and showed no abnormal symptoms, suggesting that a normal diet satisfies the calcium requirements of *CaBP-9k* KO mice. To

compare calcium homeostasis ability in both KO and WT mice, these mice were fed low-, normal- and high-calcium diets during the growth period from 3 to 10 weeks of age. Duodenal *TRPV6* and *CaBP-9k* mRNA levels in WT mice increased inversely with the diet calcium concentration (Brown *et al.,* 2005). In the low- and normal-calcium-diet groups, *TRPV6* transcript levels in WT mice were higher than those of KO mice; however, further studies are necessary to verify whether CaBP-9k directly regulates *TRPV6* gene transcription or whether other factors are involved in. Although the levels of duodenal *PMCA1b* and *NCX1* mRNAs fluctuated with dietary calcium levels, no significant differences between WT and KO mice were detected (Lee *et al.,* 2007). Duodenal *PMCA1b* mRNA levels in *VDR* KO mice were reduced by a low-calcium diet (Van Cromphaut *et al.,* 2003). However, no specific differences were found in our study, suggesting that duodenal calcium-transport genes may participate in calcium absorption in spite of CaBP-9k depletion (Lee *et al.,* 2007).

Calcium-transport genes are differentially expressed in an organ-specific manner depending on gender. Although duodenal *TRPV6, CaBP-9k* and NCX1 mRNAs were highly expressed in WT female mice fed a low-calcium diet, no induction of *TRPV6* or *NCX1* genes was detected in *CaBP-9k*-null mice (Lee *et al.,* 2007). In the kidney, all calcium transport genes were highly expressed in female mice. Renal *TRPV6* was not significantly induced in WT male mice receiving a regular diet, but *TRPV6* mRNA levels in female mice were increased. These distinct patterns in renal *TRPV5,* renal *CaBP-28k,* duodenal *TRPV6* and duodenal *CaBP-9k* expressions imply that the response of calcium transport genes is more sensitive in females than in males (Lee *et al.,* 2007). The up-regulation of calcium-absorption genes is higher in female mice due to circulating sex steroid hormone levels (Song *et al.,* 2003); however, gene expression in the kidney and duodenum was unaffected by estrous cycle in parallel with our unpublished data. A further study examining protein levels is needed to determine definitively whether there is gender specific compensatory change (Choi and Jeung, 2008).

In the recent study, the role of TRPV6 and CaBP-9k on intestinal calcium absorption was examined in the KO mice of TRPV6, CaBP-9k and TRPV6/CaBP-9k, demonstrating that significant active intestinal calcium transport occurs in the absence of TRPV6 and calbindin-D9k in these null mutant mice and suggesting that TRPV6 and CaBP-9k may be not essential for vitamin D-induced active intestinal calcium transport (Benn *et al.,* 2008). On the other hand, the requirement of TRPV6 for vitamin D-dependent intestinal calcium absorption in vivo was examined in vitamin D-deficient TRPV6 null and WT mice (Kutuzova *et al.,* 2008). This previous study together with microarray results indicated that TRPV6 is not required for vitamin D-induced intestinal calcium absorption and may not carry out a significant role in this process. These results obtained from CaBP-9k null and other calcium transport mutant mice illustrated that exact molecular events in the intestinal calcium transport in response to the active form of vitamin D are warranted to be elucidated (Kutuzova *et al.,* 2008).

Uterine CaBP-9k regulated by endogenous and exogenous progesterone is strongly and uniformly induced in the luminal epithelium prior to implantation, but specifically decreases at the site of embryo attachment during implantation (Nie *et al.,* 2000; An *et al.,* 2003; An *et al.,* 2003; Hong *et al.,* 2003; Lee *et al.,* 2003; An *et al.,* 2004; An *et al.,* 2004; Hong *et al.,* 2004; Lee *et al.,* 2004; Lee *et al.,* 2005). Furthermore, *CaBP-28k* is expressed in a pattern similar to *CaBP-9k* during early pregnancy (Luu *et al.,* 2004), and uterine CaBP-9k and -28k during early pregnancy have been thought to play a critical role in implantation (Nie *et al.,* 2005). The antisense oligonucleotides of *CaBP-9k* completely blocked embryo implantation in CaBP-28k-null mice with normal fertilization (Luu *et al.,* 2004). In the study, the numbers of corpora lutea and litter sizes of CaBP-9k-null mice were within the normal WT range. These observations imply that CaBP-9k may not be an essential factor for embryo implantation, or may be important but dispensable, and that CaBP-9k function can be compensated by the induction of other calcium-related genes such as *CaBP-28k*.

CONCLUDING REMARKS

Duodenal *CaBP-9k* may act as a gatekeeper in duodenal active calcium transport. The expression of this gene was up-regulated in both types of KO mice, and Dex directly affected duodenal *VDR* transcription, a treatment which may decrease *TRPV6* and *CaBP-9k* levels in the duodenum of all genotypes. CaBP-9k should be considered as an important factor in active calcium transport; however, it can be compensated for by other calcium-transport genes that maintain normality in CaBP-9k-null mice, including intestinal *TRPV6* and *PMCA1b* during pre-weaning, and renal *TRPV6* and *TRPV5* during growth and development. TRPV6 is known as epithelial calcium channel 2 (EcaC2) and calcium transporter 1 (CaT1), while TRPV5 is also called EcaC1 and CaT2. In addition, CaBP-9k may participate as a rate-limiting factor in duodenal and renal active calcium transport by interacting with *TRPV6, TRPV5* and *PMCA1b* genes. Calcium-transport

proteins play systematic, but complicated roles within a single cellular compartment, such that genetic inactivation of one gene does not disrupt the whole-body calcium homeostasis. Generation of double or triple knockouts of *CaBP-9k* plus other calcium-transport genes may shed further light on the active calcium transport mechanism. Taken together, a conclusive explanation indicates that the dynamic calcium transport pathways may be differentially controlled by various calcium transporter genes in the duodenum and kidney as shown in Fig. 1.

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