

Chapter 14

TRPV5 and TRPV6 in Transcellular Ca²⁺ Transport: Regulation, Gene Duplication, and Polymorphisms in African Populations

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Abstract TRPV5 and TRPV6 are unique members of the TRP super family. They are highly selective for Ca²⁺ ions with multiple layers of Ca²⁺-dependent inactivation mechanisms, expressed at the apical membrane of Ca²⁺ transporting epithelia, and robustly responsive to 1,25-dihydroxivitamin D₃. These features are well suited for their roles as Ca²⁺ entry channels in the first step of transcellular Ca²⁺ transport pathways, which are involved in intestinal absorption, renal reabsorption of Ca²⁺, placental transfer of Ca²⁺ to fetus, and many other processes. While TRPV6 is more broadly expressed in a variety of tissues such as esophagus, stomach, small intestine, colon, kidney, placenta, pancreas, prostate, uterus, salivary gland, and sweat gland, TRPV5 expression is relatively restricted to the distal convoluted tubule and connecting tubule of the kidney. There is only one *TRPV6*-like gene in fish and birds in comparison to both *TRPV5* and *TRPV6* genes in mammals, indicating *TRPV5* gene was likely generated from duplication of *TRPV6* gene during the evolution of mammals to meet the needs of complex renal function. TRPV5 and TRPV6 are subjected to vigorous regulations under physiological, pathological, and therapeutic conditions. The elevated TRPV6 level in malignant tumors such as prostate and breast cancers makes it a potential therapeutic target. *TRPV6*, and to a lesser extent *TRPV5*, exhibit unusually high levels of single nucleotide polymorphisms (SNPs) in African populations as compared to other populations, indicating *TRPV6* gene was under selective pressure during or after humans migrated out of Africa. The SNPs of *TRPV6* and *TRPV5* likely contribute to the Ca²⁺ conservation mechanisms in African populations.

TRPV5 and TRPV6 are unique members of the TRP super family with three major common features that distinguish them from other members: highly selective for Ca²⁺, distributing to apical membranes in Ca²⁺ transporting epithelial cells, and

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highly responsive to 1, 25-dihydroxyvitamin D₃ (1,25[OH]₂D₃). These features also make them suitable for their roles as Ca²⁺ entry channels in the first step of the transcellular pathway, which is involved in many processes such as Ca²⁺ absorption in the intestine and reabsorption in the kidney. In this chapter, I will summarize the evolution of the *TRPV5* and *TRPV6* genes, the features of TRPV5 and TRPV6 in function and regulation to meet the needs for Ca²⁺ entry channels in the transcellular Ca²⁺ transport pathways, their regulation under pathophysiological and therapeutic conditions, and the unusual high allele frequency of *TRPV6* (and to a lesser extent *TRPV5*) single nucleotide polymorphisms (SNPs) in African populations. Due to the scope of this chapter, it is impossible to cover all important work. Readers are directed to relevant chapters of this series and recent reviews [1–12] for further details about various aspects of TRPV5 and TRPV6.

14.1 Identification – Results of Seeking Ca²⁺ Transporters in Transcellular Pathways

TRPV5 and TRPV6 were identified as results of searching for genes responsible for Ca²⁺ transport in the transcellular pathway in the kidney [13] and intestine [14], respectively. Until the end of the twentieth century, calbindins had remained to be the main molecules in the transcellular Ca²⁺ transport pathway with little information available about the apical entry mechanism. To tackle this problem, an expression cloning approach with *Xenopus laevis* oocytes was employed to identify Ca²⁺ transport proteins by two groups independently. Using mRNA isolated from connecting tubule and cortical collecting duct cells immunodissected from rabbit kidney, Hoenderop, Bindels and colleagues [13] constructed a cDNA library and identified a cDNA clone by functional screening of the library using radio-tracer Ca²⁺ uptake assay. The resultant cDNA encodes a protein of 730 amino acids, which was named ECaC (Epithelial Ca²⁺ channel) [13]. In contrast, we chose intestinal epithelial tissues as the source to clone an intestinal Ca²⁺ transporter [14]. We observed significant increases in Ca²⁺ uptake in oocytes injected with mRNAs from cecum or duodenum from rats fed on Ca²⁺ deficient diet for 2 weeks. A 10–20% reduction in serum Ca²⁺ was observed in these rats. From the rat duodenal cDNA library, we identified a cDNA encoding a protein of 727 amino acids, which we named CaT1 (Ca²⁺ transport protein subtype 1). The two proteins share 74% amino-acid identity. ECaC and CaT1 were renamed later on as TRPV5 and TRPV6, respectively. Other alternative names include CaT2 [15] and ECaC1 [16] for TRPV5, and CaT-L [17] and ECaC2 [18] for TRPV6. Only two cloned proteins share primary sequence similarity to TRPV5 and TRPV6 at the time of cloning, the capsaicin receptor VR1 (TRPV1), a heat activated channel in the pain pathway [19] and OSM-9, a *C. elegans* protein involved in olfaction, mechanosensation, and olfactory adaptation [20]. The subsequent identification of VRL-1 (TRPV2) [21, 22], OTRPC4/VR-OAC/Trp12/VRL-2 (TRPV4) [23–26] and TRPV3 [27–29], completed the TRPV family. TRPV5 and TRPV6 share approximately 40–45% amino-acid identity with the other 4 members and are the only

calcium selective channels in the family; the other four members are non-selective cation channels, they serves as sensors for heat (TRPV1-3) or osmolarity (TRPV4).

14.2 Evolution of the Genes: From One to Two

No proteins similar to TRPV members were identified in *E. coli* or yeast. Five TRPV proteins were identified in *C. elegans*, namely *osm-9*, *ocr-1*, *ocr-2*, *ocr-3* and *ocr-4* [30]. Among them, *ocr-4* is closer to TRPV5 and TRPV6 (25.9 and 25.4% identity, respectively) than other TRPV proteins in *C. elegans* (20.3–23.6% identity). *Ocr-4* is also closer to TRPV5 and TRPV6 among all the 6 mammalian TRPV members. This indicates that TRPV5 and TRPV6 in vertebrates were likely evolved from *Ocr-4* in invertebrate.

The phylogenetic tree of TRPV5 and TRPV6 from vertebrates is shown in Fig. 14.1. One interesting observation is that both TRPV5 and TRPV6 are present in mammals, but only one of the two, i.e., TRPV6, is present in birds and fish based on

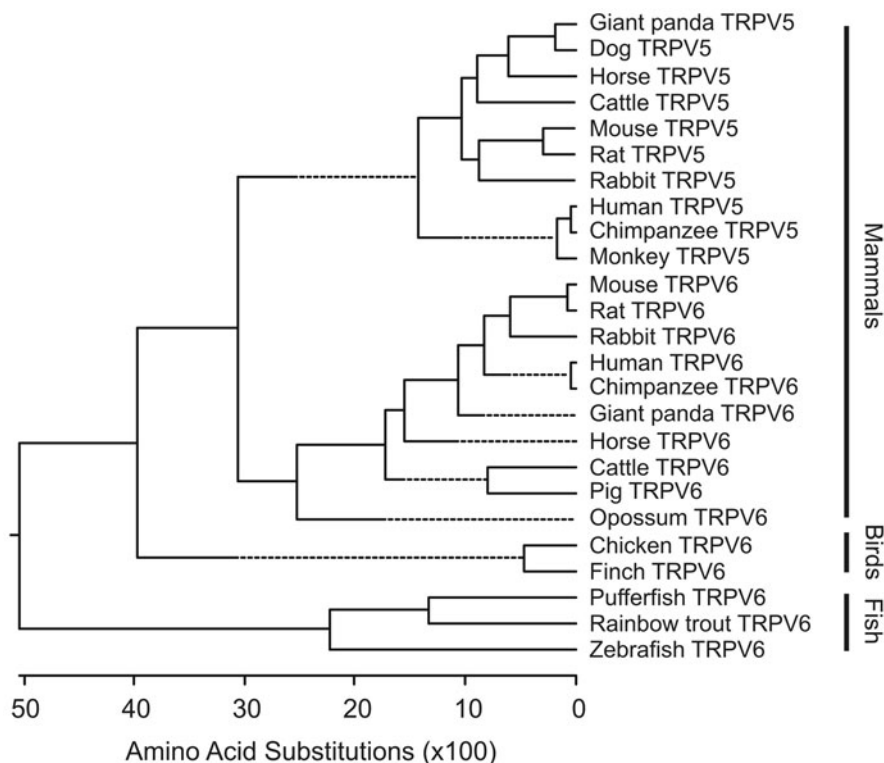


Fig. 14.1 Phylogenetic tree generated from alignment of TRPV5 and TRPV6 proteins from various species. Clustal W method was used to align the proteins with MegAlign program in DNASTAR Lasergene software (DNASTAR Inc., Madison, WI). TRPV6-like proteins are present in fish and birds, whereas both TRPV5 and TRPV6 are present in mammals

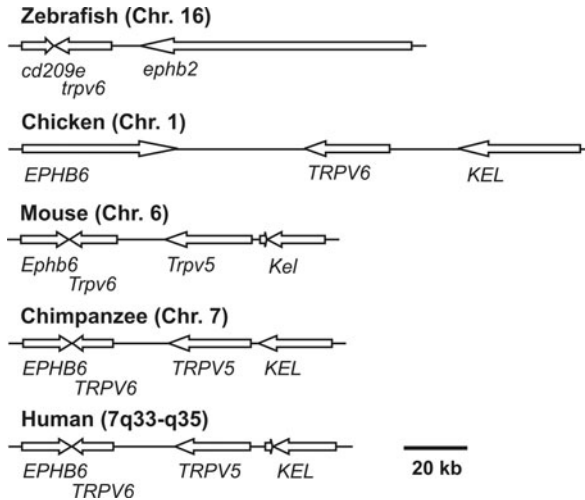


Fig. 14.2 *TRPV5* and *TRPV6* and their flanking genes in zebrafish, chicken, mouse, chimpanzee, and human genomes. Only *TRPV6* is present in zebrafish and chicken genomes, whereas both *TRPV5* and *TRPV6* are present in mouse, chimpanzee and human genomes. *Cd209e*, CD209E antigen-like protein E-like, current official gene symbol *LOC563797*; *Ephb2*, ephrin (EPH) receptor B2-like, current official gene symbol *LOC572411*; *KEL*, Kell blood group, metallo-endopeptidase; *EPHB6*, EPH receptor B6

the current available databases (Fig. 14.1). It has been noted by Qiu and Hogstrand that there is only one *TRPV5/6*-like gene in pufferfish genome [31]. This is not a single incident because the same is true in zebrafish (Fig. 14.2). Furthermore, in the chicken genome, only *TRPV6* gene is present. In contrast, both *TRPV5* and *TRPV6* genes are present in mouse, chimpanzee, and humans (Fig. 14.2). All mammals examined so far have both *TRPV5* and *TRPV6* genes in their genomes. In human and other mammal genomes, *TRPV5* and *TRPV6* genes situate side by side (Fig. 14.2). The two genes are completely conserved in the exon size in the coding region [32]. Thus, it is likely *TRPV5* gene was generated by duplication of *TRPV6* gene. As both birds and mammals are evolved from reptiles, it is likely that gene duplication event occurred during evolution of mammals from reptiles. *TRPV6* has a broader tissue expression pattern whereas *TRPV5* expression is relatively restricted to the kidney (see Sections 14.3.1 and 14.4), therefore, the difference in complex kidney function between reptiles, birds and mammals [33] is likely the driving force behind the generation of the kidney specific *TRPV5* gene.

14.3 Roles as Ca^{2+} Entry Channel in Active Intestinal/Renal Ca^{2+} Re/absorption

Transcellular Ca^{2+} transport pathway is a 3-step process: entry across the apical membrane, transfer of Ca^{2+} from the site of entry to the site of extrusion, and extrusion of Ca^{2+} across the basolateral membrane. This active pathway is

involved in intestinal Ca²⁺ absorption, renal Ca²⁺ reabsorption, and many other processes. While vitamin D responsive Ca²⁺ binding protein calbindins may facilitate intracellular Ca²⁺ transfer and the Na⁺-Ca²⁺ exchanger and Ca²⁺ ATPase mediate the extrusion of Ca²⁺, TRPV5 and TRPV6 serve the role for Ca²⁺ entry as the first step of the transcellular pathway.

14.3.1 Expression in Ca²⁺ Transporting Epithelia

The major Ca²⁺ transporting epithelia reside in the intestine and kidney, where Ca²⁺ absorption and reabsorption take place, respectively. In the intestine, TRPV6 is the major Ca²⁺ entry channel whereas TRPV5 is barely detectable. Due to the high sequence similarity between TRPV5 and TRPV6, strong signals could be detected in the rat or rabbit duodenum using TRPV5 cDNA probe in Northern blot analyses [13, 15], but this is likely due to cross hybridization with TRPV6 mRNA [15]. In small intestine, TRPV6 mRNA expression decreases from duodenum to ileum in rats [14] and humans [34]; TRPV6 mRNA is also detectable in large intestine in rats and humans [14, 34]. TRPV6 protein is localized to the apical membrane of intestinal and colonic epithelial cells [35]. Even though TRPV6 mRNA is barely detectable in the ileum in mice and rats, immunostaining with an antibody against TRPV6 indicates the presence of TRPV6 protein in ileum [35]. It is worth noting that in sheep, TRPV6 mRNA and protein level are more abundant in jejunum than in duodenum [36]; higher expression of TRPV6 mRNA in proximal jejunum than duodenum was also documented in horse [37], suggesting a more important role of jejunum in active Ca²⁺ absorption in some animals. TRPV6 mRNA and protein are also weakly expressed in ovine rumen [36], where active Ca²⁺ absorption also occurs in sheep and goats [38].

In mice and rats, TRPV5 mRNA is much more abundant than that of TRPV6 in the kidney [39, 40]. TRPV5 mRNA level is approximately 10 to 20 times higher than that of TRPV6 in mouse kidney [39]. In humans, we have observed much higher mRNA level of TRPV6 than TRPV5 in the kidney [32]. Similarly, in horse kidney, TRPV6 is also much abundant than TRPV5 [37]. Thus, TRPV6 may play more significant roles in some species such as humans and horses.

TRPV5 is localized to the apical membrane of the tubular cells in the late segment of distal convoluted tubule and connecting tubule in mouse kidney [41]. In the rabbit, much TRPV5 is distributed in the connecting tubule [13]. With regards to TRPV6, the tubular distribution is less clear and controversial. Using an RT-PCR approach with microdissected mouse kidney tubules, TRPV6 signal was detected in the medullary thick ascending limb [42]. Due to its low level of expression and the lack of efficient antibodies for TRPV6, TRPV6 protein is difficult to detect in rodent kidney; however, it has been reported that TRPV6 is distributed to mouse distal convoluted tubules (DCT2), connecting tubules, and cortical and medullary collecting ducts using an immunohistochemistry approach [43]. We found a higher mRNA level of TRPV6 in outer medulla than cortex of human kidney, opposite to that of TRPV5 (unpublished observations). Using a TRPV6 specific antibody, we found TRPV6 protein is more abundant in the outer medulla, much less in the cortex, little

or none in inner medulla (Zhang *et al.*, unpublished observations). This is consistent with TRPV6 protein being mostly distributed to the thick ascending limb and/or the proximal tubule in the kidney.

14.3.2 Function and Regulation as Ca^{2+} Entry Channels

TRPV5 and TRPV6 share very similar functional properties. Being identified by $^{45}\text{Ca}^{2+}$ uptake assay, TRPV5 and TRPV6 mediate Ca^{2+} influx in the absence of any exogenous ligand, altered membrane potential, mechanical stress, or other cues. This is different from other members of the TRPV family, including TRPV1-4, which are activated by ligands, heat, and/or reduction of osmolarity. At macroscopic level, this constitutive Ca^{2+} transport activity makes them act as facilitative transporters that transport solute down its concentration gradient. The Ca^{2+} uptake mediated by TRPV5 or TRPV6 is saturable, with apparent K_m values at sub-millimolar range, which are well suited for the luminal environment in the intestine and the distal tubule [13–15, 34].

At microscopic level, TRPV5 and TRPV6 exhibit single channel activities. The single channel activity could only be detected in the absence of extracellular divalent cation, such as Ca^{2+} and Mg^{2+} , using Na^+ or K^+ as charge carrier [44–46]. Single channel activity could not be detected with Ca^{2+} as charge carrier due to very small conductance. One common feature is that TRPV5 and TRPV6 are highly selective for Ca^{2+} , with permeability ratio between Ca^{2+} and Na^+ higher than 100:1 [45, 47]. This sets TRPV5 and TRPV6 apart from other TRPV members, which are non-selective cation channels.

TRPV5 and TRPV6 likely function as tetramers [48]. Five ankyrin repeats are present in the N-terminal region of TRPV5 and TRPV6, and it is believed that the third ankyrin repeat initiates a molecular zippering process necessary for the assembly of TRPV6 channels [49]. Regions in N- and C-terminal of TRPV5 were also found important to TRPV5 assembly [50]. However, the crystal structure of ankyrin repeats of TRPV6 indicates that ankyrin repeat domain of TRPV6 does not form a tetramer, challenging its role in channel assembly [51].

TRPV6 outer pore structure is likely similar to that of K^+ channel with a single filter preceded by a pore helix [52]. An aspartate residue at 542 (D542) from each subunit of the TRPV5/6 tetramer likely forms the filter for Ca^{2+} [53–55]. This residue is also responsible for the Mg^{2+} blockade [53, 56].

TRPV5 and TRPV6 exhibit Ca^{2+} -dependent inactivation as feedback control mechanisms via intrinsic channel structure, interaction with Ca^{2+} binding proteins, and phosphatidylinositol 4,5-bisphosphate (PIP_2). TRPV6 activity is tightly regulated by intracellular Ca^{2+} [57]. It has a fast inactivation mechanism compared to TRPV5 [58]. The intracellular loop between transmembrane domains 2 and 3 is the molecular determinant of this mechanism [58]. A CaM binding site is present in the C-terminal of TRPV6 [59], and it mediates a slow phase of Ca^{2+} -dependent inactivation of TRPV6 [59–61]. The interaction between Ca^{2+} /CaM and TRPV6 increases dynamically as intracellular Ca^{2+} level rises [60]. Calbindin- $\text{D}_{28\text{k}}$, a vitamin D regulated Ca^{2+} binding protein, buffers Ca^{2+} entered through TRPV5 to prevent

inactivation of the channel [62]. TRPV5 activity is also regulated by a Ca²⁺ sensor 80 K-H [63]. In addition, PIP₂ increases TRPV5 and TRPV6 activity and prevents their Ca²⁺-dependent inactivation [64–67]. The activation of phospholipase C by Ca²⁺ depletes PIP₂ and this contributes to the Ca²⁺-dependent inactivation of the channel [65]. ATP stabilizes TRPV6 and prevents it from rundown, and this action is counteracted by PKC βII [68].

TRPV5 and TRPV6 are also regulated by extracellular cues. Both TRPV5 and TRPV6 activities increase at alkaline pH [13, 14, 69]. Glutamate residues in the pore helix and in the extracellular domain close to the pore of TRPV5 sense the pH and alter channel activity via conformational changes of the pore [70–73]. In addition, extracellular pH also regulates the dynamic of plasma membrane delivery of functional TRPV5 proteins from intracellular vesicles [74]. Furthermore, tissue kallikrein, a serine protease secreted in the connecting tubule, enhances TRPV5 plasma membrane abundance by delaying its retrieval via the PLC/DAG/PKC pathway upon activating the bradykinin 2 receptor [75]. Finally, TRPV5 and TRPV6 can be stabilized at the plasma membrane by deglycosylation via Klotho (see Section 14.3.3).

The activity, stability and trafficking of TRPV5 and TRPV6 are also regulated by associated proteins [for reviews see [7, 8]].

14.3.3 Robust Regulation by Calcitrophic Hormones

14.3.3.1 Vitamin D

TRPV6 gene is one of the genes most responsive to 1,25(OH)₂D₃. *TRPV6* mRNA was up-regulated as early as 2 h after administration of 0.1 μM of 1,25(OH)₂D₃ in intestinal cell line Caco-2 and a 10-fold increase was achieved by 24 h [76]. Compared to calbindin-D_{9k}, a vitamin D responsive Ca²⁺ binding protein, the response of TRPV6 to 1,25(OH)₂D₃ was more rapid and robust [76]. In vitamin D receptor (*VDR*) knockout mice, Duodenal TRPV6 mRNA was reduced by 90%; however, TRPV5 mRNA level in the kidney appeared to be unchanged. In contrast, TRPV5 mRNA and protein abundance was decreased in vitamin D depleted rats [77]. A single injection of 1,25(OH)₂D₃ in vitamin D deficient mice induced a ~10-fold increase of duodenal mRNA of TRPV6 which peaked at 6 h after injection, and ~three fold to fourfold increase in TRPV5 and TRPV6 mRNA in the kidney that peaked at 12 h after injection [39]. This suggests that TRPV5 and TRPV6 may be differently regulated by vitamin D in the kidney and intestine, respectively.

In humans, duodenal *TRPV6* transcript was initially found not significantly correlated with vitamin D metabolites [18]; but later studies by Walter and colleagues indicate that duodenal TRPV6 level was positively correlated with plasma 1,25(OH)₂D₃ in a *VDR* genotype group [78], in men and likely in younger women, but not in older women [79]. This is likely due to the lower level of *VDR* in older women [79]. In isolated human duodenal specimens, TRPV6 transcript was very responsive to 1,25(OH)₂D₃ [80, 81]. TRPV6 was also responsive to 25-hydroxyvitamin D [25(OH)D], both in Caco-2 cells [82] and in human duodenal

specimens [81]. This is likely due to the expression of 25(OH)D 1 α -hydroxylase (CYP27B1), which converts 25(OH)D to 1,25(OH)₂D₃ [81, 82].

The most unequivocal evidence that *TRPV6* is a direct target of vitamin D action came from the identification of vitamin D responsive elements (VDREs) from *TRPV6* promoter by Pike and colleagues [83, 84]. Three active VDREs were identified in a 7 kb *TRPV6* promoter at -1.2, -2.1 and -4.3 kb from the transcriptional start site [83]. Similar sites are present in mouse *Trpv6* promoter [84]. VDR/RXR (retinoid X receptor) heterodimer binds to TRPV6 promoter, and then recruits steroid receptor coactivator 1 and RNA polymerase II; and this is accompanied by a broad change in histone 4 acetylation [83, 84]. The presence of VDREs in *TRPV6* gene was also confirmed by another study [85]. With regards to TRPV5, it is suggested that there exist VDREs in human *TRPV5* promoter [77]; however, experimental evidence is yet to be provided for these potential VDREs [86].

Finally, evidence exist to support the involvement of TRPV6 in the non-genomic action of 1,25(OH)₂D₃ to stimulate Ca²⁺ absorption in chicken intestinal cells [87]. 1,25(OH)₂D₃ stimulated β -glucuronidase secretion in a protein kinase A (PKA) dependent pathway. In response to β -glucuronidase administration, TRPV6 was translocated to the brush border membrane where it mediates Ca²⁺ influx. The non-genomic effect of 1,25(OH)₂D₃ on Ca²⁺ uptake was blocked by siRNA for either β -glucuronidase or TRPV6 [87]. Thus, 1,25(OH)₂D₃ appears to stimulate TRPV6 expression and trafficking via genomic and non-genomic pathways, respectively. TRPV6 remains the most vitamin D-responsive Ca²⁺ channel identified thus far.

14.3.3.2 Parathyroid Hormone

Parathyroid hormone (PTH) is well known to regulate renal Ca²⁺ reabsorption. The regulation of TRPV5 by PTH is twofold: expression and activity. In parathyroidectomized rats and calcimimetic compound NPS R-467 infused mice that had a lower PTH level, renal TRPV5 (calbindin-D_{28k}, and NCX1) level was decreased; and the PTH supplementation restored the level of TRPV5 and other Ca²⁺ transport proteins [88]. Inhibition of TRPV5-mediated Ca²⁺ influx by ruthenium red decreased the levels of other Ca²⁺ transport proteins, suggesting the major effect of PTH on Ca²⁺ transport machinery is to enhance TRPV5 expression, and the expression of other Ca²⁺ transport proteins depends on TRPV5-mediated Ca²⁺ influx [88].

PTH is also capable of increasing Ca²⁺ transport in the distal tubule acutely [89]. Both PKA and protein kinase C (PKC) are involved in this regulation [90]. Indeed, activation of TRPV5 by PTH via both PKA and PKC has been reported [91, 92]. PTH activated the cAMP-PKA signaling cascade and increased TRPV5 open probability via phosphorylation of threonine-709 of TRPV5 [91]. This regulation required a strong intracellular buffering of intracellular Ca²⁺. However, the PKA phosphorylation site in TRPV5 (threonine-709) is not conserved in human TRPV5. To what extent this mechanism is applicable to humans remains to be determined.

In another study, the increase of TRPV5 activity by heterogeneously expressed PTH receptor was prevented by PKC inhibitor [92]. Mutation of PKC phosphorylation sites serine-299/serine-654 in TRPV5 abolished the regulation.

Caveolae-mediated endocytosis of TRPV5 appears to be inhibited via PKC-dependent pathway in response to PTH [92].

14.3.3.3 Calcitonin

Calcitonin affects renal Ca²⁺ reabsorption through the thick ascending limb [93, 94]. Thus, it is not surprising that this action is independent of TRPV5 because its localization to distal convoluted tubule and connecting tubule. This was confirmed using the *Trpv5* KO mice, as the effects of calcitonin on urinary excretion of Ca²⁺ (and Na⁺ and K⁺) was not different between wild-type (WT) and *Trpv5* KO mice [95]. To what extent TRPV6 is involved in calcitonin-mediated regulation of Ca²⁺ reabsorption is yet to be examined.

14.3.3.4 Klotho

KL gene product Klotho functions to suppress aging process [96]. The two forms of Klotho, membrane and secreted forms, function distinctively (see [97] for a recent review). While the membrane Klotho interacts with fibroblast growth factor (FGF) receptors to form a high affinity receptor for FGF23, a hormone regulating phosphate excretion, the secreted form acts as a hormonal factor that regulates the stability of TRPV5/6 [98–100] and renal outer medullary potassium (ROMK) channel [101]. Klotho is expressed in the distal convoluted tubule where it colocalizes with TRPV5 [98]. Because Klotho exhibits β -glucuronidase activity [102], it was thought that Klotho modifies TRPV5 glycan through this activity [98]. Like Klotho, β -glucuronidase also activated TRPV5 and TRPV6 but not related TRP channels TRPV4 and TRPM6 [99]. Alternatively, Klotho removed terminal sialic acids from their glycan chains of TRPV5 and exposes disaccharide galactose-N-acetylglucosamine, which bound to galactoside-binding lectin galectin-1 [100]. Galectin-1 linked TRPV5 proteins are likely resistant to endocytosis, but the underlying mechanism is not well understood. However, the removal of N-glycan by endoglycosidase-F, which completely removes N-glycans, also increased TRPV5 and TRPV6 activity [99]. Under this condition, galectin-1 may not be able to bind to TRPV5. Although the mechanisms are still to be clarified, the regulation of TRPV5/6 by Klotho represents a novel area of ion channel regulation. The dysregulation of TRPV5/6 may be responsible for the increased excretion of Ca²⁺ in *KL* deficient mice, in which PTH stimulated Ca²⁺ reabsorption in the connecting tubule is impaired [103].

14.3.4 Regulation Under Physiological Conditions

14.3.4.1 Low Dietary Ca²⁺

The biosynthesis of TRPV6 has been shown to be the most responsive to the body's need for Ca²⁺ under conditions such as low Ca²⁺ diet, pregnancy, and lactation.

Restriction of dietary Ca^{2+} drastically increases duodenal TRPV6 level, and to a less extent, renal TRPV5 and TRPV6 in mice [39, 40], and in rats [104]. At least part of the effect of low Ca^{2+} diet to up-regulate TRPV6 is independent of VDR [105].

14.3.4.2 Pregnancy and Lactation

During pregnancy, duodenal *TRPV6* transcript level was up-regulated by 12-fold in either WT or *VDR* KO mice, whereas calbindin- $\text{D}_{9\text{k}}$ and Ca^{2+} -ATPase PMCA1 were only moderately increased in WT mice [106]. This observation was confirmed with global microarray analysis in pregnant *VDR* KO mice [107]. Similarly, a 13-fold increase in duodenal *TRPV6* transcript was observed in both mouse lines during lactation [106]. The increase of prolactin during lactation causes a positive genomic effect on the *TRPV6* transcription along with paracellular claudins; however, TRPV6 appears not to be involved in the nongenomic effect of suckling induced prolactin surge on intestinal Ca^{2+} absorption [108].

14.3.4.3 Sex Hormones

TRPV5 and TRPV6 levels are also controlled by sex hormones. In estrogen receptor β KO mice, TRPV6 level was not altered, however, a 55% reduction of duodenal TRPV6 mRNA level was observed in estrogen receptor α KO mice. Pharmacological doses of estrogens increased TRPV6 mRNA by fourfold and eightfold in ovariectomized WT and *VDR* KO mice, respectively [106]. At the same level of $1,25(\text{OH})_2\text{D}_3$, female mice had a higher Ca^{2+} absorption and TRPV6 mRNA level than male mice. The response of duodenal TRPV6 mRNA to $1,25(\text{OH})_2\text{D}_3$ in female mice was twofold of that in male mice even though the *VDR* mRNA level was the same [109]. In ovariectomized rats, both TRPV5 mRNA and protein levels were increased by pharmacological doses of 17β -estradiol and this effect was independent of $1,25(\text{OH})_2\text{D}_3$ [110]. In aromatase deficient (ArKO) mice, deficiency in estrogen caused hypercalciuria together with decreased levels of renal TRPV5 and TRPV6 [111]. Additionally, 17β -estradiol exerted a nongenomic effect to enhance TRPV6 activity in human colonic T84 cells [112] and TRPV5 activity in rat cortical collecting duct cells [113].

We have previously observed that TRPV6 expression was negatively regulated by androgen in LNCaP cells [114]. Whether this effect of androgen on TRPV6 is related to the lowered intestinal Ca^{2+} absorption and TRPV6 level in male mice is unclear [109]. Interestingly, male mice also have a higher urinary Ca^{2+} excretion and a lower renal TRPV5 level than female mice [115]. TRPV5 level was increased in orchidectomized mice, which excreted less Ca^{2+} in their urine [115]. Males in general have a higher bone mineral density than females; therefore, a higher intestinal Ca^{2+} absorption is expected for males.

14.3.4.4 Exercise

Endurance swimming stimulated Ca^{2+} transport in the small intestine and cecum and decreased that in the proximal colon in rats [116]. The increased Ca^{2+}

absorption were associated with increased mRNA levels of VDR and Ca²⁺ transport proteins (such as TRPV6 and TRPV5) in transcellular and paracellular pathways [116]. In contrast, TRPV6 and TRPV5 mRNA levels dropped along with decreased 1,25(OH)₂D₃ level in immobilized rats, leading to a reduction in intestinal Ca²⁺ absorption [117]. These studies suggest that TRPV6 (and to a lesser extent TRPV5) participate in exercise-induced increase in Ca²⁺ absorption in a vitamin D-dependent manner.

14.3.4.5 Aging

Decreased Ca²⁺ absorption and increased urinary Ca²⁺ excretion are associated with aging. TRPV6 (and also TRPV5) mRNA level in adult (12-month old) rats was less than half of that in young (2-month old) rats [104]. In mice, intestinal absorption decreased and urinary Ca²⁺ excretion increased with age; this was associated with decreased TRPV6 and TRPV5 in the duodenum and kidney, respectively [118]. The same is true for VDR [118]. In women, both TRPV6 and VDR transcripts were significantly decreased in older age (> 50) [79]. Thus, reduction in TRPV5 and TRPV6 may contribute to a negative Ca²⁺ balance as one ages.

In addition to the above physiological conditions, TRPV6 expression is up-regulated by short-chain fatty acids, fermentation products of indigestible oligosaccharides in rat large intestine [119]. This is likely involved in the increase of Ca²⁺ absorption by the indigestible oligosaccharides such as fructooligosaccharides [120].

14.3.5 Gene Knockout Studies

The roles of TRPV5 or TRPV6 in vitamin D-regulated Ca²⁺ transport pathways have been evaluated by gene knockout studies. The major phenotypes of *Trpv5* and *Trpv6* KO mice are listed in Table 14.1. The common features of the *Trpv5* and *Trpv6* KO mice include elevated serum 1,25(OH)₂D₃, increased urinary Ca²⁺ excretion, and some degree of deficiency in the bone [121, 122]. Urinary Ca²⁺ excretion increased by sixfold in *Trpv5* KO mice [122]. Intestinal Ca²⁺ absorption is decreased in *Trpv6* KO mice [121], increased in *Trpv5* KO mice as duodenal TRPV6 and calbindin-D_{9k} mRNAs are increased due to an elevated 1,25(OH)₂D₃ level [122]. However, the serum Ca²⁺ levels are largely normal, even though the PTH level is elevated in *trpv6* KO mice [121], and in older *Trpv5* KO mice [118].

It is worth noting, however, active Ca²⁺ transport did occur in the absence of TRPV6 alone or together with calbindin-D_{9k} [123]; and the increase of intestinal Ca²⁺ absorption in response to 1,25(OH)₂D₃ appeared to be intact in the absence of TRPV6 [124]. While Ca²⁺ balance studies are needed to verify the results, it is not surprising as transcellular Ca²⁺ transport contributes only a small fraction of total Ca²⁺ absorbed when the dietary Ca²⁺ level is adequate or high due to the short sojourn time of digested food in the duodenum [125]. The transcellular pathway plays a significant role only when Ca²⁺ in diet is low. The main driving forces for Ca²⁺ absorption through the paracellular pathway are transepithelial potential and

Table 14.1 Phenotype of *Trpv5* KO and *Trpv6* KO mice

Phenotype	KO mouse	Alteration	Mechanism/explanation	References
Serum 1,25(OH) ₂ D ₃	<i>Trpv5</i>	Elevated (2.9-fold)	Compensatory response	[122]
	<i>Trpv6</i>	Elevated (2.4-fold)	Compensatory response	[121]
	<i>Trpv5</i>	Elevated only in older mice	Compensatory response	[118]
	<i>Trpv6</i>	Elevated (3.8-fold)	Compensatory response	[121]
	<i>Trpv5</i>	No increase in the absence of 25(OH)D ₃	Due to the increased 1,25(OH) ₂ D ₃ and in turn TRPV6, calbindin-D _{9k}	[122, 126]
Intestinal Ca ²⁺ absorption		Increased (~30% more)		
		1 α -hydroxylase		
	<i>Trpv6</i>	Decreased (~60% less)	Trpv6 plays a role in Ca ²⁺ absorption	[121]
	<i>Trpv5</i>	Increased (~sixfold)	Trpv5 plays a role in Ca ²⁺ reabsorption	[122]
	<i>Trpv6</i>	Increased (>twofold)	Trpv6 plays a role in Ca ²⁺ reabsorption	[121]
Placental Ca ²⁺ transport	<i>Trpv5</i>	Not determined		
	<i>Trpv6</i>	Decreased	Trpv6 is involved in the active transport of maternal Ca ²⁺ to fetus	[134]
	<i>Trpv5</i>	Increased urine volume reduced urine pH	Activation of CaSR in the collecting duct	[122, 127]
	<i>Trpv6</i>	Increase urine volume	Defect in urine concentrating mechanism	[121]
	<i>Trpv5</i>	Reduced bone thickness	Prolonged elevation of 1,25(OH) ₂ D ₃	[122, 128]
Bone	<i>Trpv6</i>	9.3% decrease in BMD	Prolonged elevation of 1,25(OH) ₂ D ₃	[121]
		Reduced bone formation/mineralization on low Ca ²⁺ diet		[129]
	<i>Trpv5</i>	Not determined		
	<i>Trpv6</i>	Disrupted skin Ca ²⁺ gradient.	Trpv6 is involved in transporting Ca ²⁺ into the epidermal keratinocytes	[121]
	Skin	~20% developed alopecia and dermatitis		

Ca²⁺ concentration gradient. As Ca²⁺ concentration in the plasma is around 1 mM, the driving force for Ca²⁺ absorption via paracellular pathway would be decreased when dietary Ca²⁺ level is low. In contrast, there is a huge (10,000-fold normally) Ca²⁺ gradient across the brush border membrane, even when Ca²⁺ in the diet is scarce, the transcellular pathway will still function properly. When dietary Ca²⁺ is limited, 1,25(OH)₂D₃ will be elevated in the body, which in turn will induce the transcription of TRPV6 and related Ca²⁺ transport proteins [39]. Low dietary Ca²⁺ also increases TRPV6 transcription independent of the genomic action of vitamin D [105]. Thus, TRPV6 would be an important component for Ca²⁺ absorption when dietary Ca²⁺ is restricted; when the dietary Ca²⁺ is adequate, it may play a regulatory role to fine tune the level of Ca²⁺ absorption. This role is also indispensable because 1,25(OH)₂D₃ level is elevated in the absence of TRPV6.

14.4 Roles Beyond Intestinal/Renal Ca²⁺ Re/absorption

14.4.1 TRPV6 in Maternal-Fetal Ca²⁺ Transport

Both TRPV5 and TRPV6 are all expressed in the placenta [17, 34, 130–132], however, TRPV6 mRNA level is ~1,000 times higher than that of TRPV5 [32]. TRPV6 is expressed in the trophoblasts and syncytiotrophoblasts of human placenta [17]. In human syncytiotrophoblasts, TRPV6 and TRPV5 were detected in both apical and basal membranes [130]. Ca²⁺ transport in human syncytiotrophoblasts was insensitive to voltage and L-type Ca²⁺ channel modulators but is sensitive to TRPV5/6 blocker Mg²⁺ and ruthenium red [132]. In cultured human trophoblasts isolated from term placenta, TRPV6 and TRPV5 expression correlated with the Ca²⁺ uptake potential along the differentiation of the trophoblasts [131]. Cyclophilin B, a member of immunophilin family, was associated with TRPV6 in human syncytiotrophoblasts and increased TRPV6 activity in vitro [133].

The role of TRPV6 in maternal-fetal Ca²⁺ transport was confirmed by the *Trpv6* KO model [134]. TRPV6 mRNA and protein were detected mainly in intraplacental and the visceral layer of extraplacental yolk sac, where maternal-fetal Ca²⁺ transport takes place in mice. A 14-fold increase was observed in mouse placenta in the last 4 days of gestation to meet the need of Ca²⁺ required for fetal bone mineralization. The maternal-fetal Ca²⁺ transport was 40% lower in *Trpv6* KO fetuses than in WT. As a result, *Trpv6* KO fetuses exhibited low blood Ca²⁺ level and low ash weight [134]. Thus, TRPV6 represents an important Ca²⁺ entry route for maternal-fetal Ca²⁺ transport pathway. A significant reduction of Ca²⁺ transport along with decreases of TRPV5, TRPV6 and other Ca²⁺ transport proteins was found in primary preeclamptic syncytiotrophoblasts, suggesting that TRPV5 and TRPV6 are involved in the abnormal placental Ca²⁺ transport in preeclampsia [135].

14.4.2 TRPV6 Expression and Regulation in the Uterus

The expression of TRPV6 in uterine tissue was studied in mice [136, 137], rats [137, 138], and pigs [139]. TRPV6 mRNA was detected in rat uterine endometrium and glandular endometrium and was highly up-regulated at diestrus compared with proestrus [138]. In mice, uterine TRPV6 mRNA increased in mid- and late-pregnancy, and TRPV6 mRNA was responsive to 17β -estradiol in the luminal and glandular epithelia [136]. In rats, uterine TRPV6 was regulated by progesterone via its receptor [138], whereas TRPV6 appears to be dependent on estrogen receptors in mice [137]. Uterine and placenta TRPV6 levels changed cyclically during pregnancy, suggesting a role of TRPV6 in the embryo implantation process [137]. TRPV6 mRNA was only expressed in placenta-unattached areas of the uterus, and in the labyrinth and spongy zone of placenta. In pig uterus TRPV6 was localized mainly to luminal epithelial cells and to a lesser extent to glandular epithelial cells and chorionic membrane during pregnancy [139]. TRPV6 was higher during pregnancy than in estrous cycle [139]. These observations indicate that TRPV6 plays a role in establishing and maintaining pregnancy as well as in Ca^{2+} transfer between embryo and placenta [137, 139].

14.4.3 TRPV5 in Bone Resorption

TRPV5 was shown to be expressed in the ruffled border membrane in mouse osteoclasts [140]. In the absence of TRPV5, there were increased osteoclast numbers and osteoclast area; however, urinary bone resorption marker deoxypyridinoline was reduced [140]. This is consistent with the impaired bone resorption by osteoclasts derived from *Trpv5* KO mice in an in vitro bone marrow culture system [140]. However, bone resorption inhibitor alendronate normalized the reduced bone thickness in *Trpv5* KO mice, even though it specifically increased bone TRPV5 expression in mice [128]. This indicates that although TRPV5 may play a role in bone resorption, this process is still functional in the absence of TRPV5. Thus the defect of bone in *Trpv5* KO mice is largely due to other compensatory mechanisms as a result of urinary Ca^{2+} wasting.

14.4.4 TRPV5 and TRPV6 in Maintaining Ca^{2+} Gradient in the Inner Ear

The low Ca^{2+} concentration of mammalian endolymph in the inner ear is required for normal hearing and balance. Marcus and colleagues showed that TRPV5 and TRPV6 may play a role in the function of inner ear [141–144]. Both TRPV5 and TRPV6 were detected in primary cultures of semicircular canal duct (SCCD) epithelial cells from neonatal rats, and TRPV5 transcript was responsive to $1,25(\text{OH})_2\text{D}_3$ [144], however, the protein level was not up-regulated [141]. TRPV5 and TRPV6 were detected in native SCCD, cochlear lateral wall and stria vascularis (TRPV5

only) of adult rats along with other Ca²⁺ transport proteins [141]. TRPV5 protein was localized close to the apical membrane of strial marginal cells; and both TRPV5 and TRPV6 were found in outer and inner sulcus cells of the cochlea and in the SCCD of the vestibular system [141]. TRPV5 and TRPV6 immunostaining was detected in mouse inner ear as well, and the levels of TRPV5 and TRPV6 decreased in older mice [145]. Mutations in pendrin (Slc26a4, an anion exchanger) cause the most common form of syndromic deafness. Reduced pH and utricular endolymphatic potential and increased Ca²⁺ concentration were found in pendrin KO mice [142, 143]. The reduced pH likely blocks the activity of TRPV5 and TRPV6, whose Ca²⁺ transport activity is reduced at low pH, similar to what was observed in primary SCCD cells [143]. The elevation of endolymphatic Ca²⁺ level in pendrin KO mice may inhibit sensory transduction necessary for hearing and promote the degeneration of the sensory hair cells, which is necessary for the development of normal hearing [142]. Similar mechanisms may be responsible for loss of hearing in patients with Pendred syndrome.

14.4.5 TRPV6 in Exocrine Organs

Human TRPV6 transcripts are most abundant in exocrine organs including pancreas, prostate, mammary, salivary and sweat glands [17, 34, 35]. In pancreas, TRPV6 protein is prominently distributed to the apical membrane and granules of the secretory pole in both mice and humans [35]. In human mammary glands, TRPV6 was strongly expressed in the apical membrane of ductal epithelial cells. Apical membrane localization of TRPV6 was also observed in human prostate epithelial cells [35]. In human sweat gland, TRPV6 appears to be expressed in both apical and basolateral membranes [35]. TRPV6 mRNA is expressed in human parotid and submandibular glands, and TRPV6 protein is localized to the basolateral plasma membrane of acinar cells [146]. While TRPV6 might be involved in salivary Ca²⁺ secretion which is important in mineralization of dental enamel and exposed dentin [146], details of TRPV6 in exocrine function are largely unavailable although it is well known that Ca²⁺ plays important role in secretion. It is possible that TRPV6-mediated Ca²⁺ influx stimulates exocytosis; more likely, TRPV6 may replenish cellular Ca²⁺ by apical re-uptake of released Ca²⁺ following the secretory events, or from extracellular fluid via the basolateral membrane.

In addition, TRPV6 may play a role in Ca²⁺ induced keratinocyte differentiation [147]. Both TRPV5 and TRPV6 are expressed in retina pigment epithelium and they may mediate Ca²⁺ influx from subretinal space to regulate the Ca²⁺ level in light/dark transitions [148].

14.5 Regulation Under Pathological and Therapeutic Conditions

TRPV5 and TRPV6 undergo changes in response to diseases and therapies. Tables 14.2 and 14.3 summarize the changes in TRPV5 and TRPV6 mRNA or protein level under disease and therapeutic conditions, respectively. The regulation

of TRPV6 and TRPV5 in cancers, pseudohypoaldosteronism Type II (PHA II), and under treatments of glucocorticoids and diuretics are summarized in the following.

14.5.1 TRPV6 in Cancer

TRPV6 level is elevated in prostate, breast, thyroid, colon and ovarian carcinomas [35]. In prostate cancer samples, TRPV6 mRNA level increases as tumor grade advances as defined by Gleason score [17, 114, 149]. Furthermore, TRPV6 expression also correlates with pathological stage and extraprostatic extension; and androgen-insensitive tumors exhibited decreased TRPV6 levels compared to untreated tumors [149]. Thus, TRPV6 represents a prognostic marker for advanced prostate cancer.

TRPV6 appears to be a component of the store-operated Ca^{2+} channels in prostate LNCaP cells [150, 151] while evidence exists against this [152]. Ca^{2+} store depletion induced the expression of TRPC1, TRPC3, and TRPV6 in LNCaP cells, but these channels were not sufficient to stimulate store-operated Ca^{2+} entry [153]. The store-operated current was down-regulated when LNCaP cells differentiated to androgen-insensitive, apoptotic-resistant neuroendocrine phenotype [149]. TRPV6 expression activated Ca^{2+} /NFAT pathways that enables LNCaP cells to proliferate at high rate and to become resistant to apoptosis [154]. The expression of intermediate-conductance Ca^{2+} -activated K^+ channels, which are preferentially expressed in human prostate cancer tissues, hyperpolarized the cells to drive TRPV6-mediated Ca^{2+} entry, and in turn, proliferation of cancer cells [155]. Reducing Ca^{2+} entry via blocking the K^+ channels prevented proliferation [155]. Therefore, TRPV6 represents a potential therapeutic target to treat prostate cancer.

The potential role of TRPV6 in breast cancer was recently put forward by Hediger and colleagues [156, 157]. Seven out of 12 patients exhibited 2–15-fold increases of TRPV6 mRNA in breast cancer tissues over normal tissues as control [156]. Interestingly, estrogen receptor antagonist tamoxifen not only partially blocked TRPV6 expression in breast cancer cell line T47D, but also inhibited TRPV6-mediated Ca^{2+} uptake activity in *Xenopus* oocytes [156]. In MCF-7 breast cancer cells, TRPV6 activity was blocked by tamoxifen in the presence of estrogen receptor antagonist and the effect of tamoxifen on TRPV6 remained intact in estrogen receptor-negative MDA-MB-231 cells [157]. Activation of PKC blocked the effect of tamoxifen [157]. Thus, tamoxifen-mediated inhibition of TRPV6 might be part of the therapeutic effects of tamoxifen in both estrogen sensitive and insensitive breast cancers.

In a *Citrobacter rodentium*-induced transmissible murine colonic hyperplasia model, a 10- to 20-fold increase of TRPV6 mRNA was observed during the induction of colonic hyperplasia, while VDR, calcium sensing receptor, calbindin- $\text{D}_{9\text{k}}$ and TRPV5 mRNA levels were unaltered [158]. High Ca^{2+} diet abrogated TRPV6 expression and hyperplasia [158]. Elevated TRPV6 expression was associated with early-stage colon cancer in humans, and blocking TRPV6 expression by siRNA

Table 14.2 Alteration of TRPV5/TRPV6 expression under disease conditions

Disease conditions	Model	Tissue/cell type	Alterations	References
Arterial calcifications	TGF1alpha-deficient kidneys	Kidney	TRPV5/6mRNA ↑	[186]
Cancer	Human specimens	Prostate, breast, thyroid, colon and ovarian carcinomas	TRPV6 mRNA/protein ↑	[17, 35, 114, 149, 156, 158]
Colonic hyperplasia	Transmissible murine colonic hyperplasia	Colon	TRPV6 mRNA/protein ↑	[158]
Crohn's disease	TNF(DeltaARE) mouse	Intestine/kidney	TRPV5/6 mRNA ↓	[187]
Familial hypomagnesemia with hypercalciuria and nephrocalcinosis	<i>Cldn16</i> ^{-/-} mice	Kidney	TRPV5/6 mRNA ↑	[188]
Preeclampsia	Primary culture from patients	Syncytiotrophoblasts	TRPV5/6 mRNA/protein ↓	[135]
Pseudohypoparathyroidism type II	<i>Wnk4</i> ^{D561A/+} knock-in mice	Kidney tubules	TRPV6 Protein ↓	[168]
Streptozotocin-induced diabetes mellitus	Rats	Kidney	TRPV5/6 mRNA ↑ TRPV5 protein ↑	[184]

Table 14.3 Alteration of TRPV5/TRPV6 expression under therapeutic conditions

Therapeutic conditions	Model	Tissue	Alterations	References
Prednisolone	Mice	Duodenum	TRPV6 mRNA↓	[172]
Dexamethasone	Mice	Duodenum	TRPV6 mRNA↓	[170, 171]
	Rats	Kidney	TRPV5 mRNA↑	[189]
Tacrolimus (FK506)	Rats	Kidney	TRPV5 mRNA↑	[189]
Hydrochlorothiazide (high dose)	Rats	Kidney	TRPV5 mRNA/Protein↓	[181]
Hydrochlorothiazide (medium dose)	Rats	Kidney	TRPV5 protein↑	[178]
Chlorothiazide (acute, low dose)	Mice	Kidney	TRPV5 mRNA↑	[180]
Furosemide	Mice	Kidney	TRPV5/6 mRNA↑	[183]
Gentamicin	Mice	Kidney	TRPV5/6 mRNA↑	[183]
Sap of acer mono	Mice	Duodenum	TRPV6 mRNA↑	[190]

inhibited colon cancer cell proliferation and induced apoptosis [158]. Thus, elevated TRPV6 expression likely contributes to colonic hyperplasia and colon cancer cell proliferation and TRPV6 represents a therapeutic target for colon cancer.

Capsaicin, an ingredient of chili pepper and TRPV1 activator, induces apoptosis in some cancer cells. Interestingly, gastric cancer cells were more susceptible to capsaicin-induced apoptosis because of the high TRPV6 expression in cancer cells [159]. Overexpression of TRPV6 in normal cells increased capsaicin-induced apoptosis and knockdown of TRPV6 in cancer cells suppressed it [159]. This suggests a strategy to selectively induce cancer cell apoptosis based on its high level TRPV6 expression. The potential roles of TRPV6 as a cancer marker and therapeutic target are still to be further explored.

14.5.2 TRPV5 and TRPV6 in Pseudohypoaldosteronism Type II

Pseudohypoaldosteronism type II (PHAII), also known as familial hyperkalemia and hypertension (FHH) or Gordon syndrome, is a genetic disorder caused by mutations in With No lysine (K) (WNK) kinases 1 and 4 [160]. Patients carrying WNK4 mutation exhibit marked sensitivity to thiazides, hypercalciuria and low bone mineral density, indicating a defect in Ca^{2+} reabsorption [161, 162]. To understand potential roles of TRPV5 and TRPV6 in this process, we investigated their regulation by WNK kinases [163–165]. In *Xenopus* oocytes expression system, WNK4 selectively up-regulated TRPV5 while exerted little effect on TRPV6 [163]. WNK1 showed no significant effect on either of them [163]. In contrast, WNK3 was capable of up-regulating both [164]. The effects of WNK3 and WNK4 on TRPV5 are at least in part via enhancing the forward trafficking of TRPV5 to the plasma membrane through the secretory pathway [164, 165]. While the disease causing mutants of WNK4 regulated TRPV5 to similar extent as WT, the presence of

the thiazide-sensitive Na⁺-Cl⁻ cotransporter, which was down-regulated by WNK4 [166, 167], blocked the effect of WNK4 on TRPV5 in a dose-dependent manner. The blocking effect of NCC was further strengthened when WT WNK4 was replaced by the PHAII-causing Q565E mutant, suggesting a reduction of TRPV5-mediated Ca²⁺ influx under this condition [163]. This is consistent with the observation of hypercalciuria and low bone mineral density in patients carrying WNK4 Q565E mutation.

In the *Wnk4*^{D561A/+} knock-in mouse model of human PHAII, TRPV5 protein level appears to be similar to the WT littermate, but a significant increase in TRPV6 and calbindin-D_{28k} was observed, along with attenuated level of NKCC2, and increased abundance of NCC and ENaC [168]. In addition, urine Ca²⁺ excretion rate was not increased by furosemide in *Wnk4*^{D561A/+} mice as was in WT mice [168]. These data indicate decreased Ca²⁺ reabsorption in the thick ascending loop of *Wnk4*^{D561A/+} mice, and the enhanced level of TRPV6 and calbindin-D_{28k} represents a secondary adaptive mechanism in the distal nephron [168]. The effects of WNK4 mutation on NKCC2 and TRPV6 are consistent with the high abundance of WNK4 in the outer medulla [169]. Since TRPV6 is predominantly expressed in the thick ascending limb [42](our unpublished observation), the effect of WNK4 on TRPV6 expression (or indirectly via NKCC2) warrants further studies. The increased NCC level is also consistent with a potential decrease of TRPV5-mediated Ca²⁺ reabsorption through NCC-mediated inhibitory effect on the positive action of WNK4 on TRPV5 as noted earlier [163].

14.5.3 Glucocorticoids

Glucocorticoids are a class of hormones that are used widely as an anti-inflammatory and immunosuppressive drug. However, they have side effects such as inducing osteoporosis. Reduced intestinal Ca²⁺ absorption and renal Ca²⁺ reabsorption may be part of the mechanisms of glucocorticoid-induced osteoporosis. Regulation of TRPV5 and TRPV6 by glucocorticoids may occur through two mechanisms, expression regulation [170–172] and activity regulation via glucocorticoid inducible protein kinase SGK1 [173–176].

Oral application of prednisolone for a week in mice reduced intestinal Ca²⁺ absorption and TRPV6 mRNA level and calbindin-D_{9k} mRNA and protein levels but renal TRPV5 and calbindin-D_{28k} and NCX1 mRNA levels remained unaltered [172]. In calbindin-D_{9k} and calbindin-D_{28k} KO mice, compensatory elevation of TRPV6 mRNA in the duodenum was blocked by 5-day dexamethasone treatment [171]. The renal TRPV5 mRNA in calbindin-D_{9k} KO mice was elevated while the renal TRPV6 mRNA was reduced in calbindin-D_{28k} KO mice after dexamethasone administration [171]. These regulations appear to be related to the decreased duodenal VDR level by dexamethasone [171]. The effects of dexamethasone were time dependent: at day one it increased duodenal TRPV6 mRNA and renal TRPV5 mRNA while decreased renal TRPV6 mRNA; after 5-day treatment, only TRPV6

in the duodenum was robustly reduced [170]. Thus, it appears intestinal TRPV6 is the target of glucocorticoids action and this action appears to be associated with decreased VDR level in the intestine.

The activity of TRPV5 and TRPV6 could be regulated by glucocorticoid inducible SGK kinases. In *Xenopus* oocyte system, SGK1 and SGK3 up-regulated TRPV5 activity in the presence of Na⁺/H⁺ exchanger (NHE) regulating factor 2 (NHERF2), and this effect could not be reproduced by active form of protein kinase B (PKB) and SGK2 [175]. The surface abundance of TRPV5 was increased by SGK1 and NHERF2 [174]. TRPV5 C-terminal tail interacts with NHERF2, and deletion of the second PDZ domain in NHERF2 abrogates the stimulating effect of SGK1/NHERF2 on TRPV5 [174]. In contrast, TRPV6 was also up-regulated by SGK1, SGK3 and PKB/Akt; however, neither NHERF1 nor NHERF2 was required for this regulation [173, 176]. The effect of SGK1 on TRPV6 was augmented in the presence of phosphatidylinositol-3-phosphate-5-kinase PIKfyve (PIP5K3), a kinase generating phosphatidylinositol 3,5-bisphosphate [PI(3,5)P₂] [176]. TRPV6 expression at or close to the *Xenopus* oocytes surface was significantly increased by the co-expression of SGK1 and PIKfyve [176]. PIKfyve alone did not affect TRPV6, and ^{5318A}PIKfyve lacking the SGK1 phosphorylation site could not further increase the positive effect of SGK1 on TRPV6 [176]. Thus, PIKfyve requires its phosphorylation by SGK1 to act on TRPV6.

The effect of SGK1 on Ca²⁺ reabsorption and TRPV5 was evaluated in *Sgk1* KO mice [177]. Membrane expression of TRPV5 protein and cytosolic calbindin-D_{28k} protein were all decreased in the KO mice, suggesting that SGK1 play a role in the regulation of TRPV5 trafficking as observed in previous in vitro studies [174, 175, 177]. However, fractional excretion of Ca²⁺ was lower in the *Sgk1* KO mice than in the WT mice, despite normal 1,25(OH)₂D₃ and PTH levels [177]. The lowered Ca²⁺ excretion is likely due to the increased reabsorption of Ca²⁺ in the thick ascending limb because NKCC2 inhibitor furosemide dissipated the difference in Ca²⁺ excretion between the KO and WT mice [177]. Whether TRPV6 is altered in the *Sgk1* KO mice is yet to be determined.

14.5.4 Diuretics

Hypocalciuria is a well known effect of thiazide diuretics, which blocks the Na⁺-Cl⁻ cotransporter NCC. A number of studies have been undertaken to dissect the molecular mechanisms of this effect [178–181]; however, the mechanisms of thiazides-induced hypocalciuria are controversial. Application of high dose of hydrochlorothiazide (HCTZ), at 12 mg/day for a week, significantly reduced TRPV5, calbindin-D_{28k}, NCX1 and NCC mRNA and protein levels and the number of tubules expressing calbindin-D_{28k} and NCC in rat kidneys [181]. Since Na⁺ repletion prevented both volume contraction and hypocalciuria in HCTZ treated rats, it is suggested volume contraction play a critical role in thiazide induced hypocalciuria [181]. At lower dose, acute application of chlorothiazide (CTZ), at

either 25 or 50 mg/kg but not at 100 mg/kg, significantly increased TRPV5 expression in mouse kidneys [180]. Chronic treatment with CTZ at 25 mg/kg twice daily for 3 days caused no change in TRPV5, TRPV6 and calbindins, but salt supplement in drinking water alone or with CTZ significantly increased the expression of these Ca²⁺ transport proteins [180]. This study agrees that under volume contraction, increased Ca²⁺ reabsorption via paracellular pathway in the proximal tubule plays the major role; however, without volume contraction, hypocalciuria is probably achieved through increased Ca²⁺ reabsorption in the distal convoluted tubule by up-regulating the transcellular pathway [180].

Subsequent studies with the *Trpv5* KO mice indicate that enhanced passive Ca²⁺ transport in the proximal tubule rather than active Ca²⁺ transport in distal convolution is the cause of thiazide-induced hypocalciuria [179]. HCTZ-induced hypocalciuria remained unaltered in *Trpv5* KO mice, and the reabsorption of Na⁺ and Ca²⁺ was increased in the proximal tubule and the Ca²⁺ reabsorption in the distal convolution was apparently unaffected in micropuncture experiments [179]. In a recent study that evaluates the effects of HCTZ on different transporters in proximal tubule, thick ascending limb and distal convoluted tubule under high salt or high Ca²⁺ diet induced hypercalciuric rats, TRPV5, NHE3, and NKCC2 proteins were decreased in all hypercalciuric rats, whereas increased TRPV5 protein was associated with hypocalciuric effect induced by HCTZ [178]. These results indicate that thiazide-induced hypocalciuria is a result of coordinated alterations of Ca²⁺ reabsorption mechanisms in all tubular segments involved in Ca²⁺ reabsorption, including proximal tubule, thick ascending limb, and the distal convoluted and connecting tubule.

In contrast to hypocalciuric effect of thiazide diuretics, loop diuretics such as furosemide promote urinary Ca²⁺ excretion [182]. Furosemide inhibits NKCC2 in the thick ascending limb and diminishes the negative luminal potential needed to drive Ca²⁺ and Mg²⁺ reabsorption through the paracellular pathway. Lien and colleagues [183] found that acute and chronic application of furosemide increased both TRPV5 and TRPV6 levels along with that of calbindins; similar increases of TRPV5 and TRPV6 were found in hypercalciuria induced by gentamicin [183] and in streptozotocin-induced diabetes mellitus [184]. The up-regulation of Ca²⁺ transporters in these situations is likely due to the increased Ca²⁺ load to the segments [183, 184], consistent with Ca²⁺ load-dependent expression of TRPV5 in the kidney [185].

14.6 Unusual High Frequencies of SNPs in African Populations

TRPV6 and *TRPV5* have been shown to have high frequency of SNPs in African populations. By analyzing SNP data of 132 genes from 24 African-Americans and 23 European-Americans, Akey and colleagues [191] identified 4 contiguous genes under demographically robust selection, including *EPHB6*, *TRPV6*, *TRPV5*, and *KEL4*, in a 115-kb region in chromosome 7q34-35 exhibiting features of a

recent selective sweep. Similarly, using data from 151 genes, Stajich and Hahn [192] found that *TRPV6* gene shows a population-specific pattern of positive selection. All these studies utilized the public available database from SeattleSNPs programs (<http://pga.mbt.washington.edu/>), in which approximately 200 genes from 24 African-Americans and 23 European-Americans are sequenced. The visual SNP genotype of *TRPV6* (<http://pga.gs.washington.edu/data/trpv6/trpv6.prettybase.png>) clearly shows that there are many rare alleles in African-Americans whereas very few in European-Americans. The same is true for *TRPV5*. Subsequent studies [193, 194] also support that *TRPV6* locus has undergone positive selection. The selective scenario put forward by Stajich and Hahn [192] is that there were many mutations in *TRPV6* locus in early humans in Africa; as early humans migrated out of Africa, a preexisting mutation in the ancestral African population became advantageous in a new environment and rose to high frequency. Akey and colleagues found that the haplotype defined by 3 nonsynonymous SNPs (C157R, M378V and M681T, Allele frequency ~0.5 in African-Americans, ~0.02 in European-Americans from SeattleSNPs) are nearly fixed for the derived alleles in non-African populations based on genotyping data of 1,064 individuals from 52 populations [195]. Hughes and colleagues [193] further provided evidence for independent, parallel selection on *TRPV6* locus in Europeans and Asians.

Which one of the 4 genes in chromosome 7q34-35 was under the selective pressure outside of Africa is unclear; however, *TRPV6* is a good candidate as suggested by Akey and colleagues [191, 195]. What drove the selection of *TRPV6* haplotype outside of Africa is unknown; however, it might be associated with the alteration of dietary Ca^{2+} availability and the change of vitamin D status in earlier humans. Akey and colleagues [191, 195] suggested that patterns of *TRPV6* sequence variation may have been influenced by availability of dairy products due to the domestication of milk-producing animals approximately 10,000 years ago, similar to positive selection for lactase persistence [196]. As early humans migrated away from the equator their vitamin D level decreased due to the reduction of ultraviolet light exposure [197]. Because *TRPV6* is a highly vitamin D-responsive gene, the alteration of vitamin D status may be a factor. Another possibility is that the derived *TRPV6* allele may be advantageous in resistant to a pathogen humans encountered after migrated outside of Africa [195]. *TRPV6* is expressed in the brush border membrane of intestinal epithelium, thus it is likely that *TRPV6* could be a receptor for certain pathogens. Consistent with this hypothesis, M378V variation in the first extracellular loop of *TRPV6* is in direct contact with digested food and potential pathogens. The 378V residue is conserved in many species. If the extracellular loop of *TRPV6* interacts with a contagious pathogen, this pathogen is potentially transferable between animals and humans. Thus, the 378M mutation could potentially make *TRPV6* resistant to this pathogen and provide an advantage for the 378M carriers to survive disastrous infectious diseases. In this case, the derived *TRPV6* allele may not necessarily be beneficial for Ca^{2+} absorption. Due to the increased Ca^{2+} intake from the dairy products and the increased availability of vitamin D by the development of fishery, the role of *TRPV6*-mediated Ca^{2+} absorption may not be as critical for human survival outside of Africa. This hypothesis is consistent with

the fact that African–Americans have higher intestinal Ca²⁺ absorption compared to Caucasians [198].

The nonsynonymous SNPs in the ancestral haplotype (157R, 378V, 681T) may have an impact on TRPV6 function; however, the results from different groups are controversial [193, 199]. In one study the Ca²⁺ uptake activity of the ancestral TRPV6 protein (157R, 378V, 681T) was twofold that of the derived protein (157C, 378M, 681M) when expressed in *Xenopus* oocytes; and 378V, located to the first extracellular loop, appeared to be responsible for the increase in Ca²⁺ uptake activity [199]. In another study, however, no significant differences in channel function were identified for the ancestral TRPV6 [193]. We also failed in observing any functional difference between the two forms of TRPV6 (unpublished observations). An interesting observation is that ATP binds to TRPV6 and prevents rundown of channel activity and 2 arginine residues R¹⁵³R¹⁵⁴ close to 157R are involved in ATP binding [68]. The 157R in the ancestral TRPV6 is located to N-terminal region between ankyrin repeats 3 and 4. The S155 becomes a potential phosphorylation site (S¹⁵⁵P¹⁵⁶R¹⁵⁷) in the ancestral TRPV6 and phosphorylation of S155 by PKC is likely involved in the slower rundown of TRPV6 ancestral protein in the presence of ATP [68]. However, the difference is very small and may not result in significant difference in overall channel activity under normal condition.

Four nonsynonymous SNPs in *TRPV5* were identified by SeattleSNPs: three of them (A8V, A563T and L712F) were only present in African–Americans, not in European–Americans; R154H is common in both populations. In contrast to the nonsynonymous SNP variations in TRPV6 ancestral haplotype that are mostly conserved in other species, the variations in TRPV5 are newly derived as they are not commonly present in other species surveyed including chimpanzee, dog, rat and mouse, with the exceptions of 563T in dog and 8V in rat [200]. In addition, the nonsynonymous SNPs of TRPV5 are not associated with each other as are those in TRPV6. Thus, these variations more likely alter the function of TRPV5. By expression in *Xenopus* oocytes, we found that 2 of the SNPs, A563T and L712F, significantly increased TRPV5-mediated Ca²⁺ uptake by approx. 50 and 25%, respectively [200]. For A563T variant, the increased Ca²⁺ uptake activity was not associated with increased protein abundance in the plasma membrane; rather it was associated with increase apparent K_m for Ca²⁺ and increased sensitivity to extracellular Mg²⁺, suggesting increased permeation of Ca²⁺ in the cation translocation pathway of the channel [200]. The location of A563T in the last transmembrane domain of TRPV5, 20 amino-acid residues away from the D542 that forms Ca²⁺ filter in the pore, is likely close to the cation translocation path in a 3D structure.

Africans exhibit Ca²⁺ conservation mechanisms. African–Americans have lower urinary Ca²⁺ excretion than Caucasians [198, 201, 202], and the risk of kidney stone in African–American is lower than that in Caucasians [203, 204]. African–Americans have higher bone mass [205], and lower incidence of osteoporosis related fractures than whites [206]. Because of the high allele frequencies of *TRPV5* and *TRPV6* SNPs in African populations, these SNPs may contribute to the Ca²⁺ conservation mechanisms in African populations. This is yet to be tested in population studies.

The roles of *TRPV6* or *TRPV5* SNPs have been evaluated in kidney stone disease and hypercalciuria. In a study of 170 kidney stone formers, the prevalence of the *TRPV6* ancestral haplotype in stone-forming patients was significantly higher than that of non-stone-forming patients [199]. The only kidney stone patient carrying the homozygous ancestral *TRPV6* haplotype exhibited more stone episodes, much higher urinary Ca^{2+} excretion and lower plasma PTH level, suggesting a potential role of ancestral *TRPV6* protein in the enhanced Ca^{2+} absorption in this patient [199]. In a study involving 20 renal hypercalciuria patients, non-synonymous variation of *TRPV5* (A8V, R154H and A561T) and synonymous variations were identified among these patients [207]. Although A561T is very close to A563T, which has significant effect on Ca^{2+} permeation [200], no functional difference was identified between A561T variant and control *TRPV5*. This result, together with the previous screening that failed to identify *TRPV5* mutations in patients with autosomal dominant idiopathic hypercalciuria [208], indicates that *TRPV5* may not play a primary role in hypercalciuria. However, the role of *TRPV6* variations in hypercalciuria warrants further investigation.

TRPV6 expression level positively correlates with tumor grade in prostate cancer. Mortality from prostate cancer is two to three times greater in African–Americans than in Caucasians [209, 210]. It is not clear whether the high prostate cancer mortality in African–Americans is relevant to the high prevalence of ancestral *TRPV6* allele in African–Americans. However, in a recent study the frequency of *TRPV6* ancestral allele in 142 Caucasian prostate adenocarcinoma samples was not different from that in 169 Caucasian healthy control subjects [211]. The *TRPV6* ancestral allele frequency did not correlate with the onset of prostate cancer, the Gleason score, or the tumor stage [211]. Because of the low frequency of ancestral alleles in the Caucasian subjects (only one patient with homozygous *TRPV6* ancestral allele) of this study, a conclusion on a relation between *TRPV6* genotype and prostate cancer should be drawn with caution. Studies in African or African–American populations will likely provide more conclusive results.

14.7 Perspectives

Over 10 years after the identification of *TRPV5* and *TRPV6*, a much better understanding of the apical Ca^{2+} entry mechanisms in the transcellular transport pathway has been achieved. *TRPV5* and *TRPV6* provide a tightly regulated selective Ca^{2+} entry in the transcellular Ca^{2+} transport pathway that is responsible for the fine tuning of Ca^{2+} absorption and reabsorption, even though this pathway does not contribute greatly to the bulk Ca^{2+} absorption and reabsorption as does the paracellular pathway. *TRPV5* and *TRPV6* certainly are not the sole mediator of vitamin D regulated Ca^{2+} transport; however, without either of them, the body appears to be in a negative Ca^{2+} balance as indicated by the elevated $1,25(\text{OH})_2\text{D}_3$ and PTH levels. The roles of *TRPV5* and *TRPV6* in tuning Ca^{2+} homeostasis are well indicated by

their robust responses to the body's need for Ca²⁺ under physiological, pathological and therapeutic conditions. Notably, TRPV6 participates in cell proliferation and apoptosis and is emerging as a potential cancer marker and therapeutic target for cancers.

The study in renal Ca²⁺ transport has been mostly focused on TRPV5 in the past 10 more years. However, *Trpv6* KO mice also lose Ca²⁺ in the urine, even though intestinal absorption of Ca²⁺ is decreased. Although TRPV6 mRNA level is much lower than TRPV5 in rodent kidney, in humans and horses, renal TRPV6 mRNA is much higher than TRPV5, indicating a more important role of TRPV6 in renal function in these species. TRPV5 and TRPV6 are not always regulated in the same way in the kidney, indicating their overlapping yet distinct roles.

While TRPV6 mediates Ca²⁺ uptake in many tissues, TRPV5 is relatively specific to the kidney. The need of a specific Ca²⁺ transporter for the complex mammalian distal tubule likely resulted in the generation of *TRPV5* by duplication of *TRPV6*. The changed source of dietary Ca²⁺ and vitamin D status during or after humans migrated out of Africa may be part of the reasons for further evolution of the *TRPV6* gene. *TRPV6* genotype is a good genetic marker in African populations. African populations could be separated into 3 groups based on their *TRPV6* genotype because of their ~50% allele frequency of the 3 *TRPV6* nonsynonymous SNPs. The involvement of *TRPV6* and *TRPV5* SNPs in the health issues related to Ca²⁺, salt and water homeostasis in African descents will likely become a hot spot of investigation in the near future.

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