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Human skeletal dysplasia caused by a constitutive activated transient receptor potential vanilloid 4 (TRPV4) cation channel mutation

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Abbreviations: BRAC3, brachyolmia type 3; CMT2C, Charcot-Marie-Tooth disease type 2C; COPD, chronic obstructive pulmonary disease; DSMAC, distal spinal muscular atrophy congenital non-progressive; FDAB, familial digital arthropathy-brachydactyly; HEK293 cells, human embryonic kidney 293 cells; HMSN2C, hereditary motor and sensory neuropathy, type IIC; MIM, mendelian inheritance in man; MTD, metatropic dysplasia; PSTD, parastremmatic dwarfism; SEDM, spondyloepiphyseal dysplasia Maroteaux type; SMA, spinal muscular atrophies; SMDK, spondylometaphyseal dysplasias Kozlowski type; SPSMA, scapuloperoneal spinal muscular atrophy; TRP, transient receptor potential; TRPV, vanilloid receptor-related TRPs

Abstract

The transient receptor potential vanilloid 4 (TRPV4) cation channel, a member of the TRP vanilloid subfamily, is expressed in a broad range of tissues where it participates in the generation of Ca^{2+} signals and/or depolarization of the membrane potential. Regulation of TRPV4 abundance at the cell surface is critical for osmo- and mechanotransduction. Defects in TRPV4 are the cause of several human diseases, including brachyolmia type 3 (MIM:113500) (also known as brachyrachia or spondylometaphyseal dysplasia Kozlowski type [MIM:118452]), and metatropic dysplasia (MIM:156530) (also called metatropic dwarfism or parastremmatic dwarfism [MIM:168400]). These bone dysplasia mutants are characterized by severe dwarfism, kyphoscoliosis, distortion and bowing of the extremities, and contractures of the large joints. These diseases are characterized by a combination of decreased bone density, bowing of the long bones, platyspondyly, and striking irregularities of endochondral ossification with areas of calcific stippling and streaking in radiolucent epiphyses, metaphyses, and apophyses. In this review, we discuss the potential effect of the mutation on the regulation of TRPV4 functions, which are related to human diseases through deviated function. In particular, we emphasize how the constitutive active TRPV4 mutant affects endochondral ossification with a reduced number of hypertrophic chondrocytes and the presence of cartilage islands within the zone of primary mineralization. In addition, we summarize current knowledge about the role of TRPV4 in the pathogenesis of several diseases.

Keywords: genetic diseases, inborn; osteochondrodysplasias; osteogenesis; phosphorylation; TRPV cation channels

Introduction

The transient receptor potential vanilloid 4 (TRPV4) cation channel, a member of the TRP vanilloid subfamily, is expressed in a broad range of tissues, where it contributes to the generation of a Ca^{2+} signal and/or the depolarization of membrane potential (Vennekens *et al*., 2008; Everaerts *et al*., 2010; Verma *et al*., 2010; Lewis *et al*., 2011; Chun *et al*., 2012). TRPV4 is a polymodal Ca^{2+}-permeable cation channel of 871 amino acid (Delany *et al*., 2001; Watanabe *et al*., 2002b). It shows a very prominent outward rectification, rarely opening upon hyperpolarization (Voets *et al*., 2005; Loukin *et al*., 2010a). Mutational analyses suggest that outward

rectification is governed by a gating mechanism independent of the main intracellular gates (Kim *et al*., 2010; Loukin *et al*., 2010a).

The predicted TRPV4 structure harbors six membrane-spanning domains with a pore loop, an N-terminal domain with at least three ankyrin repeats, and a C-terminal domain residue within the cytoplasm (Delany *et al*., 2001; Watanabe *et al*., 2002b; Voets *et al*., 2005; Loukin *et al*., 2010a). These characteristics are common features in all six TRPVs (TRPV1-6) (Vennekens *et al*., 2008; Everaerts *et al*., 2010; Chun *et al*., 2012). Although the TRPV family shows similar characteristics, each member has its own distinguishable functions from other TRPVs (Figure 1).

The participation of TRPV4 in osmo- and mechanotransduction is relevant to several important functions, including cellular and systemic volume homeostasis, arterial dilation, nociception, bladder voiding, and the regulation of ciliary beat frequency (Suzuki *et al*., 2003b; Andrade *et al*., 2005; Becker *et al*., 2005; Earley *et al*., 2005; Birder *et al*., 2007; Hartmannsgruber *et al*., 2007). TRPV4 channel activity can be sensitized by the co-application of a variety of stimuli and by the participation of a number of cell signaling pathways, which suggests the presence of different regulatory sites (Nilius *et al*., 2004; Shin *et al*., 2012). In this regard, several proteins have been proposed to modulate TRPV4 subcellular localization and/or function, including microtubule-associated protein 7, calmodulin, F-actin, and pacsin3 (Strotmann *et al*., 2003; Suzuki *et al*., 2003a; Cuajungco *et al*., 2006; Ramadass *et al*., 2007); (Table 1). A functional and physical interaction between inositol trisphosphate receptor 3 and TRPV4 has been demonstrated, which sensitizes the latter to the mechano- and osmotransducing messenger, 5'-6'-epoxieicosatrienoic acid (Vriens *et al*., 2005; Fernandes *et al*., 2008). TRPV4 is also responsive to temperature, endogenous arachidonic acid metabolites, and phorbol esters, including 4-α phorbol 12, 13-didecanoate (4-αPDD), and participates in receptor-operated Ca^{2+} entry, thus, possessing multiple activation modes (Guler *et al*., 2002; Watanabe *et al*., 2003; Jia *et al*., 2004); (Table 2). However, the precise manner in which TRPV4 is

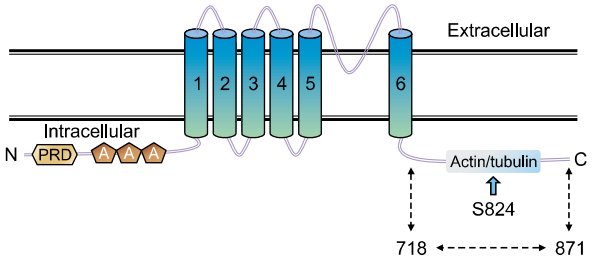

Figure 1. The TRPV4 topology and its domains. Transmembrane topology of the mouse TRPV4 (871 amino acids length). Indicated are the three ankyrin-binding repeats (ANK; as pentagon), the six trans-membrane regions (TM1-TM6), the Ca^{2+}/F-actin- or microtubule-binding site (F-actin or microtubule), and the putative SGK1 phosphorylation site (S824) which is indicated by the arrow type (WT; Gene Bank #. BC127052). The putative cytoplasmic region of TRPV4 (718-871 amino acids) is also indicated by underlining.

Table 1. Amino acid sequences of binding domain in TRPV4 for various interacting proteins

Protein	Binding site (amino acids # in 871 amino acids)
β-catenin, E-cadherin	1-235
Pacsin3 (syndapin3)	131-144
PACS-1/AP-1	171-190
Ankyrin	236-390
OS-9	438-468
MAP7	789-809
IP3 receptor	811-840
Calmodulin	811-830
Actin/tubulin	798-809
Homo tetramer	236-390
Aquaporin 4	Unknown

The protein and its binding site are summarized from the literatures in Reference Section.

Table 2. Various modification sites in TRPV4 (871 amino acids)

Modification	Site (amino acid #)	Enzyme
Phoshorylation on Ser/Thr	S88, S134, S528, S824	PKC
	S824	SGK1
Phoshorylation on Tyr	Y253	Lyn
S-nitrosylation	C853	
N-glycosylation	N651	Endoglycosidase-F
Small ligand binding site		
Gallic	C194	
4-a PDD	L584, W586	
Capsaicin	Y556	

The modification and its amino acid position are summarized from the literatures in Reference Section.

Table 3. TRPV4 mutant and its genetic disorder

	Mutation	Residue	Change in charge	Domain/motif effected	Effects on ion conductivity	Genetic disorder
1	C144T (exon 2)-	P19S	Nonpolar to polar	N-terminal	Less conductivity	Hyponatermia COPD
2	-	G78W		N-terminal		Fetal Akinesia
3	C366T (exon 2)	T89I	Polar (uncharged) to nonpolar	N-terminal	Not done	Metatropic dysplasia
4	G547A (exon 3)	E183K	Negative to plus	ARD1	Not done	SEDM-PM2
5	A590G (exon 4)	K197R	Plus to plus	ARD2	Not done	Metatropic dysplasia
6	-	L199F	Nonpolar to aromatic	ARD2	Not done	Metatropic dysplaisa
7	*G806A (exon 5)	R269H	Plus to plus	ARD3	Less membrane localization and less conductance	SMA or HMSN2C
8	C805T, *G806A (exon 5)	R269C, R269H	Plus to polar un charged	ARD3	More conductivity	CMT2C
9		G270V	Nonpolar to polar	ARD3	Less conductivity	FDAB
10		R271P	Plus to nonpolar	ARD3	Less conductivity	FDAB
11		F273L	Aromatic to nonpolar	ARD3	Less conductivity	FDAB
12	-	K276E		ARD3		Fetal Akinesia
13	-	E278K	Negative to plus	ARD3	Not done	SMDK
14	-	T295A	Polar (uncharged) to nonpolar	ARD4	Not done	Metatropic dysplaisa
15	C943T (exon 6)	R315W	Plus to aromatic	ARD4	Less conductivity	HMSN2C
16	C946T (exon 6)	R316C	Plus to polar (uncharged)	ARD4	Less conductivity	HMSN2C
17	A1080T (exon 6)	I331F	Nonpolar to aromatic	ARD5	Not done	Metatropic dysplasia
18	-	I331T	Nonpolar to polar (uncharged)	ARD5	Not done	Metatropic dysplasia
19	A992G (exon 6)	D333G	Negative to nonploar	ARD4	More conductivity	SMDK
20	-	V342F	Nonpolar to aromatic	ARD5	Not done	Metatropic dysplasia
21	-	F592L	Aromatic to nonpolar	TM4	Not done	Metatropic dysplasia
22	G1781A (exon 11)	R594H	Plus to plus	TM4	More conductivity	SMDK
23	A1805G (exon 11)	Y602C	Aromatic to polar	TM4-TM5	Not done	SEDM-PM2
24	C1812G (exon 11)	I604M	Nonpolar to nonpolar	TM4-TM5	Not done	Metatropic dyslpasia
25	G1847A (exon 12)	R616Q	Plus to polar uncharged	TM5, pore region	More conductivity	Brachylomia
26	C1851A (exon 12)	F617L	Aromatic to nonpolar	TM5, pore region	Not done	Metatropic dysplasia
27	T1853C (exon 12)	L618Q	Nonpolar to polar (uncharged)	TM5, pore region	Not done	Metatropic dysplasia
28	G858A (exon 12)	V620I	Nonpolar to nonpolar	TM5, pore region	More conductivity	Brachylomia
29	-	M625I	Nonpolar to nonpolar	TM5, pore region	More conductivity	SMDK
30	-	L709M	Nonpolar to nonpolar	TM5, pore region	More conductivity	SMDK
31	C2146T (exon 13)	A716S	Nonpolar to polar	Cytoplasmic side of TM6	Same as wild type	SMDK
32	-	T740I		C-terminal region		Fetal Akinesia
33	-	R775K	Plus to plus	C-terminal region	Not done	Metatropic dysplasia
34	-	C777Y	Polar (uncharged) to aromatic	C-terminal region	More conductivity	SMDK
35	-	E797K	Negative to plus	C-terminal region	More conductivity	SMDK, PSTD
36	-	P799R	Nonpolar to plus	C-terminal region	More conductivity	SMDK, PSTD
37	- 6	P799S	Nonpolar to polar (uncharged)	C-terminal region	Not done	Metatropic dysplasia
38	-	P799A	Nonpolar to nonpolar	C-terminal region	Not done	Metatropic dysplasia
39	C2396T (exon 15)	P799L	Nonpolar to nonpolar	C-terminal	Not done	SMDK

The disease abbreviation means below: Serum Sodium Level Quantitative Trait Locus (Hyponatermia) (the # of MIM is not available), Chronic obstructive pulmonary disease (COPD) (the # of MIM is not available), Brachyolmia type 3 (BRAC3) [MIM:113500], Metatropic dysplasia (MTD) [MIM:156530]; Distal spinal muscular atrophy congenital non-progressive (DSMAC) [MIM:600175]. (DSMAC is also called as Hereditary Motor and Sensory Neuropathy, Type IIC; HMSN2C or Spinal muscular atrophies (SMA), also known as hereditary motor neuropathies. Spondyloepiphyseal dysplasia Maroteaux type (SEDM) [MIM:184095]. TRPV4 mutant (E797K or P799R) causes both Parastremmatic dwarfism (PSTD) [MIM:168400] and Spondylometaphyseal dysplasias Kozlowski type (SMDK) [MIM:184252]. Charcot-Marie-Tooth disease type 2C (CMT2C) and Scapuloperoneal Spinal Muscular Atrophy (SPSMA) [MIM:606071]. Familial digital arthropathy-brachydactyly (FDAB): (the # of MIM is not available). MIM: Mendelian Inheritance in Man. *G806A (exon 5) mutation causes both SMA/HMSN2C and CMT2C. It is also unclear yet that the channel activity of TRPV4 R269H is increased than that of TRPV4 WT.

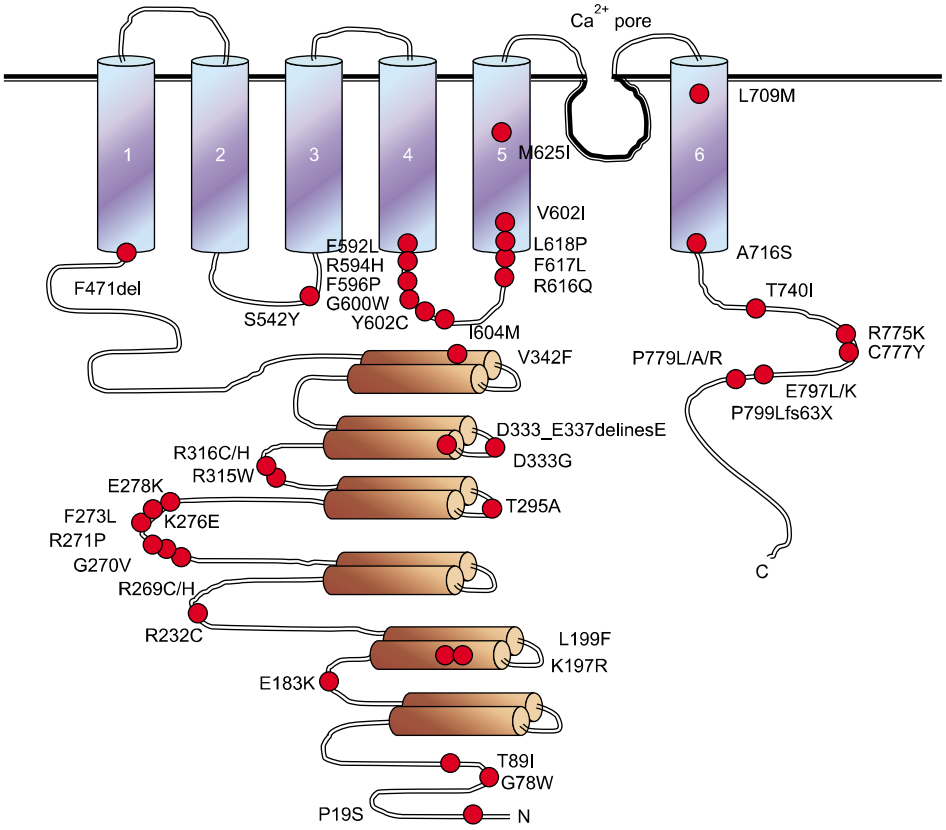

Figure 2. The naturally mutation sites on human TRPV4. Transmembrane topology of the human TRPV4 (871 amino acids length). Indicates are the three ankyrin-binding repeats (ANK; gray bar), the six trans-membrane regions (TM1-TM6), the Ca^{2+} pore and the mutation site (WT; Gene Bank #. BC127052). The putative cytoplasmic region of N-terminal (1-471 amino acids) and C-terminal (718-871 amino acids) of TRPV4 are indicated with N and C. Two "hot spots" in TRPV4 sequences are prominent, one at the pore region and the other one in the between ANK 3 and 4 (del: deletion, delines: deletion or insertion extra sequence, fs: fame shift).

regulated in the cell by these protein interactions, chemicals, and stimuli remains to be clearly elucidated.

Many mammalian cell types, including renal tubule cells, bronchial epithelia, keratinocytes, spermatocytes, and erythrocytes, encounter varying osmolarities in their environments (Strotmann *et al*., 2000; Vriens *et al*., 2004). The ability of these cells to adapt to changing osmotic conditions is critical for cellular homeostasis, and disequilibria can result in dramatic events, including apoptosis and necrosis. Therefore, a variety of cell types have evolved specialized mechanisms for volume regulation to counteract the damage induced by either swelling or shrinking of cells (Alessandri-Haber *et al*., 2003; Vriens *et al*., 2004). Under hypotonic conditions, cells increase their volume *via* water uptake mediated by the osmotic gradient. The reduction in volume to its former value is achieved by a process referred to as regulatory volume decrease (RVD), which allows cells to survive in a hypotonic environment. The direct participation of TRPV4 in RVD at the cellular level and systemic osmosensing in organisms has been demonstrated (Becker *et al*., 2005).

Although TRPV4 mutants develop several genetic bone diseases, including brachyolmia type 3 (BRAC3) [MIM:113500]; also known as brachyrachia and spondylometaphyseal dysplasia Kozlowski type (SMDK) [MIM:184252], and metatropic dysplasia (MTD) [MIM:156530]; also called metatropic dwarfism or parastremmatic dwarfism (PSTD) [MIM:168400], only abnormal osmotic regulation in TRPV4-/- mouse has been reported (Suzuki *et al*., 2003b; Verma *et al*., 2010; Kang, 2012); (Figure 2 and Table 3). It is currently unclear why the phenotype of TRPV4-/- mice is so much different than that of TRPV4 point mutants. The TRPV4 knockout mouse phenotype does not fully recapitulate any of the human phenotypes under discussion but bladder dysfunction and hearing loss have been reported (Tabuchi *et al*., 2005; Gevaert *et al*., 2007). Defects in TRPV4 are the cause of several human diseases including BRAC3 [MIM:113500]; also known as brachyrachia or SMDK [MIM:184252], and MTD [MIM:156530]; also called metatropic dwarfism or PSTD [MIM:168400] (Auer-Grumbach *et al*., 2010; Dai *et al*., 2010; Deng *et al*., 2010; Landoure *et al*., 2010; Verma *et al*., 2010; Kang, 2012; McEntagart, 2012). These bone dysplasia mutants are characterized by severe dwarfism, kyphoscoliosis, distortion and bowing of the extremities, and contractures of the large joints (Figure 3 and Table 3). These diseases are characterized by a combination of decreased bone density, bowing of the long bones, platyspondyly,

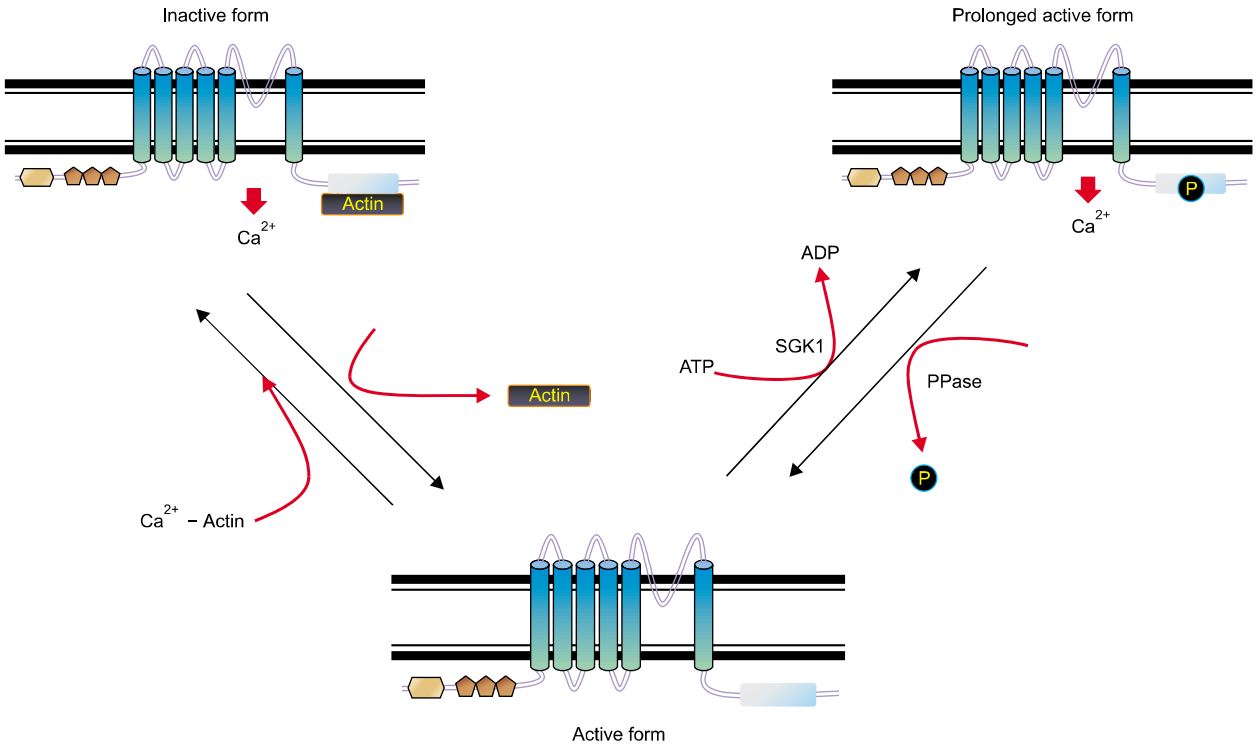

Figure 3. The scheme of proposed TRPV4 functional regulation. TRPV4 can be modulated by the putative dual (activator/inhibitor) function protein (such as F-actin or microtubule) association/dissociation from its C-terminal cytoplasmic domain (activation/inactivation) phosphorylation. After receiving a growth signal from outside, the protein kinase such as SGK1 is activated. The protein-protein interaction between TRPV4 and (F-actin or tubulin) appears to be modulated by phosphorylation on its 824 serine residue by protein kinases (right). The active TRPV4 seems to be inactivated by protein phosphatases *via* dephosphorylation on its Ser 824 residue (left). The inactivated TRPV4 seems to bind with tubulin (Chun *et al*., 2012).

and striking irregularities of endochondral ossification with areas of calcific stippling and streaking in radiolucent epiphyses, metaphyses, and apophyses (Kang, 2012; McEntagart, 2012).

Thus, in this review, the potential effect of the mutation on the regulation of TRPV4 functions is discussed, which is related to the bone diseases through its deviated function and how the mutant TRPV4 affects endochondral ossification, with reduced numbers of hypertrophic chondrocytes and the presence of cartilage islands within the zone of primary mineralization (Figure 4). With the analysis of TRPV4 mutants, we noticed the tendency that human skeletal dysplasia is caused by a constitutive activated TRPV4 mutation, but not by a constitutive inactivated mutation (Figure 5). We also realized that the seriousness of disease is dependent on its channel activity. In addition, the current knowledge about the role of TRPV4 in the pathogenesis of skeletal dysplasia is summarized (Figure 2 and Table 3).

Naturally occurring TRPV4 mutants and genetic bone disorders

Few naturally occurring TRPV4 mutants have been identified. Interestingly, most of these missense and nonsense point mutations are linked with the development of genetic disorders in humans and a detailed list of naturally occurring TRPV4 mutations and related disease has been documented (Figure 2 and Table 3). Some of these mutations that have gained importance in terms of genetic disease had been discussed (Verma *et al*., 2010; Kang, 2012; McEntagart, 2012).

BRAC3 [MIM:113500]

BRAC3 was characterized using a linkage analysis and candidate gene sequencing. Rock *et al*. (2008) found that some patients affected with brachylomia have a TRPV4 missense mutation, specifically at positions R616Q or V620I, respectively (Auer-Grumbach *et al*., 2010; Dai *et al*., 2010; Deng *et al*., 2010; Landoure *et al*., 2010). These mutations are located at the fifth transmembrane region, which is a part of the functional pore. Each of these mutations

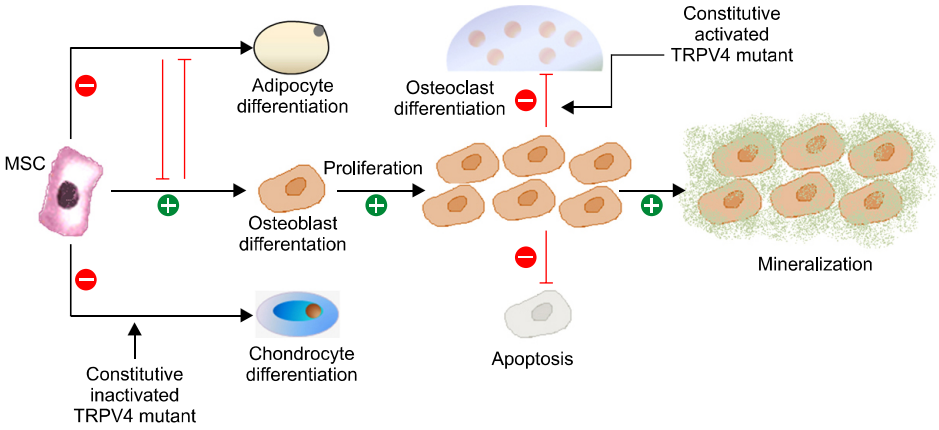

Figure 4. The osteogenesis regulation mechanisms by TRPV4 signalling. TRPV4 represses alternative mesenchymal differentiation pathways such as adipocyte and chondrocyte differentiation and promote osteoblast differentiation, proliferation, and mineralization activity while blocking osteoblast apoptosis. In this review, we hope to elucidate the etiological mechanism how the mutation in TRPV4 (E797K or P799R) causes Spondylometaphyseal dysplasias Kozlowski type (SMDK) [MIM:184252], eventually. The mutant TRPV4 which is constitutively active in HEK293 cell results in the activation of osteoclast abnormally. It is unknown yet how the constitutive active TRPV4 activates osteoclast. The research hypothesis is that the constitutive active mutant TRPV4 (such as E797K, P799R) represses the chondrocyte differentiation from MSC and promotes the osteoclast activation (or the osteoblast apoptosis) with the increase of related transcription factor, such as NFATc1 (Wegierski *et al*., 2006). Thus, the mutant TRPV4 containing the more channel activity than the TRPV4 WT causes the abnormality of backbone morphogenesis, meanwhile the constitutive inactive mutant TRPV4 (the less channel activity than the TRPV4 WT) causes the abnormality of articular morphogenesis or the neuropathy (FDAB or HMSN2C), which is similar with that of TRPV4 (-/-) mouse (Suzuki *et al*., 2003b; Verma *et al*., 2010; Kang, 2012).

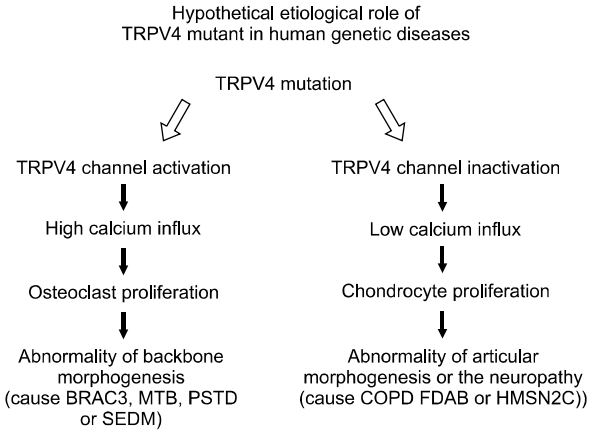

Figure 5. Hypothetical etiological role of gain-of-function TRPV4 mutant in human genetic diseases. The mutants containing the more channel activity than the TRPV4 wt lead the abnormality of backbone morphogenesis by the more Ca^{2+} influx (left). However, even though this is not completely accorded in some case yet, the mutants containing the less channel activity causes the abnormality of articular morphogenesis or the neuropathy by the less Ca^{2+} influx (right). Because both SMA/HMSN2C and CMT2C are caused by G806A (exon 5) mutation, presently, it is unclear whether the channel activity of mutant TRPV4 R269H is increased than that of TRPV4 WT.

increases basal level activity when compared to that of wild-type TRPV4 (TRPV4-WT). Additionally the response to 4-αPDD (a TRPV4 specific agonist) is greater in the mutants when compared with that in the WT. This result also indicates that these two mutations preferably stabilize TRPV4 in its "open stage" resulting in constitutive activity of the channel. BRAC3 constitutes a clinically and genetically heterogeneous group of skeletal dysplasias characterized by a short trunk, scoliosis, and mild short stature. BRAC3 is an autosomal dominant form, and patients have severe kyphoscoliosis and flattened, irregular cervical vertebrae (Loukin *et al*., 2011).

BRAC3, caused by the R616Q gain-of-function channel, possesses increased whole-cell current densities compared with those of WT channels (Loukin *et al*., 2011). A single-channel analysis revealed that R616Q channels maintain mechano sensitivity but have greater constitutive activity and no change in unitary conductance or rectification. The BRAC3 range from mild autosomal-dominant BO (brachylomia), diagnosed by a shortened spine with characteristic vertebral defects and minor defects in the long bones to MTD characterized by more prominent spine defects as well as pronounced abnormalities in the articular skeleton resulting in short dumbbell-shaped long bones, which lead to prenatal lethality in its severest form (Loukin *et al*., 2010b; Lamande *et al*., 2011; Nemec *et al*., 2012). The reason why TRPV4 R616Q (which shows greater constitutive activity and no change in unitary conductance or rectification) causes such a prominent phenotype is unknown.

MTD [MIM:156530]

MTD is a clinical heterogeneous skeletal dysplasia characterized by short extremities, a short trunk with progressive kyphoscoliosis, and craniofacial abnormalities including a prominent forehead, midface hypoplasia, and a squared-off jaw (Krakow *et al*., 2009; Camacho *et al*., 2010). Dominant mutations in the gene encoding TRPV4 were identified in all 10 of a series of MTD cases, ranging in severity from mild to perinatal lethal. MTD is a severe spondyloepimetaphyseal dysplasia characterized by short limbs with limitation and enlargement of joints and severe kyphoscoliosis (Cho *et al*., 2012). Radiologic features include severe platyspondyly, severe metaphyseal enlargement, and shortening of long bones (Andreucci *et al*., 2011). TRPV4 I331F and P799L mutations are well known to induce MTD (Krakow *et al*., 2009; Camacho *et al*., 2010). As all these mutants are naturally occurring and are not embryonically lethal (as most lethal mutants may be naturally excluded from the population). Notably, none of these mutants show complete loss of their prime function, i.e., ionic conductivity (Krakow *et al*., 2009; Cho *et al*., 2012).

Several experimental results suggest that some of these mutants even have enhanced channel opening. Furthermore, they demonstrate that the lethal form of the disorder is dominantly inherited and suggest locus homogeneity in the disease (Krakow *et al*., 2009; Camacho *et al*., 2010; Andreucci *et al*., 2011; Cho *et al*., 2012). Additionally, electrophysiological studies have demonstrated that the mutations activate the channel, indicating that the disease mechanism may result from increased calcium in chondrocytes (Camacho *et al*., 2010). Histological studies in two cases of lethal MTD revealed markedly disrupted endochondral ossification with a reduced number of hypertrophic chondrocytes and the presence of cartilage islands within the zone of primary mineralization. These data suggest that altered chondrocyte differentiation in the growth plate leads to the clinical findings of MTD (Krakow *et al*., 2009; Camacho *et al*., 2010; Andreucci *et al*., 2011; Cho *et al*., 2012).

Distal spinal muscular atrophy congenital non-progressive (DSMAC) [MIM:600175]

DSMAC (also called hereditary motor and sensory neuropathy, type IIC; HMSN2C) is a clinically variable, neuromuscular disorder characterized by a congenital lower motor neuron disorder restricted to the lower part of the body (Auer-Grumbach *et al*., 2010; Deng *et al*., 2010; Ding *et al*., 2010). Clinical manifestations include non-progressive muscular atrophy, thigh muscle atrophy, weak thigh adductors, weak knee and foot extensors, minimal jaw muscle and neck flexor weakness, flexion contractures of the knees, and pes equinovarus. However, tendon reflexes are normal (Deng *et al*., 2010; Nilius and Owsianik, 2010; Zimon *et al*., 2010).

Inheritance is autosomal dominant. The R315W mutation has been identified in an unrelated family that also had HMSN2C. Auer-Grumbach *et al*. discovered two additional TRPV4 mutations (R269H and R316C) in affected members of three additional families with these three phenotypes, indicating that they are allelic disorders (Auer-Grumbach *et al*., 2010; Zimon *et al*., 2010). All three mutations occurred at the outer helices of the ANK4 and ANK5 domains in the N-terminal cytoplasmic domain (Figure 2). *In vitro* functional expression studies in HeLa cells have shown that the mutant protein forms cytoplasmic aggregates and has reduced surface expression, as well as an impaired response to stimulus-dependent channel activity. These results suggest that the mutations interfere with normal channel trafficking and function. Furthermore, Auer-Grumbach *et al*. identified a different heterozygous mutation in the TRPV4 gene (R315W) in a patient with congenital distal SMA whose other family members with the same mutation had phenotypes consistent with hereditary motor and sensory neuropathy-2 or scapuloperoneal spinal muscular atrophy; thus, proving that these are allelic disorders with overlapping phenotypes (Auer-Grumbach *et al*., 2010; Deng *et al*., 2010; Nilius and Owsianik, 2010; Zimon *et al*., 2010).

Spondyloepiphyseal dysplasia Maroteaux type (SEDM) [MIM:184095]

SEDM is a clinically variable spondyloepiphyseal dysplasia with manifestations limited to the musculoskeletal system (Dai *et al*., 2010; Loukin *et al*., 2011). Clinical features of SEDM include short stature, brachydactyly, platyspondyly, short and stubby hands and feet, epiphyseal hypoplasia of the large joints, and iliac hypoplasia, even though patient intelligence is normal (Andreucci *et al*., 2011). Genetic mapping of the patients affected with this disease shows a missense mutation in TRPV4 of either E183K, Y602C, or E797K (Krakow *et al*., 2009; Camacho *et al*., 2010; Dai *et al*., 2010; Loukin *et al*., 2011). The channel activity of the TRPV4 E797K mutant in HEK293 cells is constitutively active, which is consistent with the argument that effects on TRPV4 are the cause of SEDM. It is a clinically variable spondyloepiphyseal dysplasia with manifestations limited to the musculoskeletal system. Both SEDM and parastremmatic dysplasia are part of the TRPV4 dysplasia family, and TRPV4

mutations show considerable variability in phenotypic expression resulting in distinct clinical-radiographic phenotypes (Krakow *et al*., 2009; Camacho *et al*., 2010; Andreucci *et al*., 2011).

PSTD [MIM:168400]

Defects in TRPV4 are also the cause of PSTD, which is a bone dysplasia characterized by severe dwarfism, kyphoscoliosis, distortion and bowing of the extremities, and contractures of the large joints (Andreucci *et al*., 2011). This disease is radiographically characterized by a combination of decreased bone density, bowing of the long bones, platyspondyly, and striking irregularities of endochondral ossification with areas of calcific stippling and streaking in radiolucent epiphyses, metaphyses, and apophyses (Andreucci *et al*., 2011; Nemec *et al*., 2012).

Nishimura *et al*. (2010) analyzed the candidate TRPV4 gene in a 7-year-old girl with PSTD and identified heterozygosity for a missense mutation (R594H) that had previously been found in patients with the Kozlowski type of SMDK (Nishimura *et al*., 2010). However, in patients with the Kozlowski type of SMDK, Krakow *et al*. (2009) identified a 1781G-A transition in exon 11 of the TRPV4 gene, resulting in an arg594-to-his (R594H) substitution in the cytoplasmic S4 domain (Krakow *et al*., 2009). Thus, both PSTD and SMDK, which are caused by the TRPV4 mutation, seem to be associated with increased basal intracellular calcium ion concentration and intracellular calcium activity. However, the Kozlowski type of SMDK is different from SEDM in the TRPV4 mutation sites (E183K Y602C, or E797K).

Charcot-Marie-Tooth disease type 2C (CMT2C) and scapuloperoneal spinal muscular atrophy (SPSMA) [MIM:606071]

CMT2C is an axonal form of Charcot-Marie-Tooth disease, a disorder of the peripheral nervous system that is characterized by progressive weakness and atrophy, initially of the peroneal muscles and later of the distal muscles of the arms (Deng *et al*., 2010; Landoure *et al*., 2010). Charcot-Marie-Tooth disease is classified into two main groups based on electrophysiologic properties and histopathology: primary peripheral demyelinating neuropathies (designated CMT1 when they are dominantly inherited) and primary peripheral axonal neuropathies (CMT2). Neuropathies of the CMT2 group are characterized by signs of axonal regeneration in the absence of obvious myelin alterations, normal or slightly reduced nerve conduction velocities, and progressive distal muscle weakness and atrophy (Bird, 1993; Nilius and Owsianik, 2010). Nerve conduction velocities are normal or slightly reduced. CMT2C and SPSMA are also known as hereditary motor and sensory neuropathy type 2 (Chen *et al*., 2010; Landoure *et al*., 2010). Patients with SPSMA are characterized by weakness of the scapularis muscle and bone abnormalities. CMT2C leads to weakness of distal limbs and vocal cords and often impairs hearing and vision. Genetic analyses of these patients have shown the presence of TRPV4 missense mutations, particularly at the R269H, R315W, and R316C positions (Bird, 1993; Deng *et al*., 2010; Landoure *et al*., 2010).

Familial digital arthropathy-brachydactyly (FDAB)

FDAB is a dominantly inherited condition characterized by aggressive osteoarthropathy of the fingers and toes and consequent shortening of the middle and distal phalanges (Amor *et al*., 2002). Lamandé *et al*. showed that FDAB is caused by mutations encoding p.Gly270Val, p.Arg271Pro, and p.Phe273Leu substitutions in the intracellular ankyrin-repeat domain of the TRPV4 cation channel (Lamande *et al*., 2011). The TRPV4 mutant in HEK-293 cells showed that the mutant proteins have poor cell-surface localization. Those authors also suggested that TRPV4 mutations that reduce channel activity cause a third phenotype, inherited osteoarthropathy, which shows the importance of TRPV4 activity in particular cartilage homeostasis. Thus, the TRPV4 mutant (G270V, R271P, and Y273L) also seems to be related with FDAB (Nilius and Owsianik, 2010; Verma *et al*., 2010; Kang, 2012; McEntagart, 2012; Nemec *et al*., 2012).

Other diseases (not included bone) with TRPV4 mutations

Serum sodium level quantitative trait locus (hyponatremia): Tian *et al*. (2009) demonstrated that the rs3742030 single nucleotide polymorphism in the TRPV4 gene (P19S) is significantly associated with serum sodium concentration (Tian *et al*., 2009). After this discovery, hyponatremia was defined as serum sodium $<$ 135 mEq/L in non-Hispanic Caucasian male populations. In heterologous expression studies in HEK293 cells, P19S mutant channels show a diminished response to hypotonic stress and to the osmotransducing lipid epoxyeicosatrienoic acid compared to that in WT channels (Carreno *et al*., 2009; Nedungadi *et al*., 2012). The P19S polymorphism affects TRPV4 function *in vivo* and likely influences systemic water balance on a population wide basis.

Chronic obstructive pulmonary disease (COPD): COPD is characterized by airway epithelial damage, bronchoconstriction, parenchymal destruction, and mucus hypersecretion (Zhu *et al*., 2009; Li *et al*., 2011). Upon activation by a broad range of stimuli, TRPV4 functions to control airway epithelial cell volume and epithelial and endothelial permeability; it also triggers bronchial smooth muscle contraction and participates in autoregulation of mucociliary transport (Obeidat *et al*., 2011). These TRPV4 functions may be important for regulating COPD pathogenesis; thus, TRPV4 is a candidate COPD gene. The TRPV4 P19S mutant, which is characterized as the cause of the hyponatremia, is also observed in COPD.

Fetal akinesia: Recently, Unger *et al*. reported that the fetal akinesia as the presenting feature of severe metatropic dysplasia, was identified as a certain TRPV4 mutant which can cause both a skeletal and a neuropathic phenotype (Unger *et al*., 2011). Three cases were detected on prenatal ultrasound because of absent movements in the second trimester. The mutation of TRPV4 were confirmed the presence of de novo heterozygous mutations predicting G78W, K276E, and T740I (Figure 2 and Table 3).

The TRPV4 regulatory mechanism

The gating diversity of the TRPV4 channel demonstrated in the current study reflects the presence of multiple physical and chemical signaling pathways that converge on the channel regardless of the mechanism (Vennekens *et al*., 2008; Everaerts *et al*., 2010; Lewis *et al*., 2011; Chun *et al*., 2012). As a consequence, the TRPV4 channel appears to function as a molecular integrator of a complex array of diverse signals (Tables 1 and 2). Because multiple environmental signals and transduction pathways converge on TRPV4, the channel may function as a molecular integrator of microenvironmental chemical and physical signals. Further study to elucidate the TRPV4 regulation mechanism is required to clearly determine the physiological role and mechanism of channel activation (Delany *et al*., 2001; Voets *et al*., 2002; Watanabe *et al*., 2002b; Loukin *et al*., 2010a).

The Ser 824 residue of TRPV4 is localized within its F-actin or microtubule-binding domain (798-809 amino acids) of TRPV4 (Suzuki *et al*., 2003a; Shin *et al*., 2012). Consequently, the functional relevance of Ser 824 residue phosphorylation in the context of cytoskeletal interaction should be emphasized (Suzuki *et al*., 2003a; Lee *et al*., 2010; Chun *et al*., 2012; Shin *et al*., 2012). Thus, phosphorylation of the TRPV4 Ser 824 residue exerts a regulatory effect on its functional Ca^{2+} entry, protein stability, and its interactions with CaM (Figures 1 and 3), F-actin or microtubules result in an expansion of cell surface area and proper subcellular localization (Suzuki *et al*., 2003a; Chun *et al*., 2012; Shin *et al*., 2012).

TRPV4 channel activation and serine phosphorylation are enhanced by exposure to the non-PKC activator 4-α PDD or by applying bradykinin, which activates protein kinase C (PKC) or PKA *via* a G-protein-coupled mechanism. This enhancement was inhibited by the PKC inhibitors staurosporine, BIM, and rottlerin, and by mutation of the serine/threonine residues S162, T175, and S189 (Table 2). Enhancement of TRPV4 activity by S824 phosphorylation is expected to be agonized or antagonized by the selective SGK1 activity modulators (IGF2 and wortmannin) (Lee *et al*., 2010). However, basal activity and sensitivity to 4-α PDD of TRPV4 S824A (an analog of unphosphorylated TRPV4 by SGK1) was higher than those of the TRPV4 WT, suggesting that the TRPV4 C-terminal domain near the serine residue 824 was assigned to regulate its function by an unknown controlling mechanism beyond the phosphorylation modification, such as a protein-protein interaction with CaM (Amor *et al*., 2002; Strotmann *et al*., 2003; Chun *et al*., 2012). Because the TRPV4 Ser824 residue has also been detected in the consensus SGK1 substrate sequences, whether or not SGK1 phosphorylates the Ser 824 residue of TRPV4, as one of its specific substrate proteins was ascertained (Figure 1 and Table 2). SGK1-mediated phosphorylation of the TRPV4 Ser 824 residue exerts a synergistic effect on functional Ca^{2+} entry, as well as reactivity to 4-α PDD, interactions with CaM, subcellular localization, and cell survival (Figures 1 and 2).

TRPV4 is modulated by phosphorylation of a Ser824 residue negative/positive feedback regulation loop. In a short-term regulation loop (Figure 3), TRPV4 WT appears to be dually modulated by the association of regulatory proteins (such as Ca^{2+} bound CaM) on its C-terminal cytoplasmic domain (Figure 1). First, the naive TRPV4 begins to open in response to several over-threshold environmental signals (e.g., mechanical chemical, temperature, and osmolality). TRPV4 is positively activated by dissociation with regulatory proteins such as CaM at low Ca^{2+} concentrations. However, the channel is negatively feedback inhibited at high Ca^{2+} concentrations by the fully active TRPV4 and returned to the inactive form; these mechanisms constitute the short-term negative/positive feedback regulation loop (Figure 3). This phenomenon also explains our observation that TRPV4 WT shows Ca^{2+} ion concentration oscillations in the cytoplasm after

activation with 4-α PDD or heat. In contrast, after the TRPV4 WT is phosphorylated on its Ser824 residue by SGK1, the prolonged active (phosphorylated) form (a TRPV4 S824D analogue), which remains active until being dephosphorylated on its Ser824 residue by a protein phosphatase and the association of a putative regulatory protein (such as Ca^{2+}-CaM). Consistent with this assumption, TRPV4 S824D (an analogue with a prolonged active form), shows high Ca^{2+} channel activity with/without 4-α PDD treatment, abrogating the protein interaction with the inhibitory factor (Amor *et al*., 2002; Strotmann *et al*., 2003; Chun *et al*., 2012). This model explains why the TRPV4 E797 mutant is constitutively active. Following 4-α PDD or heat treatment, TRPV4 S824A appears to be analogous with its active form. TRPV4 S824A, which is not associated with the inhibitory protein Ca^{2+}-CaM (as an inhibition complex) returns back to an inactive form (Figure 3). This may explain why channel activity and Ca^{2+} entry of TRPV4 S824A appear to be higher than those of TRPV4 WT after 4-α PDD treatment. However, binding of Ca^{2+}-CaM can be prevented by SGK1-mediated phosphorylation of a serine 824 residue within the CaM binding site.

Conversely, substitution of the target site (serine 824) with aspartic acid (S824D) results in a more rapid and longer activation of TRPV4-mediated currents, as Ca^{2+}-CaM binding can be prevented by phosphorylation (Amor *et al*., 2002; Chun *et al*., 2012; Shin *et al*., 2012). This convergence on a shared C-terminal domain may represent an important mechanism by which the timing and convergence of signal responses is controlled. Although Ca^{2+}-CaM as the negative feedback regulator at high Ca^{2+} concentration is the focus of this research, binding competition with other proteins on the TRPV4 C-terminal region cannot be ruled out (Figure 3). It seems to be valuable to elucidate the protein that interacts with the C-terminal region of TRPV4.

TRPV4 harbors a consensus sequence for protein-Ser 824 phosphorylation by SGK1 within the putative CaM, actin, or tubulin binding domain (811-850 amino acids). Thus, Ca^{2+}-CaM binding is modified by phosphorylation on Ser 824. In this regard, the CaM binding site on its C-termini seems to be involved in Ca^{2+}-induced conformational changes. It is likely that the Ser 824 of the C-terminal domain induces a conformational change in the TRPV4 channel protein that results in decreased (or inactivated) channel activity, as both TRPV4 S824D, and S824A (such as the TRPV4 E797 mutant regardless of phosphorylation) are more active than that of TRPV4 WT (Amor *et al*., 2002; Shin *et al*., 2012).

A variety of stimuli activate members of the various TRPV subfamilies. Vanilloid compounds such as capsaicin, the compound responsible for the spiciness of hot chili peppers, moderate heat, and protons activate TRPV4 channels and function as integrators of a variety of painful stimuli. Furthermore, TRPV4 is sensitive to noxious heat and can be constitutively activated by growth factors (Table 2).

The nitric oxide (NO) donor induces S-nitrosylation at Cys853 in the C terminus of TRPV4. S-nitrosylation at the Cys853 residue in the TRPV4 channel functionally and directly regulates channel sensitivity. We propose that NO conversely inhibits the Ca^{2+} response and direct S-nitrosylation on the Cys853 of TRPV4 as negative feedback regulation (Ding *et al*., 2010; Lee *et al*., 2011). Endothelial NO synthase and TRPV4 co-localize in the caveolar fraction of guinea pig hearts, thereby indicating close proximity of the NO donor and the target protein (Yoshida *et al*., 2006). Thus, multiple factors are responsible for making the Cys853 of TRPV4 (a target of molecule-specific and site-specific S-nitrosylation). Although TRPV5 and TRPV6 are tightly controlled by membrane potential, including a voltage-dependent open-pore block by Ca^{2+}-dependent feedback mechanism, it is unknown whether TRPV4 is also regulated in the same manner. S-nitrosylation of the TRPV4 channel seems to be a Ca^{2+}-dependent feedback mechanism (Figure 3). S-nitrosylation of native TRPV4 upon G protein-coupled ATP receptor stimulation elicits Ca^{2+} entry into endothelial cells (Yoshida *et al*., 2006; Ding *et al*., 2010). In the case of TRPV4, however, even though the increase of NO in cells also appears to enhance S-nitrosylation of native TRPV4, the nitrosylation site (C853) of TRPV4 is located in its C-terminal tail, and Ca^{2+} transport by the channel appears to be inactivated by this modification, as a negative feedback Ca^{2+} transport regulation mechanism. However, the fully active TRPV4 is inhibited by negative feedback at high Ca^{2+} ion levels and returned to the inactive form, as a negative feedback regulatory loop by NO (S-nitrosylation). Therefore, the channel properties of TRPV4 are similar those of voltage-gated Na^{+} channels. The various modifications of the TRPV channel C-terminal are summarized in Table 2.

The mode of action of TRPV4 mutants

The mode of action of the mutations in these TRPV4-related disorders has proved difficult to elucidate. Initial functional work investigating the impact of the mutations seen in the neuropathy group was conflicting, as both gain-of-function and loss-of-function mechanisms were proposed (Figures 4 and 5). This may have resulted from different

experimental protocols, as each group studied different cell lines (HEK293, HeLa, Xenopus oocytes, and dorsal root ganglion cells). Further work by Fecto *et al*. investigated three of the TRPV4 neuropathy spectrum mutations in three cell types (HEK293, HeLa, and Neuro2a cells) and proposed that they caused the channels to have an increased channel open probability and an increased sensitivity to agonists (4α-PDD) (Fecto *et al*., 2011). They suggested that this observation could have resulted from a change in channel gating or increased membrane insertion of the channels (Deng *et al*., 2010; Fecto *et al*., 2011). Cell viability studies have shown a correlation with increased calcium influx and cytotoxicity that could be reversed by a TRPV4 channel inhibitor (ruthenium red; (Watanabe *et al*., 2002a)). Interestingly Loukin *et al*. studied 14 of the skeletal dysplasia-associated TRPV4 mutations in Xenopus oocytes and found evidence for increased open probability of the mutant channels (Loukin *et al*., 2010a; Loukin *et al*., 2011). Mutant channels also show increased sensitivity to channel agonists (4α-PDD and hypotonicity). They observed a correlation between increasing levels of channel open probability and phenotype severity (Loukin *et al*., 2010a; Loukin *et al*., 2011). Hence, mutations that result in the mildest phenotype, brachyolmia, cause an approximate 2% increase in channel open probability, whereas open probability of mutant channels causing the severe phenotype MTD approached 100%. TRPV4 participates in terminal differentiation of developing osteoclasts by providing sustained calcium influx, but the mechanism by which these mutations cause the skeletal dysplasia remains unclear (Figure 4). Finally, functional studies of the TRPV4 mutations causing FDAB in HEK293 cells showed a small increase in channel open probability with a slight increase in sensitivity to channel agonists and reduced cell surface expression. The overall effect was thought to result in a reduction in channel activity (Figures 4 and 5).

The effects of these mutations on other factors such as protein folding, post translational glycosylation, protein-protein interactions, heterotetramerization, and protein trafficking may also contribute to the phenotypic expression of these TRPV4 mutations (Cuajungco *et al*., 2006; Wang *et al*., 2007; Fernandes *et al*., 2008; Ma *et al*., 2011). Consideration of the three-dimensional structure of the channel reveals that four key residues in the ARD (ankyrin repeat domains) (Table 3) associated with the neuropathy spectrum are surface mutations, not thought to affect protein folding. They cluster on the positively charged convex surface of the ARD and involve highly conserved arginine residues, whereas the skeletal dysplasia mutations affecting the ARD are situated on the concave surface and may interrupt the protein-protein interactions (Hellwig *et al*., 2005; Arniges *et al*., 2006). Thus, the mutations in each group of conditions may affect different protein functions, while overlapping conditions may result from mutations affecting residues involved in shared functions of the protein. TRPV4 forms homotetramers, but recent work has shown that it interacts with other channels including TRPC1 and TRPP2, forming heterotetramers with a 2:2 stoichiometry and alternating subunit arrangement results in distinct electrophysiological properties (Hellwig *et al*., 2005; Feng *et al*., 2011).

Further work has shown that TRPV4 interacts with the cytoskeleton and regulates microtubules and actin, which, in turn, is regulated by microtubule dynamics (Suzuki *et al*., 2003a; Ramadass *et al*., 2007; Shin *et al*., 2012). Thus, the full effects of the TRPV4 mutations may result from effects on additional functions of the protein not just on changes to the channel gating properties. Further studies are required to elucidate the pathogenic mechanisms occurring in these disorders and identify the genetic and environmental modifying factors that cause such striking variation in disease expression.

Conclusion and perspective

The TRPV4 functional Ca^{2+} channel consists of the homo tetramer subunits. TRPV4 and TRPC1 can co-assemble to form heteromeric TRPV4-C1 channels (Hellwig *et al*., 2005; Feng *et al*., 2011). Because the TRPV4 ankyrin repeat is responsible for channel self-assembly in cell lines, mutations in the TRPV4 ankyrin domain also seem to affect the channel assembly in humans, as shown in many dominant negative genetic disorders (Hellwig *et al*., 2005; Arniges *et al*., 2006).

TRPV4 was originally shown to be activated by hypotonicity, but later studies have demonstrated that activation can also be achieved by phorbol esters, arachidonic acid, and moderate heat (Guler *et al*., 2002; Watanabe *et al*., 2002a; Alessandri-Haber *et al*., 2003; Watanabe *et al*., 2003; Vriens *et al*., 2004; Birder *et al*., 2007). TRPV4 appears to be an important player in pathological sensory perception and bone growth. The potential effect of a TRPV4 functional mutation remains to be elucidated in the future. Furthermore, the role of TRPV4 in the pathogenesis of several diseases should be characterized as to how the channel protein contributes to the specific diseases. This information may be useful to cure or alleviate the human disease caused by TRPV4 mutations.

As expected, TRPV4 activation in osteoclasts

increased the number of osteoclasts and their resorption activity, thereby resulting in bone loss. During *in vitro* analysis, $TRPV4^{R616Q/V620I}$ osteoclasts showed activated Ca^{2+}/calmodulin signaling compared with osteoclasts lacking TRPV4 (Mizoguchi *et al*., 2008; Rock *et al*., 2008; Everaerts *et al*., 2010). In addition, studies in $TRPV4^{R616Q/V620I}$ mice, which lack the calmodulin-binding domain, indicate that bone loss due to TRPV4 activation was abrogated by loss of interactions between Ca^{2+}/calmodulin signaling and TRPV4. Finally, modulators of TRPV4 interactions with the calmodulin-binding domain should be investigated by proteomic analysis (Tables 1 and 2).

Bones are constantly undergoing remodeling to adapt to mechanical stress, repair (micro) fractures, and regulate mineral metabolism (Mizoguchi *et al*., 2008; Abed *et al*., 2009; Masuyama *et al*., 2012). Mechanical stress plays an important role in bone formation and remodeling. Importantly, mechanical unloading of bones leads to the suppression of bone formation and increases bone resorption, as observed in bedridden patients that develop osteoporosis (Mizoguchi *et al*., 2008; Everaerts *et al*., 2010). As TRPV4 is expressed in both osteoblasts (responsible for bone deposition), and osteoclasts (mediate bone resorption), it may play a role in sensing mechanical stress and controlling bone remodeling (Mizoguchi *et al*., 2008; Abed *et al*., 2009; Masuyama *et al*., 2012). In mice, mechanical hind limb unloading induces osteopenia, reduces bone formation rate, and stimulates bone resorption by increasing the number of osteoclasts. In contrast, these effects are suppressed in TRPV4-/- mice (Mizoguchi *et al*., 2008). Moreover, TRPV4-/- mice show mild osteopetrosis, increased trabecular bone mineral density and increased cortical thickness, resulting from reduced bone resorption as a consequence of disrupted osteoclast differentiation (Figure 4). TRPV4-mediated Ca^{2+} influx plays a crucial role in Ca^{2+}-dependent regulation of nuclear factor-activated T cells c1 (NFATc1), a transcription factor that controls osteoclast-specific gene expression (Masuyama *et al*., 2008).

TRPV4 in chondrocytes of cartilage tissue is involved in modulating chondrogenic bone formation by regulating sex determining region Y-box 9 (SOX9) activity (Muramatsu *et al*., 2007). SOX9 is a well-established chondrocyte transcription factor that regulates expression of cartilage-specific extracellular matrix proteins and controls chondrocyte differentiation and bone formation (Figure 4). TRPV4 stimulates SOX9-dependent reporter activity in mesenchymal stem cells *via* a Ca^{2+}-calmodulin dependent mechanism. The role of TRPV4 requires further elucidation in the case of late chondrogenic differentiation, in which SOX9 functions as a negative regulator. Thus, it seems that TRPV4 mutants, which are constitutively active, maintain Ca^{2+} influx and its high concentration, resulting in activated NFATc1. As a consequence, osteoclast-specific gene expression is enhanced. The eventual phenotype of those mutants appears like SEDM (Figures 4 and 5 and Table 3).

Recent observations on endothelial cells (ECs) indicate that small numbers of EC TRPV4 channels regulate vascular physiology and suggest that pathologies characterized by blood pressure reduction and vascular permeability increases (e.g., septic shock) may involve excessive activation of EC TRPV4 channels (Sonkusare *et al*., 2012). These results demonstrate that a small number of active TRPV4 channels (about three to eight per cell) mediate local Ca^{2+} signals that activate the intermediate (IK)- and the small (SK)-conductance, Ca^{2+}-sensitive potassium (K^+) channels to cause maximal dilation of resistance arteries. Similarly, in the chondrocyte, osteoblast, or osteoclast a small number of active TRPV4 channels mediate local Ca^{2+} signals that activate other channel such as IK and SK (Sonkusare *et al*., 2012). Even the increased ATP binding in E183K and E278K also contribute to constitutive TRPV4 activity, and the increase in TRPV4 activity seems to lead to TRPV4-linked spinal disease phenotypes (Phelps *et al*., 2010; Inada *et al*., 2012). Thus, the mutant TRPV4 channel that has increased activity in bone cells compared to normal causes a catastrophic effect, such as BRAC3, CMT2C, MTB, PSTD, or SEDM (Figures 4 and 5 and Table 3).

However, FDAB is not same as SEDM, because FDAB TRPV4 (G270V, R271P, Y273L) is not constitutively active compared to that of the TRPV4 WT. The mutation sites of FDAB TRPV4 are localized between ankyrin repeats 3 and 4, which seem to be responsible for membrane localization. FDAB is caused by mutations encoding p.Gly270Val, p.Arg271Pro, and p.Phe273Leu substitutions in the intracellular ankyrin-repeat domain of the TRPV4 cation channel. The TRPV4 mutant that causes FDAB is poorly localized to the cell-surface in HEK-293 cells and shows reduced channel activity (Amor *et al*., 2002; Dai *et al*., 2010; Lamande *et al*., 2011; Loukin *et al*., 2011). Therefore, it is assumed that the TRPV4 mutant causes the different phenotypic diseases, depending on mutant TRPV4 channel activity (Figure 5). The balance between osteoblasts and osteoclasts seems to be regulated by TRPV4 activity (Figure 4). The only ambiguous mutant is the G806A (exon 5) mutation which causes both SMA/HMSN2C and CMT2C (Table 3). It is not clarified yet that the channel activity of TRPV4 R269H is constitutively active or not (Amor *et al*.,

2002; Dai *et al*., 2010; Lamande *et al*., 2011; Loukin *et al*., 2011). It is not obvious why some mutations result in one phenotype over another but an intriguing possibility is that certain mutations associated with the neurological phenotype may predispose the TRPV4 transcript to act like no other TRPV isoform (trans-speciation) (Dai *et al*., 2010; Nishimura *et al*., 2010). Recent evidence also suggests that the mutations associated with a skeletal phenotype caused the increased calcium channel basal activity (Loukin *et al*., 2010a; Loukin *et al*., 2010b; Loukin *et al*., 2011). However, the same has been shown for mutations associated with the clear neurological phenotype (Deng *et al*., 2010; Fecto *et al*., 2011).

TRPV4 should be the first line of genetic investigation in any individual presenting with a predominant motor axonal neuropathy. Documentation of height and any skeletal complaints should also be determined. Those providing genetic counseling for these families need to be aware of the frequent occurrence of non-penetrance and the striking phenotypic variability (Bird, 1993; Dai *et al*., 2010). The importance of identifying the correct hereditary neuropathy subtype may have moved beyond prognostication and genetic counseling, as there is no treatment for these disorders, but the existence of known agonists and antagonists of the TRPV4 channel have prompted many suggestions that therapeutic pharmacological modification of the mutant channels may be possible.

Even though it is not completely accorded in all mutant cases yet, the mutant shown the more channel activity than the TRPV4 WT causes the abnormality of backbone morphogenesis (cause BRAC3, CMT2C, MTB, PSTD or SEDM), whereas the mutant shown the less channel activity than the TRPV4 WT causes the abnormality of articular morphogenesis or the neuropathy (FDAB or HMSN2C) (Figures 2 and 5 and Table 3). TRPV4 mutants are constitutively active and maintain high Ca^{2+} influx, resulting in activated NFATc1 and high osteoclast-specific gene expression (Wegierski *et al*., 2006). Thus, TRPV4 mutant causes the different phenotypic diseases, depending on the mutant TRPV4 channel activity. In addition, the balance of cell number between osteoblast and osteroclast seems to be regulated by the TRPV4 activity.

Several mutants studied in heterologous expression systems show constitutive basal activity and enhanced response to stimuli (Deng *et al*., 2010; Loukin *et al*., 2010a; Fecto *et al*., 2011; Loukin *et al*., 2011). These findings are consistent with cell death caused by high calcium influx such as the observed degeneration of motor neurons in neuropathies. However, it is difficult to explain how seemingly similar molecular properties lead to such diverse disease phenotypes. It is essential to elucidate how TRPV4 is regulated at the molecular level to understand the mechanisms behind these inherited diseases.

Further studies are required to understand the pathogenic mechanisms occurring in these disorders and identify the modifying genetic and environmental factors that cause such striking variation in disease expression. The reason why TRPV4 mutations, which show greater constitutive activity and no change in unitary conductance or rectification, cause such prominent spinal diseases will be elucidated. Further, among 26 members of the TRP subfamily which are expressed in a broad range of tissues, why the only TRPV4 cation channel contributes to the generation of such many genetic bone diseases should be answered.

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