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Review

The differential role of TRPV4 ion channel in osteoclast and chondrocyte

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ABSTRACT

The transient receptor potential vanilloid type 4 ion channel (TRPV4) is known to play an important role in the regulation of pain and inflammation. Pharmacological ligands of TRPV4 regulate human osteoclast formation *in vitro*, but the effects of these agents on osteoblast function have not been studied and their effects on bone loss *in vivo* are unknown. Here it was reviewed the effects of the TRPV4 mutants on mouse osteoclast and osteoblast differentiation *in vitro* and *in vivo*. The constitutive active mutants activated osteoclast formation and bone resorption in a dose dependent manner in bone marrow–osteoblast co-cultures and receptor activator of NFκB ligand-generated osteoclast cultures. We also discussed here that the constitutively active TRPV4 mutants induced bone loss in mice, with histomorphometric analysis showing activating effects on indices of bone resorption and bone formation. The chondrocyte differentiation factor SOX9 also seems to be in association with chondrocyte-like cells, suggesting that TRPV4 regulates the SOX9 pathway and contributes to the process of chondrogenesis. Furthermore, it seems to be possible that the pharmacological blockade of TRPV4 ion channels by small chemicals may inactivate osteoclastic bone resorption and protect against future bone loss.

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INTRODUCTION

The transient receptor potential vanilloid type 4 ion channel (TRPV4) is a member of a family of polymodal and nonselective cation channels that are predominately expressed by sensory nerve fibers of the somatic and autonomic afferent neurons [1, 2]. TRPV4 (among 27 TRP channels) is activated in response to various stimuli such as physical abrasion, heat, protons, and by a variety of pharmacological ligands such as capsaicin, the endocannabinoid anandamide, and arachidonic acid metabolites [3, 4]. TRPV4 ion channels have been implicated in the regulation of pain perception, inflammation, and cardiovascular homeostasis [5]. Activation of the TRPV4 ion channel using the agonist capsaicin causes desensitization of the ion channel and evokes hypothermia in mice [6].

Bones are constantly undergoing remodeling in order to adapt to mechanical stress, repair (micro) fractures, and regulate mineral metabolism. Mechanical stress has an important impact on bone formation and remodeling. Importantly, mechanical unloading of bones leads to a suppression of bone formation and increases bone resorption, as observed in bed ridden patients who developed osteoporosis. Since TRPV4 is expressed in both osteoblasts responsible

for bone deposition, and osteoclasts that mediate bone resorption, it may play a role in sensing mechanical stress and controlling bone remodeling [2, 7]. In mice, mechanical hind limb unloading induces osteopenia, reduces the bone formation rate, and stimulates bone resorption by increasing the number of osteoclasts.

A number of recent studies have also shown that TRPV4-expressing fibers innervate bone and that the pharmacological and genetic inactivation of TRPV4 reduces bone pain in animal models of cancer [6, 8]. Moreover, mice deficient in TRPV4 ion channels display a significant reduction in pain and joint swelling in response to inflammatory arthritis, indicating that pharmacological blockade of this ion channel could be of therapeutic value in patients with this disease [9]. These results suggest that TRPV4 also plays a role in bone metabolism, and indeed the ion channel is expressed by lymphocytes, osteoclasts, and osteoblast-like cells.

Moreover, the pharmacological activation of TRPV4 with agonists such as 4-αPDD, GSK 1016790A and RN-1747 has been shown to enhance osteoclast formation in human bone marrow (BM) cultures stimulated with receptor activator of NFκB ligand (RANKL) [3, 10, 11]. It has also been suggested that TRPV4 ion channel activation may partly account for the stimulatory effect of the endocannabinoid anandamide and arachidonic acid (AA) on osteoclast formation and bone turnover, since anandamide is known to activate TRPV4 ion channels [3, 11]. Thus, these reports have suggested that the RANKL pathway in osteoclast development has a relationship with TRPV4 function [5, 12]. Previously, it was reviewed how the constitutive activation of TRPV4 by a point mutation promotes the differentiation of mouse osteoclasts.

Therefore we summarize here studies that show that TRPV4 also enhances osteoclast differentiation through the RANKL signal pathway and mutants that constitutively activate TRPV4 cause several genetic diseases in humans [5, 12].

RANKL binds to its receptor RANK, which is important for the initial induction of NFATc1. NFATc1 is activated by calcium signaling and binds to its own promoter, thus switching on an auto regulatory loop that enables the robust induction of NFATc1. NFATc1, in turn, cooperates with other transcriptional partners to activate osteoclast-specific genes [13, 14]. The auto regulation of NFATc1 is controlled by an epigenetic mechanism, which has profound implications for the understanding of the general mechanism of irreversible cell fate determination. NFATc1 as a master transcription factor for RANKL-induced osteoclastogenesis has provided major insight into the molecular mechanism of osteoclast differentiation. Thus, we assumed that constitutively active TRPV4 mutants maintain Ca^{2+} influx at a high concentration, resulting in activated NFATc1 [15]. We also call attention to the RANKL signaling pathway as a strategy for suppressing the excessive osteoclast formation characteristic of a variety of bone diseases.

Further, because TRPV4 in chondrocytes is involved in the modulation of chondrogenic bone formation by SOX9 activity, we discussed how SOX9 (a well-established chondrocyte transcription factor which regulates expression of cartilage specific extracellular matrix proteins and controls chondrocyte differentiation and bone formation) is influenced by TRPV4 [1, 16].

The regulation mechanism of TRPV4

TRPV4 is known to form homo tetramers, but recent work has shown that it also interacts with other channels including TRPC1 and TRPP2 (polycystin-2), forming hetero tetramers with a 2:2 stoichiometry and alternating subunit arrangements resulting in distinct electrophysiological properties [17]. Further work has also shown that TRPV4 interacts with the cytoskeleton and regulates both the microtubule and the actin and is, in turn, regulated by microtubule dynamics [18, 19, 20]. Taken together, intracellular $[Ca^{2+}]_i$ measurements indicate that during the differentiation of osteoclasts, a gradual shift occurs from spontaneous Ca^{2+} oscillations to sustained Ca^{2+} influx via TRPV4 [7]. TRPV4 forms a complex with TRPC1 or TRPP2, and the heterotetramer is localized in the ER functioning as the store-operated calcium channel (SOCE) [6]. Thus, the regulation of the subcellular localization of TRPV4 seems to determine whether the channel forms a complex with TRPC1 (heterotetramer) or itself (homotetramer). It is interesting to note that the phosphorylation of the Ser824 residue in the C-terminal of TRPV4 seems to regulate the localization and the complex formation with TRPC1 [20, 21, 22].

Recently, Ye *et al*. reported that TRPV4 is a regulator of the oxidative metabolism of adipocytes, inflammation, and energy homeostasis [23]. They observed that TRPV4 is a negative regulator of PGC1α, a key transcriptional co-regulator of oxidative metabolism, and the morphogenesis which plays a key role in mitochondrial biogenesis in brown adipocytes. Constitutively active TRPV4 seems to down regulate PGC1α expression compared with TRPV4 WT, resulting in the lowering of the metabolic activity state in patients. TRPV4 seems to be a promising site to regulate/accelerate metabolism.

The full effects of the mutations in TRPV4 may result from the effects on the additional functions of the protein, not just the changes to the gating properties of the channel. Further studies are required to elucidate the pathogenic mechanisms occurring in these disorders and identify the modifying factors, both genetic and environmental, that cause such striking variation in disease expression.

TRPV4 channel activation and serine phosphorylation were enhanced by exposure to the non-PKC activator, phorbol 12-myristate 13-acetate (4-αPDD), or by the application of bradykinin, which activates PKC via a G-protein-coupled mechanism, or PKA [20, 21]. In experimental animal models, 4-αPDD reduces hyperalgesia induced by mechanical stimuli, or capsaicin or carrageen injections in animal models of osteosarcoma. However, in response to reports of 4-αPDD displaying species-dependent efficacies in rodent models of hyperalgesia and chronic pain, a new generation of selective TRPV4 ligand has been developed. Agents such as GSK 1016790A, RN-1747, HC-067047, and 4-αPDD are potent and highly selective TRPV4 agonists that have been shown to inhibit capsaicin-evoked current *in vitro*. Recent clinical studies are currently assessing the potential analgesic and anti-inflammatory qualities of a number of TRPV4 agonists and antagonists [24, 25].

Because the Ser824 residue of TRPV4 has also been detected in the consensus serine/threonine-protein kinase (SGK1) substrate sequences, it was ascertained that SGK1 phosphorylates the Ser824 residue of TRPV4 as one of its specific substrate proteins [20]. Previously, we observed that the SGK1-mediated phosphorylation of the TRPV4 Ser824 residue exerts a synergistic effect on its functional Ca^{2+} entry, as well as its reactivity to 4-αPDD, interactions with CaM, subcellular localization, and cell survival [20]. In line with these reports, we also expected that TRPV4 activity enhancement by S824 phosphorylation is agonized or antagonized by the selective SGK1 activity modulators IGF2 and wortmannin.

With wortmannin or IGF2 treatment, we observed changes in the TRPV4 WT single channel properties as expected. However, we also observed that the basal activity and sensitivity of TRPV4 S824A (an analog of unphosphorylated TRPV4 by SGK1) to 4-αPDD was higher than that of TRPV4 WT. This observation suggested that the C-terminal domain of TRPV4 near serine residue 824 seems to regulate its function through an unknown mechanism beyond phosphorylation modification, perhaps through its protein-protein interaction with CaM [20].

Recently our observations have indicated that TRPV4 is modulated by the phosphorylation of the Ser824 residue in a feedback regulation loop. In its short-term regulation, 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. At first, the naive TRPV4 begins to open in response to several over-threshold environmental signals (e.g. mechanical, chemical, temperature, osmolality) [20]. TRPV4 is positively activated by the dissociation of regulatory proteins such as CaM, which occurs at low Ca^{2+} ion concentration levels. However, at high Ca^{2+} ion levels with a fully active TRPV4, the channel is negatively modulated through feedback inhibition and is returned to its inactive form. These mechanisms constitute the short-term negative/positive feedback regulation loop. This phenomenon also explains our observation that after activation with 4-αPDD or heat, TRPV4 WT showed oscillations of Ca^{2+} ion concentration in the cytoplasm [26]. Consistent with this assumption, TRPV4 S824D, (an analogue with a prolonged active form) showed high Ca^{2+} channel activity with/without 4-αPDD treatment, abrogating its protein interaction with the inhibitory factor. This model is also able to explain why the TRPV4 E797 and P799 mutants are constitutively active. Following 4-αPDD or heat treatment, TRPV4 S824A appears to be analogous to its active form. TRPV4 S824A, which is not associated with the

inhibitory complex of Ca^{2+}-CaM, returns back to an inactive form. This may explain why Ca^{2+} entry and the channel activity of TRPV4 S824A appear to be higher than those of TRPV4 WT after 4-αPDD treatment. The binding of Ca^{2+}-CaM, however, can be prevented by the SGK1-mediated phosphorylation of a serine 824 residue within the CaM binding site. TRPV4 harbors a consensus sequence for the protein-Ser824 phosphorylation by SGK1 within the putative CaM, actin, or tubulin binding domain (811-850 aa). Thus, Ca^{2+}-CaM binding is modified by the phosphorylation of Ser824. In this regard, the CaM binding site on the channel′s C-terminal seems to be involved in Ca^{2+}-induced conformational changes. It seems likely that the Ser824 of the C-terminal domain induces a conformational change in TRPV4 that results in decreased (or inactivated) channel activity, as both TRPV4 S824D and S824A (such as TRPV4 E797 or P799 mutant which is regardless of the phosphorylation) are more active than TRPV4 WT. Regardless of the mechanisms, however, the gating diversity of the TRPV4 channel demonstrated in the current study is reflective of the presence of multiple physical and chemical signaling pathways that converge on the channel. As a consequence, the TRPV4 channel would appear to function as a molecular integrator of a complex array of diverse signals. 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 studies to elucidate the regulatory mechanisms of TRPV4 are required in order to determine the physiological role and activation mechanism of this channel.

TRPV4 in osteoclast cells

As TRPV4 is expressed in both osteoblasts (which are responsible for bone deposition), and osteoclasts (which mediate bone resorption), the protein may play a role in sensing mechanical stress and controlling bone remodeling. Osteoclasts develop from hematopoietic cells of the monocyte-macrophage lineage. 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. 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 [1].

The expression of TRPV4 in bone cells was determined using qPCR and Western blot analysis. Barsony *et al.* observed that osteoblasts express TRPV4 mRNA, but at significantly lower levels than cells in the lung and kidney. TRPV4 mRNA expression was also present in osteoblasts and M-CSF dependent osteoclast precursors, but at lower levels. Western blot analysis confirmed the presence of TRPV4 ion channels in osteoblasts, osteoclasts and M-CSF-dependent osteoclast precursors [5].

Activation of the TRPV4 ion channel has been implicated in the pathogenesis of bone pain associated with cancer and arthritis, but very little is known about the potential role that TRPV4 plays in bone metabolism. Recently, other researchers showed that TRPV4 was expressed by osteoclasts both *in vitro* and *in vivo* and that the TRPV4 agonist (I-RTX) inhibited human osteoclast formation *in vitro*, whereas the TRPV4 agonist resiniferatoxin increased osteoclast formation [6]. However, the role of TRPV4 in osteoblast activity and the mechanisms by which TRPV4 agonists inhibit osteoclast formation remain unclear. To address this gap, the effects of the TRPV4 ion channel agonists capsaicin and 4-αPDD on osteoclast and osteoblast activity *in vitro*, as well as the effects of 4-αPDD on ovariectomy-induced bone loss *in vivo* should be examined.

In agreement with other studies, it has been reported that TRPV4 ion channels are expressed by mature osteoclasts; in addition, this study showed for the first time that osteoblasts also express TRPV4 mRNA and protein, albeit at a lower level than cells in the lung and kidney. Further studies showed that the TRPV4 agonist 4α-PDD inhibited osteoclast formation and bone resorption in a dose dependent manner in osteoblast–bone marrow co-cultures and RANKL generated osteoclasts, whereas the TRPV4 agonist capsaicin increased osteoclast formation. The concentrations at which 4-αPDD inhibited bone resorption were significantly lower than those required to suppress osteoclast formation, indicating that the inhibitory effect of 4-αPDD *in vitro* was most likely mediated by its effect on the activity of mature osteoclasts rather than on

M-CSF dependent osteoclast precursors. Keeping with these findings, it was shown that 4-αPDD is incapable of inhibiting mRNA expression of RANKL and osteoprotegerin (OPG) in calvarial osteoblasts, suggesting that this TRPV4 agonist is unlikely to affect the role of osteoblasts in osteoclastogenesis. The OPG is a decoy receptor for RANKL, which inhibits osteoclast differentiation and function by interrupting the interaction between RANKL and RANK, a receptor of RANKL [27, 28, 29].

Since TRPV4 ion channels are known to regulate MAPK and NFκB signaling in primary afferent neurons, Facer *et al.* investigated the effects of 4-αPDD on RANKL-induced phosphorylation of IκB and ERK1/2 in osteoclasts. They showed that 4-αPDD inhibited RANKL induced NFκB and ERK1/2 MAPK activation. These effects were observed at concentrations similar to those that inhibited osteoclast formation and activity. Since the activation of NFκB and ERK1/2 MAPK signaling pathways is vital for osteoclast formation and survival as well as bone resorption, it is likely that these actions may also have contributed to the inhibitory effects on osteoclast differentiation and function that were observed in this study [30]. Previous studies, however, have shown that TRPV4 agonists such as 4-αPDD also target a number of receptors and ion channels such as the transient receptor potential melastatin 8 (TRPM8) channel, voltage-activated calcium channels, and nicotinic acetylcholine receptors [31]. In view of this, it would be of great interest in future studies to investigate the effects of 4-αPDD and other highly selective TRPV1 agonists (such as 4-αPDD, GSK 1016790A, and RN-1747) in osteoclast formation and signaling in cultures generated from TRPV4 knockout mice [32].

The mechanisms by which TRPV4 signaling regulates osteoclast activity are poorly understood. In a previous study, the TRPV4 agonist capsaicin increased intracellular calcium levels in human osteoclasts, which was reversed by I-RTX, a TRPV4 agonist. Increases in intracellular calcium have been shown to play a critical role in RANKL-induced osteoclast activation by increasing NFATc1 transcription. This is in broad agreement with the findings of other investigators, who found that I-RTX inhibited human osteoclast formation from peripheral blood cells. Both 4α-PDD and I-RTX are known to target TRPV4 ion channels and block capsaicin-induced calcium uptake acting on TRPV4 ion channels *in vitro*, with I-RTX showing greater selectivity and more potency towards TRPV4 ion channels [6]. Based on these findings, it is clear that TRPV4 blockage, whether with the traditional TRPV4 agonist 4-αPDD or the relatively more potent agonist I-RTX, is sufficient to inhibit osteoclast formation *in vitro*. Recently it has been reported that TRPV4 even regulates the SOX9 pathway and contributes to the process of chondrogenesis; it remains unclear how TRPV4 plays a role in osteoclast differentiation [1, 16, 33].

The effects of TRPV4 blockage on osteoblast number, alkaline phosphatase activity, and bone nodule formation have also been investigated *in vitro* [34]. While 4-αPDD and I-RTX had no significant effect on osteoblast growth or viability at concentrations of up to 10 μM for up to 21 days of continuous treatment, both compounds inhibited alkaline phosphatase activity and bone nodule formation at concentrations similar to those that affected osteoclast formation and function [35, 36]. Although these authors found that 4-αPDD inhibited both osteoclast and osteoblast activity *in vitro*, they observed significant inhibitory effects *in vivo* on bone loss following ovariectomy, *with* histomorphometric analysis showing a significant reduction in bone resorption and bone formation [37]. This indicates that 4-αPDD inhibited bone resorption more than bone formation, there by explaining its protective effect on ovariectomy-induced bone loss [38]. Similar findings have been reported with other anti-resorptive drugs such as bisphosphonates and activators of the RANK signaling pathway [39]. While the work presented there clearly indicates that TRPV4 agonists display anti-resorptive effects *in vivo* and therefore could be of value in the treatment of bone loss, further studies will be required to determine if genetic inactivation of TRPV4 ion channels and/or treatment with highly selective TRPV4 agonists including 4-αPDD, GSK 1016790A, and RN-1747 are effective in preventing the development and progression of bone loss in animal models [40].

The TRPV4 functional Ca^{2+} channel consists of homotetramer subunits. TRPV4 and TRPC1 can co-assemble to form heteromeric TRPV4–C1 channels.

The TRPV4 ankyrin repeat is responsible for channel self-assembly in cell lines, and mutations in the TRPV4 ankyrin domain seem to affect channel assembly in humans, as shown in many dominant negative genetic disorders [41]. 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. TRPV4 appears to be an important player in pathological sensory perception and bone growth. The potential effects of a TRPV4 functional mutation remain to be elucidated in the future. Furthermore, the role of TRPV4 in the pathogenesis of disease should be characterized as to how the channel protein contributes to the specific disease [1, 6]. This information may be useful in curing or alleviating the human consequences of TRPV4 mutations.

For example, FDAB is not same as spondyloepiphyseal dysplasia Maroteaux type (SEDM), because FDAB TRPV4 (G270V, R271P, F273L) is not constitutively active compared to that of TRPV4 WT. The mutation sites of FDABTRPV4 are localized between ankyrin repeats 3 and 4, which seem to be responsible for the membrane localization. FDAB is caused by mutations encoding Gly270Val, Arg271Pro, and Phe273Leu substitutions in the intracellular ankyrin-repeat domain of the TRPV4 cation channel [42, 43]. The TRPV4 mutant that causes FDAB is poorly localized to the cell-surface in HEK-293 cells and shows reduced channel activity. These differences suggest that different TRPV4 mutants cause different disease phenotypes depending on the specific mutation affecting TRPV4 channel activity. The only ambiguous mutant is the G806A (exon 5) mutation which causes both Spinal muscular atrophies/ hereditary motor and sensory neuropathies (SMA/ HMSN2C) and Charcot-Marie-Tooth disease type 2C (CMT2C). It has not yet been clarified whether TRPV4 R269H has a constitutively active channel [44, 45].

During *in vitro* analysis, TRPV4R616Q/V620*I* osteoclasts showed activated Ca^{2+}/calmodulin signaling compared with osteoclasts lacking TRPV4. In addition, studies of TRPV4R616Q/ V620*I* mice that lacked the calmodulin-binding domain indicated that bone loss due to TRPV4 activation was abrogated by the loss of interactions between Ca^{2+}/calmodulin signaling and TRPV4 [2]. Modulators of TRPV4 interactions with the calmodulin-binding domain should be investigated by proteomic analysis [2, 46].

It is not obvious why some mutations result in one phenotype over another, but one possibility is that certain mutations associated with a neurological phenotype may predispose the TRPV4 transcript to act like no other TRPV isoform (trans-speciation). However, there is still much to be understood. For example, recent evidence also suggests that the mutations associated with a particular skeletal phenotype caused an increase in the basal activity of the calcium channel. The same has been shown, however, for mutations associated with clear neurological phenotypes [47].

TRPV4 should be the focus of the first line of genetic investigation in any individual presenting with a predominantly motor axonal neuropathy. The documentation of height and any skeletal complaints should also be completed. Professionals providing genetic counseling for these families need to be aware of the frequent occurrence of non-penetrance and striking phenotypic variability in TRPV4-related disorders [45]. The importance of identifying the correct hereditary neuropathy subtype may be 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. Although this has not been shown in all cases, in general, mutants that show increased channel activity over TRPV4 WT results in abnormalities in backbone morphogenesis (e.g. BRAC3, CMT2C, MTB, PSTD or SEDM), whereas mutants that results in decreased channel activity compared to TRPV4 WT causes abnormalities in articular morphogenesis or neuropathies (FDAB or HMSN2C). TRPV4 mutants are constitutively active and maintain high Ca^{2+} influx, resulting in activated NFATc1 and high osteoclast-specific gene expression, although these mutants cause the different phenotypic diseases depending on the mutant TRPV4 channel activity [6, 7].

The balance of the number of osteoblast and osteoclast cells also seems to be regulated by TRPV4 activity. Indeed, stimulation of TRPV4

with 4-αPDD at room temperature evoked robust increases in $[Ca^{2+}]_i$ in large osteoclasts obtained from WT mice, which were fully abolished in TRPV4-deficient large osteoclasts [6, 48]. In contrast, the Ca^{2+}-ionophore ionomycin evoked equal responses in large osteoclasts derived from either genotype. Thus, this data indicates that TRPV4 is functional as a Ca^{2+} influx channel in large mature osteoclasts, where it allows basal Ca^{2+} influx at physiological temperatures [7]. Interestingly, a clear correlation was found between TRPV4 activity, as assessed by the response to 4α-PDD, and the differentiation state of WT osteoclasts: small osteoclasts, which generally displayed Ca^{2+} oscillations, did not respond to 4-αPDD treatment, whereas large osteoclasts, which fully lack Ca^{2+} oscillations, showed robust responses to 4-αPDD, a response that was blocked by RR (Ruthenium Red) [7, 49, 50]. In medium-sized WT osteoclasts, both Ca^{2+} oscillations and 4-αPDD responses were observed; however, the 4-αPDD response in these medium-sized cells was significantly smaller than in large osteoclasts. Osteoclasts are multinucleated cells of monocyte/macrophage origin that degrade bone matrix [51]. The differentiation of osteoclasts is dependent on a tumor necrosis factor (TNF) family cytokine, receptor activator of RANKL, as well as macrophage colony-stimulating factor (M-CSF). Congenital lack of osteoclasts causes osteopetrosis, and the investigation of this process has provided insights into the essential molecules for osteoclastogenesis, including TNF receptor-associated factor (TRAF) 6, NF-kappaB, and c-Fos [52]. In addition, genome-wide screening techniques have elucidated an additional set of gene products involved in this process such as nuclear factor of activated T cells (NFAT) c1.

In the sequence of molecular events induced by RANKL during osteoclast differentiation, RANKL first binds to its receptor RANK, which recruits adaptor molecules such as TRAF6 [53, 54]. TRAF6 activates NF-kappaB, which is important for the initial induction of NFATc1. NFATc1 is activated by calcium signaling and binds to its own promoter, thus switching on an auto regulatory loop. An activator protein (AP)-1 complex containing c-Fos is required for the auto amplification of NFATc1, enabling the robust induction of NFATc1. Finally, NFATc1 cooperates with other transcriptional partners to activate osteoclast-specific genes. NFATc1 auto regulation is controlled by an epigenetic mechanism, which has profound implications for an understanding of the general mechanism of irreversible cell fate determination [54, 55]. From the clinical point of view, the RANKL signaling pathway has promise as a strategy for suppressing the excessive osteoclast formation characteristic of a variety of bone diseases. Furthermore, the involvement of TRPV4 in the Ca^{2+}/calcineurin-NFATc1 pathway and its basolateral localization, specifically in differentiated osteoclasts, suggest that this channel may regulate Ca^{2+} influx at certain stages of osteoclast differentiation [7, 56].

In contrast, these effects are suppressed in TRPV4-/- mice. Moreover, TRPV4-/- mice show mild osteopetrosis, an increased trabecular bone mineral density, and increased cortical thickness resulting from reduced bone resorption as a consequence of disrupted osteoclast differentiation [1, 6, 57]. TRPV4-mediated Ca^{2+} influx plays a crucial role in the Ca^{2+}-dependent regulation of NFATc1, a transcription factor that controls osteoclast-specific gene expression.

Previous studies have shown that RANKL-evoked Ca^{2+} signals in osteoclasts are characterized by baseline Ca^{2+} oscillations [7, 58]. Indeed, spontaneous Ca^{2+} oscillations were readily detected in the majority of small osteoclasts from both WT and TRPV4-deficient mice [6, 7]. Oscillation persisted for several minutes in Ca^{2+}-free extracellular solution, demonstrating that the generation of Ca^{2+} spikes is not critically dependent on Ca^{2+} influx through plasma membrane channels [59]. This observation indicates that the spontaneous Ca^{2+} oscillations originate from Ca^{2+} release from the intracellular stores followed by Ca^{2+} re-uptake. These observations demonstrate that TRPV4 is not required for the generation of Ca^{2+} oscillations in osteoclasts, and that TRPV4 directly regulates osteoblast and osteoclast differentiation and function both *in vitro* and *in vivo*, and TRPV4 blockade protects against ovariectomy-induced bone loss in mice [60].

These results also suggest that TRPV4 activators may be of value in preventing bone loss and treating pain associated with inflammatory and neoplastic disorders. On the other hand, the

associated reduction in bone formation, mediated through its effect on osteoblasts, may limit the long-term usefulness of these agents. These studies will help elucidate how the osteoporosis can be best prevented. In future, it will be elucidate how TRPV4-mediated Ca^{2+} influx plays a crucial role in the Ca^{2+}-dependent regulation of NFATc1, a transcription factor that controls osteoclast-specific gene expression [7, 56].

TRPV4 in chondrogenic differentiation

TRPV4 was originally identified as a channel molecule activated by hypotonic cell swelling. Later studies showed that it could also be activated by temperature, acidic pH, and a synthetic activator such as 4-αPDD. However, the physiological stimulus for TRPV4 during chondrogenesis remains unknown. TRPV4 is considered to be a mechanosensor for shear stress. In cartilage tissue, TRPV4 in chondrocytes is involved in the modulation of chondrogenic bone formation by SOX9 activity. SOX9 is a well-established chondrocyte transcription factor that regulates the expression of cartilage specific extracellular matrix proteins and controls chondrocyte differentiation and bone formation. It has been reported that TRPV4 stimulates a SOX9-dependent reporter activity in mesenchymal stem cells via a Ca^{2+}-calmodulin dependent mechanism [61, 62, 63]. In the case of late chondrogenic differentiation, where SOX9 functions as a negative regulator, the role of TRPV4 requires further elucidation.

In murine embryonic limb bud mesenchymal cells, compressive force increases the mRNA levels of SOX9, type II collagen, and aggrecan, resulting in the promotion of chondrogenesis [64, 65, 66]. Furthermore, elevation of aggrecan mRNA by compressive forces is mediated by a transient increase in intracellular Ca^{2+} and Ca^{2+}/calmodulin levels in bovine articular chondrocytes [65]. Given these observations, it is conceivable that TRPV4 might sense mechanical stress in the articular cartilage and be involved in the maintenance of cartilage homeostasis. In the previous study, TRPV4 was identified to have an effect on SOX9-dependent transcription. This data suggested an important role for TRPV4 in early chondrogenesis. It would also be worthwhile to examine the role of TRPV4 during hypertrophic differentiation in late chondrogenesis since SOX9 functions as a negative regulator in that process as well. The transcription factor SOX9, which contains a SRY-related high mobility group box, has an essential role in the chondrocyte differentiation pathway [67].

SOX9 regulates the transcription of cartilage-specific extracellular matrix molecules such as collagen type II, IX, and XI, and aggrecan, and heterozygous mutations in the SOX9 gene seem to cause campomelic dysplasia characterized by severe chondrodysplasia [68]. SOX9 heterozygous mutant mice and mice lacking SOX9 function show impaired endochondral bone formation. SOX9 is also involved in the expression of SOX5 and SOX6, both of which form a transcriptional complex with SOX9 that controls the expression of type II collagen and aggrecan [67]. These findings indicated that SOX9 plays essential roles in chondrogenesis as a linker of TRPV4 function. Although several molecules involved in chondrocyte differentiation have been identified, the mechanism of chondrogenesis is not fully understood. The identification of the mechanisms that control expression and activity of SOX9 by TRPV4 activation may provide important insights into the regulation of chondrogenesis. New insights of TRPV4 function may also be provided by investigating the mechanisms underlying chondrogenesis and the pathogenesis of cartilage diseases [69].

Because TRPV4 in late chondrogenic differentiation required SOX9 as a negative regulator to stop its proliferation, it seems that TRPV4 mutants that are constitutively inactive and do not properly maintain Ca^{2+} influx and concentration result in a postponing of SOX9 function in chondrocyte differentiation (but not cell proliferation). Through this mechanism, mutations in TRPV4 can result in decreased SOX9 function in chondrocytes, which can lead to abnormal proliferation and joint abnormalities, ultimately resulting in diseases such as SMA/HMSN2C. The disease might be caused by abnormalities in articular morphogenesis [69, 70].

Meanwhile, TRPV4 mutants, which are constitutively active, maintain high Ca^{2+} influx and activated NFATc1 [71, 72]. As a result, osteoclast-specific gene expression is enhanced. The eventual phenotype of these mutants resembles SEDM. It has been also reported that RANKL stimulation

evokes Ca^{2+} oscillations originating from alternate Ca^{2+} release from and reloading into intracellular stores, which induces NFATc1-dependent gene transcription [7]. This oscillatory behavior is gradually lost during differentiation, and at these later stages, Ca^{2+} influx from the extracellular space via TRPV4 channels becomes necessary for Ca^{2+} signaling, NFATc1 activation, and the terminal differentiation of osteoclasts, which are concurrent with the enhancement of SOX9 function in the chondrocyte differentiation. Therefore, it seems that the Ca^{2+} influx change by TRPV4 mutation causes the difference phenotype depending on the cell context, such as NFATc1 (in osteoclasts) or SOX9 (in chondrocyte) expression [7, 34].

Human diseases by TRPV4 mutations

The mechanisms underlying the mutations in TRPV4-related disorders have proven 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. This may have resulted from different experimental protocols as each group studied different cell lines (HEK293, HeLa, Xenopus oocytes, and dorsal root ganglion cells).

The work by Fecto *et al*. identified three of the TRPV4 neuropathy spectrum mutations in three cell types (HEK293, HeLa and Neuro2a cells), and proposed that these mutations caused the channels to have an increased probability of being open and an increased sensitivity to agonists (4-αPDD) [73]. They also suggested that this observation could have resulted from a change in channel gating or increased membrane insertion of the channels. Cell viability studies showed a correlation between increased calcium influx and cytotoxicity, which could be reversed by a TRPV4 channel inhibitor (ruthenium red) [6]. Interestingly, Loukin *et al.* studied 14 of the TRPV4 mutations associated with skeletal dysplasia in *Xenopus* oocytes and found evidence that the channels had an increased probability of being open and an increased sensitivity to channel agonists (4α-PDD, hypotonicity) [48]. They also observed a correlation between increasing probabilities of the channel being open with the severity of the phenotype. Mutations that resulted in the mildest phenotype, brachyolmia, caused an approximate 2% increase in the probability of the channel being open, while the severe phenotype, metatropic dysplasia, had a probability of open channels approaching 100%. Thus, TRPV4 appears to participate in the terminal differentiation of developing osteoclasts by providing sustained calcium influx, but the mechanism by which these mutations cause the skeletal dysplasia remains unclear [34].

Functional studies of the TRPV4 mutations causing familial digital arthropathy-brachydactyly (FDAB) in HEK293 cells showed a small increase in its probability of having an open channel and its sensitivity to channel agonists, as well as a reduced cell surface expression [74]. The overall effect was thought to result in a reduction of channel activity. Examining the three-dimensional structure of the channel shows that the four key residues in the ARD (232, 269, 315 or 316 aa) 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, in contrast to the skeletal dysplasia mutations affecting the ARD which are situated on the concave surface and may well interrupt protein-protein interactions. Thus, the mutations in different groups of conditions may affect different protein functions, while overlapping conditions may result from mutations affecting residues involved in the shared functions of the protein. Thus, TRPV4 participates in the terminal differentiation of developing osteoclasts by providing sustained calcium influx, but the mechanism by which these mutations cause skeletal dysplasia also remains unclear. As expected, TRPV4 activation in osteoclasts increased the number of osteoclasts and their resorption activity, thereby resulting in bone loss.

TRPV4 appears to be an important player in pathological sensory perception. In a study conducted with the antisense treatment targeting TRPV4, Taxol-induced mechanical hypersensitivity was completely reversed [75]. As chemotherapy-induced neuropathy is a common and potentially irreversible side effect of certain chemotherapeutic agents, these results suggest a potential mechanism by which this side effect might be ameliorated.

Other studies with both knockout animals and antisense administration have indicated a role for TRPV4 in inflammatory pain, but not in normal pain sensing. These intriguing findings indicate the potentially key role of TRPV4 in chemotherapy-induced neuropathy [6, 76]. It appears relevant that pain or sensitivity due to cutaneous sensory neurons is increased through the interaction between the TRPV4 channel and F-actin, and this interaction is enhanced by phosphorylation.

This observation suggests that the regulation of the interaction between the TRPV4 channel and F-actin is a potential site for interventions that can alleviate pain. It also appears that the activation of TRPV4 by the phosphorylation of its Ser824 residue is related to pain sensitization by promoting TRPV4 to associate with F-actin [20]. Dynamic arrangements of adhesion molecules and cytoskeletal components as well as keratinocyte differentiation take place in both the early and late stages of cell-cell junction development.

A variety of stimuli activate members of the TRPV subfamilies. Vanilloid compounds (such as capsaicin, the compound responsible for the spiciness of hot chili peppers) and moderate heat are able to activate TRPV4 channels which seem to function as integrators of a variety of painful stimuli. The predominant localization of TRPV4 at the apical side of keratinocytes suggests that TRPV4 must be located in close proximity to the tight junction (TJ) barrier to exert its regulatory functions [77]. Abnormal adheren junctions (AJs) drastically impair the TJ-dependent barrier, thereby suggesting that TRPV4 deficiency-induced AJ abnormalities could result in impaired functionality of the TJ-dependent intercellular barrier. TJs are also regulated by Rho via interaction between the F-actin cytoskeleton and TJ components. These authors also suggested that TRPV4 binds to β-catenin, an adaptor protein that links intercellular adhesion molecules (E-cadherin) and the cytoskeleton (F-actin fibers).

CONCLUSION AND PERSPECTIVE

The TRPV4 functional Ca^{2+} channel consists of homo tetramer subunits. Recently, it was reported that TRPV4 and TRPC1 (TRPA5 or TRPP2) can co-assemble to form heteromeric TRPV4–C1 channels [78]. The ankyrin repeat of TRPV4 is responsible for their self-assembly into channels in cell lines, and the mutations in the TRPV4 ankyrin domain also seem to affect its channel assembly in humans as shown in many dominant negative genetic disorders [79].

Originally, TRPV4 was shown to be activated by hypotonicity, but later works have demonstrated that activation can also be achieved by phorbol esters, arachidonic acid (AA) and moderate heat [80]. TRPV4 appears to be an important player in pathological sensory perception and bone growth. The potential effects of mutations on TRPV4 function remain to be elucidated in the future. Furthermore, the role of TRPV4 in the pathogenesis of diseases should be characterized by how the channel protein contributes to the specific disease [6, 81]. This information may be useful in developing treatments that could cure or alleviate human diseases caused by TRPV4 mutations.

It was assumed that the physiological role of the S824 phosphorylation of TRPV4 by protein kinases was related to TRPV4 regulation from cell migration, adhesion, or polarity reorientation [20]. Regardless of the protein kinase involved, it seems that the phosphorylation of the S824 residue modifies several TRPV4 functions including cell surface expansion, affinity to cytoskeleton protein, and channel activity. The TRPV4 C-terminal region seems to be the binding site for its inhibitory proteins, which are not yet characterized**.** Tubulin, IP3 receptor or CaM are candidate proteins which participate in this negative regulation. Future studies will provide more information regarding how the modulation of the Ser824 residue of TRPV4 can function as a major target in treating several human diseases, including pain sensitivity. Thus, the identification of the proteins that interact with the C-terminal of TRPV4 will help to elucidate the mechanism by which TRPV4 is regulated in the normal cell and in human diseases caused by abnormal osteoclast differentiation. However, further studies of the TRPV4 regulation mechanism will be required in order to clearly elucidate the physiological role and activation mechanism of this channel. Our results strongly indicate that the interaction of TRPV4 with F-actin is enhanced via phosphorylation of the Ser824 residue, resulting in the expansion of the

cell surface area and enhanced cell survival. Originally, TRPV4 was shown to be activated by hypotonicity, but later works have demonstrated that activation can also be achieved by phorbol esters, arachidonic acid (AA) and moderate heat. Thus, the interaction between F-actin and TRPV4 appears to have structural, rather than functional, significance. TRPV4 acts further downstream in the signaling cascade. However, many physiological SGK1 substrates remain to be identified, and the manner in which they are modulated in the presence of SGK1 remains to be clearly elucidated [20].

The intensive study elucidates a role for TRPV4 in bone remodeling and shows that osteoclast differentiation is not only dependent on intracellular Ca^{2+} release, but also relies on extracellular Ca^{2+} influx mediated by TRPV4 [6, 82]. However, the outstanding questions are still unclear. What are the *in vivo* stimuli for TRPV4 activation in osteoclasts? How is the communication between Ca^{2+} influx and intracellular Ca^{2+} release mediated in osteoclasts? How is $[Ca^{2+}]_i$ regulated in large multinuclear osteoclasts? In order to elucidate the role of TRPV4 in osteoclasts, the proteins which interact with TRPV4 need to be identified and their post-translational modifications characterized. The characterization of how TRPV4 mutants cause specific bone diseases can provide clues to the channel's normal physiological roles. Since the genetic ablation of TRPV4 impaired osteoclast activity without affecting osteoblast function, resulting in increased bone mass in adult mice, the pharmacological blockade of TRPV4 function may offer a potent therapy for bone diseases caused by excessive bone resorption [7].

Based on these reports, the following working model was proposed (Fig. 1). The TRPV4 gene has once again illustrated the concept of one gene-many diseases. Within the skeletal phenotypes, the spectrum ranges are remarkably wide, from isolated digital arthropathy to lethal hyperplastic MD. In addition, some mutations have been found to result in neuromuscular phenotypes or in combined phenotypes. Genetic counseling is based on the uniformly dominant inheritance of the phenotypes and prognosis is tied to the severity of the

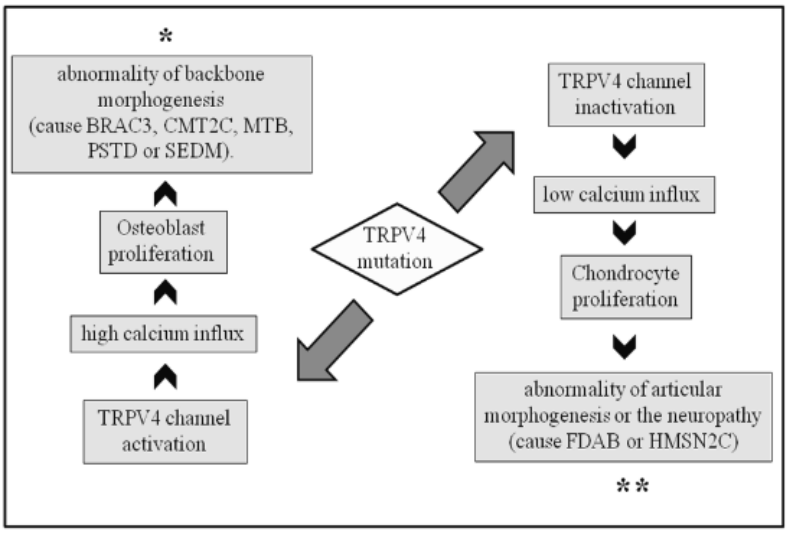

Fig. 1. Hypothetical etiological role of TRPV4 mutant in human genetic diseases.
*RANKL stimulation by TRPV4 activation evokes Ca^{2+} oscillations originating from alternate Ca^{2+} release from and reloading into intracellular stores. The event seems to induce NFATc1-dependent gene transcription in the osteoclast, resulting in the cell abnormal proliferation and the bone loss.
**The inactive mutations in TRPV4 can result in decreased SOX9 function in chondrocytes, which can lead to abnormal proliferation and joint abnormalities, ultimately resulting in diseases such as SMA/HMSN2C. The disease might be caused by abnormalities in articular morphogenesis. The enhancement of SOX9 seems to inhibit the proliferation of chondrocyte.

individual forms. In the future, a pharmacologic approach to the modulation of TRPV4 dysfunction with the perspective of diminishing its phenotypic consequences seems possible, but requires a better understanding of the underlying pathogenesis of TRPV4-related disorders. Based on the notion that TRPV4 causes many human genetic diseases, it will be valuable to study how or when the channel activity should be modulated in patients by pharmacologic approaches. The pathogenetic mechanisms leading from TRPV4 activation or dysfunction to skeletal dysplasia remain unexplained. Fortunately, the relatively specific radiographic features of these disorders allow for a confident radiographic diagnosis in most cases, which can be confirmed by molecular means.

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