Review

TRIC-B Mutations Causing Osteogenesis Imperfecta

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Received July 29, 2016

Trimeric intracellular cation (TRIC) channel subtypes, namely TRIC-A and TRIC-B, are expressed in the endoplasmic/sarcoplasmic reticulum and nuclear envelope, and likely function as monovalent cation channels in various cell types. Our studies using knockout mice so far suggest that TRIC subtypes support Ca^{2+} release from intracellular stores by mediating counter-cationic fluxes. Several genetic mutations within the *TRIC-B* locus were recently identified in autosomal recessive osteogenesis imperfecta (OI) patients. However, the molecular mechanism by which the mutations cause human disease is not fully addressed. We found that *Tric-b*-knockout mice exhibit poor bone ossification and thus serve as an OI-model animal. Studies on *Tric-b*-knockout bones and cultured cell lines derived from the patients currently reveal the main part of the pathophysiological mechanism involved in the *TRIC-B*-mutated OI form. This mini-review focuses on the essential role of TRIC-B channels in bone ossification.

Key words bone development; endoplasmic reticulum; intracellular ionic channel; osteoblast; collagen synthesis

1. TRIMERIC INTRACELLULAR CATION (TRIC) CHANNELS

Our previous immuno-proteomic screen in muscle membrane systems found novel transmembrane proteins with indirect roles in the Ca^{2+} handling of endo/sarcoplasmic reticulum (ER/SR), such as mitsugumin 29, mitsugumin 23 and junctophilins.^{1–3)} TRIC channel subtypes have also been identified in this molecular search.⁴⁾ TRIC-A is preferentially expressed in excitable tissues, particularly in skeletal and cardiac muscle cells, and localized in the SR and nuclear envelope. Meanwhile, TRIC-B is ubiquitously distributed throughout tissues at moderate expression levels. In TRIC subtypes composed of *ca.* 300 amino acid residues, three membrane-spanning segments are predicted, and the amino- and carboxyl-termini are assigned to the luminal and cytoplasmic sides, respectively.⁴⁾ Moreover, TRIC-A preparations purified from both skeletal muscle and recombinant bacteria exhibit bullet-shaped homo-trimeric complexes, and form functional monovalent cation-selective channels in artificial lipid bilayers.^{4,5)} Skeletal and cardiac muscle SR contains the well-known decamethonium-sensitive SR K^+ channel,⁶⁾ which vaguely resembles TRIC subtypes in certain electrophysiological characteristics. However, TRIC subtypes are unlikely to form the authentic decamethonium-sensitive K^+ channel, because the recording frequency and gating features of the SR K^+ channel are not altered in SR preparations from *Tric-a*-knockout mice.^{7,8)}

Knockout mice so far indicate that TRIC subtypes exert important roles during ER/SR Ca^{2+} release mediated by ryanodine (RyR) and inositol-trisphosphate receptors (IP_3R). Double-knockout mice lacking both the subtypes die during early embryonic stages, and RyR-mediated Ca^{2+} release is weakened in the mutant cardiomyocytes.⁴⁾ In *Tric-a*-knockout mice, the mutant vascular smooth muscle shows poor RyR-mediated Ca^{2+} sparks,^{9,10)} and the mutant skeletal muscle exhibits ‘alternan’ contractile responses which are likely

generated due to compromised RyR-mediated Ca^{2+} release.¹¹⁾ Moreover, *Tric-b*-knockout mice die immediately after birth due to respiratory failure, and the mutant alveolar epithelial cells exhibit insufficient handling of surfactant lipids, probably due to diminished IP_3R -mediated Ca^{2+} release.¹²⁾

Both RyRs and IP_3Rs mediate Ca^{2+} release, rapidly activating and lasting for several milliseconds under physiological conditions. Such efficient Ca^{2+} release events would not be possible if only Ca^{2+} moved across the store membrane, because the initial efflux of the divalent cations would quickly generate negative membrane potential at the luminal side to inhibit subsequent Ca^{2+} release processes. Previous studies predicted that other ion species, such as K^+ , Mg^{2+} , Cl^- and/or H^+ , may provide counter-currents to suppress the membrane potential generation.^{13,14)} However, real channels/transporters generating the counter-currents had not yet been identified. Considering that TRIC subtypes are localized to Ca^{2+} stores as monovalent cation-selective channels, the observations from the knockout mice suggest that TRIC channels could provide certain aspects of the counter-ion movement during ER/SR Ca^{2+} release (Fig. 1A). This hypothesis is also supported by data from the fluorometric membrane potential monitoring of SR vesicles from skeletal muscle.⁴⁾ Ca^{2+} -loaded *Tric-a*-knockout vesicles under ATP-containing conditions displayed a slightly negative potential compared to wild-type vesicles. This negative potential was increased by Ca^{2+} leakage triggered by a Ca^{2+} -pump inhibitor, while application of a K^+ ionophore eliminates the difference in membrane potential between the knockout and wild-type vesicles. Therefore, *Tric-a* deficiency seems to alter membrane potential due to reduced K^+ permeability in the muscle SR.

2. OSTEOGENESIS IMPERFECTA (OI)

OI is a group of divergent hereditary diseases characterized by low bone mass, and leads to increased bone fragility. The

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Table 1. Classified and Unclassified OI Forms

OI type	Defective gene	Defective protein	Primary defect in osteoblast	OI phenotype
<u>Dominant inheritance:</u>				
Classical sillence type				
I	COL1A1 (null)	$\alpha 1$ collagen	Collagen synthesis	Mild
II	COL1A1/COL1A2	$\alpha 1 / \alpha 2$ collagen	Collagen structure	Lethal
III	COL1A1/COL1A2	$\alpha 1 / \alpha 2$ collagen	Collagen structure	Progressive
IV	COL1A1/COL1A2	$\alpha 1 / \alpha 2$ collagen	Collagen structure	Moderate
COL1-mutation negative				
V	IFITM5	Interferon induced transmembrane protein 5	Interaction with prolyl isomerase FKBP11?	Mild to moderate
<u>Recessive inheritance:</u>				
Mineralization defect				
VI	SERPINF1	Pigment epithelium-derived factor	Phospholipase A2 signaling?	Moderate to severe
Hydroxylation defect				
VII	CRTAP	Prolyl-3-hydroxylase family member 5	Collagen hydroxylation	Severe to lethal
VIII	LEPRE1	Prolyl-3-hydroxylase-1	Collagen hydroxylation	Severe to lethal
IX	PPIB	Peptidyl-prolyl isomerase B	Collagen hydroxylation	Moderate to lethal
Chaperone defect				
X	SERPINH1	Chaperone Hsp47	Collagen folding	Severe to lethal
XI	FKBP10	FK506-binding protein 10	Collagen telopeptide hydroxylation	Variable
Propeptide-cleavage defect				
XII	BMP1	Metalloproteinase BMP1	BMP2/4 activation	Severe
Unclassified form				
Transcription factor defect	SP7/OSX	Zinc finger transcription factor SP7	Cellular maturation	Moderate
Cation channel defect	TRIC-B/TMEM38B	Intracellular TRIC-B channel	ER ionic homeostasis	Moderate
Wnt signaling defect	WNT1	Proto-oncogene protein Wnt-1	Cellular maturation?	Progressive
ER stress transducer defect	CREBL1	bZIP transcription factor OASIS	COL1A1 transcription?	Severe

incidence of OI is approximately 1 in 20000 births, and a vast majority of the cases are due to defects in type I collagen. Several varied manifestations are often observed in OI cases, such as persistently blue sclera, shortened and bowed limbs, and brittle opalescent teeth, demonstrating the involvement of multiple OI-causing genes.¹⁵⁾ Based on clinical characteristics, including disease severity, Sillence *et al.* defined an authentic classification of OI subtypes in 1979; OI type I is the mild and major form exhibiting blue sclera, type II is the perinatal lethal form characterized by crumpled and beaded bones, type III is the progressive form maintaining normal sclera, and type IV contains moderate to severe cases with normal sclera.¹⁶⁾ However, with an increase in newly identified causative genes, OI is now shown to be a highly divergent disease with different inheritance patterns, variable severities and multifaceted clinical conditions, and thus Sillence's classification has been repeatedly revised. In parallel, genetics-based OI classifications were proposed,^{17,18)} but it became impossible to maintain pathological correlations between Sillence's types and OI-responsible genes, as shown in Table 1. To avoid

confusing implications in clinical practice, in 2009 the International Society of Skeletal Dysplasias released a standard classification, in which Sillence's typing is maintained as the prototypical form. In this new typing, OI cases are separated into five groups, and causative genes are simply listed in each group.^{19,20)}

In developing bones, active osteoblasts extensively produce bone matrix and matrix vesicles to facilitate ossification. Type I collagen is the main protein component of bone matrix, and consists of $\alpha 1$ and $\alpha 2$ chains, encoded by *COL1A1* and *COL1A2*, respectively. Mutations in both genes affect the structure and/or amount of bone matrix, leading to bone abnormalities with subclinical to lethal phenotypes. *COL1A1* and *COL1A2* mutations in OI cases often exert dominant negative effects, because mutant α chains assemble with normal chains during collagen biosynthesis and thus possibly impair the quality of bone matrix. The functional maturation of collagen requires multiple post-translational modification and folding processes, and various mutations in processing enzymes (*CRTAP*, *LEPRE1* and *PPIB*) and chaperone genes (*FKBP10*,

Biography

Hiroshi Takeshima obtained his B.S. at Tokyo University of Agriculture and Technology in 1983 and Ph.D. at Kyoto University in 1989, then received appointments at several institutes as junior and senior faculty member for academic training. During this period, he focused on identifying and characterizing new muscle membrane components, including ryanodine receptors, junctophilins and mitsugumin 53. After his appointment as Professor at Kurume University and Tohoku University, he moved to the Graduate School of Pharmaceutical Sciences at Kyoto University in 2006, and has been studying TRIC channels and other new membrane proteins.

Fig. 1. Proposed TRIC Function and OI Mutations at *TRIC-B* Locus

(A) Schematic diagram of TRIC channels in intracellular Ca²⁺ stores. Physiological Ca²⁺ release mediated by RyRs and IP₃R probably requires counter-ion movements to stabilize ER/SR potential. Previous studies predicted H⁺, K⁺ and Cl⁻ channels in the ER/SR, but their molecular identities and biological functions are unknown. (B) Schematic diagram of OI mutations in the human *TRIC-B/TMEM38* gene on Chromosome 9. The OI mutations 1, 2, 3, 4 and 5 (ref. 21–25) are shown.

SERPINH1 and *SERPINF1*) have been also identified in OI pedigrees. Moreover, OI-causing mutations are found in several collagen-unrelated genes, such as *SP7* (osteoblast-specific transcription factor Osterix), *WNT1* (signaling peptide WNT1) and *IFITM5* (osteoblast-specific transmembrane protein). Full function of these genes is probably important for developmental maturation in osteoblasts.

Recently, homozygous mutations in the *TRIC-B* (also referred to as *TMEM38B*) locus has been identified in OI pedigrees from different races. As shown in Fig. 1B, the Arabic mutant locus encodes a truncated TRIC-B protein lacking the carboxy-terminal half (mutation 1),^{21,22} whereas the 5'-terminal region encompassing exons 1 and 2 is deleted in the Albanian mutant locus (mutation 2).²³ Furthermore, three additional loci were recently reported in Chinese and American OI patients^{24,25}; a mutation disrupting the acceptor splice site of intron 3 (mutation 3), and mutations in exon 4 (mutation 4) and exon 1 (mutation 5). These critical OI-causing mutations presumably result in functionally-defective TRIC-B channels. Based on the phenotypes of 22 cases reported to date,^{21–24} OI patients bearing the *TRIC-B* mutations develop moderately severe OI, and have various fracture frequency, mildly or moderately short stature, gray to blue sclera and no tooth defect. However, the pathophysiological mechanism remains to be addressed in these OI patients.

3. OI-LIKE PHENOTYPE IN *Tric-b*-KNOCKOUT MICE

Ablation of the *Tric-b* gene produces an OI-like bone phenotype in mouse.²⁶ Although *Tric-b*-knockout mice are slightly smaller in whole body size than wild-type controls,¹² their overall skeletal formation is macroscopically normal.

Fig. 2. OI-Like Phenotype in *Tric-b*-Knockout Mice

(A) Immunocytochemical detection of procollagen in cultured osteoblasts. Procollagen-positive green signals formed meshwork structures in most wild-type osteoblasts (left panel), while enlarged deposits packed with procollagen (>5 μm in diameter) were frequently observed in *Tric-b*-knockout osteoblasts. Scale bar, 10 μm. (B) Schematic diagram representing the pathophysiology of *Tric-b*-knockout OI-like phenotype.

However, in the knockout mice, bone mineralization was remarkably poor; both calcium and collagen depositions were obviously decreased in major bones including the skull, ribs, and femur. Based on impaired ossification in both endochondral and intramembranous bones,²⁷⁾ it was hypothesized that inactivated osteoblasts and/or hyper-activated osteoclasts may be responsible for the OI-like phenotype. However, histochemical and gene expression analyses suggested that the knockout femoral bones regularly maintain bone-related cells, including osteoblasts and osteoclasts.

In electron-microscopic analysis of bone tissues, dilated rough ER elements, regressing Golgi bodies and sparse secretory vesicles were detected in *Tric-b*-knockout osteoblasts. In biochemical experiments using primary cultured osteoblasts, *Tric-b* deficiency significantly reduced the deposition of both extracellular mineral and collagen, but increased intracellular procollagen content. The excess procollagen fibers were immunocytochemically detected as dense cytoplasmic deposits within cultured *Tric-b*-knockout osteoblasts (Fig. 2A), and these deposits were also decorated with an antibody to the ER marker KDEL. These observations in primary cultured osteoblasts were fully consistent with the reduction of both collagen matrix and mineral depositions detected in major bones from *Tric-b*-knockout mice. *Tric-b* deficiency probably induces the ER retention of procollagen, leading to severe ER dilation, and thus inhibits collagen production in osteoblasts. In the same manner as the major human OI forms, an insufficient collagen matrix seems to be the causal factor of the OI-like phenotype in the knockout mice.

TRIC subtypes likely form monovalent cation channels implicated in the maintenance of Ca^{2+} -store function. In fluorometric Ca^{2+} imaging, cultured *Tric-b*-knockout osteoblasts exhibited impaired IP_3R -mediated Ca^{2+} release and store Ca^{2+} overloading. Additionally, the transfection of *Tric-b* cDNA rescued not only the altered store Ca^{2+} handling, but also ER dilation and collagen production in the cultured knockout cells. In general terms, ER dilation is an ultrastructural sign of ER stress, and store Ca^{2+} overloading often aggravates ER stress. Gene expression profiling suggested that the unfolded protein response (UPR) and ER-associated degradation (ERAD) are activated in *Tric-b*-knockout osteoblasts. It is likely that the excess accumulation of procollagen induces both ER stress and dilation.

Our experiments could not detect any abnormal features in *Tric-b*-knockout osteoclasts. Osteoclasts exhibited regular ultrastructures in *Tric-b*-knockout mice, and primary cultured osteoclasts derived from the knockout mice preserved normal cell growth, maturation and bone resorption. Therefore, facilitated bone resorption was unlikely to contribute to the OI-like phenotype. Taken together, TRIC-B channels seem to have an essential role in the mass production of collagen in osteoblasts, and thereby *Tric-b* deficiency causes impaired collagen production and inhibits efficient bone mineralization (Fig. 2B).

4. ABNORMAL FEATURES IN CULTURED CELL LINES FROM *TRIC-B*-MUTATED OI PATIENTS

Cabral *et al.* currently reported altered features in cultured fibroblast and osteoblast cell lines derived from *TRIC-B*-mutated OI patients.²⁵⁾ Their biochemical and immunochemical experiments indicated that the *TRIC-B* mutations, correspond-

ing to mutation 1 and mutation 5 in Fig. 1B, results in functional null alleles. The human OI cells exhibited decreased synthesis, secretion and deposition of type I collagen. In addition, the assembly and lysyl-hydroxylation of procollagen fibers were impaired in the OI cells, and thus matrix collagen exhibited an unstable conformation. Moreover, the OI cells displayed enhanced ER stress; UPR-related proteins, such as activating transcription factor 4 (ATF4) and Bip/GRP78, were significantly up-regulated. These observations are consistent with the results from *Tric-b*-knockout mice.

In fluorometric Ca^{2+} measurements, the human OI cells showed impaired IP_3R -mediated Ca^{2+} release. However, in contrast to the observations in *Tric-b*-knockout osteoblasts, the resting cytosolic Ca^{2+} level was reduced and a response indicative of store Ca^{2+} overloading was not detected in the OI cells. The reason for this apparent discrepancy between the human OI cells and mouse *Tric-b*-knockout osteoblasts is unknown. However, based on the data from the OI cells, Cabral *et al.* concluded that TRIC-B mutations cause OI by dysregulation of ER Ca^{2+} homeostasis, impacting multiple collagen-specific chaperones and modifying enzymes in osteoblasts.²⁵⁾

5. FUTURE PERSPECTIVE

Current studies using knockout mice and human OI cells uncovered the essential role of TRIC-B in osteoblastic collagen production. *TRIC-B* mutations found in OI patients seem to result in functional null alleles (Fig. 1B). As in the knockout mice, an insufficient collagen matrix is reasonably predicted in patients bearing *TRIC-B* mutations. From a phenotypical point of view, it is worth mentioning the difference in lung function between the knockout mice and human OI patients. *Tric-b*-knockout mice exhibit birth asphyxia due to poor surfactant production in alveolar epithelial cells,¹²⁾ while no respiratory failure was reported in the OI patients.^{21–24)} It is unclear why this phenotypical difference is observed. In mouse alveolar cells, *Tric-a* expression is very low, and TRIC-B channels might predominantly mediate counter-ionic currents during ER Ca^{2+} release.¹²⁾ In contrast, compensatory activation of other channels/transporters, including TRIC-A channels, could preserve surfactant production in human alveolar cells. For better understanding the molecular basis of counter-ionic flows in non-excitable cells, it may be important to compare gene expression between mouse and human alveolar epithelial cells and to profile candidate channels/transporters in the ER.

In a few OI patients bearing *TRIC-B* mutations, bisphosphonate treatments seemed to effectively increase bone mineral density (BMD) and to reduce fracture incidence.²⁴⁾ However, in order to establish the clinical benefit, the efficacy of such medications needs to be objectively and statistically evaluated in model experimental systems. *Tric-b*-knockout mice probably provide a useful model system towards the development of medical treatments for human patients. As explained above, the impaired folding and excess retention of procollagen in the ER are recognized as an essential cause of the TRIC-B-mutated OI form. Although the detailed mechanism is still unclear, altered Ca^{2+} handling is suspected of contributing to the procollagen retention. A majority of ER luminal enzymes, including chaperones, are functioning in a Ca^{2+} -dependent manner, and may be inactivated under Ca^{2+} -overloaded

conditions. Therefore, disturbed procollagen processing and folding may lead to aggravated ER stress in *TRIC-B*-mutated osteoblasts. To facilitate protein-folding functions in the ER, chemical chaperones, including tauroursodeoxycholic acid and 4-phenylbutyrate,^{28,29} have been developed in recent years. Such chemical chaperones could be candidates for medication to ameliorate OI conditions.

Acknowledgments This work was supported in part by the MEXT/JSPS (KAKENHI 15H04676, 15H05652) and the Keihanshin Consortium for Fostering the Next Generation of Global Leaders in Research (K-CONNEX).

Conflict of Interest The authors declare no conflict of interest.

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