The Role of Pannexin 3 in Bone Biology

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Abstract

Cell–cell and cell–matrix communications play important roles in both cell proliferation and differentiation. Gap junction proteins mediate signaling communication by exchanging small molecules and dramatically stimulating intracellular signaling pathways to determine cell fate. Vertebrates have 2 gap junction families: pannexins (Panxs) and connexins (Cxs). Unlike Cxs, the functions of Panxs are not fully understood. In skeletal formation, Panx3 and Cx43 are the most abundantly expressed gap junction proteins from each family. Panx3 is induced in the transient stage from the proliferation and differentiation of chondrocytes and osteoprogenitor cells. Panx3 regulates both chondrocyte and osteoblast differentiation via the activation of intracellular Ca^{2+} signaling pathways through multiple channel activities: hemichannels, endoplasmic reticulum (ER) Ca^{2+} channels, and gap junctions. Moreover, Panx3 also inhibits osteoprogenitor cell proliferation and promotes cell cycle exit through the inactivation of Wnt/ β -catenin signaling and the activation of p21. *Panx3*-knockout (KO) mice have more severe skeletal abnormalities than those of *Cx43*-KO mice. A phenotypic analysis of *Panx3*-KO mice indicates that Panx3 regulates the terminal differentiation of chondrocytes by promoting vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP) 13. Based on the generation of *Panx3*^{-/-}; *Cx43*^{-/-} mice, Panx3 is upstream of Cx43 in osteogenesis. Panx3 promotes Cx43 expression by regulating Wnt/ β -catenin signaling and osterix expression. Further, although Panx3 can function in 3 ways, Cx43 cannot function through the ER Ca^{2+} channel, only via the hemichannels and gap junction routes. In this review, we discuss the current knowledge regarding the roles of Panx3 in skeletal formation and address the potential for new therapies in the treatment of diseases and pathologies associated with Panx3, such as osteoarthritis (OA).

Keywords: gap junction proteins, osteoblast proliferation and differentiation, endochondral ossification, bone formation, Wnt/ β -catenin, Panx3 ER Ca^{2+} channel signaling

Introduction

Bone is a mineralized connective tissue with numerous important functions, including mechanical protection of organs, mineral storage, and hematopoiesis. Historically, the function of bone has been considered as a supporting tissue for the body's structure. Recently, however, bone has been regarded as not only a supporting tissue but also as an endocrine organ (About et al. 2002). For example, osteocalcin, the most abundant non-collagenous bone matrix protein, regulates insulin sensitivity and secretion, and influences brain development (About et al. 2002). In addition, fibroblast growth factor 23, which is produced in bone and secreted into the circulation, can potentially regulate systemic phosphate, 1,25-dihydroxyvitamin D, and parathyroid hormone (PTH) homeostasis for maintaining Ca^{2+} homeostasis in blood (Guntur and Rosen 2012).

Skeletal formation occurs through 2 highly coordinated processes: endochondral ossification and intramembranous ossification. Endochondral ossification, observed in most bones, consists of early cartilage formation that is replaced by bone, whereas intramembranous ossification, observed in the skull and in the maxillomandibular bones, involves the direct differentiation of mesenchymal cells into osteoblasts that deposit bone matrix proteins.

Bone cells, such as osteoblasts and osteoclasts, orchestrate bone development. The initial step in endochondral ossification is the condensation of mesenchymal cells, which differentiate

into proliferative chondrocytes. This is followed by their further differentiation to hypertrophic chondrocytes, which are later replaced by bone cells. Osteoblasts originate from osteoprogenitors, which give rise to multi-potential mesenchymal stromal cells (Oury et al. 2013). Osteoblasts play an important role in the formation, deposition, and mineralization of bone tissue (DiGirolamo et al. 2012). Differentiated osteoblasts secrete bone matrix proteins consisting of collagen and non-collagenous proteins, such as glycoproteins and proteoglycans (Buckwalter et al. 1996). The deposited organic bone matrix is subsequently mineralized by replacement with hydroxyapatite, a complex of calcium phosphate $\text{Ca}_5(\text{PO}_4)_3\text{OH}$. Osteoclasts, multi-nucleated cells, are derived from mononuclear precursor

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cells of the monocyte–macrophage lineage (Long 2012). Activated osteoclasts are responsible for bone resorption.

Ca^{2+} is one of the most important second messengers regulating cell proliferation, differentiation, morphology, and function (Long 2012). The intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is maintained by membrane ion channels: the endoplasmic reticulum (ER), an intracellular Ca^{2+} storage organelle, channels such as inositol triphosphate 3 (IP_3) receptors (IP_3Rs), which are ubiquitously expressed and act as ER Ca^{2+} channels upon IP_3 binding (Boyle et al. 2003), and Ryanodine receptors (RyRs), present only in some tissues (Berridge et al. 2000). In addition, $[\text{Ca}^{2+}]_i$ is also regulated by the intracellular concentration of other ions, such as K^+ and Na^+ . The circulation of intracellular Ca^{2+} between the cytosol and the ER is indirectly regulated by the trimeric intracellular cation (TRIC) channel, which controls transmembrane K^+ flux to maintain electron neutrality across the ER membrane (Fill and Copello 2002). $\text{Ca}^{2+}/\text{K}^+$ flux plays a role in the synthesis and secretion of additional matrix components, including collagen. Type I collagen is the major scaffolding protein of the extracellular matrices of skin, tendon, and bone (Matchkov et al. 2007), and its synthesis is regulated by intracellular Ca^{2+} signaling that involves the activation of IP_3R , RyR , or the sarcoplasmic-ER Ca^{2+} -ATPase type 2b (SERCA2b), which promotes Ca^{2+} influx into the ER (Venturi et al. 2013). ER Ca^{2+} homeostasis mediates the expression and activities of multiple collagen-interacting proteins and the process of collagen biosynthesis. Thus, it is possible that $\text{Ca}^{2+}/\text{K}^+$ flux controls osteoblast proliferation and differentiation.

Cell–cell and cell–matrix communication regulates the activation of signaling pathways involved in cell function, proliferation, differentiation, and death. Gap junction proteins play important roles in such cellular communication as well as in bone development and homeostasis. Here, we review the recent findings on pannexin 3 (Panx3), a new member of the gap junction protein family, in both cartilage and bone development.

Pannexin and Connexin Gap Junction Protein Families

Gap junctions mediate intracellular signaling events, which regulate various downstream cellular and physiological functions. Gap junction proteins allow the exchange of ions and small molecules, such as Ca^{2+} , ATP, and IP_3 , between adjacent cells, and between cells and the extracellular space via hemichannels. In vertebrates, there are 2 gap junction protein families: connexins (Cxs) and pannexins (Panxs). Cxs and Panxs contain common structural features, including 4 transmembrane

Figure 1. Gap junction protein structure and activities. **(A)** Common structure of gap junction proteins (Panxs and Cxs). Each Panx and Cx monomer consists of 2 extracellular loops, 4 transmembrane domains, and 1 intracellular loop (upper). Hexamers of each subunit form a channel structure to exchange small molecules, such as ion and other messenger molecules (bottom). **(B)** Gap junction protein functions. Both Panxs and Cxs function as hemichannels and gap junctions. Panxs also function as ER Ca^{2+} channels. Secondary messenger molecules, such as Ca^{2+} , ATP, and IP_3 , are transferred between cells via gap junctions (upper) and released from the cell to the extracellular space via hemichannels (middle). Panxs function as the ER Ca^{2+} channel to increase intracellular Ca^{2+} levels released from the ER, the major Ca^{2+} storage organelle.

domains, 2 extracellular loops, 1 intracellular loop, and N- and C-terminal segments. Further, each extracellular loop in Cxs and Panxs comprises 3 and 2 cysteine residues, respectively. The tetramer of the subunit forms a channel structure, which is regulated by extracellular signaling (Fig. 1). Both Panxs and Cxs function as a gap junctions and hemichannels, transferring small molecules, such as ATP, into the extracellular space. Unlike Cxs, Panxs also function as an ER Ca^{2+} channel (Fig. 1).

The Cx family has more than 20 members and has been relatively well characterized. Ca^{2+} dysfunction and mutations in Cxs cause several human diseases, including cancer, hypertension, atherosclerosis, and developmental abnormalities. For example, mutations in Cx43 cause oculodentodigital dysplasia (ODDD), which shows multiple, variable craniofacial, limb, ocular, and dental anomalies. The Panx family, by comparison, is not well characterized and consists of only 3 members: Panx1, -2, and -3. Panx1 is ubiquitously expressed, especially in the central nervous system (Fallahi et al. 2005). Panx2 is also expressed in the central nervous system (Corbett and Michalak 2000), whereas Panx3 is expressed in skin, cochlea, and in developing hard tissues, including cartilage, bones, and teeth (Bruzzone et al. 2003).

Panx3 Functions in Chondrogenesis

In growth plates, Panx3 is expressed in the prehypertrophic and hypertrophic zones of the cartilage area. Panx3 is induced by prehypertrophic chondrocytes and promotes chondrocyte

Figure 2. Functions and its signaling pathways of Panx3 in osteoblast differentiation. The Panx3 hemichannel releases intracellular ATP into the extracellular space. This released ATP binds to purinergic receptors in an autocrine or paracrine manner. This activates phosphatidylinositol 3-kinase (PI3K)/Akt signaling, which in turn activates the Panx3 ER Ca^{2+} channel and releases Ca^{2+} from the ER. Upon Ca^{2+} binding, calmodulin (CaM) activates calcineurin (CN) signaling pathways. This activates the transcription factor nuclear factor of activated T-cells calcineurin-dependent 1 (NFATc1) by CN-mediated dephosphorylation. Activated NFATc1 then translocates into the nucleus and promotes Osterix expression. This in turn induces the expression of osteoblast genes, such as alkaline phosphatase (ALP) and osteocalcin (Ocn). Panx3 also promotes degradation of p53, an inhibitor of osteoblast differentiation, by activating the Akt/mouse double minute 2 homolog (MDM2) pathway. In addition, the Panx3 gap junction propagates intracellular Ca^{2+} to neighboring cells to promote osteoblast differentiation.

differentiation, while reducing the proliferation of chondrocytes. Panx3 can function as an ATP-releasing hemichannel, an ER Ca^{2+} channel, or a gap junction to pass intracellular Ca^{2+} to neighboring cells. During PTH-induced reduction in chondrocyte proliferation, the Panx3 hemichannel releases intracellular ATP following a reduction in intracellular cAMP levels. This causes the inhibition of PKA/cAMP response element-binding protein (CREB) signaling, which is an essential pathway for cell proliferation (see Fig. 5) (Vogt et al. 2005; Ray et al. 2006). It remains unclear how Panx3 regulates this signaling pathway to promote the differentiation of prehypertrophic chondrocytes to hypertrophic chondrocytes. However, because extracellular ATP levels regulate the differentiation of chondrocytes via increasing $[\text{Ca}^{2+}]_i$ (Penuela et al. 2007; Iwamoto et al. 2010; Ishikawa et al. 2011; Lohman et al. 2012), Panx3 may regulate $[\text{Ca}^{2+}]_i$ through the ER Ca^{2+} channel function of Panx3 in this chondrocyte differentiation process.

Panx3 Functions in Osteogenesis

In growth plates, Panx3 is also highly expressed in bone and in the perichondrium/periosteum, which includes preosteoblasts and osteoblasts (Iwamoto et al. 2010). Osteoblasts differentiate from preosteoblasts, which are derived from mesenchymal stem cells. Essential growth factors for osteoblast differentiation, such as bone morphogenetic protein 2 (BMP2), are responsible for the induction of 2 master transcription factors:

Runx2 and osterix (Osx). Their activation results in activation of downstream osteogenic marker genes and the subsequent terminal differentiation of osteoblasts. For osteogenesis, Panx3 expression is induced in the transition stage from cell proliferation to differentiation, and promotes osteoblast differentiation (Hung et al. 1997). As previously indicated, Panx3 can function as 3 different channels in bone: a hemichannel, ER Ca^{2+} channel, or gap junction (Ishikawa et al. 2011; Ishikawa et al. 2016). The initiation function of Panx3 is as a hemichannel to release intracellular ATP into the extracellular space (Fig. 2). The released ATP in the microenvironment binds to purinergic receptors in an autocrine or paracrine manner. This binding activates phosphatidylinositol 3-kinase (PI3K)/Akt signaling, which in turn opens the Panx3 ER Ca^{2+} channel to release Ca^{2+} from the ER lumen. Consequently, intracellular Ca^{2+} increases following the activation of the ER Ca^{2+} channel. Upon Ca^{2+} binding, calmodulin (CaM) activates downstream signaling molecules, such as calmodulin kinase II (CaMKII) and the phosphatase calcineurin (CN). The nuclear factor of activated T-cells calcineurin-dependent 1 (NFATc1) transcription factor is activated through dephosphorylation by CN, resulting in the nuclear translocation of NFATc1 (Tomita et al. 2002; Ishikawa et al. 2011; Ishikawa et al. 2014). Active NFATc1 can promote the expression of genes, such as Osx, a key molecule involved in osteogenesis (Koga et al. 2005; Ishikawa et al. 2011; Esseltine and Laird 2016), by inducing osteogenic markers, like alkaline phosphatase (ALP) (Beals et al. 1997; Koga et al. 2005) (Figs. 2 and 5). Additionally, Panx3 hemichannel-activated Akt stimulates the degradation of p53, a negative regulator for osteoblast differentiation through E3-ubiquitin protein ligase-mediated activation of mouse double minute 2 homolog (MDM2) (Nakashima et al. 2002; Koga et al. 2005; Ishikawa et al. 2011) (Fig. 2).

Wnt/ β -catenin signaling is important for osteoprogenitor cell proliferation and plays a role in differentiating mature osteoblasts, especially during mineralization (Ishikawa et al. 2011). BMP2 antagonizes Wnt/ β -catenin (Ogawara et al. 2002; Lengner et al. 2006), and Wnt/ β -catenin signaling is reduced in the early stages of osteoblast differentiation. However, how these changes occur is unknown.

Panx3 inhibits Wnt/ β -catenin signaling by promoting β -catenin degradation through the activation of glycogen synthase kinase 3- β (GSK3 β) (Boyden et al. 2002; Kokubu et al. 2004; Glass et al. 2005; Morvan et al. 2006; Zhang et al. 2008; Guntur and Rosen 2012) (Fig. 3). This Panx3-mediated inhibition of Wnt/ β -catenin signaling is regulated by the Panx3

hemichannel (Fujita and Janz 2007; Zhang et al. 2008; Zhang et al. 2013) (Figs. 3 and 5). Additionally, the Panx3 ER Ca^{2+} channel induces the expression and activation of p21, a cell cycle inhibitor through the CaM/Smad pathway, which promotes cell cycle exit (Ishikawa et al. 2014) (Figs. 3 and 5).

BMP2 promotes Panx3 expression, which is reduced at a later stage of osteoblast differentiation (Ishikawa et al. 2014). This reduction in Panx3 expression likely turns on Wnt/ β -catenin signaling. Thus, the antagonizing mechanism between BMP2 and Wnt/ β -catenin may explain Panx3 expression and functions. Collectively, Panx3 is a new regulator that promotes the switch from proliferation to differentiation in both osteoblasts and chondrocytes (Bellosta et al. 2003; Pardali et al. 2005) (Figs. 3 and 5).

In Vivo Functions of Panx3 in Skeletal Development

We recently generated *Panx3*-KO mice to identify the in vivo functions of Panx3. *Panx3*-KO mice show short axial and appendicular skeletal abnormalities at the newborn stage (Ishikawa et al. 2011; Ishikawa et al. 2016) (Fig. 4). These abnormalities are caused by increases in the proliferation of chondrocytes and osteoprogenitors, and the inhibition of differentiation of these cells, and these findings are consistent with the in vitro findings in which Panx3 regulates chondrogenesis and osteogenesis with its multiple functions (Iwamoto et al. 2010; Ishikawa et al. 2011; Ishikawa et al. 2014). In the growth plates of *Panx3*-KO mice, we see an increase in the number of Ki67-positive cells (a marker of proliferating cells) within the proliferative chondrocyte zone, perichondrium, periosteum, bone collar, and trabecular bone as compared with that in their WT counterparts. The number of Ki67-positive cells also increases in the calvariae of newborn *Panx3*-KO mice relative to that in WT mice. These results indicate that Panx3 deficiency increases the proliferation of chondrocytes and osteoprogenitor cells (Ishikawa et al. 2016) (Fig. 5).

Unexpectedly, we have found that *Panx3*-KO mice show inhibited terminal differentiation of hypertrophic chondrocytes (Fig. 5). Mature hypertrophic chondrocytes form a few cell layers at the end of the growth plate adjacent to the chondro-osseous junction (Iwamoto et al. 2010; Ishikawa et al. 2011; Ishijima et al. 2012; Ishikawa et al. 2016). The matrix surrounding mature hypertrophic chondrocytes becomes mineralized, and the chondrocytes are replaced with osteoblasts in endochondral ossification. This cartilage replacement is necessary to recruit osteoclasts from blood vessels, which remove the cartilage matrix and apoptotic chondrocytes. The expression of matrix metalloproteinase (MMP)-13, vascular

Figure 3. Panx3 signaling pathways in the inhibition of osteoprogenitor cell proliferation and the promotion of cell cycle exit. The Panx3 hemichannel releases intracellular ATP, which reduces intracellular cAMP after protein kinase A (PKA)/CREB signaling is inhibited. The inactivation of PKA activates glycogen synthase kinase 3- β (GSK3 β), which causes β -catenin degradation. Thus, Wnt/ β -catenin signaling is inhibited, which inhibits cell proliferation. Likewise, PKA inactivation reduces CREB activity, which also inhibits cell proliferation. Additionally, PI3K/Akt signaling, activated by extracellular ATP from the Panx3 hemichannel, stimulates the Panx3 ER Ca^{2+} channel, which activates CaM/CaMK signaling. This activates Smad and p21, a cell cycle inhibitor, and thus promotes cell cycle exit.

Figure 4. Skeletal abnormalities of newborn *Panx3*-knock out (KO) mice (Ishikawa et al. 2016). Whole-body skeletal staining, using Alizarin red for bone and Alcian blue for cartilage in newborn wild-type (*Panx3*^{+/+}; left) and KO (*Panx3*^{-/-}; right) mice. Appendicular and axial bones, such as the limbs, skull, clavicles, spines, and ribs, were shorter in *Panx3*^{-/-} mice as compared with WT mice. Mineralization defects were also observed in *Panx3*-KO cranial vaults.

endothelial growth factor (VEGF), and osteopontin (OPN), markers of mature hypertrophic chondrocytes, is reduced in the growth plates of *Panx3*^{-/-} mice relative to that in WT

Figure 5. Panx3 regulates chondrogenesis and osteogenesis by promoting cell cycle exit and cell differentiation. During chondrogenesis and osteogenesis, Panx3 is induced in the transition stage from proliferation to differentiation of both chondrocytes and osteoblasts. Panx3 promotes differentiation of hypertrophic chondrocytes. Panx3 is also required for the mature, terminal differentiation stage of hypertrophic chondrocytes. In the osteogenesis cascade, Panx3 inhibits Wnt/ β -catenin signaling-mediated proliferation of osteoprogenitor cells and results in cell cycle exit. BMP2/Runx2 signaling induces Panx3 expression. Panx3 increases the expression of osterix (Osx), which subsequently induces osteocalcin and Cx43. Other pathways are also reported. For example, Wnt/ β -catenin signaling activates bone morphogenetic protein-2 (BMP2) expression (Luft 2016). BMP-2 activates nuclear factor of activated T-cells calcineurin-dependent 1 (NFATc1) through an autoregulatory loop involving Smad/Akt/ Ca^{2+} signaling (Moon et al. 2015). BMP2 regulates Runx2 and Osx (Zhang et al. 2013).

controls (Ishikawa et al. 2016). VEGF expressed by mature chondrocytes is required for vascular invasion at the chondro-osseous boundary (Zelzer et al. 2004). In the *Panx3*^{-/-} growth plate, there is a reduction in the expression of the endothelial cell marker, CD31, and the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts, indicating reduced vascular invasion (Ishikawa et al. 2016). Although these *in vivo* analyses reveal that Panx3 regulates the differentiation of mature hypertrophic chondrocytes, the molecular mechanism and Panx3 signaling pathways involved in this differentiation process remain to be elucidated.

Recently, 3 other groups have generated and analyzed *Panx3*-KO mice. The first group generated *Panx3*-KO mice with a conditional strategy (cKO) using CMV-Cre for global KO and collagen type 2-Cre for cartilage-specific deletion. These mice showed fewer bone abnormalities than that of *Panx3*-KO mice (Moon et al. 2015). When they tested for osteoarthritis (OA) susceptibility using a surgical destabilization of the medial meniscus (DMM) model, *Panx3*-cKO mice showed fewer OA symptoms than that of WT. They found a strong increase in the expression of Panx3 in the OA model of the WT controls (Moon et al. 2015; Oh et al. 2015; Caskenette et al. 2016). Thus, the authors expect that Panx3 is a targeted molecule for the development of OA therapy.

The second group, using the same *Panx3*-cKO mice, reported bone abnormalities. They found that *Panx3*-cKO mice produced phenotypic effects in the femurs and humeri (Moon et al. 2015). The third group used a cKO strategy using EIIa-Cre, which is expressed from the early embryonic stage and in all cell types. Their *Panx3*-cKO mice displayed an abnormal differentiation of chondrocytes and a delay in osteoblast differentiation and mineralization. They also generated a knockdown of Panx3 in zebrafish using an antisense morpholino, which revealed cartilage deformities and delayed osteoblast differentiation and mineralization like that in the *Panx3*-cKO mice (Moon et al. 2015).

Although several phenotypic differences of *Panx3*-KO have been reported, the differing results may reflect variances in the Cre-mediated recombination or mouse strains.

Panx3 and Cx43 Regulate Skeletal Development through Their Distinct Expression Patterns and Functions

Panx3 and Cx43 are the 2 major gap junction proteins that are highly expressed during bone growth. *Cx43*-KO and *Cx43*-cKO mice have bone abnormalities, particularly, a delay in skull formation in the *Cx43* KO mice; although body and limb sizes in these mice appear normal at the newborn stage (Caskenette et al. 2016). Compared with *Cx43*-KO mice, *Panx3*-KO mice show obvious bone abnormalities at the newborn stage (Fig. 4). *Cx43* expression is reduced in the limbs and calvariae of *Panx3* KO newborn mice; however, Panx3 expression is normal in *Cx43*-KO mice, indicating that Panx3 is upstream of Cx43 (Oh et al. 2015) (Fig. 5). Interestingly, *Panx3*^{-/-} and *Cx43*^{-/-} mice show a similar body size to that of *Panx3*-KO mice; this also supports Panx3 being upstream.

Panx3 regulates Cx43 expression through Wnt/ β -catenin signaling and Osx pathways. Panx3 inhibits Wnt/ β -catenin signaling by promoting β -catenin degradation via GSK3 β activation at the osteoprogenitor proliferation stage. Panx3 expression is gradually reduced as immature osteoblasts differentiate into mature osteoblasts. This causes a resumption of Wnt/ β -catenin signaling because the synthesis of β -catenin mRNA continues during osteoblast development. Wnt/ β -catenin signaling regulates Cx43 expression (Lecanda et al. 2000; Ishikawa et al. 2016), and this resumption causes an increase in Cx43 expression. Furthermore, Panx3 increases Osx expression by upregulating intracellular Ca^{2+} levels, which activates CaM/NFAT

signaling. *Osx* increases Cx43 expression, and thus these multiple activations of Wnt/ β -catenin signaling and *Osx* by Panx3 regulate Cx43 expression during osteoblast differentiation.

Functional differences between Panx3 and Cx43 have also been identified. Panx3 functions via the ATP-releasing hemichannel, ER Ca^{2+} channel, and gap junction to transfer intracellular Ca^{2+} to neighboring cells. By contrast, Cx43 only has hemichannel and gap junction activities (Ishikawa et al. 2016). We previously showed that Panx3, but not Cx43, is localized in the ER and functions as an ER Ca^{2+} channel (van der Heyden et al. 1998). The structural differences in Panx3 and Cx43 may be responsible for their functional difference. However, it is still unclear why there is a difference in the ER Ca^{2+} channel activity between these 2 proteins (Ishikawa et al. 2016).

Rescue experiments for osteoblast differentiation using calvariae from *Panx3*-KO and *Cx43*-KO in cultures reveal that Panx3 can replace Cx43 function, but Cx43 is unable to replace Panx3 function (Ishikawa et al. 2011). This is because Panx3 regulates Cx43 expression, and the Panx3 ER Ca^{2+} channel is essential for osteoblast differentiation. Cx43 likely plays an important role during mineralization in the mature osteoblast stage. Another distinct difference between Panx3 and Cx43 is that Panx3, but not Cx43, is expressed in cartilage. This difference is reflected in the shortened limbs of *Panx3*-KO mice and the normal limb sizes of *Cx43*-KO mice at the newborn stage. Though the body size of *Panx3*^{-/-}; *Cx43*^{-/-} mice is like that of *Panx3*-KO mice, *Panx3*^{-/-}; *Cx43*^{-/-} mice show more severe skeletal abnormalities than *Panx3*-KO or *Cx43*-KO mice. Thus, Cx43 is likely controlled by regulatory mechanisms other than Panx3.

Ubiquitously expressed IP3R1, -2, and -3 function as ER Ca^{2+} channels upon IP3 binding (Caskenette et al. 2016). RyRs also function as ER Ca^{2+} channels in some tissues (Ishikawa et al. 2016). All IP3Rs, but not all RyRs, are expressed in the osteoblast lineage (Mikoshihara 2007). Unlike Panx3, IP3Rs are expressed continuously from the mesenchyme during osteogenesis. Although mice lacking IP3R2, IP3R3, or both, are born with no obvious skeletal abnormalities (Fill and Copello 2002), it is still possible that IP3Rs may play a role in bone formation and in homeostasis with Panx3 and Cx43.

Gap Junction Proteins in Tooth Development

Tooth abnormalities of patients with ODDD caused by Cx43 include small teeth, enamel hypoplasia, and multiple cavities, which results in early tooth loss (Powell et al. 2001). Cx43 is expressed in both dental epithelial cells and mesenchymal cells and regulates both ameloblast and odontoblast differentiation (Futatsugi et al. 2005; Kuroda et al. 2008). Cx43 functions as an ATP-releasing hemichannel and a gap junction to transfer Ca^{2+} to neighboring cells (Paznekas et al. 2003). These functions of Cx43 regulate the proliferation and differentiation of dental epithelial and mesenchymal cells (Kagayama et al. 1995; About et al. 2002; Toth et al. 2010). For example, in odontoblast differentiation, Cx43 plays a role in the mineralization of dentin though its gap junction function (Lecanda et al. 2000; Saez et al. 2005; Cherian et al. 2008). However, the

molecular mechanisms of the Cx43 in both ameloblast and odontoblast differentiation remain unclear. In *Cx43*-KO mice, the incisors are less prominent at birth, although teeth are present (Furlan et al. 2001; Gramsch et al. 2001; Toth et al. 2010).

On the other hand, Panx3 is only expressed in dental mesenchymal cells and in odontoblasts (unpublished findings). Panx3 is expressed in the transition stage from cell proliferation to differentiation and is induced during odontoblast differentiation, as seen in chondrocyte and osteoblast development. In *Panx3*-KO mice, tooth eruption is delayed, and tooth size is also small. Further in vivo and in vitro analyses are needed to identify the how Panx3 functions in odontoblast development and in tooth morphogenesis. Earlier reports indicated that Panx3 is expressed in human odontoblast-like cells and functions as an ATP releasing hemichannel (About et al. 2002). It would be interesting to further study the Panx3 signaling pathways in odontogenesis.

Human Disease or Genetic Disorder

Human diseases linked to the *Panx* family, and especially to *PANX1*, are ischemia, stroke, overactive bladder, HIV infections, Crohn's disease, and platelet aggregation. Recently, a germline variant in *PANX1* has been associated with intellectual disabilities, severe hearing loss, and multiple other multi-system defects. The *PANX1* mutation may also be involved in Perrault syndromes and Woodhouse-Sataki syndrome (Shao et al. 2016). Panx3, on the other hand, has been linked to OA in a surgically induced mouse model of disabling degenerative joint disease associated with cartilage destruction, subchondral bone remodeling, and inflammation of the synovial membrane (Lecanda et al. 2000). Currently, there are no disease-modifying therapies for OA. In an OA mouse model, Panx3 is expressed in articular cartilage (Fu et al. 2015). Furthermore, in human OA patients, *Panx3* expression is highly upregulated (Moon et al. 2015). Thus, *Panx3* may be a potential target for new OA therapy. To date, human genetic disorders caused by *Panx3* mutations have not been reported. However, current reports may increase the focus on *Panx3* as a candidate gene contributing to genetic diseases and, by applying or inhibiting the multiple ways in which Panx3 functions, new medicines and therapies may be generated.

Conclusion and Future Perspectives

Recent in vitro and in vivo studies of Panx3 have introduced new concepts regarding skeletal formation based on how Panx3 promotes chondrogenesis and osteogenesis, including how Panx3 regulates signaling cascades, inhibits Wnt/ β -catenin signaling, and acts downstream of BMP2. Panx3 is upstream of Cx43, and these proteins have distinct roles in regulating each other. The animal studies described provide new evidence for the important differences between Panx and Cx43. Although there are no reports yet about the direct involvement of Panx3 in human disease or in genetic disorders, accumulating knowledge may ultimately provide new therapies and drug applications for diseases associated with ectopic

cartilage and bone formation. In addition to skeletal tissues, Panx3 is also expressed in other tissues, such as the epidermis and hair follicles. It would be interesting to study the role of Panx3 in the regeneration of these tissues. In addition, the roles of Panx3 in odontoblast development and in tooth morphogenesis remain to be studied. The molecular mechanisms of Panx3 in odontogenesis may be different from those in chondrogenesis and osteogenesis. Finally, it remains unknown how Panx3 channels are regulated. The phosphorylation and glycosylation of Panx3 may regulate the activities of Panx3 channels. Channel activity analyses of site-specific mutations at potential active sites of Panx3 phosphorylation and glycosylation will address these questions.

Author Contributions

M. Ishikawa, contributed to conception and design, drafted the manuscript; Y. Yamada, contributed to conception and design, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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