ability [44] to reduce the production costs and developing a genetically engineered mutant growth factors to improve the binding property to the extracellular matrix in order to prevent rapid diffusion.

BMPs have been shown to increase the formation of bone nodules *in vitro* [45–56] and stimulate bone formation *in vivo*. But the dosage applied in these clinical studies was several orders of magnitude higher than the concentration of naturally occurring BMPs [57]. It is believed that small animals require a much lower dose of BMPs to bridge bone defects in comparison to larger animals, although this correlation is expected to change appropriately for specific carriers [58]. Hydroxyapatite, natural bone mineral, collagen, gelatin hydrogels and other biodegradable polymers compose the range of carriers that are currently being investigated as vehicles for the implantation of osteogenic factors. More recently, the attention of researchers in the biomaterial field was directed at the relationship between tissue engineering and bone morphogenesis. The fundamental principle governing these investigations is the production of more "intelligent" materials that could influence protein pharmacokinetics to modulate the delivery of rhBMP-2 at the site of implantation, or to enhance osteogenesis with these factors by altering the geometry of the environment [59–62].

Recently several groups have developed genetically modified mutant rhBMP-2, which is generated from *E-coli*, and this rhBMP-2 possesses an improved binding property to the extracellular matrix in order to prevent its rapid diffusion [42,43]. Wurzler et al. reported a genetically engineered mutant rhBMP-2 (rhBMP-2 T4), which was developed with two additional repeats of a positively charged epitope, called the heparin-binding domain, in the N-terminal sequence. Bing et al. reported a genetically engineered collagen targeting rhBMP-2 (rhBMP-2-v), which was fused with collagen bonding peptide to the N-terminal of rhBMP-2. Their rhBMP-2-v contained a collagen-binding domain which modified from von Willebrand factor, and this collagen-binding domain was flanked by linker regions to minimize steric hindrance. Both genetically engineered mutant rhBMP-2 have been shown to have higher extracellular matrix binding and stronger osteoinductivity than the wild-type rhBMP-2 *in vitro* and *in vivo*. By concentrating at the targeted wound site, these BMP-2 mutants can be avoids being washed away by extracellular fluids, which will eventually lead to not only a more effective osteogenesis but also a reduction of the undesirable systemic side effects.

Therefore, many questions must be answered before the growth factors can attain widespread clinical usage. Knowledge of the cellular and molecular basis of the bone regenerative signaling pathways, and the development of appropriate carriers will certainly stimulate a great revolution in dentistry, thus allowing the dominance of regenerative over cicatricial processes. However, the number of well-designed blind and randomized clinical trials is still too small to establish the clinical protocols for the improvement of a recipient bone bed prior to implant placement, or to enhance the integration process of an implant. The dissimilarities in the experimental designs, as well as, the use of nonstandardized concentration of growth factors, or the type of carriers of growth factors by different authors make it difficult to compare the outcomes of the growth factor applications in implant dentistry. A better-designed RCT, using growth factors for intra-oral bone augmentation, especially longitudinal clinical studies with a wider patient population, is necessary. However, these studies in this paper will set a golden standard for examining the effect of following new therapeutics.

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Pannexin 3 promotes osteoblast differentiation through its function as an endoplasmic reticulum (ER) Ca^{2+} channel, a hemichannel and a gap junction

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Abstract

The pannexin proteins represent a new gap junction family. However, the cellular functions of pannexins remain largely unknown. Here, we demonstrate that pannexin 3 (Panx3) promotes differentiation of osteoblasts and ex vivo growth of metatarsals. Panx3 expression was induced during osteogenic differentiation of C2C12 cells and primary calvarial cells, and suppression of this endogenous expression inhibited differentiation. Panx3 functioned as a unique Ca^{2+} channel in the endoplasmic reticulum (ER), which was activated by purinergic receptor/PI3K/Akt signaling, following activation of calmodulin signaling for differentiation. Panx3 also formed hemichannels that allowed release of ATP into the extracellular space and activation of purinergic receptors following the activation of PI3K/Akt signaling. Panx3 also formed gap junctions and propagated Ca^{2+} waves between cells. Blocking the Panx3 Ca^{2+} channel and gap junction activities inhibited osteoblast differentiation. Thus, Panx3 appears to be a new regulator that promotes osteoblast differentiation by functioning as an ER Ca^{2+} channel and a hemichannel, and by forming gap junctions.

Introduction

Gap junctions mediate intracellular signaling events, which in turn regulate various downstream cellular and physiological functions (Bennett and Verselis, 1992; Scemes et al., 2007). Gap junction proteins allow ions and small molecules to pass between adjacent cells via gap junctions, and between cells and the extracellular space via hemichannels (Bruzzone et al., 2001; Unger et al., 1999). In vertebrates, gap junction proteins are categorized into two families, connexins (Cxs) and pannexins (Panxs) (Vinken et al., 2006). The connexin family has more than 20 members and has been relatively well characterized. Dysregulation and mutations of connexins cause several human diseases, including cancer, hypertension, atherosclerosis and developmental abnormalities (Laird, 2006). The pannexin family is less well known and consists of only 3 members, Panx1, 2, and 3 (Baranova et al., 2004; D'Hondt et al., 2009; Panchin et al., 2000). Panx1 is ubiquitously expressed, especially in the central nervous system. Panx2 is also expressed in the central nervous system (Bruzzone et al., 2003). Panx3 is expressed in skin, cochlea, and in developing hard tissues including cartilage and bone (Iwamoto et al., 2010; Penuela et al., 2007; Penuela et al., 2008; Wang et al., 2009). Panx3 is induced in the prehypertrophic zone in developing growth plates, and it inhibits PTH-mediated chondrocyte proliferation through its hemichannel activity and promotes differentiation in culture (Iwamoto et al., 2010). Panx3 expression is also known to inhibit proliferation of keratinocytes (Celetti et al., 2010), although the underlying mechanism has not yet been established.

Ca^{2+} is a universal intracellular signaling molecule that regulates cell proliferation, differentiation, morphology, and function (Berridge et al., 2000b). Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) can rise more than 5-fold via Ca^{2+} influx from the extracellular space and/or release from the endoplasmic reticulum (ER), an intracellular Ca^{2+} storage organelle, when cells are activated by extracellular stimuli. Inositol trisphosphate 3 (IP3) receptors (IP3Rs) are ubiquitously expressed and act as ER Ca^{2+} channels upon IP3 binding (Mikoshiba, 2007). IP3 synthesis for activation of IP3R ER channels can be induced by many stimuli. For example, external ATP can bind purinergic receptors (P2Rs) in the plasma membrane, and this triggers activation of phospholipase C (PLC) and subsequent IP3 generation. Ryanodine receptors are also known to function as ER Ca^{2+} channels in some tissues (Fill and Copello, 2002). More recently, Panx1 was unexpectedly found to function as an ER Ca^{2+} channel in prostate cancer cells (Vanden Abeele et al., 2006).

The Ca^{2+} binding protein calmodulin (CaM) is one of the major Ca^{2+} signaling mediators (Berridge et al., 2000b), and the CaM pathway regulates osteoblast differentiation (Zayzafoon, 2006). Osteoblasts differentiate from mesenchyme stem cells and form bone through endochondral and intramembranous ossification. Growth factors such as BMP2 induce the master osteogenic proteins Runx2 and osterix (Osx/Sp7). This leads to the activation of osteogenic marker genes, and subsequently, to terminal differentiation of osteoblasts (Fujita et al., 2004; Mukherjee and Rotwein, 2009). Many signaling molecules have been identified that positively or negatively regulate osteoblast differentiation. For example, PI3K/Akt signaling is crucial for osteoblast differentiation (Fujita et al., 2004; Mukherjee and Rotwein, 2009), whereas p53 is a

negative regulator for osteogenesis (Wang et al., 2006). In the case of CaM, binding to Ca^{2+} activates downstream signaling molecules such as calmodulin kinase II (CaMKII) and calcineurin (CN), and promotes osteoblast differentiation (Zayzafoon et al., 2005).

Our previous study showed that Panx3 mRNA is expressed in osteoprogenitors and osteoblasts and prompted us to explore in more detail the role of Panx3 in osteoblast differentiation. In the present study, we demonstrate that Panx3 is induced during osteoblast differentiation and promotes differentiation. We found that Panx3 functions as an ER Ca^{2+} channel and is regulated through a PI3K/Akt pathway. The Panx3 Ca^{2+} ER channels regulate CaM pathways and promote osteogenic differentiation. Panx3, acting as a hemichannel, also promotes the release of ATP into the extracellular space. The released ATP may bind to P2Rs following activation of PI3K/Akt signaling. Furthermore, Panx3 gap junctions propagate a Ca^{2+} wave between cells and enhance osteoblast differentiation. Our results reveal that Panx3 plays a multifunctional role as a new regulator of osteoblast differentiation.

Results

Panx3 expression and localization in growth plates and differentiating osteoblasts

We previously reported that Panx3 mRNA is expressed in prehypertrophic chondrocytes, in the perichondrium/periosteum, and in osteoblasts in the growth plate (Iwamoto et al., 2010). Immunohistochemistry showed that the Panx3 protein was expressed in prehypertrophic and hypertrophic chondrocytes, as well as in the perichondrium/periosteum, which are progenitors for osteoblasts (arrows in Fig. 1 Ab). Immunostaining of osteocalcin (Ocn), an osteoblast marker, showed a distinct boundary between cartilage and bone. Panx3 was also expressed in osteoblasts (Fig. 1 Ac-d).

To study the role of Panx3 in osteoblast differentiation, we analyzed the expression of Panx3 mRNA in the osteoprogenitor cell line C2C12, and in primary calvarial cells from newborn mice (Fig. 1 B). Panx3 mRNA was not detectable in undifferentiated cells. However, when the cells differentiated into osteoblasts, Panx3 expression was found concomitantly with the induction of osteoblast marker genes such as Runx2, osterix, alkaline phosphatase (ALP) and Ocn (Fig. 1 Ba, b). Similar induction of Panx3 mRNA expression was observed during osteogenic differentiation of MC3T3-E1 and C3H10T1/2 cells (data not shown).

We next examined subcellular localization of the Panx3 protein in C2C12 cells using fluorescence confocal microscopy (Fig. 1 C). In differentiating C2C12 cells, Panx3 was localized to the plasma membrane and within cell-cell contact areas, as well as diffusely in the cytosol. Calnexin, an ER marker, was colocalized with Panx3, suggesting that Panx3 is localized in the ER (Fig. 1 Cf). Quantitative analysis revealed that 60% of

the Panx3 protein was colocalized with calnexin, while 90% of calnexin was colocalized with Panx3 (Fig. 1 Cb). These results suggest that Panx3 may function at multiple subcellular regions.

Panx3 promotes osteoblast differentiation

For further elucidation of the Panx3 function during osteoblast differentiation, we examined whether Panx3 expression promotes osteogenic differentiation of C2C12 cells. Following transfection with the Panx3 expression vector (pEF1/Panx3) (Fig. S1A and B), C2C12 cells were induced to differentiate, and mRNA expression of osteoblast marker genes was analyzed over time using real-time qPCR (Fig. 2 A). The expression of osterix, ALP, and Ocn was increased in Panx3-overexpressing cells compared to control cells transfected with vector pEF1, whereas Runx2 expression was unaffected level in both cell types (Fig. 2 A). When endogenous Panx3 expression was inhibited by shPanx3 RNA transfection (Fig. S1A and B), the induction levels of the osterix, ALP and Ocn, but not of Runx2, were reduced (Fig. 2 B). Panx3 overexpression also promoted ALP activity and mineralization, while suppression of endogenous Panx3 by shPanx3 inhibited these processes (Figs. 2 C and D). We observed similar results in primary calvarial cells (Fig. S2A, B and C). Taken together, these findings indicated that Panx3 promotes osteoblast differentiation processes.

Panx3 promotes metatarsal bone growth

Since Panx3 was expressed in the perichondrium/periosteum and osteoblasts, we analyzed Panx3 functions further with respect to ex vivo growth of the metatarsus.

Metatarsal bones from newborn mice were cultured and infected with Panx3 expression adenovirus (AdPanx3). AdPanx3 promoted growth in the length and width of both cartilage and bone in metatarsus after 3 days culture compared to the growth in the control adenovirus-infected metatarsus, as shown in videos and histology sections (Figs. 3 Aa and Ba,b; Video 1 and Video 2 in Fig. S4, and histology in the bottom panels). AdPanx3 infection increased not only the expression of Panx3, but also the expression of the osteoblast marker genes osterix, ALP, and Ocn (Fig. 3 C). To inhibit endogenous Panx3 activity, we used an inhibitory Panx3 peptide from the extracellular domain of Panx3, which blocked hemichannel activity and the release of ATP release (Fig. 6), as described previously (Iwamoto et al., 2010). Addition of the Panx3 peptide to a metatarsus culture inhibited the growth in length and width compared to the growth without the peptide or growth in a culture with a control scrambled peptide (Figs. 3 Ab, Ba,b; Video 3 and Video 4 in Fig. S4). The Panx3 peptide inhibited the expression of osteoblast marker genes (Fig. 3 C), indicating that Panx3 regulates growth plate expansion.

Panx3 functions as an ER Ca^{2+} channel

Since the intracellular Ca^{2+} level ($[Ca2^{+}]_i$) plays an important role in osteoblast differentiation (Seo et al., 2009; Zayzafoon et al., 2005), we examined whether intracellular Ca^{2+} signaling is involved in Panx3-promoted osteoblast differentiation. The ER serves as the main intracellular Ca^{2+} storage compartment. In the ER membrane, the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) takes up Ca^{2+} from the cytosol to the ER, while the ubiquitous IP3 receptors (IP3R1, 2 and 3), as well as the ryanodine

receptors (RyRs) in certain cell types, serve as ER Ca^{2+} channels for Ca^{2+} release from the ER (Fill and Copello, 2002; Futatsugi et al., 2005; Keller and Grover, 2000). Extracellular ATP can bind to purinergic receptors (P2Rs) and subsequently activate downstream intracellular signaling cascades. These signaling pathways are known to promote IP3 production, thereby activating IP3R ER Ca^{2+} channels and increasing $[Ca^{2+}]_i$ (Solini et al., 2008). In C2C12 cells, all IP3Rs are expressed, with IP3R3 showing the highest expression level (Powell et al., 2001), whereas RyRs are not expressed (Fig. S1C)(Biswas et al., 1999). No significant change was observed in the expression levels of IP3Rs during osteogenic differentiation of C2C12 (Fig. S1C). Since Panx3 was induced during differentiation of C2C12 and calvarial cells and was localized in the ER, Panx3 may function as an ER Ca^{2+} channel, thereby increasing $[Ca^{2+}]_i$ and subsequently promoting osteoblast differentiation. To explore Panx3 Ca^{2+} ER channel activity, Panx3 overexpressing C2C12 and calvarial cells were loaded with a UV excitable intracellular Ca^{2+} indicator, Fura-2 AM, in Ca^{2+}-free media. The cells were then stimulated by ATP, and increases in the $[Ca^{2+}]_i$ level were measured over a time course and compared with $[Ca^{2+}]_i$ levels in control cells lacking Panx3 (Fig. 4 Aa and b). $[Ca^{2+}]_i$ was approximately two-fold higher in Panx3 overexpressing C2C12 and calvarial cells than in control cells. This suggests that Panx3 may act as an ER Ca^{2+} channel.

To measure steady state $[Ca^{2+}]_i$ levels during differentiation, C2C12 cells were cultured with BMP2 and $[Ca^{2+}]_i$ was analyzed without ATP-stimulation (Fig. 4 Ac). The level of $[Ca^{2+}]_i$ in undifferentiated C2C12 cells was approximately 100 nM, a concentration typically observed in cells at rest. At 5 days after differentiation by BMP2, $[Ca^{2+}]_i$ was increased to ~180 nM, and this $[Ca^{2+}]_i$ increase was further enhanced to ~225

nM in Panx3 overexpressing C2C12 cells. Inhibition of differentiation by shPanx3 reduced $[Ca^{2+}]_i$. Taken together, these results suggest that Panx3 regulates $[Ca^{2+}]_i$. Similar $[Ca^{2+}]_i$ increases in calvarial cells were observed during osteogenic differentiation (Fig. S2D).

Panx3 activates the calmodulin pathways for differentiation

Intracellular Ca^{2+} activates and affects many signaling pathways that modulate cell differentiation (Berridge et al., 2000a). Upon Ca^{2+} binding, calmodulin (CaM) activates many downstream signaling molecules such calmodulin kinase II (CaMKII) and the phosphatase calcineurin (CN), and promotes osteoblast differentiation (Berridge et al., 2000b; Seo et al., 2009). NFATc1 is activated through dephosphorylation by CN and can promote expression of genes such as osterix, a key molecule involved in osteogenesis (Beals et al., 1997; Koga et al., 2005; Nakashima et al., 2002; Zayzafoon, 2006). We first examined the CaMKII/CN signaling pathways in pEF1/Panx3 transfected C2C12 (Fig. 4 B) and calvarial cells (Fig. 4 C), after short osteogenic induction. We found that the phosphorylation of CaMKII and the levels of CN protein were strongly increased, and that dephosphorylated NFATc1 (active form) levels also were increased in both cell types compared to control cells. The increase in NFATc1 protein levels is likely due to the enhanced NFATc1 transcription levels through the CaMKII/c-fos pathway (Koga et al., 2005; Zayzafoon et al., 2005). In a subsequent experiment involving the inhibition of endogenous Panx3 expression, shPanx3- and shControl-transfected cells were cultured under induction conditions for 1 day to induce endogenous Panx3 and the activation of

these factors was measured. Inhibition with shPanx3 reduced activation levels of CaMKII and NFATc1 in C2C12 and calvarial cells (Fig. 4 B and C).

Activation mechanism of Panx3 ER Ca^{2+} channel

Next, we determined whether any differences existed between the activation mechanisms of the Panx3 and IP3R ER Ca^{2+} channels, by using inhibitors of the IP3R function (Vanden Abeele et al., 2006). In control undifferentiated C2C12 cells, 2-APB, an inhibitor of IP3-induced Ca^{2+} release (Maruyama et al., 1997), completely blocked Ca^{2+} release from the IP3R channel in control cells (Fig. 5 Aa). In Panx3 overexpressing undifferentiated C2C12 cells, 2-APB treatment resulted in a reduction in the $[Ca^{2+}]_i$ levels, which closely corresponded to the $[Ca^{2+}]_i$ levels in control cells (Fig. 5 Aa). This indicates that 2-APB inhibited the IP3R ER Ca^{2+} channel, but not the Panx3 ER Ca^{2+} channel. In control cells, U-73122, a selective inhibitor of the phospholipase C (PLC), which catalyzes DAG and IP3 synthesis (Bleasdale et al., 1990), completely blocked the IP3R Ca^{2+} channel (Fig. 5 Ab), however, it only partially inhibited the Panx3 ER Ca^{2+} channel (Fig. 5 Ab). Inhibition of endogenous IP3R3 expression by siRNA reduced the IP3R Ca^{2+} channel but not the Panx3 ER Ca^{2+} channel (Fig. 5c). Thapsigargin, a highly selective inhibitor of the SERCA ER Ca^{2+} pumps responsible for loading Ca^{2+} stores (Inesi and Sagara, 1992), partially inhibited IP3R-mediated Ca^{2+} release from the ER in control cells (Fig. 5 Ad), whereas it completely blocked Ca^{2+} release from the ER in Panx3 overexpressing cells (Fig. 5 Ac). This suggests that the Panx3 ER Ca^{2+} channel continuously released Ca^{2+} from the ER following thapsigargin treatment. Therefore, the activation mechanism for the Panx3 ER Ca^{2+} channel appears to be different from that of

the IP3R.

Since Panx3 and IP3R ER Ca^{2+} channels are both activated by external ATP, we tested the differences in ATP receptors involved in the activation of these channels. ATP receptors (purinergic receptors, P2Rs) consist of two subtypes, the ligand-gated cation channels, P2Xs, and the G protein–coupled receptors, P2Ys (Burnstock and Knight, 2004). During osteoblast differentiation, a series of P2X and P2Y subtypes are expressed (Hoebertz et al., 2000; Orriss et al., 2006; Panupinthu et al., 2008). To examine whether specific subtypes of P2Rs are required for the activation of the Panx3 ER Ca^{2+} channel, the P2R antagonists PPADS and suramin, were tested for their effects on ATP-stimulated changes in $[Ca^{2+}]_i$ in Panx3 overexpressing and control cells (Fig. 5 B). PPADS inhibited Panx3 ER Ca^{2+} channel activity in Panx3 overexpressing C2C12 cells (Fig. 5Ba), while it did not inhibit the IP3R Ca^{2+} channel in control cells (Fig. 5 Ba). Suramin completely inhibited the IP3R Ca^{2+} channel in control cells (Fig. 5 Bb), and partially inhibited the Panx3 ER channel in Panx3 overexpressing cells (Fig. 5 Bb). A combination of PPADS and suramin completely inhibited both Panx3 and IP3R ER channels (Fig. 5 Bc). These results indicated that the Panx3 ER Ca^{2+} channel is activated through PPADS-sensitive P2Rs that are distinct from the IP3R channel.

Since PPADS specifically inhibited the Panx3 ER Ca^{2+} channel, we tested whether it would also inhibit Panx3-mediated CaM activation. For these experiments, we treated control and Panx3 overexpressing C2C12 cells with BMP-2 for 1 hour and then analyzed the phosphorylation of CaMKII and Smad1/5, both of which are targets of CaM (Pardali et al., 2005; Wicks et al., 2000) (Fig. 5 C). PPADS inhibited phosphorylation of CaMKII induced by Panx3 overexpression (Fig. 5 Ca). Without BMP2 treatment, Panx3

overexpression resulted in activation of Smad1/5 (left panel in Fig. 5 Cb). BMP2 induced phosphorylation of Smad1/5 in control cells, and Panx3 overexpression further increased phosphorylation levels of Smad1/5 (middle panel), while this stimulation was blocked by PPADS (right panel). These results suggest that the activation of specific P2Rs may be involved in Panx3-mediated signaling for osteoblast differentiation through CaMKII and Smad1/5 pathways.

Panx3 activates the Akt pathway

In addition to P2R-mediated activation of the PLC/PIP2/IP3/IP3R pathway, P2Rs also activate phosphoinositide 3-kinase (PI3K) signaling. Since Akt downstream from PI3K is crucial for osteoblast differentiation (Fujita et al., 2004; Mukherjee and Rotwein, 2009), we explored the involvement of Akt in Panx3-promoted osteoblast differentiation. We found that Panx3 overexpression increased phosphorylation of Akt and Akt-downstream MDM2, and promoted the degradation of p53 in both C2C12 and calvarial cells (Fig. 6 Aa and b). p53 is a negative regulator of osteogenesis (Lengner et al., 2006) and inhibits Runx2 and osterix expression (Lian et al., 2006). Akt-mediated MDM2 phosphorylation leads to p53 ubiquitination (Ogawara et al., 2002). Our results indicate that Panx3 expression activates Akt and promotes p53 degradation through MDM2 activation.

An Akt inhibitor reduced the expression of osterix mRNA in control and pEF1/Panx3 transfected C2C12 cells. The dominant negative Akt vector (Akt-DN) also inhibited osterix expression, whereas the activated Akt vector increased the expression (Fig. 6 Ba). Similar results were found for ALP expression (Fig. 6 Bb). These results suggest that Panx3 promotes osteoblast differentiation in part through the Akt pathway.

Akt activates the Panx3 ER Ca^{2+} channel

We next examined the involvement of Akt in the activation of a Panx3 ER Ca^{2+} channel. We found that the Akt inhibitor abolished ATP-stimulated Panx3 ER Ca^{2+} channel activity in Panx3 overexpressing C2C12 cells (Fig. 7 Aa). The remaining Ca^{2+} released from the ER was likely through the IP3R ER Ca^{2+} channel, which showed an activity level similar to that in control cells.The Akt inhibitor did not inhibit the IP3R ER Ca^{2+} channel in control cells.. Dominant negative Akt-DN also inhibited the ATP-stimulated Panx3 ER Ca^{2+}-release, but not IP3R ER Ca^{2+}-release (Fig. 7 Ab). In contrast, activated Akt-CA increased the activity of the Panx3 ER Ca^{2+} channel, but not the activity of the IP3R ER Ca^{2+} channel (Fig. 7 Ac). In control cells, neither Akt vector affected the IP3R ER Ca^{2+} channel (Fig. 7 Ab and c). LY294002, an inhibitor of PI3K, inhibited both Panx3 and IP3R ER channels (Fig. 7 Ad). The reason for this was that PI3K activates Akt as well as the IP3 synthesis pathway (Carpenter CL, 1996). These results suggest that ATP-stimulated activation of the Panx3 ER Ca^{2+} channel is mediated through a PI3K/Akt activation that is distinct from the IP3-dependent activation of IP3R ER Ca^{2+} channels.

To further confirm the Akt-mediated activation of the Panx3 ER Ca^{2+} channel, the $[Ca^{2+}]_i$ concentration was measured without ATP stimulation (Fig. 7 B). The steady state $[Ca^{2+}]_i$ concentration in Panx3 overexpressing C2C12 cells was ~140 nM, which was higher than that of control cells (90 nM). The activated Akt enhanced the $[Ca^{2+}]_i$ concentration in Panx3 overexpressing C2C12 cells by about two-fold (280 nM), compared to that in cells without the Akt vector. Akt DN reduced the steady state $[Ca^{2+}]_i$ concentration in Panx3 overexpressing C2C12 cells to a level similar to that seen in the

control cells. These results indicated that the Panx3 ER Ca^{2+} channel is regulated by Akt signaling, and that its mechanism is independent of IP3R activity.

We further examined whether Akt-mediated Panx3 ER Ca^{2+} channel activation regulates the CaM/CaMKII/NFATc1 signaling pathways (Fig. 7 C). Panx3 overexpressing C2C12 cells were transfected with the Akt CA or Akt DN vector and were treated with BMP2 for 1 hr. Western blotting analysis showed that Akt CA increased phosphorylation of CaMKII (P-CaMKII) and activation of NFATc1 (reduced P-NFATc1), while Akt DN inhibited CaMKII and NFATc1 activation (Fig. 7 C). These results support the notion that the Akt signaling pathway regulates the Panx3 ER Ca^{2+} channel.

Panx3 releases intracellular ATP though its hemichannel activity

The activation of P2Rs may be caused in part by binding of ATP released from cells through Panx3 hemichannel activity. To test this possibility, we first analyzed the intracellular ATP levels in control and Panx3 overexpressing C2C12 cells by fluorescence imaging using confocal microscopy (Fig. 8 Aa). The cells containing caged luciferin were illuminated by a flash of two-photon light, which photolysed the caged luciferin, allowing the uncaged luciferin to bind to intracellular ATP and resulting in an increase in fluorescence emissions. The fluorescence intensity at 5 and 15 sec after photolysis was much higher (red) in control C2C12 cells than in Panx3 overexpressing cells, suggesting that Panx3 expression reduced intracellular ATP levels. To analyze ATP release, control and Panx3 overexpressing C2C12 and calvarial cells were incubated in the presence of luciferin for 2 min, and the fluorescence intensity was measured (Fig. 8

Ab and c). The extracellular ATP level was approximately two-fold higher in Panx3 expressing cells than in control cells. Similar results were obtained when the cells were treated with KGlu to depolarize the cell membrane, except that the level of ATP release was much higher. Addition of anti-Panx3 antibody, but not IgG, to the cell culture assay inhibited Panx3-mediated ATP release (Fig. 8 Ba). To test whether this inhibition was specific to Panx3, we used the inhibitory Panx3 peptide from the extracellular domain of Panx3, which had been used as an antigen for the Panx3 antibody (Iwamoto et al., 2010). The peptide, but not a scrambled peptide, blocked the inhibitory activity of the antibody for the ATP release in a dose-dependent manner (Fig. 8 Ba). In addition, the Panx3 peptide alone showed inhibitory activity for the ATP release, as described previously in chondrogenic ATDC5 cells (Iwamoto et al., 2010). Under differentiation conditions, suppression of endogenous Panx3 by shRNA inhibited ATP release in C2C12 cells (Fig. 8 Bb). These results indicated that Panx3 caused the release of intracellular ATP into the extracellular space though the hemichannel.

To test the effects of the Panx3 hemichannel on osteoblast differentiation, control and Panx3 overexpressing C2C12 cells were induced to differentiate by a 2-day treatment with BMP2, in the presence or absence of the anti-Panx3 antibody, and mRNA levels for osterix and ALP were analyzed (Fig. 8 C). The Panx3 antibody inhibited the induction of osterix and ALP expression in both control and Panx3 overexpressing cells. Since the antibody and peptide blocked the Panx3 hemichannel activity, these results suggest that the Panx3 hemichannel plays a critical role in osteoblast differentiation.

Panx3 gap junction function during osteoblast differentiation

An increase in $[Ca^{2+}]_i$ in one cell can be transmitted to neighboring cells, resulting in Ca^{2+} waves that spread within cell networks. This Ca^{2+} wave propagation is mediated by gap junctions and promotes cell-cell communication for cellular processes and differentiation (Barbe et al., 2006). Therefore, we examined Panx3 gap junction activity in C2C12 cells and its effect on osteoblast differentiation (Fig. 9). First, Ca^{2+} wave propagation in Panx3 overexpressing C2C12 cells was analyzed and compared with that in control cells. The cells were loaded with a photosensitive caged form of Ca^{2+} and Ca^{2+}-sensitive Fluo-4. While performing live cell Ca^{2+}-imaging, a single cell within a cell network was subjected to two-photon photolysis. The uncaged Ca^{2+} fluctuations were analyzed by live-cell fluorescence confocal microscopy (Fig. 9 Aa; Video 5 and Video 6 in Fig. S5). We also observed similar Ca^{2+} wave propagation in Panx3 overexpressing calvarial cells (data not shown). In Panx3 overexpressing cells, Ca^{2+} waves were propagated into neighboring cells (red in high Ca^{2+}), while in control cells lacking Panx3, the Ca^{2+} fluctuations were restricted to the single uncaged cell. This Ca^{2+} wave propagation was inhibited by the gap junction inhibitor carbenoxolone (CBX) (Fig. 9 Ab; Video 7 and Video 8 in Fig. S5). Since CBX may disrupt the cell membrane and affect activation of P2 receptors, thereby inhibiting the Ca^{2+} wave, apyrase (an ATP receptor antagonist) was added to the Ca^{2+} wave assay at a concentration, which inhibited Panx3-mediated activation of Akt signaling (Fig. S3). We found that apyrase did not inhibit the Ca^{2+} wave, suggesting that Panx3 gap junction mediates Ca^{2+} wave propagation (Fig. 9Ac and Video 9 and Video 10 in DataViewer). To test the effect of Panx3-mediated gap junction activity on C2C12 cell differentiation, Panx3 overexpressing and control cells were induced to differentiate by BMP2 treatment in the