presence of CBX, and ALP activity was measured (Fig. 9 B). Panx3 overexpression promoted ALP activity, as shown in Fig. 2, but CBX inhibited this ALP activation. Similarly, Panx3 shRNA inhibited ALP induction, as shown in Fig. 2, and CBX further inhibited ALP activity. These results indicated that Panx3 can function as a gap junction and propagate Ca^{2+} waves from cell to cell, and that this activity also promotes osteoblast differentiation.

Discussion

In our present study, we demonstrated for the first time that Panx3 regulates osteoblast differentiation through its multiple functions as a hemichannel, an ER Ca^{2+} channel, and a gap junction, as illustrated in Fig.10. All three Panx3 activities are associated with and involved in osteoblast differentiation. We showed that Panx3 is induced during osteogenic differentiation and is localized in the plasma membrane, and that it functions as the hemichannel that releases ATP into the extracellular space. The ATP released from the cells binds to purinergic receptors (P2Rs) in the plasma membrane in an autocrine and paracrine manner, and thereby activates P2R/PI3K/Akt signaling for the Panx3 channel and P2R/PLC/PIP2/IP3 for the IP3R channel. Akt-mediated Panx3 ER Ca^{2+} channel activation induces Ca^{2+} release from the ER into the cytosol, which subsequently activates CaM signaling pathways, including CaMKII/Smad, CaMKII/c-Fos and CN/NFATc. The Akt activation also increases p53 degradation through activation of MDM2. The activation of these pathways promotes differentiation. In addition, the increased $[Ca^{2+}]_i$ level that originated in cells due to the hemichannel in turn propagates its wave into surrounding cells through a Panx3 gap junction, subsequently

promoting Ca^{2+} signaling among cell populations for differentiation. Because blocking of the Panx3 hemichannel inhibits osteoblast differentiation, this hemichannel activation may be the first step in Panx3-mediated differentiation processes.

Ca^{2+} is one of the most important second messengers and regulates many cellular processes (Berridge et al., 2000b), and the CaM pathway is critical for osteoblast differentiation (Zayzafoon, 2006). However, the mechanism for controlling $[Ca^{2+}]_i$ during osteoblast differentiation is not yet clear. We found that Panx3 functions as a Ca^{2+} channel in the ER, through which it regulates $[Ca^{2+}]_i$. C2C12 and calvarial cells express IP3Rs. Both Panx3 and IP3Rs function as ER Ca^{2+} channels, however their activation mechanisms are different. We found that the Panx3 ER Ca^{2+} channel was activated through Akt signaling (Fig. 7 A), distinct from IP3-mediated activation of ubiquitous IP3R ER Ca^{2+} channels (Mikoshiba, 2007). In addition, siRNA for IP3R3 and 2-APB, which is an inhibitor of IP3R-mediated Ca^{2+} release, inhibited IP3R ER Ca^{2+} channel activity, but did not inhibit the Panx3 channel (Fig. 5 A). The role of IP3Rs in osteogenic differentiation is also not yet clear. The inhibition of endogenous Panx3 by shRNA resulted in substantial inhibition of osteogenic differentiation in C2C12 cells, despite IP3R expression, while the inhibition of IP3Rs by siRNA and 2-APB showed limited inhibition of C2C12 cell differentiation (data not shown). In addition, mice lacking either IP3R2 or IP3R3 were viable and had no obvious abnormalities. Mice lacking both IP3R2 and IP3R3 were born with a normal appearance, but began losing body weight after weaning due to defects in exocrine secretion (Futatsugi et al., 2005). Both receptors may function as ER Ca^{2+} channels with distinct activation mechanisms during osteogenic differentiation. Since the Panx3 hemichannel likely triggers activation of these Panx3 and

IP3R ER Ca^{2+} channels, Panx3 may play a major role upstream of IP3Rs in osteogenic differentiation.

Extracellular ATP modulates cellular functions by binding in autocrine and paracrine manners to two subtypes, $P2XR_{1-7}$ and $P2YR_{1-12}$, of a large family of purinergic receptors (Corriden and Insel, 2010). P2YRs are G protein–coupled receptors that activate signaling pathways responsible for releasing Ca^{2+} from the ER, whereas P2XRs are non-selective cation channels that permealize Ca^{2+}, Na^{+} and other cations (Burnstock and Knight, 2004). During osteoblast differentiation, many P2X and P2Y members are expressed and implicated in bone formation (Hoebertz et al., 2000; Orriss et al., 2006; Panupinthu et al., 2008). Both Panx3 and IP3R ER Ca^{2+} channels were activated by ATP (Fig. 4). PPADS and suramin function to commonly inhibit some P2Ys and P2XRs (e.g. P2X1, P2X3 and P2Y6), however they also inhibit specifically (e.g., PPADS for P2Y1 and P2Y4; suramin, for P2X2, P2X5, and P2Y2) (P2 receptors, Sigma-RBI eHandbook). Panx3 ER Ca^{2+} channels, but not IP3R ER Ca^{2+} channels, were inhibited by PPADS (Boyer et al., 1994; Ziganshin et al., 1994), while suramin inhibited both channels (Fig. 5 B). These results suggest that the Panx3 and IP3R ER Ca^{2+} channels may be regulated through different purinergic receptors. The ATP-induced $[Ca^{2+}]_i$ experiments were performed in cell culture without external Ca^{2+} in the medium to measure $[Ca^{2+}]_i$ released from the ER. However, in vivo, external Ca^{2+} may also contribute to an increase in $[Ca^{2+}]_i$ through the ATP-P2X cation channels. Indeed, P2X7 knockout mice displayed reduced periosteal bone formation and response to mechanical loading (Ke et al., 2003; Li et al., 2005).

Panx3 expression increased phosphorylation of Akt (Fig. 6A). Akt signaling is required for osteoblast differentiation, bone formation and prevention of osteoblast apoptosis (Kawamura et al., 2007; Mukherjee and Rotwein, 2009). One of the Akt target pathways is the MDM2/p53 signaling pathway (Lengner et al., 2006; Wang et al., 2006). Panx3 expression promoted phosphorylation of MDM2 and enhanced the degradation of the p53 protein (Fig. 6A). These results indicate that Panx3-promoted osteogenic differentiation occurs in part due to reduction of the p53 level through enhancement of the Akt/MDM2/p53 pathway (Fig. 10). We found that Panx3 also stimulated phosphorylation of Smad1/5 (Fig. 5 C). Previous reports have shown that CaM/CaMKII activates Smad1/5 (Scherer and Graff, 2000), and therefore, Panx3 ER Ca^{2+} channel/CaM/CaMKII signaling activates Smad1/5. The anti-Panx3 antibody and PPADS inhibited the Panx3-promoted Smad1/5 activation, suggesting that the Panx3 hemichannel and P2Rs are involved in these processes.

Panx1 and Panx2 were expressed in C2C12 and primary calvarial cells at a very low level and were not induced at all during osteogenic differentiation of these cells (data not shown). Therefore, Panx3 is the major pannexin protein expressed during osteoblast differentiation. Among the connexin gap junction proteins, Cx43 is the most highly expressed in osteoblasts (Civitelli et al., 1993; Gramsch et al., 2001; Jiang et al., 2007; Su et al., 1997), and is implicated in osteoblast differentiation and mineralization (Chung et al., 2006; Lecanda, 2000; Inose, 2009; Stains, 2005). Cx43 knockout mice showed cranial abnormalities and delayed ossification, while axial and appendicular elements were normal at birth (Lecanda et al., 2000). Mice with conditional Cx43 deletion in osteoblasts displayed a normal appearance at birth, but developed a low bone-density osteopenia

phenotype with age (Chung et al., 2006). Panx3 overexpression in differentiated C2C12 cells promoted Cx43 expression, while suppression of endogenous Panx3 by Panx3 shRNA inhibited Cx43 expression (data not shown). These results suggest that Panx3 and Cx43 may play distinct roles in osteogenic differentiation.

In summary, we have provided evidence that Panx3 acts as a multifunctional protein that promotes osteoblast differentiation by regulating Akt and Ca^{2+} signaling through its hemichannel, ER channel and gap junction activities.

Material and methods

Reagents

Rabbit anti-Panx3 antibody, inhibitory Panx3 and scrambled peptides, Panx3 expression vector (pEF1/Panx3) and control vector (pEF1), shRNA vector for Panx3 (shPanx3) and control vector (sh control) were described previously (Iwamoto et al., 2010). Control adenovirus (Ad-Cont) and Panx3 expression adenovirus (Ad-Panx3) were prepared and purified by Welgen Inc. Constitutively active Akt (Akt-CA) and dominant negative Akt (Akt-DN) vectors were obtained from Addgene. Antibodies for P-Rb, CaM, CaMKII, P-CaMKII, CN, p53, Akt, p-Akt, Smad1 and P-Smad1/5 were obtained from Cell Signaling Technology, Inc; Rb, P-NFATc1 and MDM2 from Santa Cruz; NFATc1 from BD Biosciences; α-Tubulin from Sigma; osteocalcin from Biomedical Technology; calnexin from Pierce Biotechnology. 2-APB, U-73122, Thapsigargin, PPDA, suramin, carbenoxolone (CBX) and Apyrase were obtained from Sigma; Akt inhibitor from Calbiochem; LY294002, Fura-2AM from Invitrogen; BMP2 from Humanzyme; iQ SYBR Green Supermix from BioRad. Horseradish peroxidase (HRP)- conjugated goat anti-mouse and goat anti-rabbit IgG were obtained from US Biological (Swampscott, MA)

Cell Culture

C2C12 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS) (HyClone) at 37°C under 5% CO_2. For osteoblast differentiation, cells (~90% confluency) were transiently or stably transfected

with pEF1/Panx3, control vector, Panx3 shRNA or sh control vector and cultured in the presence of 300 ng/ml BMP2 (Humanzyme) and 2% FBS. The media were replaced every 3 days. Primary calvarial cells were prepared from calvaria of newborn mice and cultured in α-minimum essential medium (Invitrogen) with 10% FBS (HyClone, Logan, UT), 100 U/ml of penicillin and 100 μg/ml of streptomycin as previously described (Matsunobu et al., 2009). Primary calvarial cells were transiently transfected with Nucleofector (Amaxa). For the differentiation assay, primary calvarial cells were induced by the addition of 50 μg/mL ascorbic acid (Sigma) and 5mM β-glycerophosphate (Sigma).

Immunostaining

After deparaffinization and rehydration, sections were digested with pepsin (Biocare Medical). Cultured cells were blocked and reacted for two hours at room temperature with primary antibodies. Primary antibodies were detected by Alexa488 (Invitrogen) or Cy-3 (Jackson ImmunoResearch Laboratories) conjugated secondary antibodies. Nuclear staining was performed with Hoechst dye (Sigma-Aldrich). Analysis was performed on a LSM 510 inverted confocal microscope and an Axiovert 200 fluorescence microscope (Carl Zeiss MicroImaging, Inc.). Colocalization was analyzed by MetaMorph (Molecular Devices).

RT-PCR

Total RNA was extracted using QG-810 and QuickGene RNA cultured cell HC kit S (Fujifilm). Total RNA (1 μg) was used for reverse transcription to generate cDNA, which

was used as a template for PCRs with gene-specific primers (Supplemental Table 1), as previously described (Iwamoto et al., 2010). cDNA was amplified with an initial denaturation at 95 °C for 3 min; then 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 30 cycles; and then with a final elongation step at 72 °C for 5 min before being separated on agarose gels. Real time PCR amplification was performed with iQ SYBR Green Supermix (BioRad) and a Chromo4 thermocycler (BioRad). Real-time PCR was performed for 40 cycles, at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. Gene expression was normalized to the housekeeping gene Hprt.

ATP flux

ATP flux was determined by luminometry as described previously (Iwamoto et al., 2010). Cells were depolarized by incubation in KGlu (Iwamoto et al., 2010). 140 mM KGlu, 1 0mM KCl and 5.0 mM TES, pH 7.5) solution for 4 min and then assayed with luciferase/luciferin (Promega). The luminescence was measured using a multimode plate reader, Mithras LB 940 (BERTHOLD). For inhibition by the Panx3-antibody, the cells were incubated with 1.5 ng/ml affinity-purified antibody or control IgG for 30 min prior to assay.

Western blot analysis

Cell lysates were prepared as previously described (Iwamoto et al., 2010). A 10 μg sample of each protein was electrophoresed in 4–12% SDS-polyacrylamide gel (Invitrogen) and transferred onto a polyvinylidine difluoride membrane using iBlot

(Invitrogen). The membranes were immunoblotted with antibodies using standard protocols.

Alkaline phosphatase assay

C2C12 cells and primary calvarial cells were plated into 12 or 96 well culture plates and grown to 100% confluence. After they were confluent the cells were induced into osteoblast by BMP2 (300 ng/ml). ALP activity was measured in cell layers using a *p*-nitrophenyl phosphate substrate and an incubation temperature of 37°C or was determined by the TRACP & ALP double-stain kit (Takara Bio Inc.). The protein concentration was determined by the BCA protein assay method (Pierce).

Alizarin Red S staining

C2C12 cells were fixed with 60% isopropanol and stained with 1% (w/v) Alizarin Red S (Sigma). For quantification of calcium deposition, cultures were extracted from the Alizarin Red S stain with 10% cetylpyridinium chloride (Sigma) and the OD was measured at 550 nm, as previously described (Mukherjee and Rotwein, 2009).

Ex vivo metatarsal bone culture

Metatarsal bones were isolated from newborn C57BL/6 mice and were cultured in DMEM containing 0.5% bovine serum albumin, 50 μg/ml ascorbic acid (sigma) and 1 mM β-glycerol phosphate (sigma) at 37°C in a humidified atmosphere of 5% CO_2 as previously described (Mukherjee and Rotwein, 2009). One day after starting the culture, the metatarsal bones were infected with Ad-Cont or Ad-Panx3 ($1X10^9$ PFU/ml) for 3

days. For peptide inhibition assay, metatarsal culture were added the Panx3 antigen peptide or a scramble peptide (100 μg/ml) and incubated for 3days. Live images were captured with a Lumenera Infinity2 camera attached to a Zeiss Axiovert25 microscope. Images were analyzed and adjusted using MetaMorph (Molecular Devices) and NIH Image J software. Metatarsals were fixed in 4% paraformaldehyde overnight at 4°C and stored in 70% ethanol. Bones were embedded in paraffin blocks and sectioned. Sections were stained with hematoxylin and eosin. Images were captured with Zeiss microscope camera AxioCam attached to a Zeiss Axiovert135 microscope.

$[Ca^{2+}]_i$ measurements

C2C12 cells grown in a 96-well plate for 3days were loaded with 5μM Fura-2AM (Invitrogen) prepared in HBSS for 45 min at 37°C in 5% CO_2. After 3days of transfection, transiently transfected primary calvarial cells were loaded at the same condition as the C2C12 cells. Ca^{2+} transients were recorded as the 340/380 nm ratio (=R) of the resulting 510 nm emissions using a Mithras LB 940 plate reader. For the stimulation, 200 μM ATP were injected into cells automatically by the Mithras 940. For inhibition experiments, cells were incubated for 30 min prior to analysis with following each inhibitor: 5 M U-73122 for IP3 synthesis inhibition; 100 μM 2-APB for blocking IP3R ; 0.5 μM thapsigargin for SERCA ER Ca^{2+} pump; 100 μM PPADS, and 300 μM Suramin for P2Rs inhibition; 5 μM Akt inhibitor; 100 μM LY294002 for PI3K . siIP3R-3 (Dharmacon) was transfected transiently to C2C12 cells, which had stably transfected with either pEF1/Panx3 or control vector. After 3days of transfection, $[Ca^{2+}]_i$ was measured. $[Ca^{2+}]_i$ levels were calculated as previously described (Grynkiewicz et al.,

1985). Using the equation $[Ca^{2+}]_i = K_d (R - R_{min})/(R_{max} - R)(F^{380}{}_{max}/F^{380}{}_{min})$, where R_{min} is the ratio at zero Ca^{2+}, R_{max} is the ratio when Fura-2 is completely saturated with Ca^{2+}, $F^{380}{}_{min}$ is the fluorescence at 380 nm for zero Ca^{2+}, and $F^{380}{}_{max}$ is the fluorescence at saturating Ca^{2+} and $K_d = 224$ nM.

Imaging of single cell intracellular ATP concentrations and Ca^{2+} wave propagation

For the imaging of single cell intracellular ATP, cells attached to glass-bottomed dishes (MatTek Corporation, Ashland, MA, USA) were loaded with 25 μM caged D-Luciferin AM (Invitrogen) in HBSS containing 10 mM HEPES for 20 min at room temperature, followed by washing. The cells were then incubated with KGlu solution for 10 min. For Ca^{2+} waves propagation, cells seeded on a glass bottom dish were incubated in HBSS containing 4 μM of the $Ca2^{+}$ indicator Fluo-4 AM, 10 μM pluronic F-127 (Invitrogen), 0.1% OxyFluor (Oxyrase) and 2.5 μM caged reagent NP-EGTA AM (Invitrogen) for 30mins at room temperature, followed by washing and incubation with Ca^{2+}-free HBSS. Both Luciferin AM and Fluo-AM were imaged using a Zeiss LSM 510 NLO META equipped with A-Plan-Apochromat 63X (1.4 N.A.) objectives (Zeiss, Thornwood, NY). The 488 nm Argon laser line was used for excitation. Cells were imaged every 1 second for approximately 1 minute following uncaging. D-Luciferin AM and NP-EGTA AM were uncaged using a two-photon laser set at 730 nm. A nucleus-sized region of interest was used for uncaging within single cells using the Zeiss Zen software's bleach function. To block the gap junction channels, cells were incubated with CBX (25 μM). To inhibit ATP receptors, cells were incubated with Apyrase (20U).

Focal microscopy images were obtained using the same two-photon confocal microscope described above. A 488 nm argon, a 543 nm HeNe1, and a 633 nm HeNe2 were used to excite Cy2, Cy3 and Cy5 fluorophores, respectively. The pinholes for each laser line were aligned for optimal confocality. For DAPI illumination, the two-photon laser was tuned to 730 nm and used at ~3-8% power.

Data Analysis

Each experiment was repeated several times. The data were analyzed by Prism 5 software. Statistical differences between two groups of data were analyzed with Student's *t*-test. One-way ANOVA was used for the quantification of Alizarin Red S staining. $P<0.05$ was considered statistically significant.

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