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Involvement of PRIP (Phospholipase C-Related But Catalytically Inactive Protein) in BMP-Induced Smad Signaling in Osteoblast Differentiation

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ABSTRACT

Phospholipase C-related but catalytically inactive protein (PRIP) was first isolated as an inositol 1,4,5-trisphosphate binding protein. We generated PRIP gene-deficient mice which exhibited the increased bone mineral density and trabecular bone volume, indicating that PRIP is implicated in the regulation of bone properties. In this study, we investigated the possible mechanisms by which PRIP plays a role in bone morphogenetic protein (BMP) signaling, by analyzing the culture of primary cells isolated from calvaria of two genotypes, the wild type and a mutant. In the mutant culture, enhanced osteoblast differentiation was observed by measuring alkaline phosphatase staining and activity. The promoter activity of Id1 gene, responding immediately to BMP, was also more increased. Smad1/5 phosphorylation in response to BMP showed an enhanced peak and was more persistent in mutant cells, but the dephosphorylation process was not different between the two genotypes. The luciferase assay using calvaria cells transfected with the Smad1 mutated as a constitutive active form showed increased transcriptional activity at similar levels between the genotypes. The expression of BMP receptors was not different between the genotypes. BMP-induced phosphorylation of Smad1/5 was robustly decreased in wild type cells, but not in mutant cells, by pretreatment with DB867, an inhibitor of methyltransferase of inhibitory Smad6. Furthermore, BMP-induced translocation of Smad6 from nucleus to cytosol was not much observed in PRIP-deficient cells. These results indicate that PRIP is implicated in BMP-induced osteoblast differentiation by the negative regulation of Smad phosphorylation, through the methylation of inhibitory Smad6. J. Cell. Biochem. 116: 2814–2823, 2015.

KEY WORDS: BONE MORPHOGENETIC PROTEIN; KINASE; OSTEOBLAST; PHOSPHORYLATION; Smad

Phospholipase C-related but catalytically inactive protein (PRIP) was first identified in the brain cytosol fraction as a novel D-*myo*-inositol 1,4,5-trisphosphate binding protein [Kanematsu et al., 1992; 1996; 2000]. The molecule is similar to phospholipase C-δ1 but is catalytically inactive, which is the reason for its name, and is expressed predominantly in the brain [Yoshida et al., 1994; Kanematsu et al., 1996,2000; Matsuda et al., 1998]. Later, an isoform with relatively broad tissue distribution, including the brain, was reported, comprising PRIP-1 and -2 [Kikuno et al., 1999; Otsuki et al., 1999; Uji et al., 2002]. Subsequent studies revealed that there are a number of binding partners, including GABARAP [γ-aminobutyric acid type A ($GABA_A$) receptor-associated protein] [Kanematsu et al., 2002], the catalytic subunit of protein phosphatase 1 and 2A [Yoshimura et al., 2001; Terunuma et al., 2004; Kanematsu et al., 2006; Yanagihori et al., 2006; Gao et al., 2012], the phosphorylated (active) form of Akt [Fujii et al., 2010], the β subunits of the $GABA_A$ receptor [Terunuma et al., 2004; Kanematsu et al., 2006; Kanematsu et al., 2007] and SNARE proteins, syntaxin 1 and SNAP-25 [Gao et al., 2012; Zhang et al., 2013], in addition to inositol 1,4,5-trisphosphate.

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We further generated PRIP gene-deficient mice, in each subtype or both and performed analyses of the phenotypes for information regarding the physiological functions mainly using PRIP-1 and -2 double knock out (KO) mice, since binding properties to some of the above-mentioned molecules provided little difference between PRIP-1 and -2. Binding partners with known functions helped the analyses: inositol 1,4,5-trisphosphate-mediated Ca^{2+} signaling [Takeuchi et al., 2000; Harada et al., 2005], $GABA_A$ receptor signaling [Kanematsu et al., 2002, 2006, 2007; Terunuma et al., 2004; Mizokami et al., 2007, 2010; Fujii et al., 2010], phospho-regulation through phosphatase swapping [Sugiyama et al., 2012] and negative regulation of exocytosis [Gao et al., 2012; Zhang et al., 2013].

During the course of mutant mice maintenance, we noticed decreased litter events and litter size in the mutant couples, indicating dysfunction of reproduction. We then found by cross-mating experiments that this dysfunction is attributed to the mutant female, showing longer estrus days, and higher serum gonadotropins but lower serum progesterone and estrogen to a lesser extent [Matsuda et al., 2009]. Estrogen insufficiency in postmenopausal women frequently leads to osteoporosis, the most common skeletal disorder [Jilka et al., 1992], and ovariectomized mice exhibited an osteoporotic bone phenotype. Therefore, we compared KO mice with wild-type (WT) mice in an investigation regarding the bone properties influenced by PRIP deficiency. Three-dimensional analysis of the femurs of female mice, histomorphometrical assay of bone formation parameters, including the bone formation rate, mineral apposition rate, osteoid thickness, and osteoblast number, and biochemical analysis using primary preosteoblast cell cultures were performed. All these results indicated increased bone mass in KO mice, caused by enhanced bone formation, indicating that PRIP is implicated in the regulation of bone properties, independently of general hormonal regulation [Tsutsumi et al., 2011].

In this study, we explored the possible mechanisms by which PRIP-KO mice exhibit enhanced bone formation. Since bone morphogenetic protein (BMP) signaling is one of the most important pathways for the regulation of bone formation, particularly osteoblast differentiation, we investigated the BMP signaling pathway using osteoblastic cells prepared from newborn calvaria, comparing the genotypes. The results suggest that PRIP is negatively involved in BMP-induced osteoblast differentiation by the regulation of Smad phosphorylation probably through the methylation of inhibitory Smad6.

MATERIALS AND METHODS

ANIMALS

PRIP-KO mouse strains and the corresponding WT were generated as described previously [Kanematsu et al., 2006]. In brief, PRIP-1 KO mice and PRIP-2 KO mice back-crossed against the C57BL/6J (Charles River Laboratories Japan, Inc., Tokyo, Japan) background were crossed to generate a PRIP-KO mouse strain and corresponding WT. Homozygous PRIP-KO and WT mice were intercrossed to obtain the required number of mice, and only F1 generations of both genotypes were used for experiments. The handling of mice and all procedures were approved by the Animal Care Committee of Kyushu University, following the guidelines of the Japanese Council on Animal Care.

CELL CULTURE AND PREPARATION OF CELL MEMBRANE FRACTION

Primary osteoblasts isolated by a routine method from the calvaria of newborn mice were cultured for 72 h in the presence or absence of 10 ng/ml BMP4 (R&D Systems, Minneapolis, MN). Cells were suspended in a homogenizing buffer (50 mM Tris–HCl at pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM NAF, 10 mM $Na_4P_2O_7$, 20 μM β-glycerophosphate, 1 mM Na_3VO_4, and a cocktail of protease inhibitors containing aminoethyl benzylsulfonyl fluoride, leupeptin, aporotinin, and pepstatin A), followed by homogenization by 3 cycles of 10 strokes in a Teflon homogenizer. The lysate were passed through a 27G needle 15 times and centrifuged at 5,000 rpm for 3 min at 4°C to obtain a nuclear pellet and a supernatant. The supernatant was centrifuged at 100,000*g* for 10 min at 4°C (himac CS 100GX; HITACHI, Tokyo, Japan) to separate the membrane fraction from the cytosol supernatant. The pellet was resuspended in 1% Triton X-100 diluted with homogenizing buffer. The suspension was rotated for 2 h at 4°C and centrifuged at 15,000 rpm for 15 min at 4°C to obtain the membrane fraction in the supernatant.

WESTERN BLOTTING

Osteoblasts differentiated from each genotype were extracted with lysis buffer (25 mM Tris–HCl at pH 7.5, 150 mM NaCl, 1% Triton X-100, and 5 mM EDTA, a cocktail of protease inhibitors). Phosphatase inhibitors such as 10 mM NaF and 1 mM Na_3VO_4 were also included in the lysis buffer. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), followed by transferring to the polyvinyldifluoride membrane and immunoblotting. The primary antibodies used were BMPR1A (Abcam, Cambridge, MA); BMPR2 and Smad6 (Santa Cruz Biotechnology, Santa Cruz, CA); β-actin (Sigma–Aldrich, St Louis, MO); Smad1 and Phospho-Smad1(Ser463/465)/Smad5(Ser463/465)/Smad8(Ser426/428) (Cell Signaling Technology, Danvers, MA). Blots were developed with horseradish peroxidase-coupled secondary antibodies and visualized using an ECL system (GE Healthcare, Little Chalfont, UK). Density was quantified by an Image Quant TL (ImageQuant LAS 4000; GE Healthcare) and is shown as a relative value after normalizing with that of β-actin or Smad1 in each experiment.

OSTEOBLAST DIFFERENTIATION ASSAYS

Pre-osteoblasts from the calvaria of newborn mice were seeded at a density of 1×10^4 cells/well in a 96-well plate, followed by cultivation in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA). The cells were treated with 10 ng/ml BMP4 for 3 days. After treatment, the cells were fixed with an acetone/ethanol mixture (50:50, v/v), followed by an alkaline phosphatase (ALP) assay by incubating with a substrate solution containing 0.1 M diethanolamine (pH 8.5), 1 mM $MgCl_2$, and 10 mg/ml *p*-nitrophenyl phosphate. The reaction was terminated by adding 5 M NaOH to the final concentration of 1.67 M, and then absorbance was measured at 405 nm using a microplate

reader. For histochemical analysis of ALP activity, cells were stained for enzyme activity as described previously [Katagiri et al., 1994].

RNA EXTRACTION, SEMI-QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

RNA was extracted from cells of both genotypes of mice by an RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. After cDNA synthesis using total RNA (1 μg) with Rever Tra Ace (TOYOBO, Tokyo, Japan), semiquantitative PCR amplifications were performed in 20 μl with 2 μl of obtained cDNA and 0.5 μM of each primer using a PCR amplification kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer's protocols. Each primer was added to a final concentration of 0.3 μM. PCR was performed using 27 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Primer sequences (forward and reverse) were as follows: Osteocalcin 5′-AAGCAGGAGGGCAATAAGGT and 5′-TTTGA-TAGCTCGTCACAAGC; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ACCACAGTCCATGCCA TCAC and 5′-TCCAC-CACCCTGTTGCTGTA. Three independent experiments were performed to estimate the value for each culture condition.

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY

For the measurement of Id1 promoter activity, luciferase reporter plasmid driven by the Id1 promoter (Id1WT4F) [Katagiri et al., 2002] and pRL-SV40 (Promega, Madison, WI) was transfected using Lipofectamine LTX (Invitrogen, Carlsbad, CA) into the cells prepared from calvaria of newborn mice of both genotypes. Cells were cultured for 24 h after transfection followed by stimulation for 12, 24, 36 h by BMP4 (10 ng/ml). For the examination of Id1 transcriptional activity by constitutive active Smad1, calvaria cells were co-transfected with Id1WT4F and wild-type Smad1 or constitutively-active form of Smad1 [Smad1(DVD)] plasmid in which C-terminal serine residues were substituted with aspartic acid in N-terminal pFLAG-CMV expression vector as described previously [Nojima et al., 2010]. Luciferase activities in the cell extracts were determined with the Dual-Glo Luciferase Assay System (Promega) using Wallac 1420 Victor Plate Reader (Berthold Technologies, Bad Wildbad,

Germany). Experiments were repeated at least 3 times. Relative luciferase activities were normalized against Renilla luciferase activity. The mean values of the normalized ratios were compared.

EXTRACTION OF NUCLEAR AND CYTOSOLIC FRACTIONS FROM CELLS

The cells prepared from calvaria of newborn mice were cultured in the presence or absence of DB867 (100 μM) for 8 h and then stimulated with BMP4 (10 ng/ml) for 40 min. Those cells were harvested to separate nuclear and cytosolic fractions using Nuclear/Cytosol Fractionation Kit (Bio Vision, Milpitas, CA) according to the manufacturer's instructions, followed by Western blot analysis.

IMMUNOFLUORESCENT STAINING

Pre-osteoblasts from the calvaria of newborn mice were seeded on coverslips in 24 well plates and cultured in the presence or absence of DB867 (100 μM) for 8 h and then stimulated with BMP4 (10 ng/ml) for 40 min. The cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. The samples were washed with PBS and permeabilized using 0.2% Triton X-100 in PBS for 8 min. Then the cells were blocked with 1% bovine serum albumin in PBS for 1 h in the dark place at room temperature. The coverslips were incubated with rabbit polyclonal antibodies to Smad1 or goat polyclonal antibodies to Smad6 (1:100, respectively) overnight at 4°C. After washed with PBS, the cells were incubated with Alexa Fluor 488 donkey anti-rabbit IgG (1:1000) or anti-goat IgG (1:1000, respectively) (Life Technologies) for 1 h in the dark place at room temperature. Nuclei were counterstained with Hoechst 33258 (1:3000) (Sigma-Aldrich) for 20 min. Slides were visualized on a microscopy system (BZ-9000; KEYENCE, Osaka, Japan).

STATISTICAL ANALYSIS

Results are presented as the means ± standard error (SE) of data from at least three independent experiments. Student's *t*-tests were used and significance is represented by * or ** for $P < 0.05$ or $P < 0.01$, respectively.

RESULTS

OSTEOBLAST DIFFERENTIATION IN PRIMARY CULTURES OF MOUSE CALVARIA CELLS

BMP signaling is an important pathway for the regulation of osteoblast differentiation. Therefore, primary cells isolated from calvaria of newborn mice were cultured to differentiate into osteoblasts for 3 days in a medium supplemented with BMP4, followed by an assay of ALP activity and the expression of osteocalcin, typical markers of osteoblast differentiation. The number of ALP-positive cells was slightly but significantly abundant in the culture of PRIP-KO cells even in the absence of BMP4, and the stimulation with BMP more robustly increased the positive cells in KO cells (Fig. 1A). Similar patterns between WT and KO with or without BMP were also observed in assays of the enzyme activity and the expression of osteocalcin (Fig. 1B, C).

We then measured the promoter activity of an immediate early BMP responsive gene, Id1 in primary cultures of calvaria from newborn mice of both genotypes. Luciferase reporter plasmid driven by Id1 promoter (Id1WT4F) was transfected into the calvaria cells, followed by BMP4 stimulation for up to 36 h. Id1 transcriptional activity was slightly increased by BMP in WT cells for 12 and 24 h and then decreased 36 h after stimulation (Fig. 2). Similar changes in response to BMP stimulation were also observed with the cells from KO mice, but it was noted that the basal activity without stimulation was higher and the stimulation was more robust (Fig. 2), consistent with the results shown in Figure 1. The results indicate that KO cells exhibit higher basal parameters as mature osteoblasts, and are more accelerated signaling by BMP stimulation.

PHOSPHORYLATION OF Smad1/5 IN RESPONSE TO BMP STIMULATION

Smad1/5 is the downstream effector as a transcription factor in BMP signaling. They have conserved N- and C-terminal regions known as Mad homology (MH) 1 and MH2 domains, and the regions are connected by a variable linker region [Wrana, 2000].

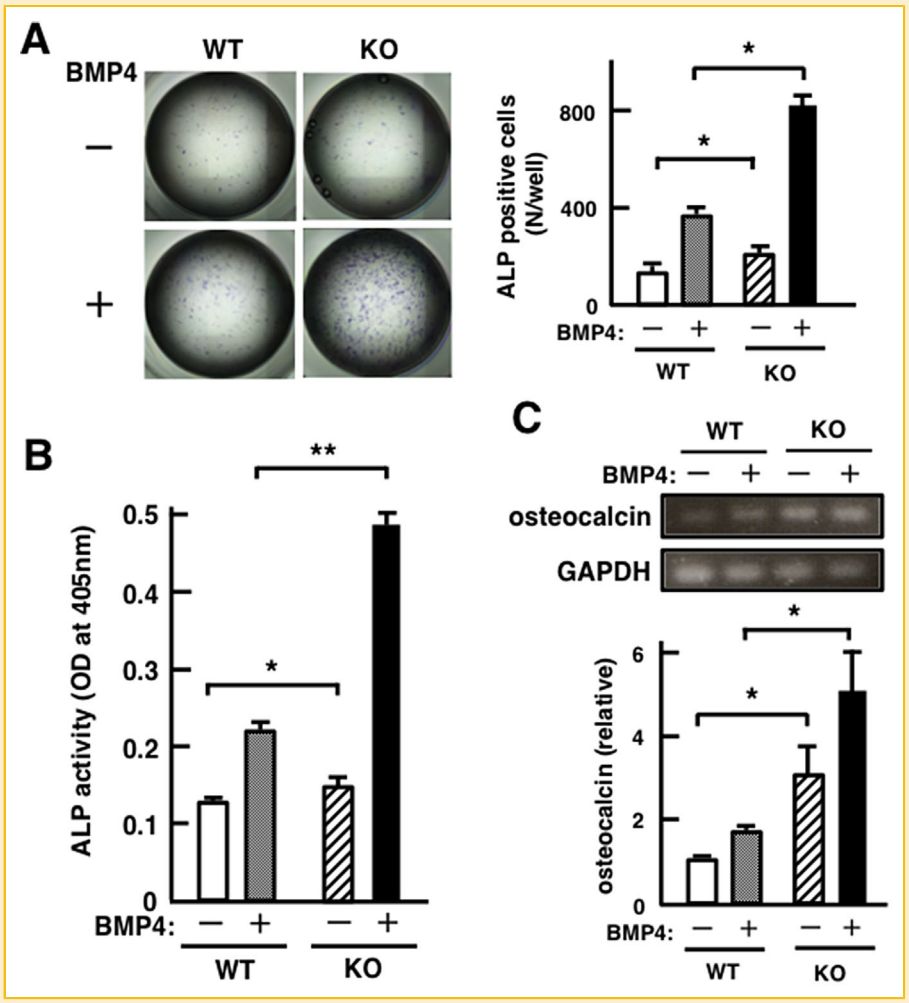

Fig. 1. Osteoblast differentiation in primary cultures. Cells prepared from calvaria of newborn WT and KO mice were cultured in the presence of BMP4 (10 ng/ml) for 3 days. (A) A typical image of ALP staining and the number of ALP-positive cells. (B) ALP activity using *p*-nitrophenylphosphate as a substrate. (C) Osteocalcin gene expression by RT-PCR analysis. Graphs are the means ± SE of data from at least four mice performed in triplicate. * or ** for $P < 0.05$ or $P < 0.01$ compared with WT, respectively.

Smad1/5 is a main factor for BMP signaling and the phosphorylation of Smad1/5 C-terminal is an initial step to transmit the BMP signaling cascade following BMP binding to the receptors [Massague et al., 2005; Katagiri, 2008]. We previously reported that Smad1/5 phosphorylation by BMP stimulation in KO calvaria cells was more evident than that in WT cells; particularly after the peak than at a peak, the results led us to assume that PRIP might be involved in the dephosphorylation process [Tsutsumi et al., 2011]. Here we re-evaluated the phosphorylation by BMP4 stimulation assessed by immunoblotting with the phospho-specific antibody. The only slight difference from the previous report was that the peak value at 60 min was higher in KO cells, but the phosphorylation levels after the peak up to 150 min remained higher, as in the previous report (Fig. 3).

To investigate the dephosphorylation process of Smad1/5 after BMP stimulation, calvaria cells prepared from both genotypes were stimulated with BMP for 30 min, followed by further incubation after the removal of BMP for up to 90 min. BMP receptor 1A inhibitor, LDN193189, was included in the medium to stop BMP stimulation, as only the removal of BMP from the culture medium did not provide a consistent result, even after extensive washing. The decrease of Smad1/5 phosphorylation with the time course was not apparently different between WT and KO mice (Fig. 4).

The linker region between two MH domains of Smad1/5 is also known to be phosphorylated by BMP stimulation, but there was little difference during the time course in the stimulation and stopping between WT and KO mice, indicating no involvement of PRIP in the phosphorylation (data not shown).

DOWNSTREAM PROCESS OF Smad PHOSPHORYLATION

Phosphorylation of Smad1/5 is followed by entry into the nucleus along with a co-stimulator, Smad4, escaping from the actions of inhibitory Smad6 and 7 for transcriptional activity [Miyazono et al., 2005]. We first analyzed the amount of other Smads (Smad4, 6 and 7)

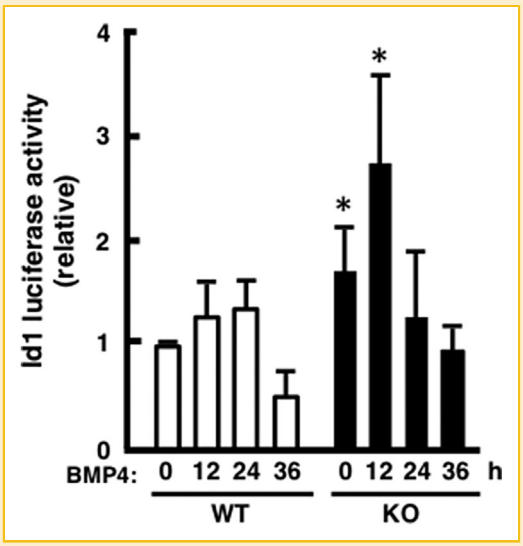

Fig. 2. Id1 gene promoter activity in response to BMP stimulation. Calvaria cells from newborn WT and KO mice were cultured for 24 h after transfection with Id1WT4F reporter plasmid and pRL-SV40, followed by stimulation with BMP4 (1 ng/ml) for 12, 24 and 36 h. The cells were lysed and luciferase activity was measured by a luminometer. Results are shown as the means ± SE of data from at least four independent experiments, each performed with duplicate samples. * for $P < 0.05$ compared with WT.

by quantitative real-time PCR assay and found no difference in their amounts at the mRNA level between the two genotypes. We then investigated the involvement of PRIP in transcriptional activity in response to BMP stimulation. In order to bypass the phosphorylation of Smad, we here used a mutant Smad1 expression plasmid, Smad1 (DVD), in which the two serine residues at the C-terminal (Ser463 and Ser465) are substituted with aspartic acids, mimicking phosphoresidues of the serine and thus gaining the function of being constitutively active [Nojima et al., 2010]. The mutant Smad1(DVD) or the wild type was co-transfected into calvaria cells with the Id1WT4F reporter plasmid to perform a luciferase assay 24 h after transfection. Smad1(DVD) increase the luciferase activity, or Id1 transcriptional activity without BMP and the activity was not significantly different between WT and KO cells (Fig. 5). The result indicates that PRIP is not involved in the regulation of signaling following Smad1/5 phosphorylation. The accerelation of Id1 transcriptional activity may be significantly different between both genotypes in the case PRIP has some distinct effects after BMP phosphorylation.

EXPRESSION AND FUNCTION OF BMP RECEPTORS IN CALVARIA CELL CULTURES

BMP signaling is transduced by BMP receptors (BMPR), which consist of two different types of transmembrane serine/threonine kinase

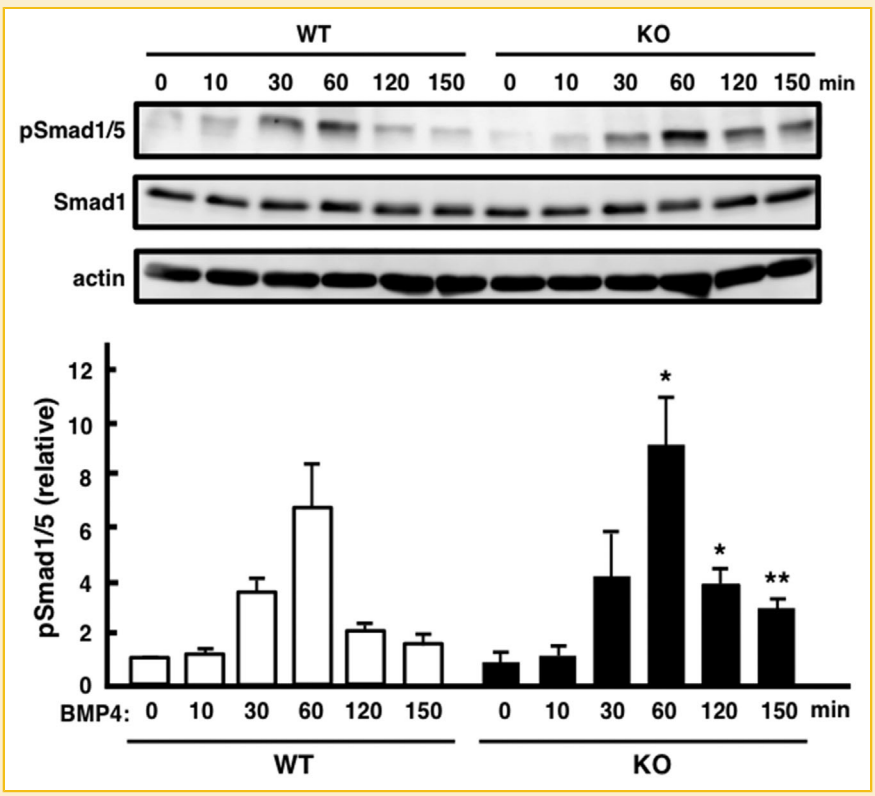

Fig. 3. Phosphorylation of Smad1/5 in response to BMP4 stimulation. Primary cells isolated from calvaria of WT and KO mice were cultured in the presence of BMP4 (10 ng/ml) for the time indicated. Cells were lysed and subjected to SDS–PAGE and the following Western blotting using antibodies against Smad1, phosphorylated Smad1/5 and β-actin. The upper blots are typical and the data are the means ± SE of data from five independent experiments. * or ** for $P < 0.05$ or $P < 0.01$ compared with WT, respectively.

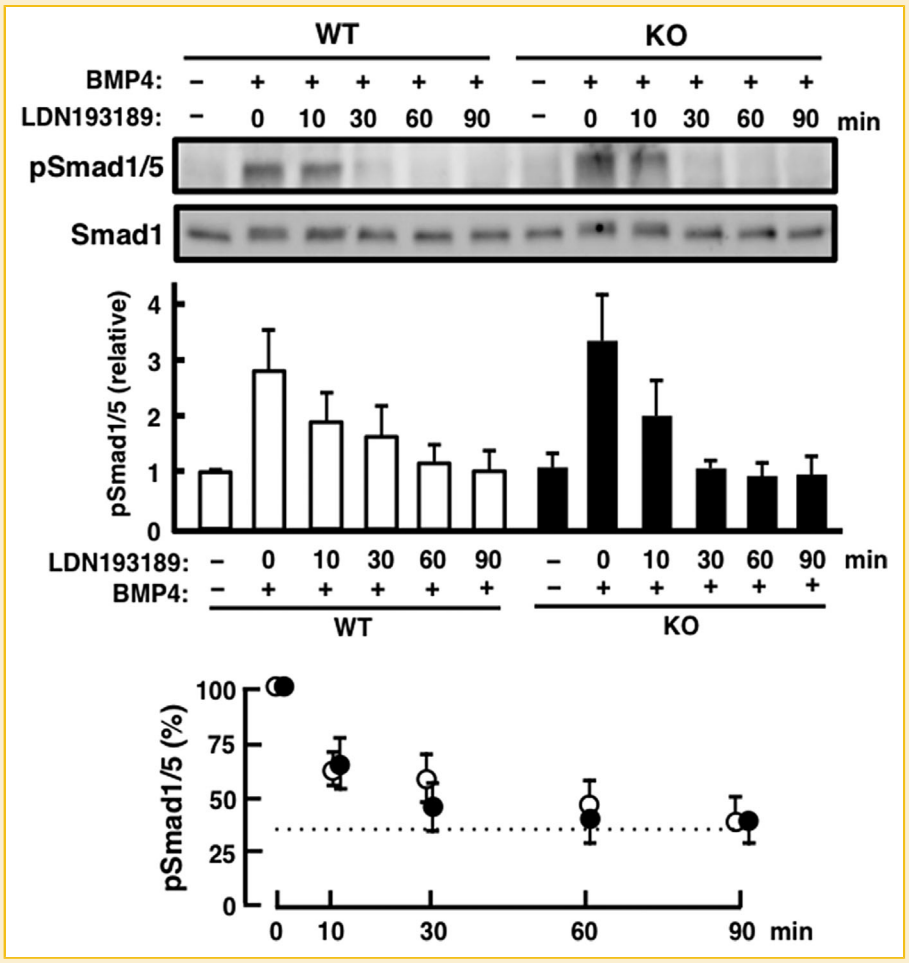

Fig. 4. Dephosphorylation of Smad1/5 after BMP4 stimulation. Primary cells isolated from calvaria were cultured in the presence of BMP4 (1 ng/ml) for 30 min, followed by removal of the medium and extensive washing. BMP receptor 1A inhibitor, LDN193189 (800 ng/ml), was added to the medium. To determine the optimal concentration of the inhibitor, we performed several experiments, changing the concentration from 100 ng/ml to 1,600 ng/ml. Cells lysates were separated by SDS–PAGE, followed by Western blotting for the detection of Smad1 or phosphorylated Smad1/5. The upper blots are typical and the data are the means ± S.E. of data from at least four independent experiments. The bottom graph shows the decrease in the phosphorylation, taking the value at the end of stimulation as 100%. Dotted line indicates the basal level.

receptors, termed type I and II receptors [Miyazono et al., 2005; Wan and Cao, 2005]. BMP4 binds to BMPR1A and BMPR2 [ten Dijke et al., 1994]. The expression of BMPR1A and BMPR2 was examined by Western blot analysis and the significant difference of both expression levels was not observed between the two genotypes (Fig. 6).

We then examined the involvement of PRIP in functional activation of BMPR mediated by Smad6 methylation [Xu et al., 2013], leading to phosphorylation of smad1/5. Calvaria cells prepared from both genotypes were preincubated with a methy-transferase inhibitor, DB867 in prior to the BMP4 stimulation, followed by Western Blot analysis for Smad1/5 phosphorylation. DB867 robustly decreased BMP-induced phosphorylation of Smad1/5 in WT cells, while little decrease was observed in KO cells (Fig. 7A). Immunofluorescent analysis was performed to investigate the subcellular localization of Smad1 and Smad6. Endogenous Smad1 translocated to nucleus in response to BMP stimulation, which was partly inhibited by pre-treatment with DB867 in WT cells. On the other hand, translocation of Smad1 in KO cells by BMP stimulation was little inhibited by DM867 (Fig. 7B). Nuclear localization of Smad1 was also assessed by immunoblotting analysis of subcellular fractions; nuclear Smad1 triggered by BMP stimulation was significantly decreased by DB867 in WT cells, whereas that was less inhibited in KO cells (Fig. 7C). Translocation of Smad6 from the nulceus to the cytosol was increased by BMP4, which was partly inhibited by DB867 in WT cells, while Smad6 remained in the nucleus even after BMP stimulation in KO cells (Fig. 7B). It was also noteworthy that KO cells exhibited more localization of Smad1 than the control cells before the stimulation with BMP4 (Fig. 7B). These results indicate that PRIP is involved in the methylation of inhibitory Smad6 probably through the regulation of Smad6 translocation by BMP stimulation, triggering the functional activation of Smad network, followed by the translocation of transcriptional Smad.

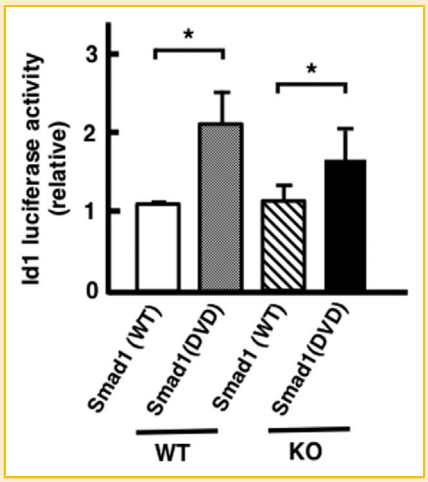

Fig. 5. Transcriptional activity by phosphorylated Smad. Myc-Smad1 (WT) or myc-Smad1(DVD) was co-transfected into calvaria cells with Id1WT4F transporter plasmid and pRL-SV40. After 24 h culture, cells were lysed and luciferase assay was performed. Results are shown as the means ± SE of data from at least five independent experiments, each performed with duplicate samples.

DISCUSSION

We reported that the increased bone mass observed in PRIP-KO mice was caused by enhanced bone formation, and BMP stimulation of newborn calvaria cells caused the prolonged phosphorylation of Smad1/5, probably explaining the enhanced bone formation as one of the many underlying mechanisms [Tsutsumi et al., 2011]. The present study was undertaken to investigate the mechanisms of how PRIP is implicated in the BMP signaling pathway.

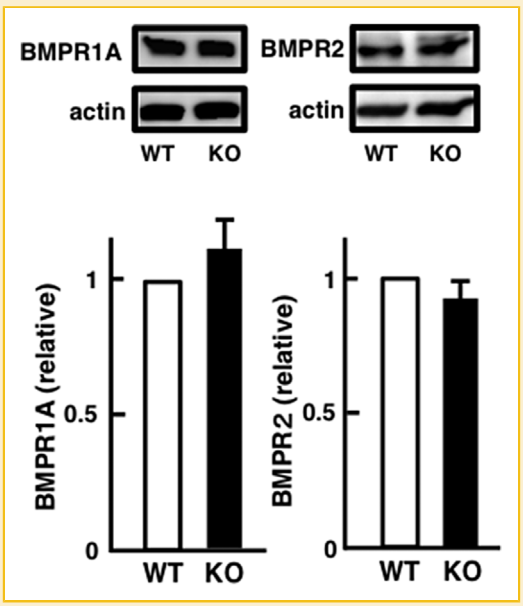

Fig. 6. The expression of BMP receptors in calvaria cell cultures. Membrane fraction was isolated from the calvarial cells of newborn mice to use for Western blotting. Results are the means ± SE of data from at least three independent cultures for both analyses.

We first confirmed the previous results: the prolonged phosphorylation of Smad1/5 at the C-terminal. Smad1/5 C-terminal phosphorylation is balanced between kinases and phosphatases; regulation by two receptor kinases termed BMPR1A and BMPR2, and two Smad phosphatases, small C-terminal domain phosphatase 1 (SCP1) and protein phosphatase magnesium-dependent 1A (PPM1A) [Shi, 2009; Kokabu et al., 2010, 2011]. We then assumed that the phosphatases might be primarily involved because the enhancement of Smad phosphorylation was prolonged rather than the initial stimulation, and furthermore, we have reported that PRIP is involved in the regulation of protein phosphatase 1 and -2A (PP1 and PP2A) by direct interaction, leading to the phospho-regulation of specific proteins [Yoshimura et al., 2001; Terunuma et al., 2004; Kanematsu et al., 2006; Gao et al., 2012; Sugiyama et al., 2012]. However, the dephosphorylation process triggered by the removal of BMP and the addition of kinase inhibitor was not different between the genotypes. It is thus likely that PRIP is not involved in the dephosphorylation process, although we have not yet analyzed whether PP1 and -2A in addition to SCP1 and PPM1A are capable of catalyzing the dephosphorylaton of Smad, and PRIP along with PP1 and -2A are present in the vicinity of Smad for the action.

The linker region of Smad1/5 could be also phosphorylated by extracellular signal-regulated kinase (ERK), which is activated by BMP, leading to ubiquitination followed by proteasomal degradation [Lai and Cheng, 2002; Fuentealba et al., 2007; Sapkota et al., 2007]. The dephosphorylation is catalyzed by PP2A [Bengtsson et al., 2009], whose activity is regulated by PRIP as mentioned above. However, little difference was observed in linker phosphorylation between the two genotypes. Our previous study showed that the phosphorylation of ERK was comparable between the genotypes [Tsutsumi et al., 2011]. These results indicate that PRIP is not involved in linker phosphorylation.

BMP receptors (BMPR1 and 2) are both serine/threonine kinases, and the activation in response to BMP leads to the phosphorylation of Smad1/5. PP2A is reported to dephosphorylate BMPR2 to suppress BMPR1 activation, resulting in decreased BMP signaling [Fuentealba et al., 2007]. PRIP might affect the regulation of BMPR activation through the interaction with PP2A, as mentioned above. We tried to examine the possibility of the phosphorylation difference and the activity levels of BMPR1 and 2 with calvaria cells from two genotypes by multiple methodologies as described below, but we failed to detect the phosphorylation and activity of the receptors prior to investigating the difference: (i) Immunoprecipitation of BMP receptors by anti-BMPR1 and BMPR2 was performed using cells metabolically labeled with [^{32}P]orthophosphate after BMP stimulation because of the lack of anti-phosho-BMPR1A and 2 antibodies; (ii) Membrane fractions from calvaria cells stimulated with BMP4 were prepared, followed by the kinase assay in medium containing Mg-ATP plus recombinant Smad1/5; (iii) Membrane fractions from calvaria cells stimulated with BMP4 were prepared, followed by regular SDS–PAGE, but containing 0.1 mM Phos-tag which shifts the phosphorylated band to the upper side to discriminate from the non-phosphorylated band.

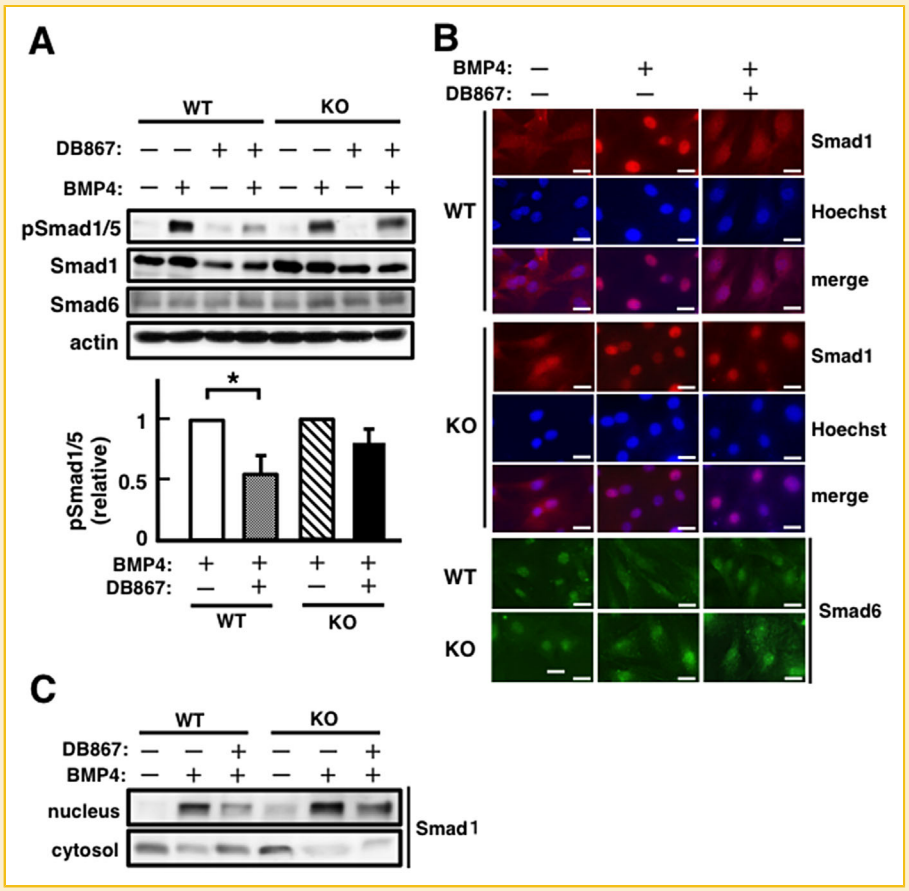

Fig. 7. BMP-induced phosphorylation and translocation of Smad1/5 in calvaria cells pre-treated with DB867. (A) Western blot analysis using the cell extracts and antibodies against Smad1 and phosphorylated Smad1/5. Blot for Smad6 was also included. The blots are typical and the graph shows the summary of three independent Western blots (means ± SE). The density of pSmad1/5 (BMP4+, DB867+) individual bands normalized with Smad1 was represented relative to that of pSmad1/5 (BMP4+, DB867−), in each genotype. * for $P < 0.05$. (B) Immunofluorescent study of Smad1 nuclear translocation triggered by BMP4, using anti-Smad1 antibody. The cells pre-treated with DB867 were stimulated with BMP4, and immunostained with Smad1 antibody (red). Nuclei were stained with Hoechst 33258 (blue). Scale bars, 20 μm. Pictures shown were those of typical among five independent experiments. Localization of Smad6 was also shown in green. (C) Nuclear localization of Smad1 triggered by BMP and the effect of DB867. Subcellular fractions of the nucleus and cytosol were immunoblotted by anti-Smad1 antibody. The blot shown is that of typical from three blots.

As an alternative mechanism for BMPR activation in response to BMP, we examined whether Smad6 methylation is involved, hinted by a recent paper of Xu et al. (2013) which reported that BMP triggers the activation of BMPR2-bound methyltransferase to methylate inhibitory Smad6 bound to BMPR1A and then liberate Smad6 from BMPR1A, resulting in the kinase activation of BMPR1A. WT, but not KO cells showed a significant inhibition of the phosphorylation and translocation to nucleus of Smad1/5 by a methyltransferase inhibitor, DB867, indicating that the regulation of Smad1/5 phosphorylation by methylation of Smad6 is inoperative in the absence of PRIP. This might be caused by either Smad6 focally interacting to BMPR1A is in a smaller amount in KO cells than WT cells, albeit Western blot analysis showed no difference between the genotypes, or Smad6 is more methylated in KO cells before stimulation by unknown mechanisms. This, in either case provides the higher basal activities of ALP and Id1 transcription and more presence of Smad1 in nucleus before stimulation with BMP4. Translocation of Smad6 to BMPR1A is impaired in KO cells to explain the smaller amount at BMPR1A; Smad6 is localized in cells at the nuclei, cytosol and plasma membrane in the absence of BMP, and BMP triggers further recruitment of Smad6 to the plasma membrane at the vicinity of BMPR1A [Miyazono et al., 2010; Xu et al., 2013]. Smad6 migration to cytosol was less in KO cells than WT in response to BMP. PRIP might be involved in the recruitment of Smad6 to the vicinity of BMPR1A at the plasma membrane. Our previous studies reported that PRIP is required for insulin-induced $GABA_A$ receptor transport to the plasma membrane by recruiting active Akt to the receptor complex [Fujii et al., 2010].

The basal levels of ALP-positive cells and the enzyme activity were higher and Id1 transcriptional activity was also higher in KO cells without any stimulation, as described above. These results may be also elucidated by the possibility of Smad1/5 to access easily to BMPR1A at basal level because of inadequate inhibitory function of Smad6 at least at plasma membrane in PRIP-deficient cells. Further

studies are apparently needed to elucidate the roles of PRIP in the association of Smad6 with BMPR1A with or without BMP stimulation, and Smad6 methylation.

Canonical Wnt signaling is also known to play an important role in the enhancement of bone formation [Krishnan et al., 2006; Kamiya et al., 2008]. Canonical Wnt (Wnt1 and Wnt3a) and BMP cooperatively induce osteoblast differentiation through a GSK3β-dependent mechanism [Fukuda et al., 2010]. We examined the involvement of Wnt signaling in BMP induced-accelerated differentiation in KO osteoblasts, and the transcriptional activity of LEF/TCF which binds to the target DNA induced by Wnt3a was observed at equivalent level in both genotypes (data not shown). We further performed experiments using calvaria cells stimulated with Wnt3a or Wnt5a for the canonical or non-canonical pathway, respectively. In these stimulations, the significantly difference of the number of cells stained with ALP and the activity was not observed between the two genotype cells (data not shown). These results suggest the involvement of PRIP in the regulation of not Wnt but BMP signaling.

Previously, it was reported that activated IGF-1R increases Id1 expression in mouse embryonic fibroblasts [Belletti et al., 2002]. Because PRIP deficiency also increases Id1 promoter activity, PRIP may be involved in the transcriptional regulation of Id1 gene and bone formation through not only BMP signaling but also other pathways such as IGF-1.

In summary, we indicated that PRIP is implicated in the initiation step of BMP signaling in osteoblast differentiation in a negative manner, probably through BMP-induced the receptor activation regulated by Smad6 methylation. Thus, PRIP deficient cells are less affected by Smad6 methylation and BMP receptor activation is enhanced, leading to the up-regulation of BMP signaling mediated by Smad1/5 phosphorylation on osteoblast differentiation and bone formation in KO mice.

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