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Wnt5a signaling is a substantial constituent in bone morphogenetic protein-2-mediated osteoblastogenesis

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ABSTRACT

Wnts are secreted glycoproteins that mediate developmental and post-developmental physiology by regulating cellular processes including proliferation, differentiation, and apoptosis through β-catenin-dependent canonical and β-catenin-independent noncanonical pathway. It has been reported that Wnt5a activates noncanonical Wnt signaling through receptor tyrosine kinase-like orphan receptor 2 (Ror2). Although it appears that Wnt5a/Ror2 signaling supports normal bone physiology, the biological significance of noncanonical Wnts in osteogenesis is essentially unknown. In this study, we identified expression of Wnt5a in osteoblasts in the ossification zone of the tibial growth plate as well as bone marrow of the rat tibia as assessed by immunohistochemistry. In addition, we show that osteoblastic differentiation mediated by BMP-2 is associated with increased expression of Wnt5a and Ror2 using cultured pre-osteoblasts, MC3T3-E1 cells. Silencing gene expression of Wnt5a and Ror2 in MC3T3-E1 cells results in suppression of BMP-2-mediated osteoblastic differentiation, suggesting that Wnt5a and Ror2 signaling are of substantial importance for BMP-2-mediated osteoblastic differentiation. BMP-2 stimulation induced phosphorylation of Smad1/5/8 in a similar fashion in both siWnt5a-treated cells and control cells, suggesting that Wnt5a was dispensable for the phosphorylation of Smads by BMP-2. Taken together, our results suggest that Wnt5a/Ror2 signaling appears to be involved in BMP-2-mediated osteoblast differentiation in a Smad independent pathway.

1. Introduction

Wnts are a family of 19 secreted glycoproteins that mediate developmental and post-developmental physiology by regulating cellular processes including proliferation, differentiation, and apoptosis [1]. Wnts are classified into two sub-families based on downstream mediated signaling. The Wnt1 sub-family (e.g. Wnt1, Wnt3a, and Wnt8) activates the canonical Wnt/β-catenin pathway, whereas the Wnt5a sub-family (e.g. Wnt5a and Wnt11) activates noncanonical pathways [1].

The canonical Wnt/β-catenin signaling pathway has been well-characterized and implicated in promotion of bone formation [1,2]. Loss-of-function mutations of low-density lipoprotein receptor related protein (LRP)-5, a co-receptor for Wnt/β-catenin signaling, leads to low bone mass accompanied by fractures causing osteoporosis pseudoglioma syndrome [3,4]. LRP-5 gain-of-function mutations in humans result in high bone mass syndrome [5,6]. On the other hand, noncanonical Wnts activate the β-catenin-independent signaling pathway, including the Wnt/Ca^{2+} pathway and Wnt/planar cell polarity (PCP) pathway, and are supposed to have crucial functions in the regulation of cell migration and polarity during embryogenesis [7]. It has been reported that receptor tyrosine kinase-like orphan receptor 2 (Ror2), a member of the Ror-family of receptor tyrosine-protein kinases [8], acts as a receptor or co-receptor for Wnt5a [9,10]. Ror2 by itself or in combination with Frizzled protein through its Frizzled-like cysteine-rich domain [9] mediates diverse Wnt5a signaling by activating the Wnt-c-Jun N-terminal kinase PCP pathway [9] and inhibiting the β-catenin-T cell factor/lymphoid enhancer factor pathway [10]. Loss-of-function mutations in Wnt5a and Ror2 cause autosomal recessive Robinow syndrome, characterized by midfacial hypoplasia, limb bone shortening, and genital abnormalities [11,12], suggesting that the Wnt5a/Ror2 signaling pathway is important in human craniofacial and skeletal development. Overexpression of Ror2 increases osteoblast differentiation of human mesenchymal cells and osteoblastic

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MC3T3-E1 cells [13]. Wnt5a induces osteoblastogenesis through attenuation of peroxisome proliferator-activated receptor-γ-induced adipogenesis in mesenchymal stem cells of bone marrow [14]. Thus, it is likely that noncanonical Wnt signaling supports normal bone physiology. However, a specific role of Wnt5a in osteogenesis has not been determined.

Bone morphogenetic proteins (BMPs) are structurally related to the transforming growth factor-β superfamily and were originally identified by their capacity to induce ectopic bone formation in rodents [15]. Among the BMP family members, BMP-2 has been extensively studied for its various biological functions, particularly during osteogenic differentiation [16].

In this study, we demonstrated that osteoblastic differentiation mediated by BMP-2 is associated with increased expression of Wnt5a and Ror2 *in vivo* and *in vitro,* and that silencing gene expression of Wnt5a and Ror2 results in the suppression of BMP-2-induced expression of alkaline phosphatase (ALP) and osteocalcin (OCN), suggesting that Wnt5a and Ror2 signaling is a substantial constituent for BMP-2-mediated osteoblastic differentiation.

2. Materials and methods

2.1. Reagents

Recombinant human BMP-2 was purchased from R&D systems (Minneapolis, MN, USA). Ascorbic acid, Triton X-100, phenylmethylsulfonyl fluoride (PMSF), and *p*-nitrophenyl phosphate (*p*NPP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Tissue preparation and immunohistochemistry (IHC)

Serial sections (5-μm) of the tibia from male Wistar rats (Kumagai-shigeyasu Co., Sendai, Japan) in their second postnatal week were used for IHC, as previously described [17]. Fixed specimens, with 4% paraformaldehyde in phosphate-buffer saline (PBS) at 4 °C overnight, were decalcificated with 10% EDTA in PBS at 4 °C for 2 weeks. After dehydration using a graded series of ethanol solutions and xylene, specimens were embedded in paraffin tissue blocks. After deparaffinization, tissue sections were blocked for endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol, and antigen retrieval was performed using Sodium citrate solution (10 mM, pH 6.0) in a microwave oven for 20 min. Sections were incubated with rabbit polyclonal antibody to Wnt5a (Abcam, Cambridge, MA, USA) diluted 1:50 in PBS containing 5% normal goat serum and 0.05% Triton X-100 overnight at 4 °C. Next, sections were incubated with 1:1000 diluted biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) for 1 h, and then visualized using the Vectastain ABC kit (Vector). Sections were counterstained with methyl green solution. For negative controls, the primary antibody was omitted.

2.3. Cell culture

A murine pre-osteoblastic cell line, MC3T3-E1, was purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in α-minimal essential medium (MEM) (Gibco™/Life technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco™/Life technologies), 100 U/ml penicillin G, and 100 μg/ml streptomycin. All tissue culture reagents were from Gibco™/Life technologies.

2.4. Reverse transcription and real-time quantitative polymerase chain reaction (PCR)

Total cellular RNA was extracted using RNeasy® (QIAGEN Inc., Valencia, CA, USA) in accordance with the manufacturer's instructions, and treated with DNAase (DNA-free™, Ambion Inc., Austin, TX, USA). Total RNA was reverse-transcribed using a Transcriptor First Strand cDNA Synthesis Kit® (Roche Diagnostic Co., Indianapolis, IN, USA), and cDNA, which is theoretically converted from 100 ng of total RNA, was used for PCR amplification. Primer sequences for each murine gene encoding Wnt5a, Ror2, ALP, OCN, and glyceraldehyde 3-phosphate dehydrogenate (GAPDH) were as follows (forward/reverse): *Wnt5a* (5′-TCAGAACCAGCCACTTAGG-3′/5′-GCACAAATGGAAAGTCTAAACG-3′); *Ror2* (5′-AATGT GCTGGTG TACGAC-3′/5′-ATGGAGAACTTTCCATACATGAC-3′); *Alp* (5′-GGGGACATGCAGTATGAGTT-3′/5′-GGCCTGGTAGTTGTTGTGAG-3′); *OCN* (5′-TGAACAGACTCCGGCG-3′/5′-GATACCGTAGATGCGTTTG-3′); *Gapdh* (5′-ACCACAGTCCATGCCATCAC-3′/5′-TCCACCACCCTGTTGCTGTA-3′). For real-time PCR, the amplification profile was 40 cycles at 95/60; 55/30; and 72/30 [temperature (°C) /time (sec)]. PCR was performed in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with iQ SYBR Green Supermix® (Bio-Rad). After amplification, one cycle of linear temperature gradient from 55 to 95 °C at a transition rate of 0.5 °C/30 s was performed to assess the specificity of the PCR products. Relative expression levels of transcripts were shown after normalization to the corresponding sample expression level of GAPDH.

2.5. Gene silencing with small interfering RNA (siRNA)

Monolayer cells at 50–70% confluence were transfected with 20 nM of small interfering RNA (Silencer® Select Pre-designed siRNA; Ambion Inc.) against mouse Wnt5a (ID: s76087) and mouse Ror2 (ID: s77265) or negative control siRNA (Silencer® Select Negative control #1 siRNA) in the presence of Lipofextamine®2000 (Invitrogen™/Life technologies), according to the manufacturer's instructions. The day after 48 h of transfection was designed day 0.

2.6. ALP activity

Confluent monolayer cells in a 96-well culture plate were washed twice with ice-cold PBS and lysed by repeating freeze and thaw three times. Whole cell lysate in a well was assayed by adding 1 mg/ml of pNPP as a substrate in 0.1 M glycine buffer (pH 10.4) containing 1 mM $ZnCl_2$ and 1 mM $MgCl_2$ for 60 min at 37 °C. The reaction was stopped by adding NaOH (final 0.6 N) and absorbance was read spectrophotometrically at 405 nm. Enzyme activity was expressed as OD_{405}/h/well.

2.7. Western blotting

Confluent monolayer cells were harvested with Cell Lysis Buffer® (Cell Signaling Technology, Beverly, MA, USA) in accordance with the manufacturer's instructions. Cell lysates were separated by SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan) using a semidry transblot system (ATTO). The blot was blocked with 0.5% (w/v) non-fat dried milk and 0.1% (v/v) Tween 20 in PBS at room temperature for 1 h, followed by incubation for 1 h at room temperature with rabbit anti-phospho-Smad1/5/8 (Cell Signaling Technology) or anti-β-actin antibodies (Cell Signaling Technology) at 1:1000. The blot was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology) at 1:2000 for 1 h at room temperature. The blot was then treated with Western blotting detection reagent ECL Plus® (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA), and a chemiluminescent signal was detected using a luminescent image analyzer ChemiDoc XRS Plus (Bio-Rad).

2.8. Statistical analysis

All experiments in this study were performed three times to test the reproducibility of the results, and representative findings are shown. Experimental values are given as means ± SD. The significance of differences between control and treatment experiments was evaluated by one-way ANOVA. *P* values less than 0.05 were considered significant.

3. Results

3.1. Expression of Wnt5a on osteoblasts and its induction upon stimulation with BMP-2

We investigated the expression of Wnt5a on osteoblasts in the tibia from two-week-old Wistar rats by IHC. Expression of Wnt5a was detected in osteoblasts at the ossification zone (OZ) of the tibial growth plate (Fig. 1A). Osteoblasts in bone marrow (BM) of the rat tibia also showed strong immunoreactivity, while no expression was detected in osteocytes (arrowhead) located in bone lacunae. (Fig. 1B) Negative control sections showed no immunoreactivities as shown in Fig. 1C. Because Wnt5a was expressed by osteoblasts, we examined expression of Wnt5a during osteoblast differentiation in MC3T3-E1, a murine pre-osteoblastic cell line. MC3T3-E1 cells were differentiated into osteoblasts by culturing with BMP-2. Real-time PCR analysis revealed that treatment with BMP-2 significantly increased gene expression levels of Wnt5a at a dose-dependent increase (Fig. 1D). The time kinetics experiment showed that a significant increase in gene expression levels of Wnt5a was detected at day 3 after BMP-2 stimulation, and was maintained at the same levels thereafter (Fig. 1E). Since it has been reported that Ror2 acts as a receptor or co-receptor for Wnt5a [9,10], we investigated whether Ror2 expression may be associated with alterations in Wnt5a expression during osteoblast differentiation. BMP-2 significantly increased gene expression levels of Ror2 at a dose of 30 ng/ml and increased in a dose-dependent manner in a similar fashion to Wnt5a expression (Fig. 1E).

3.2. Wnt5a signaling is of substantial importance for BMP-2-mediated osteoblastic differentiation

Expression of Wnt5a and increased expression of Wnt5a associated with osteogenic differentiation implicate its significance in osteoblastic functions. In order to examine the role of Wnt5a signaling for BMP-2-mediated osteoblastic differentiation, cells were pretreated with Wnt5a siRNA to reduce expression of Wnt5a mRNA and then incubated with 100 ng/ml BMP-2 for 3 days to evaluate gene expression levels of ALP and OCN. Fig. 2A shows that expression of Wnt5a mRNA in cells pretreated with Wnt5a-siRNA for 48 h was significantly lower (89% reduction) than that in control siRNA pretreated-MC3T3-E1 cells. Gene expression levels of ALP and OCN, which are osteoblast-related markers, were upregulated upon stimulation with BMP-2 in control cells, but increased expression was significantly inhibited in Wnt5a siRNA treated cells. (Fig. 2B and C). We also evaluated ALP enzymatic activity under the same condition. ALP activity of MC3T3-E1 cells increased upon stimulation by BMP-2 as previously reported [18], while pretreatment with Wnt5a-siRNA significantly inhibited BMP-2-mediated ALP activity in MC3T3-E1 cells (Fig. 2D). It was noted that this result was not a reflection of alterations in cell number because BMP-2 treatment caused no significant effect on the proliferative response as assessed by a colorimetrical assay using Cell Counting

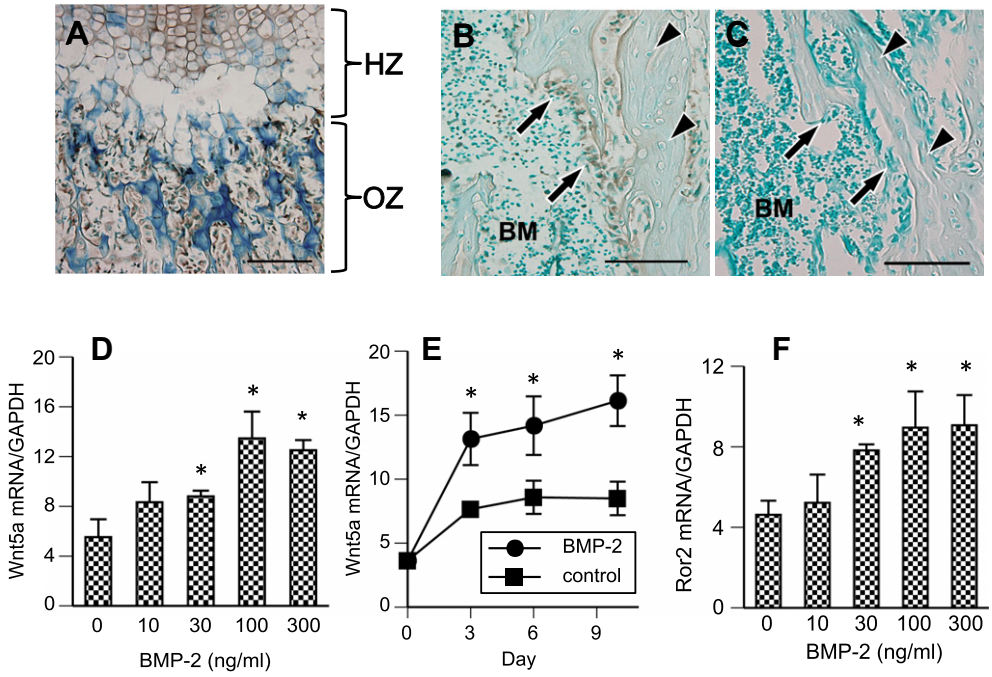

Fig. 1. Expression of Wnt5a on osteoblasts *in vivo* and *in vitro*. Immunolocalization of Wnt5a in the rat tibia. (A) Expression of Wnt5a was detected in osteoblasts at the ossification zone (OZ) of the tibial growth plate. (B) Osteoblasts in bone marrow (BM) of the rat tibia show strong immunoreactivity, while no immunoreactivity was detected in osteocytes (arrowhead) located in bone lacunae. (C) No immunoreactivities are detected in negative control sections performed without using a primary antibody for Wnt5a. Arrow: osteoblast; Arrowhead: osteocyte; HZ: hypertrophic zone and OZ: ossification zone in the growth plate; BM: bone marrow; Bars = 20 μm. (D and F) MC3T3-E1 cells, at monolayer confluence, were stimulated with the indicated concentrations of recombinant human BMP-2 for 3 days. (E) MC3T3-E1 cells, at monolayer confluence, were stimulated with 100 ng/ml of recombinant human BMP-2 for the indicated days. Total cellular RNA was extracted and transcripts were analyzed by real-time PCR. Representative data of three separate experiments are shown as means ± SD of triplicate assays. Significance is indicated (*$P < 0.05$ compared with respective BMP-2-untreated control).

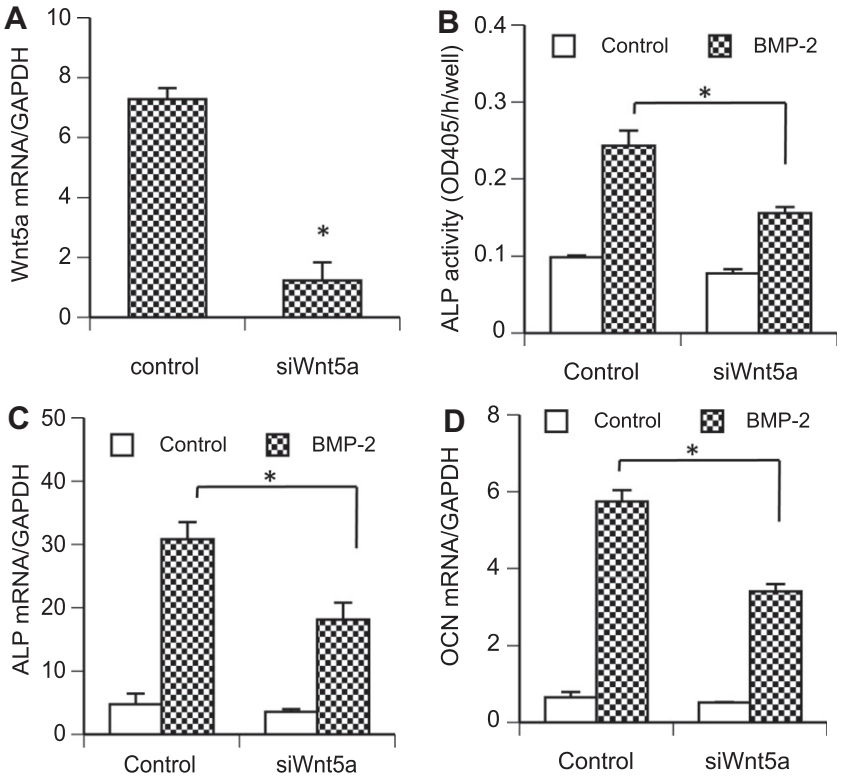

Fig. 2. Wnt-5a signaling is of substantial importance for BMP-2-mediated differentiation of MC3T3-E1 cells. (A) MC3T3-E1 cells were transfected with small interfering RNA against mouse Wnt5a or negative control siRNA, and cultured for 48 h. (B) After 48 h of transfection, cells were stimulated with 100 ng/ml of recombinant human BMP-2 for 3 days, and ALP activity on the whole cell lysate was measured as described in Section 2. Enzyme activity was expressed as OD_{405}/h/well. (C and D) After 48 h of transfection, cells were stimulated with 100 ng/ml of recombinant human BMP-2 for 3 days. (A–D) Total cellular RNA was extracted and transcripts were analyzed by real-time PCR. Representative data of three separate experiments are shown as means ± SD of triplicate assays. Significance is indicated ($^*P < 0.05$ compared with negative control).

Kit-8® (Dojindo, Kumamoto, Japan, data not shown). These findings suggested that Wnt5a signaling is a substantial constituent for BMP-2-mediated osteoblastic differentiation.

3.3. Ror2 signaling is also of substantial importance for BMP-2-mediated osteoblastic differentiation

Since Ror2 acts as a receptor or co-receptor for Wnt5a, we verified that Ror2 signaling was involved in BMP-2-mediated osteoblastic differentiation; cells were treated with Ror2 siRNA and then incubated with 100 ng/ml BMP-2 for 3 days to evaluate gene expression levels of ALP and OCN as well as ALP activity. Expression of Ror2 mRNA was significantly lower with Ror2 siRNA than that with control siRNA in MC3T3-E1 cells. Gene expression levels of ALP and OCN as well as ALP activity, all of which increased upon stimulation with BMP-2 in control cells, were significantly inhibited in Ror2 siRNA-pretreated cells. (Fig. 3B–D). These findings suggest that Ror2 signaling as well as Wnt5a signaling is important for BMP-2-mediated osteoblastic differentiation.

3.4. Wnt5a signaling is not required for phosphorylation of Smad1/5/8 induced by BMP-2

In the intraceller signaling of BMP-2, eight Smad proteins have been identified to play critical roles in mammals [19]. The receptor Smad, consisting of Smad1, Smad2, Smad3, Smad5, and Smad8, are phosphorylated directly by BMP receptor type I and then form complexes with the Co-Smad, Smad4, and move into the nucleus, where they bind to the regulatory regions of target genes [19]. In order to examine whether Wnt5a signaling is required for the process where Smad signaling is activated by BMP-2, siWnt5a pretreated- and negative siRNA pretreated-cells were stimulated with BMP-2 for 20 min and then the phosphorylated status of Smad1/5/8 was evaluated by Western blotting. Fig. 4 shows that BMP-2 was able to induce phosphorylation of Smad1/5/8 in a similar fashion in both siWnt5a cells and negative control cells, suggesting that Wnt5a signaling was not required for phosphorylation of Smad1/5/8 induced by BMP-2, and that Wnt5a/Ror2 signaling may be crosstalk with the BMP-2 cascade independent of Smad phosphorylation in osteoblast differentiation.

4. Discussion

We show that Wnt5a was expressed by osteoblasts in the ossification zone of the growth plate as well as bone marrow in the rat tibia, but was not expressed by osteocytes in bone lacunae. Furthermore, we demonstrate that expression levels of Wnt5a and Ror2 on cultured osteoblasts were upregulated by stimulation with BMP-2. Alterations in expression levels during osteoblast differentiation shown in this study are consistent with previous *in vitro* studies using conventional osteogenic medium containing dexamethasone, ascorbic acid, and glycerophosphate. These studies show that Wnt5a was up-regulated during osteoblastic differentiation compared to uncommitted mesenchymal stem cells, and declined at a late stage of differentiation [20–22], and that Ror2 expression increases during early stages of osteoblast differentiation and declines during terminal osteocytic differentiation [23].

It has been reported that Wnt5a has a potential dual role in the maintenance of mesenchymal stem cells in bone marrow and enhancing osteogenesis [24], and that Wnt5a potentiates osteogenic differentiation of mesenchymal stem cells in a Ror2-depen-

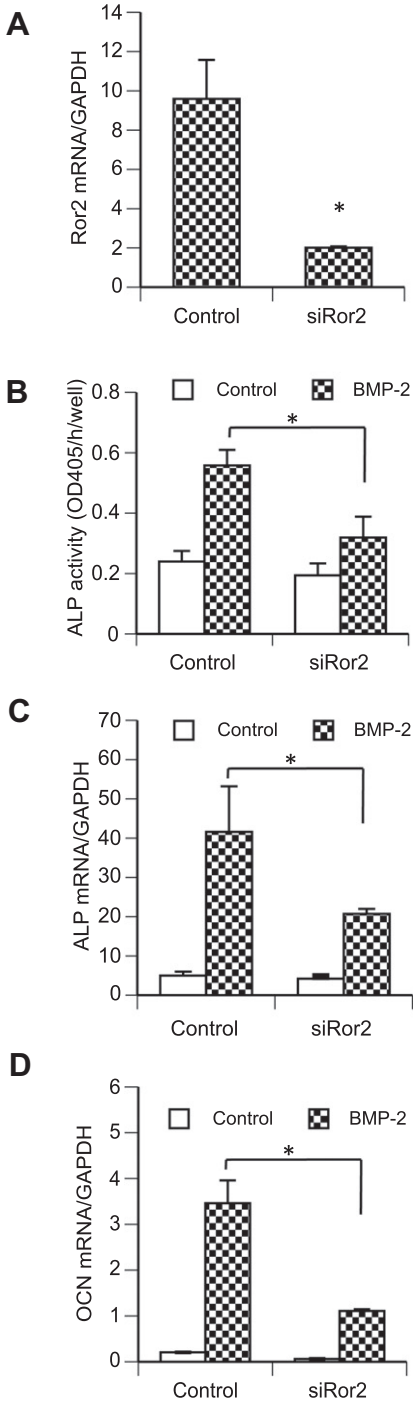

Fig. 3. Ror2 signaling is also of substantial importance for BMP-2-mediated differentiation of MC3T3-E1 cells. (A) MC3T3-E1 cells were transfected with small interfering RNA against mouse Ror2 or negative control siRNA, and cultured for 48 h. (B) After 48 h of transfection, cells were stimulated with 100 ng/ml of recombinant human BMP-2 for 3 days, and ALP activity on the whole cell lysate was measured as described in Section 2. Enzyme activity was expressed as OD_{405}/h/well. (C and D) After 48 h of transfection, cells were stimulated with 100 ng/ml of recombinant human BMP-2 for 3 days. (A–D) Total cellular RNA was extracted and transcripts were analyzed by real-time PCR. Representative data of three separate experiments are shown as means ± SD of triplicate assays. Significance is indicated (*$P < 0.05$ compared with negative control).

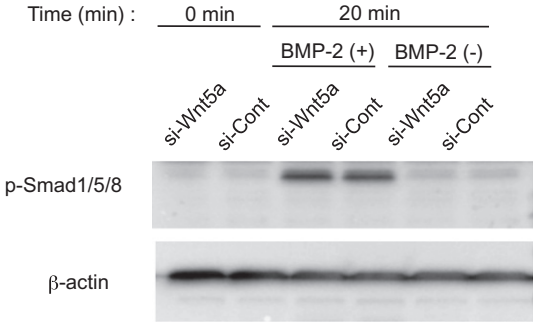

Fig. 4. Wnt5a signaling is not required for phosphorylation of Smad1/5/8 induced by BMP-2. Cells were transfected with small interfering RNA against mouse Wnt5a or negative control siRNA. After 48 h of transfection, monolayer cells were stimulated with 100 ng/ml of recombinant human BMP-2 for 20 min. Cell lysates were analyzed by Western blotting with anti-phospho-Smad1/5/8 antibody to detect phosphorylation of Smad1/5/8. An antibody against β-actin was used as a control. Findings are representative of three independent experiments.

dent manner and enhanced bone formation in mouse calvarial bone explant cultures [25]. In this study, our findings that Wnt5a is expressed by control osteoblasts and is upregulated in association with osteogenic differentiation led us to investigate the role of Wnt5a signaling by means of reducing expression levels of Wnt5a, but not by addition of Wnt5a or forced expression of Wnt5a. We show for the first time that BMP-2-mediated osteoblastic differentiation was inhibited in siWnt5a-treated osteoblasts as well as siRor2-treated osteoblasts over control cells, as assessed by gene expression of ALP and OCN and ALP activity, indicating that Wnt5a and Ror2 signaling are important for BMP-2-mediated osteoblast differentiation. A similar observation using Wnt5a-deficient mice [20] has been reported where genes regulating osteoblastic differentiation including runt-related transcription factor-2, osterix, and ALP are down-regulated in calvarial cells. Concomitant with down-regulation, follistatin, an extracellular BMP antagonist, is upregulated [20], which may imply that the possible action of BMP-2 is restricted at the extracellular level. In this study, we show that BMP-2 activates the Smad1/5/8 signaling pathway in siWnt5a-treated cells to a similar level in control cells, suggesting that it is unlikely that inhibition of cell differentiation in siWnt5a-treated cells is due to production of BMP antagonists. This finding also suggests that it is unlikely that expression levels of BMP receptors may be altered in siWnt5a-treated cells. At this point, it is unclear which signaling pathway(s) and which transcription factor(s) may be affected by silencing Wnt5a and Ror2 genes and this point has to be clarified.

It is well established that Ror2 acts as a receptor or co-receptor for Wnt5a [9,10] and the molecular mechanism of Wnt5a/Ror2 signaling has been extensively studied [8–10,25–27]. Meanwhile, Wnt5a can bind to several receptors, including Ror2 and Frizzled proteins depending on the cellular context, and activates diverse signaling pathways [7]. Therefore, inhibition of osteoblastic differentiation by treatment with siWnt5a shown in this study is not necessarily connected solely to its function in the Ror2 signaling pathway. On the other hand, it has been reported that Ror2 physically interacts with even canonical Wnts, Wnt1 and 3, and modulates the function of β-catenin signaling [23]. In our experiments, gene expression of Wnt3a was not detected on control MC3TC-E1 cells, and not even on BMP-2 stimulated cells or siWnt5a-treated cells (data not shown), suggesting that it is unlikely that Wnt3a/Ror2 signaling is involved in our finding that osteoblastic differentiation induced by BMP-2 is inhibited by siWnt5a.

In summary, we demonstrate that Wnt5a and/or Ror2 expression levels are related to osteoblastic differentiation status by *in vivo* and *in vitro* studies, and that Wnt5a and Ror2 signaling are of substantial importance for BMP-2-mediated osteoblastic differentiation in a Smad independent pathway.

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