Rspo1 promotes osteoblast differentiation via Wnt signaling pathway

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R-spondin (Rspo) proteins are a new group of Wnt/beta-catenin signaling agonists. These signaling molecules are known to be involved in the developmental stages of skeletal system. Recent studies in various murine osteoblast models have proposed that Rspo1 may interact with Wnt signaling pathway to induce differentiation in osteoblasts. Though findings in murine osteoblasts implicate a synergistic role of Rspo1 with Wnt signaling, still no study has addressed the similar role in more clinically applicable osteoblast models i.e., human cell lines or primary cells. Therefore, in the present study, we investigated the possible role of Rspo1 during differentiation process of human in vitro osteoblast cell models like primary osteoblasts or human osteoprogenitor cell line hFOB1.19 along with murine preosteoblast cell line MC3T3 E-1. Our results showed increase in Rspo1 at transcript level during differentiating phase of human primary osteoblasts and human FOB1.19 cells. We also found that Rspo1 (100 ng/mL) acts additively with Wnt3a to activate Wnt signaling, as confirmed by luciferase activity after transfection of TOPFLASH construct to hFOB1.19 cells. Similar additive role of Rspo1 and Wnt3a was apparent in alkaline phosphatase (ALP) activity analysis of human primary cells. Moreover, a reduction in ALP activity was observed with knock-down of Rspo1 by transfected shRNA in hFOB1.19 cells. These results suggested the possibility of autocrine regulation by Rspo1 on the osteogenic activities in human in vitro osteoblast models. Furthermore, these results were corroborated in MC3T3-E1, murine osteoblast cell model. Osteoblastic differentiation was induced by transfection of Rspo1 which was confirmed by increased ALP staining and qRT-PCR analysis of osteogenic markers, such as Runx2 and osteocalcin. In conclusion, present study highlights the role of Rspo1 in bone remodeling where it activates Wnt signaling to induce differentiation, as shown in human as well murine in vitro osteoblast cell models.

**Keywords:** Rspo1, Wnt signaling, Osteoblast, beta-Catenin

Wnt signaling is involved in numerous aspects of embryonic development and plays pivotal role in regulating diverse biologic processes, such as regulation of mesenchymal cell differentiation and bone physiology1. Wnt ligands are a family of secreted proteins that regulate beta-catenin turnover through interactions with frizzled family of receptors (there are 10 of these seven-transmembrane-span proteins in humans) and low-density lipoprotein receptor–related protein 5 (LRP-5) or LRP-62,3. Activated beta-catenin drives the transcription activation by binding to the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors4. Recently, it has been shown that Wnt/beta-catenin signaling plays a critical role in the regulation of bone mass and is involved in many bone disorders5. Moreover, family of Wnt proteins have been shown to play a role in distinct aspects of cartilage and bone biology, such as cartilage matrix synthesis and turnover, bone formation and turnover and mechanobiology of both cartilage and bone6,7.

The primary event of bone formation is osteoblast differentiation which is exemplified by the synthesis, deposition and mineralization of the extracellular matrix. During this event, Wnt/beta-catenin signaling is one of the mechanisms which increases bone formation via stimulation of osteoblast
proliferation and differentiation. Furthermore, Wnt/β-catenin signaling in osteoblasts has been reported to coordinate post-natal bone acquisition by controlling the activity of osteoclasts.

R-spondins (Rspo) protein family is a group of four distinct secreted proteins that are isolated from human, mouse and Xenopus. (Rspo) are shown to act as agonist of canonical Wnt signaling pathway at the receptor level and induce β-catenin/TCF dependent gene activation. Moreover, the expression of (Rspo) protein is reported to overlap with Wnt proteins during mouse development. Hence, (Rspo) may have specific functions and regulation during developmental process. The Wnt proteins regulate intracellular β-catenin accumulation by activating co-receptor complex consisting of the frizzled family of proteins and (LRP-5) or LRP-6. 

Among (Rspo) family, Rspo3 interacts with frizzled-8 and LRP-6 to enhance Wnt ligand signaling, whereas Rspo1 enhances Wnt signaling by interacting with LRP-6 and inhibiting Dickkopf (DKK)-mediated receptor internalization. Rspo2 knock-out mice have shown early death rate and limb patterning defects associated with altered β-catenin signaling.

Recently, Rspo1 has been shown to potentiate Wnt3a-mediated osteoblast differentiation in mouse primary cell cultures and myoblast C2C12 cell line. It is also reported to induce osteoblast differentiation and expression of osteoprotegerin (OPG) in TNFα-transgenic mouse model of arthritis. It is also shown recently that Rspo2 expression is reduced and is responsible, at least in part for reduced Wnt/β-catenin signaling and abnormal mineralization in osteoarthritic (OA) osteoblasts, whereas Rspo1 expression is not found altered in normal and OA osteoblasts.

Till now, the role of Rspo1 in osteoblast differentiation has been addressed by utilizing murine osteoblasts models. The cloning of Rspo1 gene into the pcDNA3 plasmid and expression plasmid encoding human β-catenin (S33A) was done as described earlier. The Wnt signaling reporter construct TOPFLASH was obtained from Upstate Biotechnology (Lake Placid, NY). Luciferase assay system was from Promega (Madison, WI). Alkaline phosphatase (ALP) assay kit was from Pierce (Rockford, IL) and ALP staining kit was procured from Sigma-Aldrich (St. Louis, MO). RT-PCR kit was obtained from Invitrogen (Carlsband, CA). All tissue culture media and serum were obtained from Gibco (Grand Island, NY). All the other chemicals were from Sigma-Aldrich.

### Isolation of human primary osteoblast

An approved method for isolation of primary human osteoblast cells from trabecular bone from the femoral head of patients undergoing hip arthroplasty was performed using a modified protocol. The experimental protocol was approved by the Ethics Committee of Hallym University/Chuncheon Sacred Heart Hospital (Number: 2009-41, Approval date: 11-16-2009), South Korea. Under sterile conditions, trabecular bone chips were dissected from the femoral head and minced into small bone chips. The bone chips were then washed several times in fresh α MEM medium and placed in a 150 mm cell culture flask containing α MEM medium, supplemented with 10% fetal bovine serum (FBS), 50 mg/mL penicillin and 50 mg/mL streptomycin at 37°C in 5% CO2. Within 2 weeks, cell migration from the bone chips occurred. Cells consistently exhibited osteoblast morphology. To induce osteogenic differentiation, cells were incubated with 10 mM β-glycerol phosphate and 50 μg/mL ascorbic acid for 5 days.
Cell culture and conditioned medium

The mouse preosteoblastic cell line MC3T3-E1 cells, human osteoprogenitor cell line hFOB1.19 cells and primary human osteoblasts were taken to examine the contribution of human Rspo1 signaling to osteoblast differentiation. Mouse preosteoblastic cell line MC3T3-E1 cells, Wnt3a-secreting L cells and control L cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA, http://www.atcc.org). Wnt3a-secreting L cells and control L cells were cultured in Dulbecco’s minimum essential medium (DMEM) containing 10% FBS (Gibco, Grand Island, NY), 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza, Basel, Switzerland).

The human fetal osteoblastic cells (hFOB1.19-ATCC number: CRL-11372) were cultured according to ATCC protocol in a 1:1 mixture of phenol-free DMEM/Ham’s F-12 medium (Gibco, Grand Island, NY) supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at either 33°C or 39°C. hFOB was developed by conditionally immortalizing human fetal osteoblasts with a temperature-sensitive mutant of the SV40 large T antigen (ts-SV40LTA) gene. At the permissive temperature of 33°C, the ts-SV40LTA was active and the cells proliferated rapidly, whereas at the non-permissive temperature of 39°C, the ts-SV40LTA was inactive and the cells differentiated into mature osteoblasts. Wnt3a conditioned medium (CM) and L cell control CM were prepared as described previously.

Alkaline phosphatase (ALP) staining

MC3T3-E1 cells were seeded in 24-well plates and transfected with human Rspo1, human β-catenin (S33A) or pcDNA3 using FuGENE 6 reagent (Roche Applied Science, Indianapolis, IN), according to the manufacturer’s protocol. After differentiation for 7 days, cells were fixed with 4% paraformaldehyde and stained with ALP staining kit (Sigma-Aldrich, St. Louis, MO), according to the manufacturer’s protocol. Stained cells were photographed with a Zeiss AxioCam digital camera.

Luciferase reporter assay

For analysis of β-catenin activity, 3 × 10^4 cells were seeded in each well of 24-well plates. After overnight culture, cells were transiently transfected with 50 ng of Wnt/β-catenin signaling reporter construct TOPFLASH and expression plasmid. After 12 h incubation, cells were treated with Rspo1 and Wnt3a CM. The luciferase activity was measured 24 h later using a luciferase assay kit (Promega, Sunnyvale, CA) according to the manufacturer’s protocol. The luminescence was detected on a luminometer (Glomax-20/20, Promega, Sunnyvale, CA).

Real-time RT-PCR

Total RNA was isolated from cultured cells using TRizol reagent (Invitrogen Carlsband, CA) according to the manufacturer’s instructions and digested with RNase-free DNase I to remove genomic DNA contamination. The first-strand cDNA was synthesized with Super-Script II (Invitrogen, Carlsband, CA) and oligo (dT) primer in a 20 µL reaction mixture containing 2 µg total RNA. One-tenth of the cDNA was used for each PCR in a total volume of 25 µL over 40 cycles of denaturing step at 94°C for 20 s, annealing at 60°C for 20 s and extending at 72°C for 25 s. The real-time RT-PCR was performed using express SYBR Geen qPCR supermix (BioPrince, Seoul, Korea) and carried out in the Roter-Gene 3000 system (Qiagen, Hilden, Germany). The level of transcripts was normalized to GAPDH levels. The forward and reverse primer sequences are listed in Table 1.

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<th>Table 1—Primers for real-time RT-PCR</th>
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<td><strong>Target</strong></td>
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<td><strong>Human</strong></td>
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<td>Rspo1</td>
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shRNA transfection and measurement of ALP

Cells (hFOB1.19) in 24-well plates were transfected with shRNA against human Rspo1 (Thermo scientific, Rockford, IL) or control shRNA using FuGENE 6 reagent and incubated for 2 days. At the time of cell harvesting, the medium was removed and cell monolayer was gently washed twice with phosphate buffer saline (PBS). The cells were then lysed with RIPA buffer (20 mM Tris-HCl,
200 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, pH 7.5) and the lysate was centrifuged at 14000 × g for 5 min. The 20 µL of cleared supernatant was mixed with 100 µL of chemiluminescent alkaline phosphatase substrate, CSPD (Roche, Mannheim, Germany) in a luminometer tube, vortexed and incubated for 30 min. The luminescence was detected on a luminometer (Glomax-20/20, Promega, Sunnyvale, CA). Cell lysates were analyzed for protein concentrations using a Bio-Rad protein assay kit and ALP activity was normalized for total protein content in each well. For evaluation of total amount of Rspo1 in transfected cells, RNA expression of Rspo1 was determined by RT-PCR. The PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide.

**Statistical analysis**

All experiments were performed thrice and results were presented as ± standard errors of means (SEM). Data were analyzed by Student’s t test (prism 4.0).

**Results**

**Transcript level of Rspo1 increased during differentiation of human FOB1.19 and primary osteoblast cells**

Initially, we tried to analyze the expression level of Rspo1 in differentiating human primary osteoblast cells (Fig. 1A) and human FOB1.19 cells (Fig. 1B) in comparison to undifferentiated cells for 1, 2 and 3 day intervals. As shown in Fig. 1A & B, real-time RT-PCR analysis showed 2-fold and 5-fold increase in Rspo1 at transcript level in both primary human osteoblast and human osteoprogenitor hFOB1.19 cells, respectively under the condition of osteoblastic differentiation.

**Rspo1 activates Wnt signaling to induce ALP activity in osteoblasts**

Rspo1 has been shown to be capable of activating Wnt/β-catenin signaling. On the other hand, Wnt3a can induce osteoblast differentiation through a mechanism involving the activation of Wnt/β-catenin signaling. Therefore, to confirm the role of Rspo1 in osteoblasts differentiation, we treated human FOB1.19 cells, transfected with TOPFLASH luciferase construct, with Rspo1 in the presence as well absence of Wnt3a CM. Treatment of hFOB1.19 cells with Rspo1 alone at the concentration of 100 ng/mL resulted in 1.2-fold increase in TOPFLASH luciferase activity, whereas 50% of Wnt3a CM alone resulted in 5-fold increase. When treated with Rspo1 protein along with Wnt3a CM, hFOB1.19 cells displayed 7-fold increase in TOPFLASH luciferase activity (Fig. 2A). These results implied that Rspo1 can stimulate Wnt signaling. Furthermore, we tried to observe the effect of Rspo1 and Wnt3a proteins on early differentiation marker for osteoblast differentiation i.e ALP. As shown in Fig. 2B, Rspo1 (100 ng/mL) alone induced ALP activity of human primary osteoblast by 25%, whereas treatment of Wnt3a (50 ng/mL) induced 180% of ALP activity, as compared to control. On combined treatment of Wnt3a (50 ng/mL) with Rspo1 (100 ng/mL), an increase of more than 200% in ALP activity was observed.

**shRNA for Rspo1 reduced amount of ALP activity in differentiating human FOB cells**

To clearly establish the inducible role of Rspo1 on osteoblast differentiation process, we analyzed the ALP activity for differentiating hFOB1.19 cells after inhibition of Rspo1 expression. For this, hFOB1.19 cells were transfected with shRNA against Rspo1 and ALP activity was analyzed after 48 h. In differentiating hFOB1.19 cells, about
50-fold of ALP activity was reduced after transfection by shRNA for Rspo1, as compared to control (Fig. 3).

Rspo1 increased amount of ALP stained cells and expression of bone related genes in MC3T3-E1 cells

To determine whether Rspo1 had similar effect as Wnt3a on osteoblastic differentiation, we further examined the effect of Rspo1 on various parameters of differentiation in other osteoblast cell model, murine preosteoblastic cell line MC3T3-E1. The process of osteoblast differentiation is represented by up-regulation of osteogenic genes, such as Runx2 and osteocalcin along with induction of mineralization at terminal stages. Therefore, we examined the changes in ALP activity by staining and real-time RT-PCR analysis of osteogenic markers such as Runx2 and osteocalcin to monitor osteogenic activities under Rspo1 effect. As shown in Fig. 4A,
MC3T3-E1 cells transfected with Rspo1 or β-catenin (S33A) showed increasing amount of ALP staining. Also, MC3T3-E1 cells transfected with Rspo1 or β-catenin (S33A) showed increasing expression of bone-related genes, including Runx2 and osteocalcin, cultured for 7 days (Fig. 4B and C).

Discussion
Bone plays an essential role in the structure, protection and movement of the body. For a normal bone homeostasis in an adult skeleton, bone remodeling is a critical process. Bone remodeling depends on the equilibrium of bone resorption and formation and is efficiently regulated by osteoclasts and osteoblasts. The bone homeostasis is further dependent on the balance of pivotal molecular regulators of bone, such as agonists and antagonists of the Wnt signaling pathway as well as the RANKL/OPG system. Wnt/β-catenin signaling has been shown to play a substantial role in the control of bone development and remodeling. Agonists and antagonists of this delicate and complex pathway are very well-regulated for normal development and maintenance of bone physiology.

R-spondin proteins have been shown to express along with Wnt proteins during mouse development. Recent studies in murine osteoblast cell models have implicated the role of Rspo1 in inducing osteoblast differentiation. According to these studies, Rspo1 synergizes with Wnt signaling to induce osteogenesis. But, still no study has testified these findings in human in vitro osteoblast models, which not only reduce the accountability of interspecies differences but also are more clinically applicable. Thus, to clarify the role of Rspo1 in osteoblast development, we first analyzed the expression level of Rspo1 in differentiating human osteoblasts. Our result clearly showed elevated expression of Rspo1 in differentiating human osteoblasts and hFOB1.19 cell lines (Fig. 1). The expression of Rspo1 in human osteoblasts also suggested their autocrine role in regulation for osteoblast differentiation.

Wnt and R-spondin share many features in terms of the activation of Wnt/β-catenin signaling. Also, current studies have shown that Rspo1 synergizes with Wnt signaling to promote osteoblast differentiation in murine cells. In accordance to these studies, hFOB1.19 transfected with TOPFLASH reporter construct showed increased luciferase activity, when treated with Wnt3a CM (Fig. 2A). Treatment of Rspo1 alone to these cells also increased luciferase activity, compared to the untreated cells and on co-treatment with Wnt3a CM, an additive increase in luciferase activity was observed. However, Wnt3a was more potent than Rspo1 in activation of Wnt signaling activity in hFOB1.19 cells.

Furthermore, additive inducing effect of Rspo1 and Wnt3a was also observed on ALP activity of human primary osteoblast cells, apart from their individual inductive effect (Fig. 2B). These results strongly indicated that Rspo1 was responsible for the activation of Wnt signaling in human osteoblast cells and thus had inducing effect on differentiation of osteoblast cells. The significant role of Rspo1 in promoting osteoblasts differentiation was further authenticated by a decrease in ALP activity on inhibition of Rspo1 by shRNA in hFOB1.19 cells (Fig. 3). This result established that expression of Rspo1 under the differentiating conditions participated in osteoblastic differentiation, such as ALP activity.

To further validate the results obtained, we testified the role of Rspo1 in murine preosteoblast cell line MC3T3 E-1. MC3T3 E-1 cells transfected with Rspo1 and β-catenin (S33A) showed increase in ALP staining (Fig. 4A). When other parameters of differentiation like Runx2 and osteocalcin were analyzed at mRNA level, these cells showed increased level of expression, as compared to control (Fig. 4B & C). These results further substantiated the role of Rspo1 in differentiation process, as observed in human osteoblasts cells. As Rspo1 can affect the various parameters of differentiation in murine and human osteoblasts, it was clear that it essentially had an important role in regulation of osteogenesis.

In conclusion, the study demonstrated that Rspo1 protein can activate canonical Wnt signaling, leading to the activation of osteogenic gene expression in both human and murine osteoblast cell models. Moreover, the results of Rspo1 knock-out by shRNA strongly established the role of Rspo1 in regulation of osteoblast differentiation. With its unique feature in inducing osteoblast differentiation along with Wnt proteins, Rspo1 may have a potential therapeutic application in bone disorders, such as osteoporosis and non-union condition of fracture healing. However, further studies are required to find out the precise mechanism by which Rspo1 induces Wnt signaling, osteoblast differentiation and also osteoclast inhibition through osteoprotegerin expression.
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