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R-spondin1 synergizes with Wnt3A in inducing osteoblast differentiation and osteoprotegerin expression

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Abstract R-spondins are a new group of Wnt/β-catenin signaling agonists, however, the role of these proteins in bone remains unclear. We reported herein that R-sponin1 (Rspo1) acted synergistically with Wnt3A to activate Wnt/β-catenin signaling in the uncommitted mesenchymal C2C12 cells. Furthermore, we found that Rspo1 at concentrations as low as 10 ng/ml synergized strongly with Wnt3A to induce C2C12 osteoblastic differentiation and osteoprotegerin expression. These events were blocked by Wnt/β-catenin signaling antagonist Dickkopf-1. Finally, we demonstrated that Rspo1 synergized with Wnt3A to induce primary mouse osteoblast differentiation. Together, these findings suggest that Rpos1 may play an important role in bone remodeling.

Keywords: R-spondin1; Wnt3A; Wnt signaling; Osteoblast differentiation; Osteoprotegerin

1. Introduction

Studies in the past several years have established that Wnt/β-catenin signaling plays a critical role in the regulation of bone mass and is involved in many disorders of bone [1]. Osteoblast differentiation is the primary event of bone formation, exemplified by the synthesis, deposition, and mineralization of the extracellular matrix. One of the mechanisms whereby Wnt/β-catenin signaling increases bone formation is via stimulation of the development of osteoblasts [1]. Furthermore, Wnt/β-catenin signaling in osteoblasts is able to coordinate postnatal bone acquisition by controlling the differentiation and activity of osteoclasts [2–6]. Osteoprotegerin (OPG) is a direct target gene of the β-catenin–T-cell factor (TCF) complex in osteoblasts [5,7], and acts as a decoy receptor blocking the binding of receptor activator of NF-kappaB ligand (RANKL) to its cognate signaling receptor RANK on hematopoietic cells, thereby inhibiting osteoclast formation as well as activity.

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Abbreviations: ALP, alkaline phosphatase; CM, conditioned medium; Dkk1, Dickkopf-1; LRP6, low density lipoprotein receptor-related protein 6; OCN, osteocalcin; OPG, osteoprotegerin; Rspo1, R-spondin 1; RANKL, receptor activator of NF-kappaB ligand

R-spondins have been recently identified as a new group of Wnt/β-catenin signaling agonists [8]. There are four members in this group in human and mouse. R-spondin1 (Rspo1) has been identified as a potent and specific mitogen for the gastrointestinal epithelium [9,10]. In addition, human Rspo1 is the gene disrupted in a recessive syndrome characterized by XX sex reversal, palmoplantar hyperkeratosis, and predisposition to squamous cell carcinoma of the skin [11]. Although Wnt/β-catenin signaling is critical for bone development and remodeling, the role of R-spondins in bone is unclear. In the present study, we have utilized the uncommitted mesenchymal C2C12 cells and primary mouse osteoblasts to examine the contribution of Wnt/β-catenin signaling to osteoblast differentiation and OPG expression induced by human Rspo1.

2. Materials and methods

2.1. Materials

Human Rspo1 plasmid pcMV6-XL5-hRspo1 was obtained from OriGene Technologies, Inc. Plasmid pGST-E-cadherin was provided by Dr. Gail Johnson (University of Alabama at Birmingham, Alabama). The Wnt signaling reporter construct TOPFLASH was from Upstate Biotechnology. A β-galactosidase-expressing vector was from Promega. The preparation of human recombinant Rspo1 protein has been described before [9]. Human recombinant Dkk1 protein and monoclonal anti-OPG were obtained from R&D Systems. Monoclonal anti-phosphorylated-LRP6 was purchased from Cell Signaling Technology. Monoclonal anti-β-catenin and anti-actin were obtained from BD Biosciences and Sigma, respectively. Peroxidase labeled anti-mouse antibody and ECL system were purchased from Amersham Life Science. The dual luciferase and β-galactosidase assay systems were from Promega. Alkaline phosphatase (ALP) assay kit was from Pierce. RNA-Bee was from Tel-Test. The ProSTARTM Ultro HF RT-PCR Kit was obtained from Strategene. All tissue culture media, serum, and plastic-ware were obtained from Life Technologies Inc. Immobilon-P transfer membrane was purchased from Millipore. Rainbow molecular weight markers were purchased from GE Healthcare. Proteinase inhibitor cocktail Complete™ was obtained from Boehringer Mannheim.

2.2. Cell culture and conditioned media

Primary osteoblasts were isolated from calvariae of newborn mice as described before [12]. The procedures involving mice are conducted in accord with standards of humane animal care. Isolated osteoblasts were cultured in minimal essential medium α-modification containing 10% heat-inactivated fetal bovine serum. C2C12 cells, HEK293 cells, Wnt3A-secreting L cells, Wnt5A-secreting L cells, and control L cells were obtained from American Type Culture Collection. Wnt3A-secreting L cells and Wnt5A-secreting L cells were cultured in Dulbecco's minimum essential medium containing 10% of fetal bovine serum, 2 mM of L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 350 μg/ml of G418. HEK293 cells and C2C12 cells were

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cultured in the same above medium without G418. Wnt3A-conditioned medium (CM), Wnt5A CM, and L cell control CM were prepared according to the manufacturer's specifications. For Rspo1 CM and control CM, HEK293 cells were cultured in 10 cm dishes, and transiently transfected with pcMV6-XL5-hRspo1 or a control vector. The media were changed with fresh Dulbecco's minimum essential medium containing 10% fetal bovine serum 24 h after transfection. After further 48 h incubation, the media were collected, centrifuged to remove cell debris, and stored at −80 °C.

2.3. Luciferase reporter assay

C2C12 cells were plated into 12-well plates. After overnight culture, the cells were transiently transfected with 0.2 μg of the Wnt signaling reporter construct TOPFLASH, and 0.2 μg of β-galactosidase-expressing vector. After 24 h incubation, cells were treated Rspo1 and/or Wnt3A CM. Cells were then lysed 24 h later and both luciferase and β-galactosidase activities were determined. The luciferase activity was normalized to the β-galactosidase activity.

2.4. Western blotting

Cells in 6-well plates were lysed in 0.5 ml of lysis buffer (phosphate-buffered saline containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) at 4 °C for 30 min. Equal quantities of protein were subjected to SDS–PAGE under reducing conditions. Following transfer to immobilon-P transfer membrane, successive incubations with anti-β-catenin, anti-OPG, anti-LRP6, or anti-actin, and horseradish peroxidase-conjugated secondary antibody were carried out for 60–120 min at room temperature. The immunoreactive proteins were then detected using the ECL system. Films showing immunoreactive bands were scanned by Kodak Digital Science DC120 Zoom Digital Camera.

2.5. GST-E-cadherin binding assay

The GST-E-cadherin binding assay was carried out exactly as described in [13,14]. Uncomplexed β-catenin present in 100 μg of total cell lysate was subjected to SDS–PAGE and detected using the monoclonal antibody to β-catenin.

2.6. Measurement of ALP activity

C2C12 cells in 12-well plates or primary mouse osteoblasts in 24-well plates were treated for indicated amounts of various reagents indicated in each figure legend. Cells were harvested for assay of ALP activity by determining the amount of *p*-nitrophenol synthesized from *p*-nitrophenylphosphate after 30 min of incubation at room temperature as described in [15]. 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.

2.7. RT-PCR and real-time RT-PCR

Total RNA was isolated from cell cultures using RNA-Bee, and first-strand cDNA synthesis was performed using ProSTARTM Ultro HF RT-PCR Kit primed with oligo(dT) primer in a 20 μl reaction mixture containing 1 μg total RNA. For analysis of Rspo1 expression, the PCR was carried out using 1 μl of cDNA in a total volume of 25 μl over 40 cycles of denaturing step at 94 °C for 40 s, annealing at 60 °C for 30 s

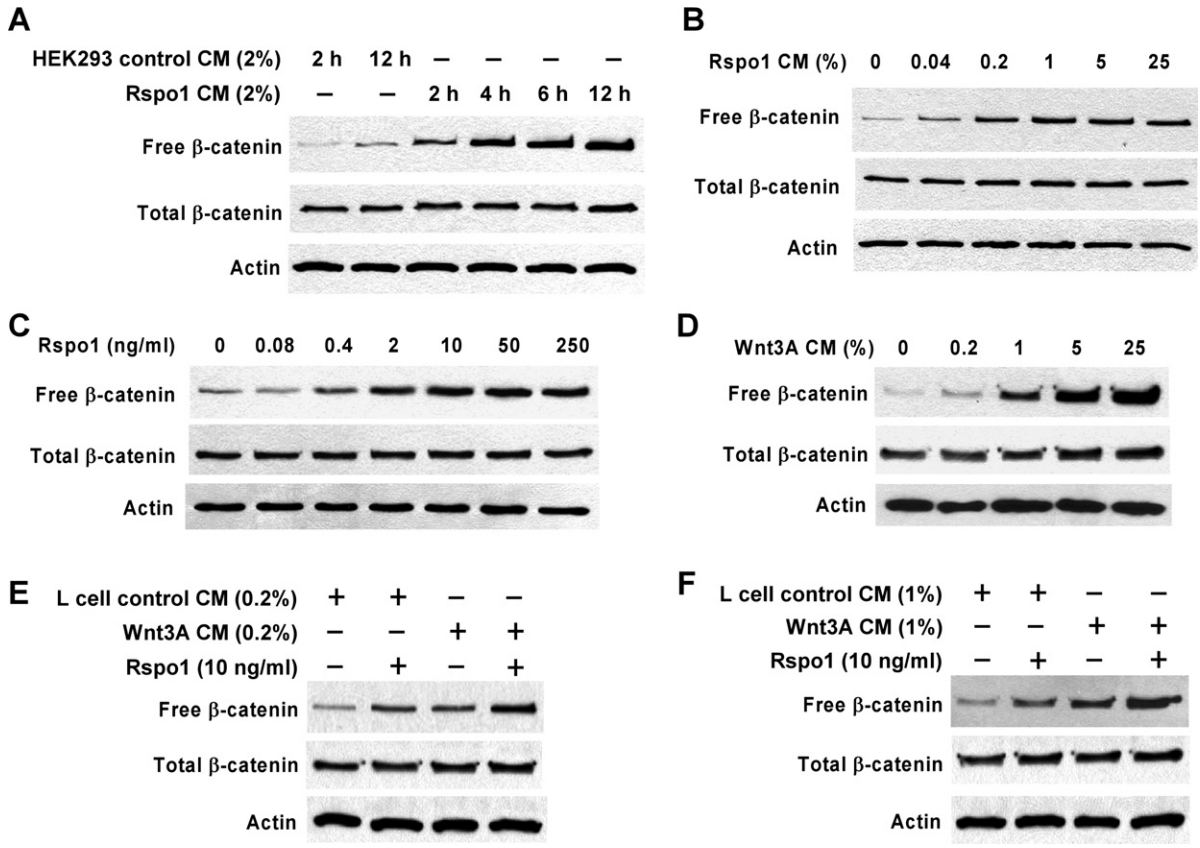

Fig. 1. Rspo1 acts synergistically with Wnt3A to stabilize cytosolic free β-catenin in C2C12 cells. (A) Time course of cytosolic free β-catenin stabilization induced by Rspo1. C2C12 cells in 6-well plates were incubated with 2% of Rspo1 CM for 0–12 h, and the levels of cytosolic free and total cellular β-catenin were then analyzed by Western blotting as described in Section 2. (B,C) Concentration dependent stabilization of cytosolic free β-catenin induced by Rspo1. In (B), C2C12 cells were treated with Rspo1 CM for 4 h. In (C), C2C12 cells were treated with recombinant Rspo1 protein for 4 h. (D) Concentration dependent stabilization of cytosolic free β-catenin induced by Wnt3A. C2C12 cells were treated with Wnt3A CM for 2 h. (E,F), Rspo1 and Wnt3A synergistically induced stabilization of cytosolic free β-catenin. In (E), C2C12 cells were treated with 10 ng/ml of Rspo1 and/or 0.2% of Wnt3A CM for 4 h. In (F), C2C12 cells were treated with 10 ng/ml of Rspo1 and/or 1% of Wnt3A CM for 4 h. All the samples for each Western blotting were also probed with anti-actin antibody to verify equal loading.

and extending at 72 °C for 45 s. The forward and reverse primers for mouse Rspo1 are 5′-GGCTGTGAGCTCTGTTCAGAAG-3′ (+609 to +630) and 5′-CCGGAAACCGCACAGCTTCCT-3′ (+969 to +989), respectively. The PCR product (381 bp) was loaded onto a 1.5% agarose gel and stained with ethidium bromide. For analysis of osteocalcin (OCN) expression, real-time RT-PCR was performed as described in [16].

3. Results

Recent studies reveal that Wnt/β-catenin signaling plays a substantial role in the control of bone metabolism [1]. Rspo1 has been shown to be capable of activating Wnt/β-catenin signaling [9,17,18]. We employed C2C12 cells to examine the effects of Rspo1 on Wnt/β-catenin signaling in osteoblasts. C2C12 cells are uncommitted mesenchymal progenitor cells that can be differentiated into osteoblasts upon the activation of Wnt/β-catenin signaling [19]. Uncomplexed cytosolic β-catenin (free β-catenin) is an active form of β-catenin and can enter the cell nucleus and associate with the transcription factors of the T-cell factor/lymphoid enhancing factor family, leading to the transcription of Wnt target genes [13,14,20]. We examined cytosolic free β-catenin levels with GST-E-cadherin pull-down assay to monitor Wnt/β-catenin signaling after Rspo1 treatment. As shown in Fig. 1A, Rspo1 induced the stabilization of cytosolic free β-catenin in a time-dependent manner. After 4 h treatment with 2% of Rspo1 CM, the level of cytosolic free β-catenin in C2C12 cells was significantly increased. Furthermore, C2C12 cells were very sensitive to Rspo1 treatment. Rspo1 induced the stabilization of cytosolic free β-catenin in a dose-dependent manner to about 1% of Rspo1 CM or 10 ng/ml of recombinant Rspo1 protein. However, higher doses of Rspo1 did not further increase the levels of cytosolic free β-catenin (Fig. 1B and C). In contrast to Rspo1, Wnt3A induced the stabilization of cytosolic free β-catenin in a dose-dependent manner up to 25% of Wnt3A CM (Fig. 1D). Importantly, Rspo1 and Wnt3A at low concentrations acted synergistically to stabilize cytosolic free β-catenin in C2C12 cells (Fig. 1E and F).

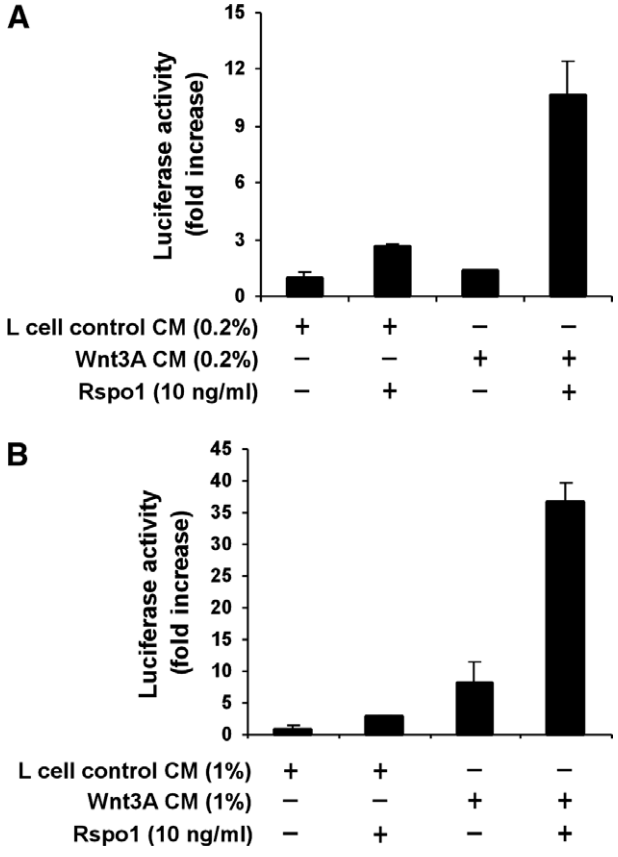

Fig. 2. Rspo1 acts synergistically with Wnt3A to activate Wnt signaling in C2C12 cells. C2C12 cells in 12-well plates were transiently transfected with 0.2 μg of the TOPFLASH luciferase construct and 0.2 μg of the β-galactosidase-expressing vector in each well. After 24 h incubation, cells were treated with Rspo1 (10 ng/ml) and/or Wnt3A CM. The luciferase activity was then measured 24 h later as described in Section 2. In (A), 0.2% of Wnt3A CM was used. In (B), 1% of Wnt3A CM was used. Values are the average of triple determinations with the S.D. indicated by error bars.

To further characterize Rspo1-induced Wnt/β-catenin signaling, C2C12 cells were transiently transfected with the Wnt/β-catenin signaling reporter construct TOPFLASH. As shown in Fig. 2, treatment of C2C12 cells with Rspo1 alone at the concentration of 10 ng/ml resulted in an 2–3-fold increase in TOPFLASH luciferase activity, and that treatment of C2C12 cells with 0.2% or 1% of Wnt3A CM alone resulted in 1.4-fold and 8-fold increases, respectively. However, when treated with 10 ng/nl of recombinant Rspo1 protein plus 0.2% or 1% of Wnt3A CM, C2C12 cells displayed 11.7-fold and 36.8-fold increases in TOPFLASH luciferase activity, respectively. Together, these results indicate that Rspo1 at low concentrations synergized strongly with Wnt3A to activate Wnt/β-catenin signaling in C2C12 cells.

Wnt3A can induce osteoblast differentiation through a mechanism involving the activation of Wnt/β-catenin signaling [1,15,19]. To determine whether Rspo1 is also able to induce osteoblastic differentiation, we examined the ALP activity in C2C12 cells. ALP is a commonly used marker of osteoblast differentiation. As shown in Fig. 3A, only small amounts of ALP were detectable in C2C12 cells without Wnt stimulation. Treatment of C2C12 cells with Wnt3A, but not Rspo1, for 2 days greatly induced them to produce ALP. For example, treatment of C2C12 cells with 1250 ng/ml of recombinant Rsp1 protein or 25% of Rspo1 CM resulted in only 2–3-fold increases in ALP activity, while treatment with 25% of Wnt3A CM resulted in an ~17-fold increase in ALP activity (Fig. 3A and B).

As we have demonstrated that Rspo1 at low concentrations synergized strongly with Wnt3A to activate Wnt/β-catenin signaling in C2C12 cells (Fig. 2), we then tested whether Rspo1 acts with Wnt3A to induce ALP production. As shown in Fig. 3C and D, Rspo1 at low concentrations was able to synergize strongly with Wnt3A to promote C2C12 osteoblastic differentiation. Treatment of C2C12 cells with 10 ng/ml of recombinant Rspo1 protein, 0.2% of Wnt3A CM or 1% of Wnt3A CM resulted in ~1–3-fold increases in ALP activity, while treatment of C2C12 cells with 10 ng/ml of recombinant Rspo1 protein plus 0.2% or 1% of Wnt3A CM resulted in ~9.5-fold and ~17-fold increases in ALP activity, respectively (Fig. 3C).

To confirm the effect of Rspo1 on C2C12 osteoblastic differentiation, we examined expression of OCN, another commonly used marker of osteoblast differentiation. As shown in Fig. 4, treatment with recombinant Rspo1 protein (10 ng/ml) plus Wnt3A CM (1%) led to a significant increase of OCN expression in C2C12 cells.

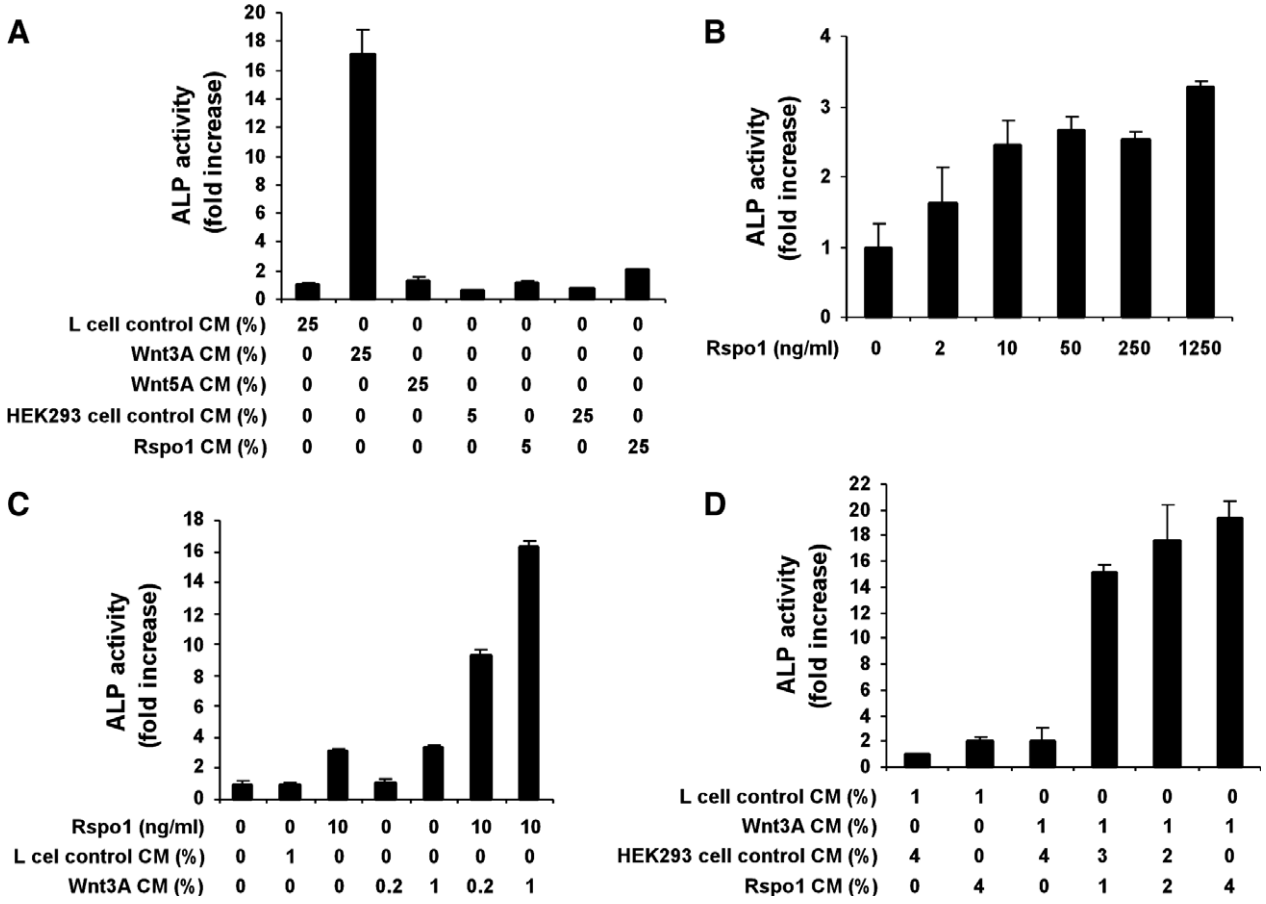

Fig. 3. Rspo1 acts synergistically with Wnt3A to induce C2C12 ALP production in C2C12 cells. (A) ALP activity in C2C12 cells was induced by Wnt3A CM and Rspo1 CM. C2C12 cells in 12-plates were incubated with 25% of L cell control CM, Wnt3A CM, Wnt5A CM, or Rspo1 CM at the indicated concentrations. Cells were harvested 48 h later for ALP activity assay as described in Section 2. (B) ALP activity in C2C12 cells was induced by recombinant Rspo1 protein. (C,D) Rspo1 acts synergistically with Wnt3A to induce C2C12 ALP production. In (C), recombinant Rspo1 protein (10 ng/ml) and Wnt3A CM (0.2% or 1%) were used. In (D), Rspo1 CM (1–4%) and Wnt3A CM (1%) were used. All the values are the average of triple determinations with the S.D. indicated by error bars.

Like C2C12 cells, primary mouse osteoblasts express Rspo1 (Fig. 5A). To confirm the role of Rspo1 in osteoblast differentiation, we treated primary mouse osteoblasts with Rspo1 in the presence or absence of Wnt3A CM. While recombinant Rspo1 protein (20 ng/ml) or Wnt3A CM (1%) alone had no effect on ALP activity in primary mouse osteoblasts, treatment of primary mouse osteoblasts with recombinant Rspo1 protein plus Wnt3A CM resulted in a significantly increase of ALP activity (Fig. 5B). Moreover, the expression of OCN was also significantly increased after the cells were treated with Rspo1 plus Wnt3A (Fig. 5C).

Recent studies have demonstrated that OPG is a direct target gene of Wnt/β-catenin signaling in osteoblasts [5,7]. We then examined OPG expression in C2C12 cells after Rspo1 treatment. We found that Wnt3A, but not Wnt5A, induced OPG expression in C2C12 cells (Fig. 6A), and that the effect of Wnt3A on OPG expression was dose dependent (Fig. 6B). Similar to ALP production, Rspo1 alone was unable to induce OPG expression in C2C12 cells even at the concentration as high as 1250 ng/ml (Fig. 6C) or 25% of Rspo1 CM (Fig. 6B). However, Rspo1 at low concentrations synergized with Wnt3A to induce OPG expression in C2C12 cells. Treatment of C2C12 cells with 10 ng/ml of recombinant Rspo1 protein plus 0.2% or 1% of Wnt3A CM for 2 days induced them to produce OPG

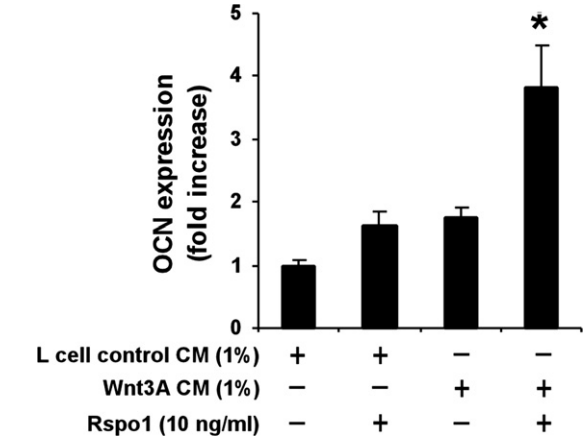

Fig. 4. Rspo1 acts synergistically with Wnt3A to induce OCN expression in C2C12 cells. C2C12 cells in 6-well plates were incubated with recombinant Rspo1 protein (10 ng/ml), Wnt3A CM (1%), or Rspo1 plus Wnt3A CM for 4 days, and the media were changed every 2 days. At the end of treatment, cells were harvested and OCN mRNA was determined by real-time RT-PCR and normalized to the message levels of β-actin mRNA. All the values are the average of triple determinations with the S.D. indicated by error bars. $^{*}P < 0.05$ indicates a significant difference compared with cells incubated with Rspo1, Wnt3A CM, or L cell control CM alone.

(Fig. 6E and F). Similarly, C2C12 cells expressed OPG after the treatment with 1–4% of Rspo1 CM plus 1% of Wnt3A CM for 2 days (Fig. 6G).

Dkk1 is a potent Wnt/β-catenin antagonist which binds the low density lipoprotein receptor-related protein 6 (LRP6) with a high affinity [21–23]. LRP6 is an essential co-receptor for the Wnt/β-catenin signaling pathway. LRP6 phosphorylation is critical for the activation of Wnt/β-catenin signaling [20]. Very recently, it has been reported that Rspo1 induces LRP6 phosphorylation in MEF cells [18]. LRP6 is expressed in C2C12 cells, while LRP5 expression is undetectable by RT-PCR (data not shown). To test whether Dkk1 affects Rspo1-induced Wnt/β-catenin signaling activation in C2C12 cells, we treated the cells with either Rspo1, Wnt3A CM, or Rspo1 plus Wnt3A CM in the presence or absence of Dkk1 and examined the levels of LRP6 phosphorylation and cytosolic β-catenin stabilization in C2C12 cells. It was found that Rspo1 (10 ng/ml) synergized with Wnt3A CM (1%) to induce LRP6 phosphorylation in C2C12 cells, and that Dkk1 was able to block LRP6 phosphorylation and cytosolic free β-catenin accumulation induced by Rspo1, Wnt3A CM, or Rspo1 plus Wnt3A CM (Fig. 7A). Moreover, Dkk1 treatment significantly reduced ALP production (Fig. 7B) and completely abolished OPG expression induced by Rspo1 plus Wnt3A (Fig. 7C).

4. Discussion

In recent years, Wnt/β-catenin signaling has been shown to play a substantial role in the control of bone development and remodeling. Previous study has identified Rspo1 as a potent and specific mitogen for the gastrointestinal epithelium and demonstrated a potential therapeutic application for this protein in mouse models of cancer therapy-induced mucositis [9,10]. Rspo1 is expressed in primary mouse osteoblasts and C2C12 cells as examined by RT-PCR. We demonstrated herein for the first time that human Rspo1 synergized strongly with Wnt3A to induce osteoblasts differentiation and OPG

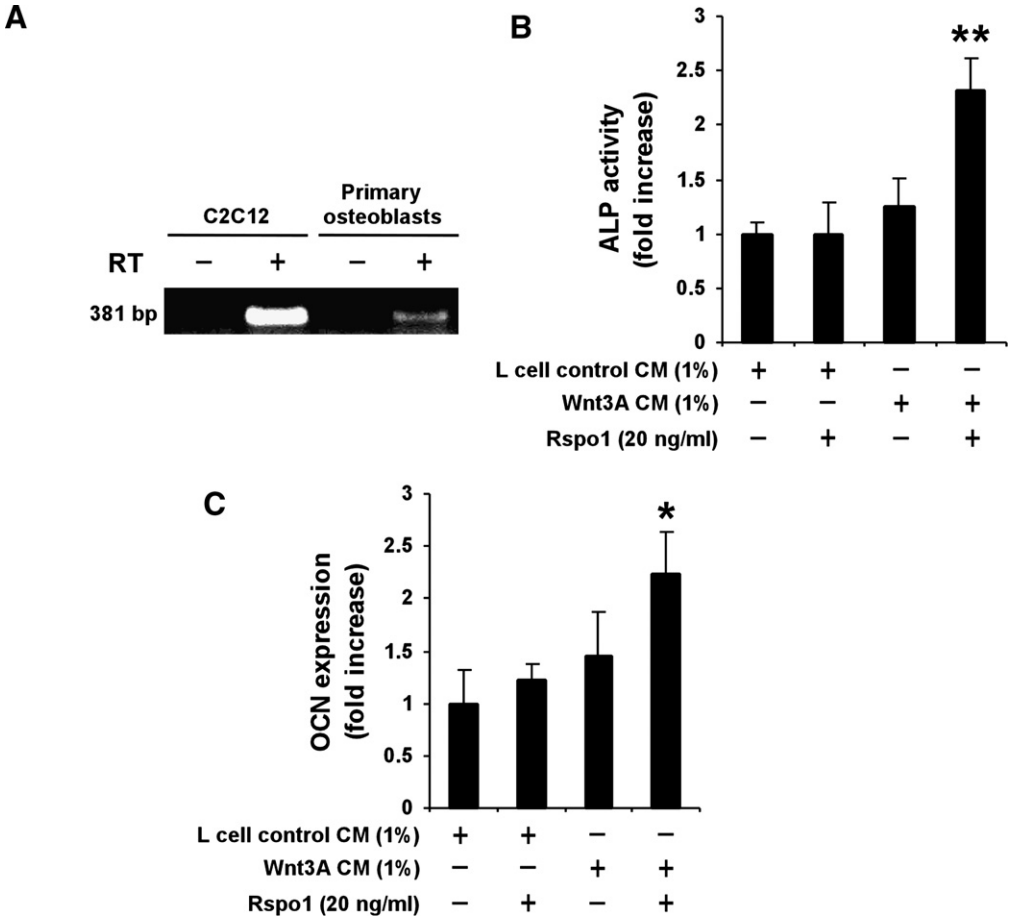

Fig. 5. Rspo1 acts synergistically with Wnt3A to induce primary mouse osteoblast differentiation. (A) Rspo1 expression in primary mouse osteoblasts and C2C12 cells. Rspo1 mRNA was examined in total RNA from primary mouse osteoblasts and C2C12 cells by PCR with or without reverse transcription (RT) as described in Section 2. (B) Rspo1 acts synergistically with Wnt3A to induce ALP production. Primary mouse osteoblasts in 24-well plates were incubated with recombinant Rspo1 protein (20 ng/ml), Wnt3A CM (1%), or Rspo1 plus Wnt3A CM for 6 days, and the media were changed every 2 days. At the end of treatment, cells were harvested for ALP activity assay as described in Section 2. All the values are the average of triple determinations with the S.D. indicated by error bars. $^{**}P < 0.01$ indicates a significant difference compared with cells incubated with Rspo1, Wnt3A CM, or L cell control CM alone. (C) Rspo1 acts synergistically with Wnt3A to induce OCN expression. Primary mouse osteoblasts in 6-well plates were incubated with recombinant Rspo1 protein (20 ng/ml), Wnt3A CM (1%), or Rspo1 plus Wnt3A CM for 8 days, and the media were changed every 2 days. At the end of treatment, cells were harvested and OCN mRNA was determined by real-time RT-PCR. All the values are the average of triple determinations with the S.D. indicated by error bars. $^{*}P < 0.05$ indicates a significant difference compared with cells incubated with L cell control CM alone.

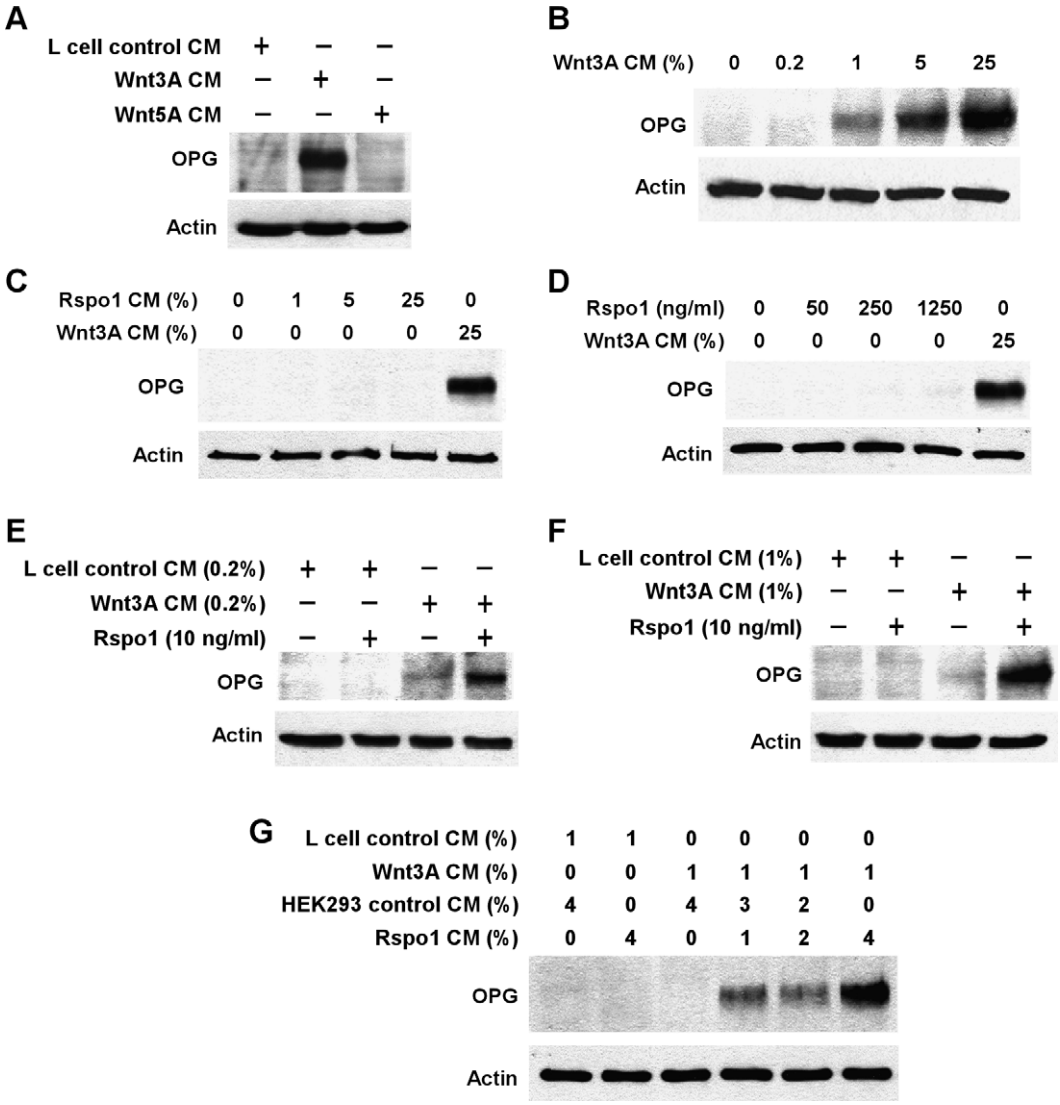

Fig. 6. Rspo1 acts synergistically with Wnt3A to promote OPG expression in C2C12 cells. (A) Wnt3A induced OPG expression in C2C12 cells. C2C12 cells in 6-well plates were incubated with 25% of L cell control CM, Wnt3A CM, or Wnt5A CM for 48 h. The levels of total cellular OPG were then analyzed by Western blotting. (B) Concentration dependent induction of OPG expression by Wnt3A. C2C12 cells in 6-well plates were incubated with 0–25% of L cell Wnt3A CM for 48 h. (C,D) Rspo1 alone was unable to induce OPG expression in C2C12 cells. Wnt3A CM (25%) was served a positive control. In (C), Rspo1 CM was used. In (D), recombinant Rspo1 protein was used. (E-G) Rspo1 acts synergistically with Wnt3A to induce OPG expression in C2C12 cells. In (E), 10 ng/ml of Rspo1 and 0.2% of Wnt3A CM were used. In (F), 10 ng/ml of Rspo1 and 1% of Wnt3A CM were used. In (G), 1–4% of Rspo1 CM and 1% of Wnt3A CM were used. All the samples for each Western blotting were also probed with anti-actin antibody to verify equal loading.

expression, suggesting that Rspo1 may play an important role in bone remodeling.

Wnt and R-spondin share many features in terms of the activation of Wnt/β-catenin signaling [9,17,18]. In the present study, we found that both Rspo1 and Wnt3A were able to induce cytosolic free β-catenin stabilization. However, Wnt3A was more potent than Rspo1 in activation of the Wnt/β-catenin pathway in C2C12 cells. For example, Rspo1 induced cytosolic free β-catenin stabilization at low concentrations (10 ng/ml or 2% of Rspo1 CM), but higher doses of Rspo1 did not further increase the levels of cytosolic free β-catenin. In addition, Wnt3A alone, but not Rspo1 alone, was able to significantly enhance ALP production and OPG expression in C2C12 cells. Critically, on the other hand, we also found that Rspo1 at the concentrations as low as 10–20 ng/ml synergized strongly with Wnt3A to activate Wnt/β-catenin signaling, and to induce osteoblast differentiation and OPG expression. These results suggest that Rspo1 may play important roles in regulation of osteoblast differentiation and osteoclast function at pathophysiological conditions. With its unique feature in inducing osteoblast differentiation and OPG expression with Wnt proteins at low concentrations, Rpo1 may have a potential therapeutic application in bone disorders such as osteoporosis.

LRP6 is an essential co-receptor for the Wnt/β-catenin signaling pathway and is subjected to modulation by many secreted proteins [20]. It has been reported that the activation of Wnt/β-catenin signaling induced by R-spondins was enhanced by LRP6 co-expression [17,18], and blocked by LRP6 inhibitor Dkk1 [9,17,18]. Furthermore, Binnerts et al. very recently

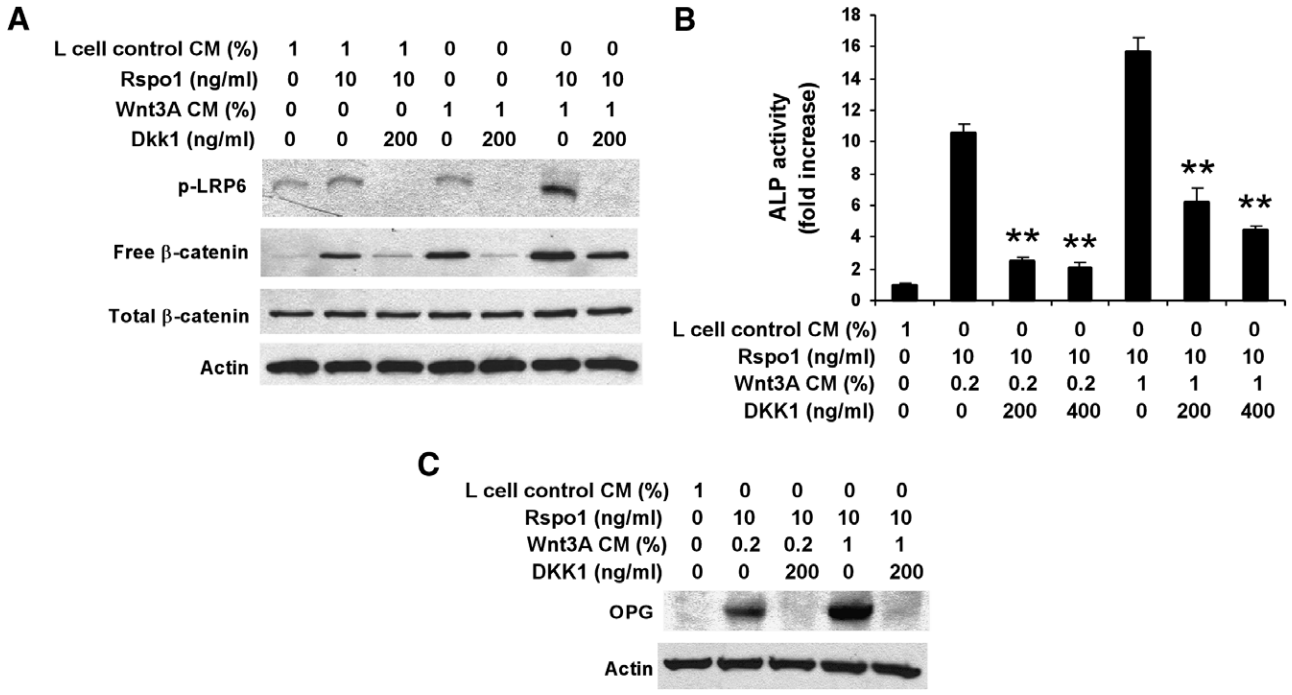

Fig. 7. Dkk1 blocked LRP6 phosphorylation, β-catenin stabilization, osteoblastic differentiation, and OPG expression induced by Rspo1 and Wnt3A. (A) Dkk1 blocked LRP6 phosphorylation and β-catenin stabilization induced by Rspo1 and Wnt3A. C2C12 cells in 6-well plates were pretreated with or without Dkk1 (200 ng/ml) for 2 h, and then incubated with Rspo1 (10 ng/ml) and/or Wnt3A CM (1%) for 4 h. The levels of phosphorylated LRP6, cytosolic free β-catenin and total cellular β-catenin were then analyzed by Western blotting. All the samples for each Western blotting were also probed with anti-actin antibody to verify equal loading. (B) Dkk1 blocked osteoblastic differentiation induced by Rspo1 and Wnt3A. C2C12 cells in 12-well plates were incubated with Rspo1 (10 ng/ml) and/or Wnt3A (0.2% or 1%) in the presence or absence of Dkk1 (200 ng/ml or 400 ng/ml). Cells were harvested 48 h later for assay for ALP activity. All the values are the average of triple determinations with the S.D. indicated by error bars. $^{**}P < 0.01$ verse cells treated with Rspo1 plus Wnt3A. (C) Dkk1 blocked OPG expression induced by Rspo1 and Wnt3A. C2C12 cells in 6-well plates were incubated with Rspo1 (10 ng/ml) and/or Wnt3A (0.2% or 1%) in the presence or absence of Dkk1 (200 ng/ml) for 48 h. The levels of total cellular OPG were analyzed by Western blotting. All the samples for each Western blotting were also probed with anti-actin antibody to verify equal loading.

reported that Rspo1 regulates Wnt signaling by modulating levels of LRP6 on the cell surface, through inhibition of Dkk1-dependent internalization of LRP6 [24]. Although LRP6 is critical for R-spondin action, the direct interaction between LRP6 and R-spondins remains controversial [17,18,24]. Dkk1 binds LRP6 with a high affinity, and is a potent Wnt/β-catenin inhibitor [21–23]. In the present study, we demonstrated that Dkk1 blocked LRP6 phosphorylation, cytosolic free β-catenin stabilization, ALP production, and OPG expression induced by Rspo1 and/or Wnt3A in C2C12 cells, suggesting that Rspo1 function is dependent on LRP6 activity. In the future, we should dissect precise mechanisms by which Rspo1 at low concentrations cooperates with Wnt to induce Wnt/β-catenin signaling, osteoblast differentiation, and OPG expression.

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