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R-spondin 2 facilitates differentiation of proliferating chondrocytes into hypertrophic chondrocytes by enhancing Wnt/β-catenin signaling in endochondral ossification

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ABSTRACT

Endochondral ossification is a crucial process for longitudinal growth of bones. Differentiating chondrocytes in growth cartilage form four sequential zones of proliferation, alignment into column, hypertrophy, and substitution of chondrocytes with osteoblasts. Wnt/β-catenin signaling is essential for differentiation of proliferating chondrocytes into hypertrophic chondrocytes in growth cartilage. R-spondin 2 (Rspo2), a member of R-spondin family, is an agonist for Wnt signaling, but its role in chondrocyte differentiation remains unknown. Here we report that growth cartilage of *Rspo2*-knockout mice shows a decreased amount of β-catenin and increased amounts collagen type II (CII) and Sox9 in the abnormally extended proliferating zone. In contrast, expression of collagen type X (CX) in the hypertrophic zone remains unchanged. Differentiating chondrogenic ATDC5 cells, mimicking proliferating chondrocytes, upregulate Rspo2 and its putative receptor, Lgr5, in parallel. Addition of recombinant human Rspo2 to differentiating ATDC5 cells decreases expressions of *Col2a1, Sox9, and Acan*, as well as production of proteoglycans. In contrast, lentivirus-mediated knockdown of Rspo2 has the opposite effect. The effect of Rspo2 on chondrogenic differentiation is mediated by Wnt/β-catenin signaling, and not by Wnt/PCP or Wnt/Ca^{2+} signaling. We propose that Rspo2 activates Wnt/β-catenin signaling to reduce *Col2a1* and *Sox9* and to facilitate differentiation of proliferating chondrocytes into hypertrophic chondrocytes in growth cartilage.

1. Introduction

Endochondral ossification is an essential process during fetal development of the mammalian skeletal system by which bone tissue is created for longitudinal growth. Unlike intramembranous ossification, chondrocytes play an essential role in endochondral ossification. At the light microscope level, five distinct zones can be histologically recognized: zones of (i) resting cartilage (RZ), (ii) proliferation/cell columns (PZ), (iii) maturation/hypertrophy of chondrocytes (HZ), (iv) calcification; and (v) ossification by bone-forming cells (osteoblasts). Chondrocytes sequentially differentiate in the order of RZ, PZ, HZ, and substitution with osteoblasts. Therefore, dysregulation of the chondrocyte differentiation leads to skeletal dysplasias in both mouse and human cartilage [1,2].

Sequential differentiations of chondrocytes are tightly regulated by transcription factors, which are associated with substantial remodeling of cartilage-specific extracellular matrix proteins. In chondrocytes in PZ, a master transcription factor, Sox9 (sex-determining region Y-type high mobility group box protein), is expressed to regulate early chondrocyte differentiation [3]. Accompanied with the differentiation, cells start to produce matrix proteins such as collagen type II (CII encoded by *Col2a1*) and aggrecan (*Acan*). Chondrocytes undergoing hypertrophy and

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terminal differentiation express another transcriptional factor, Runx2, which promotes further chondrocyte differentiations to facilitate secretion of collagen type X (CX) [4].

Sequential differentiations of chondrocytes are also regulated by several signaling pathways including Wnt signaling pathways [5]. Glycogen synthase kinase 3 (GSK3) in the Wnt signaling pathway is involved in chondrocyte differentiation *in cellulo* and in endochondral ossification *in vivo* [6]. With secreted Wnt ligands and its receptors, frizzled (Frz) family and LRP5/6, three distinct intracellular signaling cascades are activated: canonical Wnt/β-catenin signaling, non-canonical Wnt/PCP signaling, and non-canonical Wnt/Ca^{2+} signaling [7]. Sox9 and Wnt/β-catenin control chondrocyte differentiation in a mutually antagonistic manner [3], and Wnt/β-catenin signaling promotes chondrocyte hypertrophy, which leads to bone mineralization [8]. Involvement of non-canonical Wnt signaling is less well characterized compared to canonical Wnt signaling, except that Wnt5a for Wnt/PCP signaling inhibits CII expression without activation of β-catenin [9]. Wnt/Ca^{2+} pathway is crucial to inhibit chondrocyte differentiation in mesenchymal stem cells [10].

R-spondins (Rspos) constitute a family of four secreted ligands [11], and are initially characterized as activators for Wnt/β-catenin signaling [12]. Rspos form a complex with two cell surface receptors: Lgr4-6 (leucine-rich repeat containing G protein-coupled receptors) and RNF43 (ring finger protein 43)/ZNRF3 (zinc and ring finger 3). RNF43 and ZNRF3 are homologs, and RNF43/ZNRF3 is a Frizzled-specific E3 ubiquitin ligase. In Wnt signaling, insertion of an Rspo protein between Lgr and the E3 complex forms a ternary Lgr-Rspo-E3 complex, and attenuates the E3 ligase activity by its internalization, which otherwise degrades LRPs and Frizzleds [13–17]. R-spondin 2 (Rspo2) has been characterized as an important factor for developments and diseases of skeletal tissues. *Rspo2*-knockout (KO) mice display craniofacial malformation, abnormal dorsoventral patterning of limb buds, and perinatal death due to lung hypoplasia [18–20]. Osteoblasts derived from patients with osteoarthritis (OA) have significantly lower expression levels of *Rspo2* compared to controls, reduced β-catenin expression, and reduced mineralization [21]. Genome-wide association study in Japanese populations shows that Rspo2 is one of candidate genes associated with ossification of the posterior longitudinal ligament (OPLL) of the spine [22]. Although *in situ* hybridization of *Rspo1-4* in mouse development demonstrated expression of Rspo2 in growth cartilage [23], the function of Rspo2 in growth cartilage has not been dissected.

The purpose of this study is to investigate functions of Rspo2 in endochondral ossification. Here, we show that Rspo2 critically reduces expressions of CII and Sox9 by upregulating accumulation of β-catenin in chondrocytes in growth cartilage. Overexpression and knocking-down of Rspo2 in cultured chondrocytes revealed that Rspo2 activates Wnt/β-catenin signaling and reduces expressions of *Col2a1*, *Sox9*, *Acan*, and proteoglycans in chondrocytes during endochondral ossification.

2. Material and methods

2.1. Animal studies

All animal studies were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine. The day on which a vaginal plug was observed in a female mouse at noon was considered embryonic day 0.5 (E0.5). Embryos were delivered with caesarian sections because *Rspo2*-KO mice die shortly after birth. Genotyping was performed by PCR with genomic DNA isolated from yolk sac of fetuses as described elsewhere [19].

2.2. Immunohistochemistry

Immunohistochemistry was performed as previously reported [24]. Briefly, fetal tissue specimens were immersed in 80% ethanol for one day at 4 °C. Specimens were decalcified with 10% EDTA for five days at 4 °C. Formalin-fixed 5-μm coronal paraffin sections of the growth cartilage of proximal tibia at E18.5 were deparaffinized and hydrated in Lemosol (Wako). The sections were then treated with a blocking buffer containing 2% goat serum in 0.5% Triton-X 100 for 60 min, and incubated with rabbit anti-β-catenin (dilution 1:100, 9587S, Cell Signaling Technology), anti-CX (1:100, ab58632, Abcam), anti-Sox9 (1:100, AB5535, Millipore) at 4 °C for 24 h. On the next day, after multiple washings with 0.05% Tween 20 in PBS, the sections were incubated with goat anti-rabbit antibody conjugated with Alexa 488 (1:1000, A21206, Molecular Probes) at room temperature for 1 h, mounted in VectaShield containing 1.5 μg/ml DAPI (Vector Laboratories, Peter-borough, UK). CII was immunostained with a modified streptavidin-biotin method using a Collagen type II Staining Kit (Chondrex, Inc., Redmond, WA) and with DAB system (Cell signaling) following the manufacture's protocols. The numbers of Sox9-positive cells were counted by two blinded observers. Similarly, the intensities of β-catenin signals were estimated by the MetaMorph software (Molecular Devices) by two blinded observers. The longitudinal length of CII- and CX-positive areas was also measured by two blinded observers, and was normalized by the longitudinal length of the growth cartilage.

2.3. Western blotting

Right femur, right tibiae, both humers, both radiuses, and both ulnas of three embryos at E18.5 were individually isolated. *Rspo2*-KO mice showed severely hypoplastic left lower hindlimb. Growth cartilages of each isolated bone were dissected under a microscope. Growth cartilages of three embryos were mixed together, and were lysed with ice-cold RIPA Lysis Buffer (Santa Cruz) supplemented with 0.1 mM dithiothreitol, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprontinin using a Multi-Beads Shocker (Yasui Kikai). The cell lysates were separated on SDS-PAGE, and transferred to nitrocellulose membrane followed by immunoblotting with rabbit anti-β-catenin (dilution 1:1000, 9587, Cell signaling), anti-CII (1:500, LSL-LB1297, Cosmo Bio), anti-CX (1:500, LSL-LB0092, Cosmo Bio), anti-Sox9 (1:2000, AB5535, Millipore), and anti-GAPDH (1:5,000, G9545, Sigma–Aldrich) antibodies. We used donkey anti-rabbit IgG antibody (dilution 1:5,000, NA934V, GE Healthcare) as a secondary antibody. The blots were detected with the Amersham ECL Western blotting detection reagent (GE Healthcare) and quantified with the ImageJ program (http://imagej.nih.gov/ij/). For quantitative analysis, we performed Western blotting with three different samples arising from 9 embryos for both wild-type and *Rspo2*-KO mice.

2.4. ATDC5 cell culture

ATDC5 cells were purchased from the Riken BioResourse Center. ATDC5 cells were cultured in DMEM/F12 (a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium; Sigma–Aldrich) supplemented with 5% fetal bovine serum (Thermo Scientific). To induce differentiation of ATDC5 cells, the medium was added with

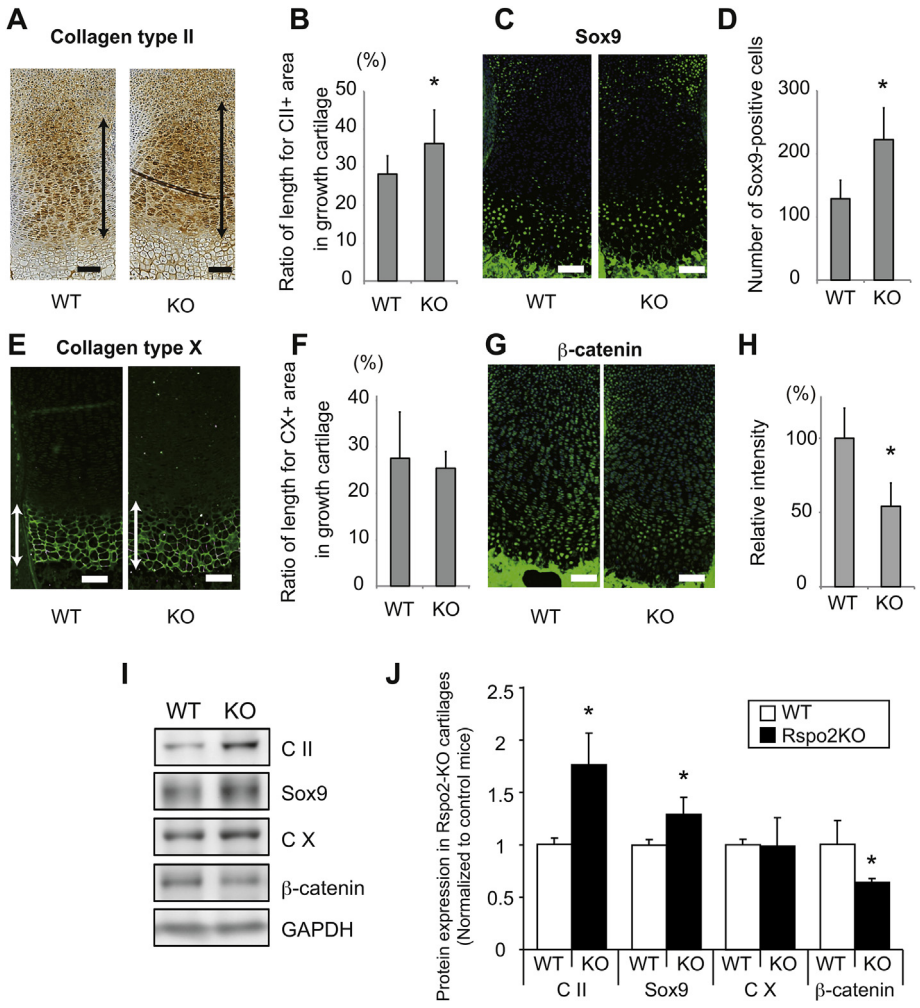

Fig. 1. Lack of Rspo2 causes increased collagen type II and Sox9, and decreased β-catenin in growth cartilage. Analysis of growth cartilages in fetal tibiae of either wild-type (WT) or *Rspo2*-knockout (KO) mice at E18.5. **(A–H)** Immunohistochemistry and quantification of collagen type II (CII) (**A, B**), Sox9 **(C, D)**, collagen type X (CX) **(E, F)**, and β-catenin **(G, H)**. Arrows in **A** and **E** indicate CII- and CX-positive areas, respectively. Quantifications in **B, D, F, H**, and **J** were performed by two blinded observers. **(H)** Cumulative signal intensity of β-catenin in growth cartilage was normalized by the area of growth cartilage and also by wild-type mice. **(I)** Western blotting for CII, Sox9, CX, and β-catenin expressed in growth cartilage at E18.5. **(J)** Signal intensities of Western blots are normalized to the mean of wild-type mice. Mean and SEM are indicated ($n = 3$ in **B**, **D**, **F**, **H** and $n = 4$ in **J**). $^{*}p < 0.05$ versus wild type by Student's *t*-test. Scale bars = 100 μm.

1% insulin-transferrin-selenite (ITS, Invitrogen) with or without 100 ng/ml recombinant human Rspo2 protein (rhRspo2; R&D systems), unless otherwise stated. Under the differentiation condition, the medium was changed every other day. To make shRNA-expressing lentiviral vectors, the following double strand shRNA oligonucleotides were synthesized and cloned into pLenti-CMV-GFPx2-DEST. shRspo2 (shR2): 5′-GATCCCGCTTGAAATTAATCCTAAATTCAAGAGATTTAGGATTAATTTCAAGCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGCTTGAAATTAATCCTAAATCTCTTGAATTTAGGATTAATTTCAAGCGG-3′. shControl (ShCont): 5′-GATCCCTAAGGCTATGAAGAGATACTTCAAGAGAGTATCTCTTCATAGCCTTATTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAATAAGGCTATGAAGAGATACTCTCTTGAAGTATCTCTTCATAGCCTTAGG-3′.

pLenti-CMV-GFPx2-DEST was kindly provided by Dr. Eric Campeau at the University of Massachusetts Medical School. ATDC5 cells were infected by lentivirus carrying ShR2 in the growth medium. *Rspo2* gene in the cells was silenced using 2 μg/ml doxycycline 24 h before harvesting cells. To examine the signaling pathways involved in Rspo2-induced expressions of *Col2a1* and *Sox9*, ATDC5 cells were cultured in the differentiation medium for 4 days, and incubated with or without 100 ng/ml rhRspo2 protein for 24 h. Then, 10 μM of Quercetin (ChromaDex, Inc.), SP600125 (Sigma–Aldrich), or KN93 (Sigma–Aldrich) was added into the differentiation medium, and cultured for 24 h.

2.5. Total RNA extraction and real-time RT-PCR analysis

Total RNA was isolated from the ATDC5 cells using Trizol reagent (Invitrogen). The first strand cDNA was synthesized with ReverTra Ace reverse-transcriptase (Toyobo). We quantified mRNA levels of *Rspo2*, *Col2a1*, *Acan*, *Cola10a1*, *Sox9*, *Axin2*, *Lgr4*, *Lgr5*, *Lgr6*, *Znfr3* and *Rnf43* using LightCycler 480 Real-Time PCR (Roche) and SYBR Ex Taq (Takara). mRNA levels were normalized for *Gapdh*. Primer sequences are shown in Table S1.

2.6. Alcian blue staining

ATDC5 cells were added with or without rhRspo2 to examine

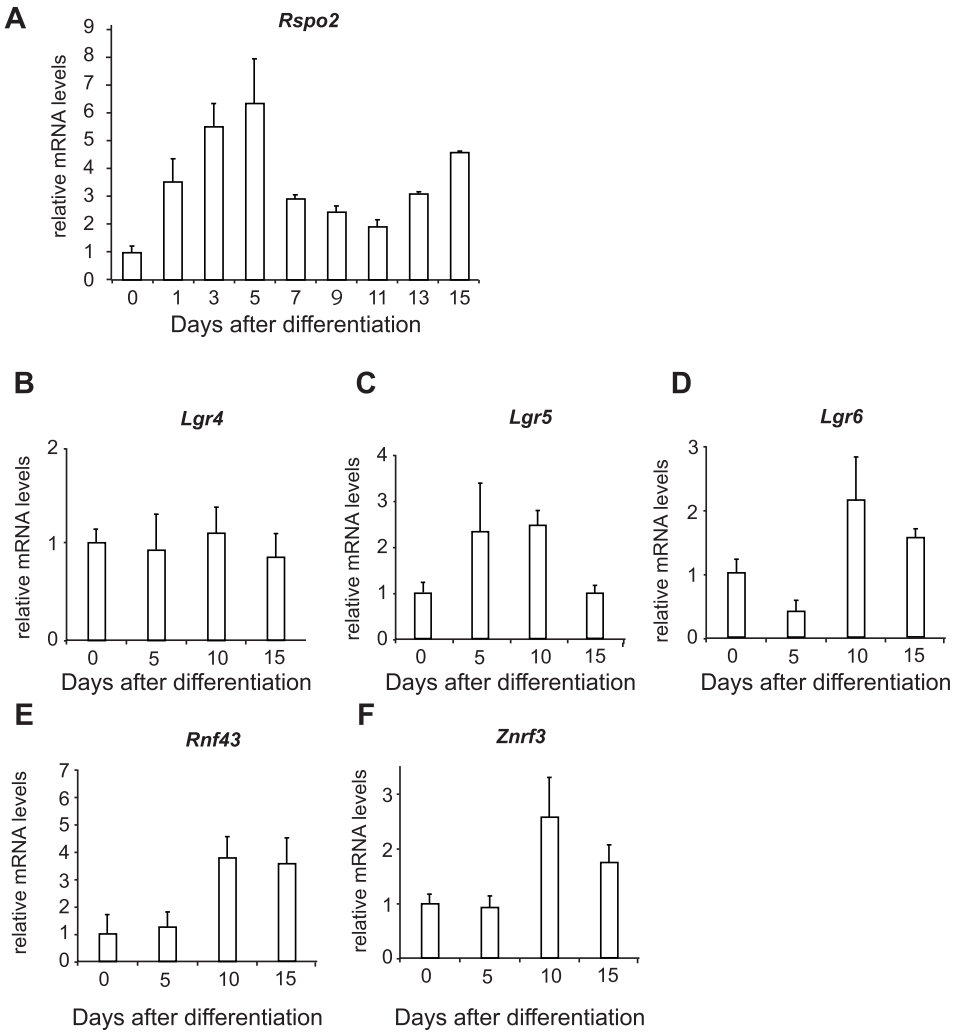

Fig. 2. Expressions of *Rspo2* and *Lgr5* are induced in parallel in chondrogenic differentiation of ATDC5 cells. ATDC5 cells were differentiated from day 0 by ITS treatment in the presence of 100 ng/ml recombinant human Rspo2 protein (rhRspo2). Gene expression levels of *Rspo2* **(A)**, *Lgr4* **(B)**, *Lgr5* **(C)**, *Lgr6* **(D)**, *Rnf43* **(E)**, and *Znrf3* **(F)** were estimated by quantitative RT-PCR, and were normalized to the mean of untreated cells on day 0. Mean and SEM are indicated ($n = 3$).

the effect of over expression of Rspo2. Other ATDC5 cells were infected with lentivirus carrying ShR2 or shCont, and added with 2 μg/ml doxycycline to examine the effect of downregulation of Rspo2. Four groups of ATDC5 cells were added with 1% ITS to induce differentiation for 15 days. The cells were fixed with 100% methanol for 30 min at −20 °C and stained overnight with 0.5% Alcian blue 8GX (Sigma) in 1 N HCl. For quantitative analysis, the Alcian blue-stained cells were lysed in 200 μl of 6 M guanidine HCl for 6 h at room temperature. The optical density of the extracted dye was measured at 630 nm using PowerScan4 (DS Pharma Biomedical).

2.7. Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined either by unpaired Student's *t*-test or one-way ANOVA followed Tukey's post-hoc test. P-values less than 0.05 were considered significant. The statistical analysis was performed with EZR (Saitama Medical Center, Jichi Medical University) [25].

3. Results

3.1. Rspo2-KO mice show excessive expressions of CII and Sox9, and a reduced amount of β-catenin in chondrocytes of growth cartilage

To dissect the functions of Rspo2 in chondrocytes *in vivo*, we compared morphologies and proteoglycan productions of chondrocytes in the right proximal tibial growth cartilages in *Rspo2*-KO and wild-type mice. We estimated the amount of extracellular matrix glycosaminoglycans by Alcian blue staining combined with hematoxylin and eosin staining. Growth cartilage of wild-type mice showed three distinct zones classified by the shapes of chondrocytes: the resting zone (RZ), the proliferating zone (PZ), and the hypertrophy zone (HZ). *Rspo2*-KO cartilage showed slight disarrangements of chondrocyte alignments in all zones (Fig. S1A), although the cell numbers and the length of each zone were not significantly different compared to those of wild-type mice (Fig. S1B and C). Immunostaining of tibial growth cartilage of *Rspo2*-KO mice revealed that the length of CII-positive area was

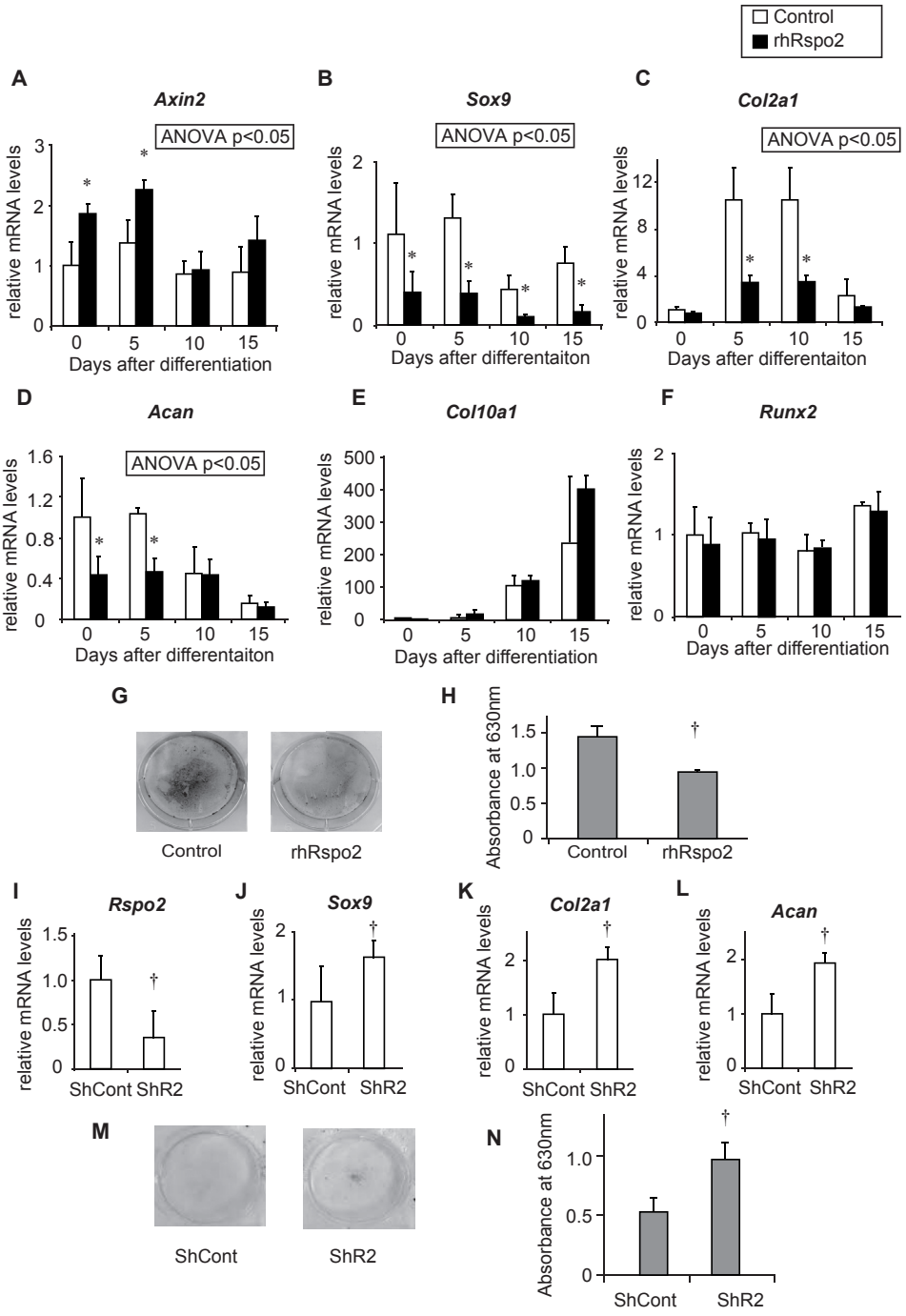

Fig. 3. Rspo2 enhances expression of *Axin2*, and reduces expressions of *Sox9*, *Acan*, *Col2a1*, and proteoglycans in differentiating ATDC5 cells. **(A–H)** ATDC5 cells were differentiated from day 0 by ITS treatment with or without rhRspo2. **(A–F)** Gene expression levels were estimated by quantitative RT-PCR, and were normalized to the mean of untreated cells on day 0. Significant statistical difference by two-way repeated measures ANOVA is indicated in an inset in each graph. $^{*}p < 0.05$ by Bonferroni post-hoc test compared to control. **(G, H)** Alcian blue staining of proteoglycans and its quantification of ATDC5 cells on day 15. $^{\dagger}p < 0.05$ by Student's *t*-test. **(I–N)** ATDC5 cells were infected with lentivirus carrying either ShCont or ShR2, and were differentiated from day 0 by ITS treatment. **(I–L)** ShRNA was induced by doxycycline on day 4, and ATDC5 cells were harvested on day 5. Gene expression levels estimated by quantitative RT-PCR were normalized to the mean of ShCont. $^{\dagger}p < 0.05$ by Student's *t*-test. **(M, N)** Alcian blue staining of proteoglycans and its quantification of ATDC5 cells on day 15. $^{\dagger}p < 0.05$ by Student's *t*-test. Mean and SEM ($n = 3$) are indicated for all graphs.

increased and the number of Sox9-positive cells was also increased (Fig. 1A–D). On the other hand, the length of CX-positive area in *Rspo2*-KO mice was not significantly different from that in wild-type mice (Fig. 1E, F). Western blotting similarly showed that expressions of CII and Sox9 proteins in tibial growth cartilage were upregulated, while CX was not significantly different from that in wild-type mice (Fig. 1I, J). These results suggest that Rspo2 attenuates expressions of *Col2a1* and *Sox9* in growth cartilage, which are highly expressed in chondrocytes in PZ. We next examined the expression of β-catenin in growth cartilage. *Rspo2*-KO mice showed decreased staining of β-catenin protein in chondrocytes of growth cartilage (Fig. 1G, H). We also confirmed that the amount of β-

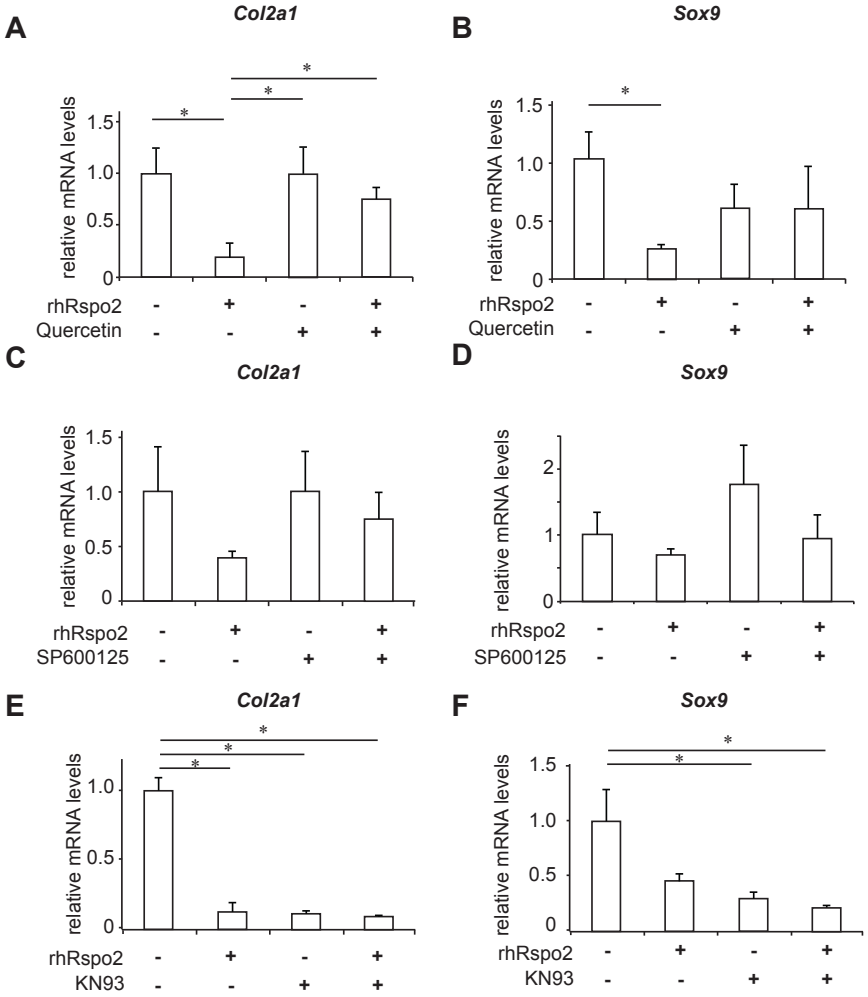

Fig. 4. Effects of Rspo2 on *Col2a1* and *Sox9* expressions are partially rescued by inhibitors for Wnt/β-catenin signaling. ATDC5 cells differentiated for 5 days were treated with rhRspo2 for 24 h, and then treated with Quercetin (an inhibitor for Wnt/β-catenin) **(A, B)**, SP600125 (a JNK inhibitor for Wnt/PCP) **(C, D)**, or KN93 (an inhibitor for Wnt/Ca^{2+}) **(E, F)** for additional 24 h. Gene expressions of *Col2a1* and *Sox9* are indicated. Values are normalized to the mean of untreated cells. $^{*}p < 0.05$ by one-way ANOVA followed by Bonferroni post-hoc test. Mean and SEM ($n = 3$) are indicated.

catenin protein was down-regulated in cartilages (Fig. 1I, J). These results indicated that Rspo2 promoted accumulation of β-catenin protein in chondrocytes of growth cartilages, which activated Wnt/β-catenin signaling.

3.2. Rspo2 and its receptor, Lgr5, are expressed in differentiating ATDC5 chondrogenic cells

We first analyzed expressions of Rspo2 and its receptors (Lgr4/5/6 and Rnf43/Znrf3) in the course of differentiation of chondrogenic cells, and also examined the effect of Rspo2 on a target molecule of Wnt/β-catenin signaling (Axin2). We used ATDC5 chondrogenic cell line, which can simulate differentiation through proliferating to hypertrophic chondrocytes in growth cartilage by addition of insulin-transferrin-selenium (ITS) to the culture medium. Quantitative RT-PCR showed that expression of *Rspo2* reached the highest level on day 5 after ITS treatment (Fig. 2A). As stated in the introduction, Rspo family members form a complex with Lgr4/5/6 and Rnf43/Znrf3 to attenuate degradation of Wnt receptors, LRPs and Frizzleds, which subsequently activates Wnt signaling [14,16,26]. We analyzed expressions of *Lgr4/5/6* and *Rnf43/Znrf3* (Fig. 2B–F) and found that the temporal profile of expression of *Lgr5*, but not *Lgr4/6* or *Rnf43/Znrf3*, was similar to that of *Rspo2* (Fig. 2C). Taken together, *Rspo2* and *Lgr5* are similarly induced in an early phase of differentiation of ATDC5 chondrogenic cells.

3.3. Rspo2 decreases Sox9, Col2a1, and Acan expressions in ATDC5 cells through Wnt/β-catenin signaling

Then we examined the effect of Rspo2 on differentiating ATDC5 cells. Temporal analysis of mRNA expressions showed that treatment of cells with rhRspo2 increased Axin2 expression in ATDC5 cells on days 0 and 5 after ITS treatment (Fig. 3A). We found that rhRspo2 decreased expression of *Sox9*, the master regulator for chondrogenesis, as well as expressions of *Col2a1* and *Acan*, marker genes in proliferating zone, on day 5 after ITS treatment (Fig. 3B–D). In contrast, rhRspo2 had no significant effects on *Col10a1* and *Runx2*, which are marker genes in hypertrophic zone and are induced in the late stage of chondrocyte differentiation (Fig. 3E and F). Rspo2 thus suppressed genes expressed in proliferating chondrocytes, but not in hypertrophic chondrocytes. The suppressive effects of Rspo2 on *Sox9*, *Col2a1*, and *Acan* peaked

around day 5 after ITS treatment. We next examined the effect of lentivirus-mediated knockdown of *Rspo2* for 24 h before harvesting cells on day 5 (Fig. 3I–L). Consistent with the results with rhRspo2, knockdown of *Rspo2* increased expressions of *Sox9*, *Col2a1*, and *Acan*. Furthermore, Alcian blue staining on day 15 after ITS treatment revealed that up- and down-regulation of Rspo2 down- and up-regulated the amount of extracellular matrix glycosaminoglycans, respectively (Fig. 3G, H, M and N). These results indicate that Rspo2 suppresses expressions of *Sox9*, *Col2a1*, and *Acan*, and decreases the amount of extracellular proteoglycans.

Akiyama et al. reported that spatiotemporal regulations of differentiation of proliferating chondrocytes are strictly regulated by the balance of activities of Sox9 and Wnt/β-catenin signaling in cartilage *in vivo* [27]. To investigate which Wnt signaling pathways are involved in Rspo2-mediated suppression of genes expressed in proliferating chondrocytes, we used chemical inhibitors for Wnt signaling pathways: Quercetin to inhibit Wnt/β-catenin signaling; SP600125 to inhibit JNK for Wnt/PCP signaling; and KN93 to inhibit Wnt/Ca^{2+} signaling. As expected, in ATDC5 cells on day 5 after ITS treatment, the effects of rhRspo2 on *Col2a1* and *Sox9* expressions were rescued with Quercetin (Fig. 4A–B), but not with SP600125 or KN93 (Fig. 4C–F). We next introduced the TOPFLASH luciferase reporter plasmid harboring six copies of β-catenin/TCF-binding regions in HCS2/8 human chondrosarcoma cells. We found that rhRspo2 activated Wnt/β-catenin signaling in a dose-dependent manner (Fig. S2). To summarize, Rspo2 suppresses differentiation of proliferating chondrocytes by activating Wnt/β-catenin signaling.

4. Discussion

4.1. Rspo2 prevents excessive expressions of Sox9 and Col2a1 in chondrocytes in growth cartilage

Previous report suggests that Rspo2 is expressed in apical ectodermal ridge and required for dorsoventral patterning of limb buds at embryonic day (E) 10.5 [20]. *Rspo2*-KO mice show hypoplastic left lower hindlimb, and frequent lack of digits of forelimbs and left hindlimb [19]. Although there was no gross alteration in growth cartilages in right hindlimb and both forelimbs at E18.5, growth cartilages showed excessive expressions of Sox9 and CII (Fig. 1A–D and I). The effects of Rspo2 on growth cartilage was corroborated in differentiating ATDC5 cells, in which addition and knockdown of Rspo2 down- and up-regulated expressions of *Sox9* and *Col2a1*, respectively (Fig. 3B, C, J, and K). *Sox9* is known to be expressed in chondrocytes of proliferating zones to regulate early chondrocyte differentiation [3]. Conditional overexpression of *Sox9* in proliferating zone, which is driven by the *Col2a1* promoter, displays disarrangements of chondrocyte alignments, similar to *Rspo2*-KO mouse [28]. The *Sox9*-knockout mice show delayed terminal differentiation of hypertrophic chondrocytes and endochondral bone formation [28]. In *Col2a1*+/− heterozygous embryos, an interface between resting and hypertrophic chondrocytes in growth cartilages becomes obscure [29], where it is preserved in *Rspo2* KO mice. These results indicate that Rspo2 suppresses expressions of *Sox9* and *Col2a1* in proliferating chondrocytes and attenuates differentiation into hypertrophic chondrocytes in endochondral ossification (Fig. S3).

Rspo2-KO mice displayed disarrangement of chondrocyte alignments (Fig. S1A), increased amounts of Sox9 and CII (Fig. 1A–D), and low amount of β-catenin proteins (Fig. 1G and H) in growth cartilage. Although other R-spondin members (Rspo1, 3 and 4) show similar expression patterns as Rspo2 by *in situ* hybridization in proliferating zone of growth cartilages [30], other Rspos in *Rspo2*-KO mice were unlikely to have rescued the phenotype. *Rspo1*-KO mice show abnormal ovarian development. Mutations in *RSPO1* in human cause palmoplantar hyperkeratosis with squamous cell carcinoma of skin and sex reversal. *Rspo3*-KO embryos die around E10 because of impaired fetal placental vascular development [31]. *Rspo4*-KO mice were generated by the KOMP project led by the Wellcome Trust Sanger Institute, but phenotypic details have not been scrutinized. Mutations in *RSPO4* in human cause autosomal recessive anonychia [32]. Although the detrimental effect of lack of Rspo2 on endochondral ossification was less prominent compared to lack of β-catenin [33] or Sox9 [3], Rspo2 is likely to play an essential role in proliferating chondrocytes of growth cartilage.

4.2. Rspo2 functions as an activator for Wnt/β-catenin signaling during chondrocyte differentiation

We then asked which signaling is activated by Rspo2 in chondrocytes. Rspo2 functions with its receptors LGR4/5/6 and RNF43/ZNRF3 by promoting stability of Wnt receptors, LRPs and Frizzleds [16]. We found that all receptors for Rspo2 and Wnts were expressed in ATDC5 cells on 5 days after ITS treatment (Figs. 2 and 3). Among the five Rspo2 receptors, the temporal profile of *Lgr5* expression was similar to that of *Rspo2* (Fig. 2C). In intestinal crypt and hair follicle, *Lgr5* expression is restricted to proliferating stem cells [34,35]. In these cells, Rspo2 secreted from adjacent cells binds to Lgr5, and enhances Wnt/β-catenin signaling [34,35]. Lgr5 is thus likely to function with Rspo2 in chondrocytes in growth cartilage.

We found that the effects of Rspo2 on chondrocytes were mediated by Wnt/β-catenin signaling and not Wnt/PCP signaling or Wnt/Ca^{2+} signaling. Our observations are consistent with previous reports showing that β-catenin and their related molecules play essential roles in endochondral ossification [1]. First, *Rspo2*-KO cartilage showed disarranged alignment of chondrocytes (Fig. S1A), which is also observed in *Col2a1*-promoter driven KO of β-catenin [36]. Second, mice deficient for *Sfrp* encoding secreted frizzled-related protein 1, which is a binding partner of Wnt ligands, show an increased amount of β-catenin and abnormal calcification of hypertrophic zone [37]. Our studies support the notion that Rspo2 activates Wnt/β-catenin signaling in chondrocytes to regulate chondrocyte differentiation during endochondral ossification.

Competing interests

The authors have declared that no competing interests exist.

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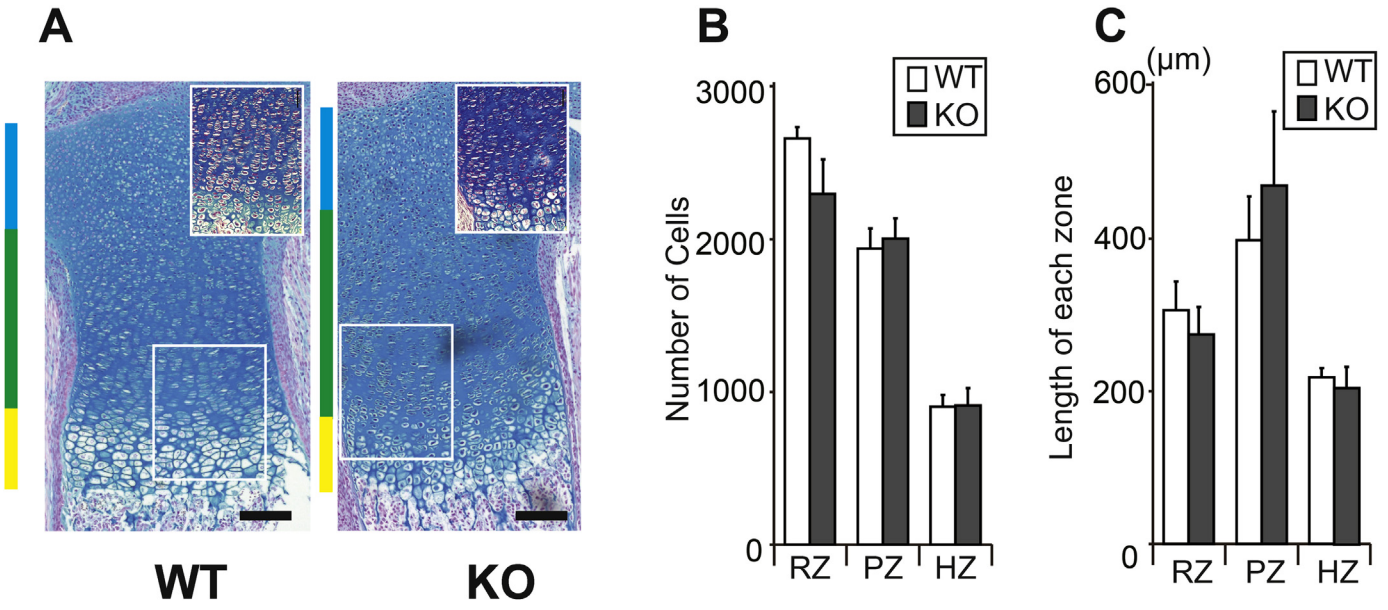
Transparency document

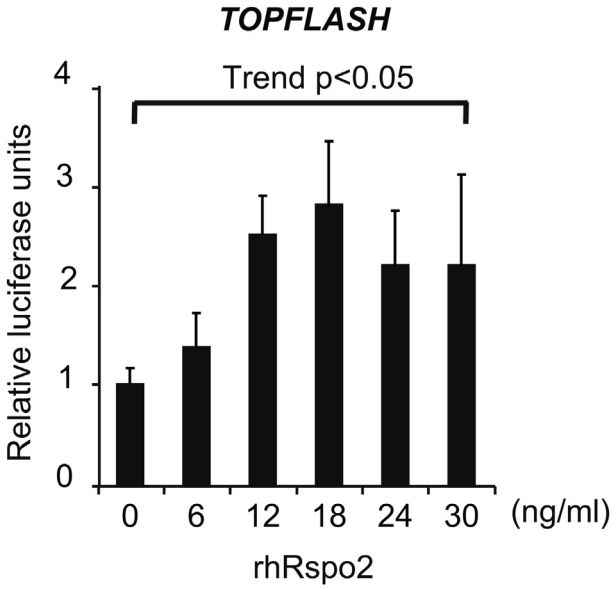
Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.03.089.

Appendix A. Supplementary data

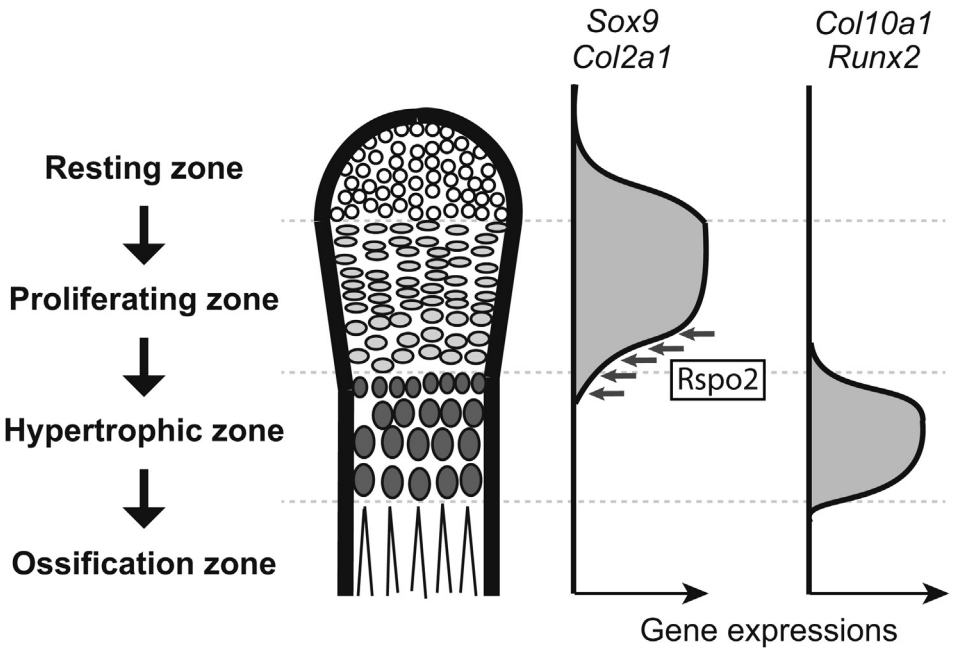
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.03.089.



Supplementary Fig. 1. In *Rspo2*-KO mice, chondrocytes in the proliferating zone of growth cartilage are small and disarranged, but the number of chondrocytes and the length of each zone are preserved. **(A)** Coronal slices of tibiae at E18.5 of wild-type (WT) and *Rspo2*-KO mice are stained with Alcian blue combined with hematoxylin and eosin staining. Three layers of resting zone (RZ; blue bar; dense small cells with less extracellular matrix), proliferating zone (PZ; green bar; cells between resting and hypertrophic zones), and hypertrophic zone (HZ; yellow bar; cells with large cytoplasmic region) are indicated. Scale bar = 100 μm. An interface between PZ and HZ is shown in a different contrast to show individual cells in an inset. Note that chondrocytes in PZ are small and disarranged in KO. **(B, C)** Cell numbers and lengths of each zone are blindly quantified, and the mean and SEM ($n = 3$) are indicated. There was no statistical difference by one-way ANOVA.



Supplementary Fig. 2. Wnt/β-catenin activity was increased by rhRspo2 treatment in a dose-dependent manner in HCS2/8 cells. Rspo2 enhances Wnt/β–catenin signaling pathway in human chondrosarcoma (HCS2/8) cells. Luciferase activities of TOPFLASH reporter in HCS2/8 cells treated with indicated concentrations of rhRspo2 for 24 h are normalized by the TK promoter-driven Renilla luciferase activity. Mean and SEM ($n = 10$) are indicated. The Jonckheere-Terpstra trend test is a statistical measure to evaluate the dose response effect of rhRspo2, and the result is indicated above the graph.



Supplementary Fig. 3. A Schematic model showing Rspo2-mediated facilitated differentiation of proliferating chondrocytes into hypertrophic chondrocytes. Sox9 is a master regulator of chondrogenesis, which is highly expressed in proliferating zone. Expression of *Col2a1* is driven by Sox9, and is a representative marker for proliferative zone. Rspo2 activates Wnt/β-catenin signaling and suppresses expressions of *Sox9* and *Col2a1* to facilitate chondrocyte differentiation.

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