A WNT/β-Catenin Signaling Activator, R-spondin, Plays Positive Regulatory Roles during Skeletal Myogenesis*

Received for publication, July 28, 2010, and in revised form, January 14, 2011 Published, JBC Papers in Press, January 20, 2011, DOI 10.1074/jbc.M110.169391

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R-spondins (RSPOs) are a recently characterized family of secreted proteins that activate WNT/β-catenin signaling. In this study, we investigated the potential roles of the RSPO proteins during myogenic differentiation. Overexpression of the Rspo1 gene or administration of recombinant RSPO2 protein enhanced mRNA and protein expression of a basic helix-loop-helix (bHLH) class myogenic determination factor, MYF5, in both C2C12 myoblasts and primary satellite cells, whereas MYOD or PAX7 expression was not affected. RSPOs also promoted myogenic differentiation and induced hypertrophic myotube formation in C2C12 cells. In addition, Rspo2 and Rspo3 gene knockdown by RNA interference significantly compromised MYF5 expression, myogenic differentiation, and myotube formation. Furthermore, Myf5 expression was reduced in the developing limbs of mouse embryos lacking the Rspo2 gene. Finally, we demonstrated that blocking of WNT/β-catenin signaling by DKK1 or a dominant-negative form of TCF4 reversed MYF5 expression, myogenic differentiation, and hypertrophic myotube formation induced by RSPO2, indicating that RSPO2 exerts its activity through the WNT/β-catenin signaling pathway. Our results provide strong evidence that RSPOs are key positive regulators of skeletal myogenesis acting through the WNT/β-catenin signaling pathway.

WNT signaling plays diverse roles in normal tissue development during embryogenesis and tissue function in adulthood. The importance of WNT signaling in skeletal myogenesis was initially demonstrated in embryonic skeletal myogenesis (1–4). The WNT1 and WNT3A ligands derived from the dorsal neural tube and the surface ectoderm positively regulate skeletal myogenesis within somites via the canonical WNT/β-catenin signaling pathway (3, 4). Furthermore, noncanonical WNT signaling, which transmits signals through the RAC/RHO-dependent planar cell polarity and calcium-PKC pathways, regulates PAX3 and subsequently MYOD expression during myogenic differentiation (5) and a directional elongation of myofibers within the myotome (6).

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

This work was supported, in whole or in part, by National Institutes of Health Grants P20 RR018789 (to D. M. Wojchowski, Program Director and J. K. Y., Project Principal Investigator) and R01 AR055278 (to J. K. Y., Principal Investigator).

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‡ The abbreviations used are: LRP, low density lipoprotein receptor-related protein; DKK1, Dickkopf-related protein 1; sFRP1, secreted Frizzled-related protein 1; TCF, T cell factor; MyHC, myosin heavy chain; GSK-3β, glycogen synthase kinase-3β; qRT-PCR, quantitative real time RT-PCR; sTopFlash; Super TOPFlash; MSTN, myostatin; FZD, Frizzled; MYOG, myogenin; SHH, sonic hedgehog.
R-spondin in Skeletal Myogenesis

In this study, we characterized the roles of the RSPO family proteins in myogenic differentiation using mouse primary satellite cells and C2C12 mouse myoblast cells. We identified that RSPO positively regulated the expression of the myogenic determination factor MYF5 in undifferentiated and differentiating C2C12 cells without affecting MYOD or PAX7 expression. Furthermore, RSPO2 promoted myogenic differentiation and hypertrophic myofiber formation. These RSPO2 effects were mediated through the WNT/β-catenin pathway. Our studies identified the RSPO family proteins as novel regulators of skeletal myogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture—The human embryonic kidney fibroblast cell line 293T was maintained in Dulbecco’s minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin under 5% CO2 at 37 °C. The mouse myoblast cell line C2C12 was obtained from ATCC (American Tissue Culture Collection, Manassas, VA) and maintained in growth medium (DMEM containing 10% FBS and 1% penicillin/streptomycin) under 5% CO2 at 37 °C. To induce myogenic differentiation, C2C12 cells were seeded near confluence and cultured overnight. Growth medium was replaced with differentiation medium (DMEM containing 2% heat inactivated horse serum). Differentiation medium was changed every 2 days. Reserve cells were prepared as described previously (19). Briefly, mononuclear reserve cells were collected by brief incubation with trypsin from C2C12 cells differentiated for 7 days. Contaminated myoblasts were further removed by filtration through a cell sieve. Primary satellite cells were prepared from the hind limb muscle of 12–14-week-old C57BL/6 mice as described previously (8). Cells were maintained in F-10 medium supplemented with 20% FBS, 1% penicillin/streptomycin, and 5 ng/ml recombinant basic fibroblast growth factor protein (Atlanta Biologicals, Lawrenceville, GA).

Animals—Mice carrying the Rspa2 null (Rspa2+/−) allele were described previously (20). A second mutant allele of the Rspa2 (Rspa2ΔZN) gene in which the LacZ and neo gene cassettes were removed by Flp-dependent recombination was generated. WNT reporter (TopGAL) mice (21) were obtained from The Jackson Laboratory (Bar Harbor, ME). The Rspa2 null and Rspa2ΔZN alleles and TopGAL transgene were genotyped by polymerase chain reaction (PCR) as described (20) and according to protocols available from The Jackson Laboratory, respectively. Mice were housed in a pathogen-free air barrier facility, and animal handling and procedures were approved by the Maine Medical Center Institutional Animal Care and Use Committee.

Whole Mount in Situ Hybridization and β-Galactosidase Staining—Whole mount in situ hybridization was performed as described (20). To visualize expression of the LacZ gene encoding β-galactosidase (β-GAL), freshly collected embryos were fixed with 0.2% glutaraldehyde for 15 min at room temperature and stained with X-Gal substrate (Invitrogen) overnight at 37 °C. The stained embryos were photographed under a StemiSV6 stereomicroscope (Zeiss) using an AxioCam digital camera (Zeiss).

Molecular Biology and Reagents—A full-length mouse Rspa1 cDNA with C-terminal HA epitope tag was excised from pcDNA3mRspa1HA DNA (16) by appropriate restriction enzymes. cDNA encoding a dominant-negative form of human TCF4 (ΔNTCF4) DNA was PCR-amplified from the CMV ΔNTCF4 expression vector (22). Rspa1HA and ΔNTCF4 cDNAs were cloned into pWZL retroviral vector (23). siRNA pools specific to the mouse Rspa2 (catalog number L-053030-09) and Rspa3 (catalog number L-049342-01) genes were purchased from Dharmacon (Lafayette, CO). 6-Bromoindirubin-3’-oxime (BIO), a specific inhibitor for GSK-3β that mimics WNT/β-catenin signaling activation (24), was purchased from Stemgent (Cambridge, MA). Recombinant RSPO2, DKK1, and sFRP1 proteins were purchased from R&D Systems (Minneapolis, MN) and used at the indicated concentrations.

Retroviral Transduction and DNA/RNA Transfection—Control WZL, WZLmRspa1HA, and WZLΔNTCF4 retroviruses were generated in the ecotropic packaging cell line BOSC23 (ATCC). Conditioned medium containing virus particles was directly used to transduce C2C12 cells in the presence of 8 µg/ml Polybrene. After overnight incubation, medium containing the virus was removed and replaced with fresh growth medium. After an additional 24 h of culture, the cells were reseeded at low density and selected in the presence of hygromycin (300 µg/ml) for 10 days with a medium change every 2 days. DNA and siRNA transfection into C2C12 cells was performed using Lipofectamine 2000 (Invitrogen) and Lipopectamine RNA Max reagent (Invitrogen) according to the manufacturer’s protocol.

RNA Isolation, Quantitative Real Time RT-PCR, and Luciferase Assay—Total RNA was isolated from cultured cells and embryonic tissues using TRIzol reagent (Invitrogen). First strand cDNA synthesis was performed using a Superscript II cDNA synthesis kit according to the manufacturer’s protocol (Invitrogen). Normally, 2 µg of total RNA was used for cDNA synthesis. cDNA (equivalent to 100 ng of total RNA) was used for quantitative real time RT-PCR (qRT-PCR). The sequences of the primers used in qRT-PCR are listed in Table 1. Luciferase assays were performed using the Dual-Luciferase assay kit (Promega, Madison, WI) following the provided procedure.

Antibodies, Western Blot Analysis, and Immunofluorescent Staining—Total cell lysates were prepared in a radioimmune precipitation assay buffer (10 mM Tris-Cl, pH 7.2, 2 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM NaF, 1% sodium deoxycholate, 1 mM PMSE, 1× protease inhibitor mixture set V (EMD Chemicals, Gibbstown, NJ), 0.2 mM sodium vanadate). Cytoplasmic lysates were prepared in a hypotonic lysis buffer (10 mM Tris, pH 4, 0.2 mM MgCl2, 1 mM PMSE, 1× protease inhibitor mixture set V, 0.2 mM sodium vanadate, 50 mM NaF). Equivalent amounts of the protein samples were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with blocking buffer (5% milk solution). After washing, the membranes were incubated with primary antibodies followed by secondary antibodies conjugated with horseradish peroxidase (HRP). The blots were developed with SuperSignal West Dura Luminol/Enhancer Solution (Thermo Scientific/Pierce) and exposed to x-ray film (Eastman Kodak Co.). Antibodies against the HA epitope (clone
myogenic differentiation, we analyzed their mRNA levels in the mouse myoblast cell line C2C12 using conventional RT-PCR. In undifferentiated, exponentially growing C2C12 cells (maintained at subconfluent density in growth medium), none of the Rspo family genes were expressed at any detectable level (Fig. 1A). When the cells were cultured in growth medium until confluent and ready to begin differentiation, robust Rspo3 expression was detected. When myogenic differentiation was induced in confluent cells for 1 day, both Rspo2 and Rspo3 expression were detected (Fig. 1A). Their expression was comparable with that of Myog, a key myogenic basic helix-loop-helix (bHLH) transcription factor tightly associated with the onset of myogenic differentiation. Interestingly, neither Rspo1 nor Rspo4 was expressed in any samples.

We further analyzed Rspo2 and Rspo3 expression by real-time quantitative RT-PCR in a time course (Fig. 1, B and C). Consistent with the conventional RT-PCR results, expression of both genes was undetectable in undifferentiated cells. Rspo3 was immediately induced during differentiation with a peak at 12 h in differentiation medium and gradually declined during later differentiation. The Rspo2 gene was induced with a slightly delayed manner as its expression peaked at 36 h during differentiation and decreased gradually as differentiation progressed. As expected, expression of Myog gradually increased during differentiation (Fig. 1D).

We also determined Rspo expression in mouse primary satellite cells. All Rspo genes but Rspo4 were expressed in satellite cells cultured in growth medium (Fig. 1E). Both Rspo1 and Rspo3 expression increased during myogenic differentiation of satellite cells, whereas Rspo2 expression remained unchanged (Fig. 1, F–H).

**Rspo2 Up-regulates Basic Helix-Loop-Helix Class Myogenic Determination Factor MYF5**—To determine the potential functional roles of Rspo during myogenic differentiation, we examined the expression of several myogenic transcription factors in C2C12 cells cultured in the presence of recombinant Rspo2 protein. In undifferentiated and exponentially growing C2C12 cells, expression of the bHLH class myogenic determination factor MYF5 was significantly increased by Rspo2 (Fig. 2A), whereas expression of another bHLH class myogenic determination factor, MYOD, was not affected. Expression of PAX7, a transcription factor critical for early myogenic determination, was not affected by Rspo2 protein (Fig. 2A).

We next incubated C2C12 cells cultured in differentiation medium with the Rspo2 protein. Similar to undifferentiated cells, Rspo2 significantly enhanced MYF5 protein expression in the early stage of myogenic differentiation (Fig. 2B). MYOD and PAX7 expression was also not significantly changed. Additionally, expression of the MEF2 family myogenic differentiation factors MEF2A and MEF2C was also not affected. Expression of the differentiation marker MYOG was not changed for 1 day, both Rspo2 and Rspo3 expression were detected (Fig. 1A). Their expression was comparable with that of Myog, a key myogenic basic helix-loop-helix (bHLH) transcription factor tightly associated with the onset of myogenic differentiation. Interestingly, neither Rspo1 nor Rspo4 was expressed in any samples.

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All Rspo family members are indistinguishable in their activity in activating WNT/β-catenin signaling (13, 16, 26). To determine whether MYF5 induction is specific to Rspo2, we generated C2C12 cell populations constitutively expressing the mouse Rspo1 gene (HA epitope-tagged) by retroviral transduc-

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**TABLE 1**

DNA sequences of PCR primers used in this study

| Rspo2 (RT-PCR) | Sense  | 5’-ATA GAG GCC GCT GCT TGG-3’ |
| Rspo2 (RT-PCR) | Antisense | 5’-AGC TGG TCC TCA TCG GTG-3’ |
| Rspo3 (RT-PCR) | Sense  | 5’-TGG CAT AAG GCC CTC TTC-3’ |
| Rspo3 (RT-PCR) | Antisense | 5’-AGC TGG TCC TCA TCG GTG-3’ |
| Rspo4 (RT-PCR) | Sense  | 5’-CCA CCT AAG GCC CTC TTC-3’ |
| Rspo4 (RT-PCR) | Antisense | 5’-AGC TGG TCC TCA TCG GTG-3’ |

**RESULTS**

Rspo Gene Expression during Myogenic Differentiation—To determine whether the Rspo family genes are expressed during
Gene/Rspo2

**Rspo2**

**A**

**GM**

**SC**

**C**

**D1**

**B**

**RSPO2**

**C**

**RSPO2**

**D**

**Rspo2**

**E**

**Rspo1**

**Rspo2**

**Rspo3**

**Gapdh**

**F**

**Rspo1**

**G**

**Rspo2**

**H**

**Rspo3**

**FIGURE 1.** mRNA expression of *Rspo* genes in C2C12 myoblast and primary satellite cells. A and E, conventional RT-PCR. mRNA expression of the Rspo family genes was analyzed in C2C12 (A) and satellite cells (E). RNA samples were isolated from undifferentiated C2C12 cells cultured in growth medium (GM) at a subconfluent (SC) or confluent (C) density, and differentiating C2C12 cells were cultured in differentiation medium for 1 day (D1). RNA samples were isolated from satellite cells cultured in growth medium. cDNAs equivalent to 50 (C2C12) and 25 ng (satellite cells) were used for PCR. PCR products were separated in a 2% agarose gel by electrophoresis and visualized by ethidium bromide staining. B–D and F–H, qRT-PCR analysis of the Rspo family genes. C2C12 (B–D) and satellite cells (F–H) were harvested for RNA isolation at the indicated time points after changing into differentiation medium. Expression of Myog (D), Rspo1 (F), Rspo2 (B and G), and Rspo3 (C and H) was analyzed. The expression level was normalized by Gapdh expression. The relative expression level was calculated against those at 12 (B–D) or 0 h (F–H). Samples were prepared in triplicate, and qRT-PCR was performed in duplicate. Error bars are presented as S.E.

**FIGURE 2.** RSPO enhances MYF5 protein expression in C2C12 and satellite cells. A, exponentially growing, undifferentiated C2C12 cells were treated with recombinant RSPO2 (200 ng/ml) protein for 24 h. Cell lysates were analyzed for MYF5, MYOD, and PAX7 expression by Western blot. The β-tubulin protein level was utilized as a loading control. B, C2C12 cells were differentiated up to 4 days (D1–D4) in the absence (BSA; 200 ng/ml) or presence of RSPO2 protein (200 ng/ml). The RSPO2 protein was added daily. In addition to MYF5, MYOD, and PAX7, expression of differentiation markers MYOG and MEF2A/C was analyzed by Western blot. C, cell lysates were prepared from undifferentiated C2C12 cells stably transduced with control WZL retrovirus (Con.WZL) or retrovirus constitutively expressing the mouse Rspo1HA gene (HA epitope-tagged) and analyzed for Rspo1HA expression by Western blot. D, cell lysates were prepared from undifferentiated (U) and differentiating (D) control and Rspo1HA C2C12 cells. Western blot analysis was performed to detect MYF5, MYOD, MYOG, and β-tubulin protein levels. E, cell lysates were prepared from exponentially growing satellite cells cultured in the presence or absence of the RSPO2 protein (200 ng/ml) for 24 h. MYF5, MYOD, PAX7, and β-tubulin protein expression was analyzed by Western blot.

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Expression of the exogenous RSPO1 protein was confirmed by Western blot using anti-HA antibody in total cell lysate (Fig. 2C). Consistent with the above RSPO2 results, MYF5 protein expression was significantly induced in C2C12 cells constitutively expressing the exogenous Rspo1 gene (C2C12/Rspo1HA) when compared with C2C12 cells established with control viral vector (Fig. 2D). MYOD expression remained largely unchanged in C2C12/Rspo1HA cells. Expression of MYOG was also reduced at the later stage of differentiation in C2C12/Rspo1HA cells.

C2C12 cells contain “reserve cells,” a population that remains mononucleated and undifferentiated during myogenic differentiation (19). This population is considered similar to satellite cells in normal muscle tissue that are critical to maintenance, growth, and repair of skeletal muscle. We isolated the reserve cell population from fully differentiated C2C12 cell cultures. RSPO2 efficiently induced MYF5 expression without changing MYOD and PAX7 protein levels in these cells (supplemental Fig. S1). Similar to C2C12 cells, MYF5 protein expression was also induced by RSPO2 in primary satellite cells cultured in growth condition, whereas MYOD and PAX7 expression was unaffected (Fig. 2E).

To determine whether the increase in MYF5 protein level by RSPO2 is a consequence of increased *Myf5* transcript, we analyzed *Myf5* mRNA expression in RSPO2-treated C2C12 and satellite cells by qRT-PCR (Fig. 3, A and D). *Myf5* mRNA expression was considerably increased in cells treated with the RSPO2 protein in both cells. Consistent with their protein expression pattern, *MyoD* mRNA expression was not affected by RSPO2 (Fig. 3, B and E). Although *Pax7* mRNA expression was not changed by RSPO2 in C2C12 cells, RSPO2 mildly but significantly inhibited *Pax7* mRNA expression in satellite cells.
likely that RSPO-dependent signaling controls any detectable level in undifferentiated cells (Fig. 1), it is not necessary for in undifferentiated C2C12 cells. However, further increased differentiation of C2C12 cells. Because Rspo3 Genes in Differentiating C2C12 Cells—The RSPO family proteins activate WNT/β-catenin signaling. To determine whether RSPO positively regulates Myf5 expression in mouse embryos lacking the Rspo2 gene by whole mount in situ hybridization. Rspo2 gene is expressed in the mesenchymal cells in the first branchial arch and developing limb buds (27) where Myf5-positive myogenic precursors for the jaw and limb muscles are located. Myf5 mRNA expression was severely compromised in the developing limbs of Rspo2 null embryos compared with wild type littermates (Fig. 4, D and E). Expression of WNT/β-catenin reporter, TopGAL transgene, was also reduced in the limbs of Rspo2 null embryos (Fig. 4, F and G). Reduced expression of Myf5 and WNT signaling target Axin2 in the developing limbs of Rspo2 mutant embryos was further confirmed by qRT-PCR (Fig. 4, H and I). Interestingly, Myf5 expression in the first branchial arch was not affected in Rspo2 mutant embryos (Fig. 4H), whereas Axin2 expression was still reduced (Fig. 4I). These results suggest that limb-specific Myf5 expression is RSPO2- and WNT/β-catenin-dependent. However, branchial arch-specific Myf5 expression is Rspo2-independent. No major abnormalities of limb muscle were noticed in Rspo2 mutants at the later stage (data not shown). It is possible that reduced Myf5 function may delay skeletal myogenesis in the limbs but is likely compensated by Myod as seen in Myf5 null mutant mice (28, 29).

RSPO2 Activates WNT/β-Catenin Signaling Pathway in Myogenic Cells—The RSPO family proteins activate WNT/β-catenin signaling in human embryonic kidney cell line 293T and other cell lines (13, 15, 16). To determine whether RSPO2 can also activate WNT/β-catenin signaling, we examined the
activation of a WNT reporter, Super TopFlash (sTopFlash) and the level of cytoplasmic β-catenin protein in C2C12 cells stimulated with the RSPO2 protein. As expected, the RSPO2 protein robustly activated sTopFlash reporter in a dose-dependent manner (Fig. 5A). Consistent with this result, the cytoplasmic β-catenin protein level was significantly higher in RSPO2-treated differentiating C2C12 cells than in untreated cells (Fig. 5B). In C2C12/Rspo1HA cells, we also detected a significantly higher level of cytoplasmic β-catenin than in control C2C12 cells (supplemental Fig. S3). Robust activation of the sTopFlash reporter was observed in C2C12/Rspo1HA cells, whereas the same reporter was not active in control cells (supplemental Fig. S3). Furthermore, RSPO2 treatment increased the β-catenin protein level in the cytoplasm of satellite cells (data not shown). Taken together, we conclude that RSPOs effectively activate WNT/β-catenin signaling in myogenic cells.

MYF5 Induction by RSPO2 Is Mediated by WNT/β-catenin Signaling—To determine whether RSPO2 activates MYF5 expression via the WNT/β-catenin pathway, we examined MYF5 expression in C2C12 cells in which WNT/β-catenin signaling was compromised. We used two experimental approaches to inhibit WNT/β-catenin signaling.

First, we cotreated C2C12 cells with RSPO2 and DKK1 or sFRP1 protein. DKK1 is an antagonist for WNT/β-catenin signaling. sFRP1 was cotreated with RSPO2, DKK1 effectively reduced sTopFlash activity and MYF5 expression induced by RSPO2, whereas sFRP1 failed to do so (Fig. 5D).

Second, we examined RSPO2 activity on WNT/β-catenin signaling and MYF5 expression in C2C12 cells (C2C12/ΔNTCF4) stably expressing a dominant-negative form of human TCF4/TCF7L2 (ΔNTCF4-Myc) (22). Both sTopFlash activity and MYF5 expression induced by RSPO2 was reversed by ΔNTCF4-Myc expression (Fig. 5, E and F). Consistent with these results, BIO, a chemical known to induce WNT signaling activation through the inhibition of GSK-3β (24), significantly enhanced both Myf5 mRNA and protein expression (supplemental Fig. S4). We conclusively...
determined that Myf5 activation by RSPO2 is mediated by WNT/β-catenin signaling.

**RSPO2 Promotes Myogenic Differentiation and Induces Hypertrophic Myotube Formation**—To determine how RSPO2 regulates myogenic differentiation of C2C12 cells, we differentiated C2C12 cells in the absence or presence of the RSPO2 protein for up to 4 days and immunostained cells for MYOG and MyHC expression. We determined the differentiation index (a percentage of MYOG- or MyHC-positive nuclei in total nuclei) and myotube fusion index (a distribution of the nucleus number in MyHC-positive cells).

The myogenic differentiation index (MyHC-positive nuclei) was increased in the presence of RSPO2 at days 3 and 4 (Fig. 6, A–C). Interestingly, a number of nuclei positive for MYOG, a marker for the onset of differentiation, did not increase in the presence of the RSPO2 protein for the first 2 days of differentiation (Fig. 6E), consistent with the Western blot analysis (Fig. 2B). However, at differentiation days 3 and 4, MYOG-positive cells were also increased in the presence of RSPO2 (Fig. 6E). Clearly, RSPO2 activity in enhancing differentiation is more potent at days 3 and 4 of differentiation. Interestingly, the overall expression level of MYOG protein detected by Western blot began to be reduced at the later stage of differentiation (Fig. 2B), whereas the number of MYOG-positive nuclei increased. It is likely that the MYOG expression level in each cell decreased by RSPO2 treatment.

In addition to promoting differentiation, RSPO2 induced the formation of hypertrophic myotubes (Fig. 6, A, B, and D). The myotubes in RSPO2-treated cultures showed increased size and a higher fusion index than those in untreated cultures at differentiation days 3 and 4. We conclude that RSPO2 stimulated myogenic differentiation and myotube hypertrophy in C2C12 cells.
Reduced Rspo Expression Attenuates Myogenic Differentiation and Myotube Formation—To determine whether expression of the endogenous Rspo genes is essential for myogenic differentiation, C2C12 cells were transiently transfected with siRNA specific to the Rspo2 and Rspo3 genes and examined for the efficiency of myogenic differentiation and myotube formation (Fig. 6, F and G). Myogenic differentiation in C2C12 cells transfected with Rspo siRNAs was decreased ~50% compared with C2C12 cells transfected with control siRNA (Fig. 6F). Additionally, myotube formation was also negatively affected as the percentage of myotubes containing more than five nuclei in cells transfected with Rspo siRNAs was reduced to about 50% of control cells (Fig. 6G). Reduced Rspo expression strongly suggests that expression of endogenous Rspo2 and Rspo3 is required for myogenesis of C2C12 cells.

RSPO2-induced Myogenic Differentiation and Hypertrophic Myotube Formation Are WNT/β-Catenin Signaling-dependent—To determine whether myogenic differentiation and hypertrophic myotube formation by RSPO2 are also WNT/β-catenin pathway-dependent, we examined myogenic differentiation and hypertrophic myotube formation in C2C12 cells overexpressing ΔNTCF4. In the absence of RSPO2 treatment, both control C2C12/WZL and C2C12/ΔNTCF4 cells showed a similar degree of myogenic differentiation (Fig. 7, A, B, and E). However, in C2C12/ΔNTCF4 cells, RSPO2 failed to enhance myogenic differentiation (Fig. 7E), suggesting that RSPO2 enhances myogenic differentiation in a β-catenin/TCF-dependent manner. When the myotube fusion index was analyzed, C2C12/ΔNTCF4 cells exhibited slightly reduced myotube fusion compared with control WZL C2C12 cells in the absence of RSPO2 (Fig. 7F). However, when C2C12/ΔNTCF4 cells were treated with RSPO2, no enhanced myotube fusion was detected (Fig. 7, C, D, and F). Therefore, we conclude that RSPO2 action on myogenic differentiation and hypertrophic myotube formation is mediated by the WNT/β-catenin pathway.

FIGURE 6. Regulation of myogenic differentiation and hypertrophic myotube formation by RSPO2. A–E, C2C12 cells were cultured in differentiation medium in the absence (BSA; 200 ng/ml) or presence of RSPO2 protein (200 ng/ml) up to 4 days (D1–D4). Cells were immunostained with anti-MyHC (A–D) or anti-MYOG antibody (E). DAPI staining was performed to visualize nuclei. A and B, fluorescent staining images of differentiating C2C12 cells cultured in the absence or presence of RSPO2 protein. C, MyHC-positive nuclei were counted and presented as a percentage of total nuclei. D, a distribution of MyHC-positive cells based on nucleus number. The number of MyHC-positive cells containing a single nucleus (Mono), two to five nuclei, and more than five nuclei was counted. The percentage of each group is presented. E, MYOG-positive nuclei were counted and presented as a percentage of total nuclei. F and G, C2C12 cells were transiently transfected with control (Con) or Rspo siRNA at differentiation day 3. MyHC-positive nuclei (F) and the cell fusion index (G) were analyzed. The experiment was performed in duplicate, and more than 1500 total nuclei were counted. Error bars are presented as S.E. p values were determined by Student’s t test.
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**DISCUSSION**

**RSPOs Are WNT Signaling Activators in Skeletal Muscle Cells**—Dynamic expression of the *Rspo* family genes during myogenic differentiation of C2C12 and primary satellite cells implicate them as attractive candidates for the extracellular WNT signaling activators in skeletal myogenesis. Indeed, RSPOs, when either overexpressed or administrated as a recombinant protein to C2C12 and satellite cells, effectively induced WNT/β-catenin signaling (Fig. 5 and supplemental Fig. S3).3 Furthermore, DKK1, a WNT signaling antagonist that acts on the LRP5/6 receptors, effectively inhibited RSPO2 activity in *Myf5* induction, whereas the sFRP1 protein that is structurally similar to the extracellular domain of the FZD receptors was unable to inhibit RSPO2 signaling function (Fig. 5). Therefore, RSPO seems to activate the canonical WNT pathway through the LRP5/6 receptors without affecting signaling through the FZD receptor in C2C12 cells.

Canonical WNT ligands such as WNT3A can simultaneously activate both the canonical and noncanonical pathways in the same cell, whereas noncanonical WNT ligands like WNT5A can only activate the noncanonical pathway without affecting the canonical pathway. Therefore, when canonical WNT3A ligand was used to mimic the activation of canonical WNT signaling, distinguishing its signal transmission through the canonical and noncanonical pathways was necessary. We failed to detect any activation of RAC1 and RHOA, two key mediators of noncanonical WNT signaling, by the RSPO proteins in C2C12 cells.4 Thus, the RSPo family proteins are a unique class of canonical WNT/β-catenin signaling activator distinct from the WNT proteins. However, it is worth noting that human RSPO1 synergizes with the specific FZD receptors in β-catenin activation in human embryonic kidney (HEK) 293T cells and *Xenopus* embryos (14). RSPO3 binds weakly to the extracellular domain of the FZD8 receptor in vitro (16). Therefore, it remains to be further determined whether RSPO can activate noncanonical WNT signaling through the specific FZD receptors in different cell contexts.

**RSPO as Positive Regulator Specific to MYF5**—MYF5 and MYOD are two key myogenic determination factors that initiate skeletal myogenesis during embryonic and postnatal myogenesis in an independent but compensatory manner. Mice lacking the *Myf5* or *Myod* gene do not develop any major defects in embryonic myogenesis (29, 33). However, mice lacking both genes display severe defects in myogenic commitment (34), resulting in a massive loss of skeletal muscle during

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3 X. H. Han and J. K. Yoon, unpublished data.

4 Y. R. Jin and J. K. Yoon, unpublished data.
embryogenesis, indicating that Myf5 and MyoD have a redundant role in myogenic commitment. Embryonic Myf5 expression within the epaxial domain of the somites is regulated by the WNT1 and WNT3A ligands derived from the dorsal region of developing neural tube in mice and chicken (1, 3, 4). An efficient activation of Myf5 gene by WNT in the somites is synergized by SHH signaling (1, 4). Several TCF binding sites as well as SHH signaling target, a GLI transcription factor binding site, were identified within the epaxial specific enhancer region of the mouse Myf5 gene (1). Mutagenesis analysis of these sites in transgenic mice showed that two TCF and GLI sites are required for robust Myf5 expression (1).

The Rspo1 and Rspo3 genes are also expressed in the dorsal neural tube in mouse embryos (27). Therefore, it is highly likely that these RSPO proteins act as a part of an inducing signal for Myf5 gene activation in the somites. It is possible that Myf5 activation by RSPOs derived from the dorsal neural tube is mediated by the TCF sites within the epaxial specific enhancer and can synergize with SHH signaling. Interestingly, no skeletal muscle phenotypes are reported in Rspo1 or Rspo3 gene knock-out mice (35, 36). Given that both genes are coexpressed in the dorsal neural tube, they likely have a redundant function. Rspo1, Rspo3 double knock-out mice need to be examined for skeletal muscle phenotype in the future.

Rspo2 expression is detected within mesenchymal cells in the developing limbs and branchial arches (27) where myogenic progenitors migrating from the hypaxial somites are localized. Reduced Myf5 expression and WNT/β-catenin signaling in the developing limbs of Rspo2 null embryos (Fig. 4, D–I) strongly suggests that Myf5 expression in limb-specific myogenic cells is regulated by Rspo2 via the WNT/β-catenin signaling pathway. The enhancer region for the limb-specific Myf5 expression was previously identified but does not overlap with the epaxial specific enhancer (37, 38). It will be interesting to investigate whether this enhancer contains potential TCF binding sites and whether RSPO2-mediated Myf5 induction is regulated through this region.

In Xenopus, Rspo2 also positively regulates embryonic myogenesis (13). Interestingly, expression of both Myf5 and MyoD is inhibited by Rspo2 knockdown in Xenopus embryos, suggesting both Myf5 and MyoD can be the targets for RSPO signaling (13). Taken together, RSPOs positively regulate Myf5 expression and skeletal myogenesis in different vertebrates.

Possible Roles of RSPOs in Adult Muscle Stem Cells—Satellite cells are mononucleated cells residing in adult muscle that serve as a stem/progenitor pool for skeletal muscle maintenance, growth, and repair (39). In adult mice, Myf5 expression, as detected by LacZ expression knocked into the Myf5 gene locus, is generally considered to be associated with quiescent and activated satellite cells (40). Pax7 is also expressed in quiescent and activated satellite cells (25). MyoD, in contrast, is not expressed in quiescent satellite cells but is induced in activated satellite cells that are committed to the myogenic lineage (41). Expression of both Myf5 and Pax7 declines as satellite cells initiate differentiation, and expression is undetectable in Myog-positive cells. In contrast, MyoD is continuously expressed in differentiated cells (41). Adult Myf5 null mice showed severely defective muscle regeneration, implicating a Myf5 role in satellite cell function during muscle regeneration (42, 43). A specific activation of MYF5 in satellite cells and C2C12 myoblasts by RSPOs strongly suggests a critical role of RSPO signaling, possibly through the β-catenin pathway, in regulating satellite cells at the stage of activation and myogenic commitment during muscle regeneration. Interestingly, PAX7 and MYOD expression is not significantly regulated by RSPOs in C2C12 and satellite cells. Therefore, it is possible that RSPO signaling only induces and regulates MYF5- but not PAX7-positive satellite cells or MYOD-positive myogenic cells derived from satellite cells. It remains to be determined whether RSPO signaling specifically regulates myogenic commitment in satellite cells.

Rspo2 in Myogenic Differentiation and Hypertrophic Myotube Formation—In addition to Myf5 activation, the Rspo2 protein promoted myogenic differentiation and induced hypertrophic myotube formation in differentiating C2C12 cells. It is currently unclear whether enhanced Myf5 activation is a primary cause of the promoted differentiation and myotube growth at the later stage of differentiation. Endogenous Myf5 expression during myogenic differentiation of C2C12 cells is quickly down-regulated as differentiation is advanced (Fig. 2B). It is reported that loss of Myf5 induces precocious differentiation in primary myoblasts as increased MYOG-positive cells were detected in Myf5 null cell culture compared with wild type myoblast culture (44), suggesting that MYF5 expression is associated with commitment to the myogenic lineage and prevents premature differentiation. Therefore, it seems that increased Myf5 expression may not directly result in enhancing differentiation.

When antisense oligomers against the Myf5 gene were delivered into differentiating C2C12 cells, they showed a strong inhibitory activity in myoblast fusion (45). This result suggests that, in addition to myogenic determination, MYF5 may be involved in myoblast fusion. However, Myf5 overexpression in C2C12 cells did not result in an increase of the myotube fusion index (46). Thus, it is possible that prolonged MYF5 expression induced by Rspo2 enhances hypertrophic myotube formation.

Two prominent signaling molecules, insulin-like growth factor 1 (IGF1) and myostatin (MSTN), were previously implicated in regulating skeletal muscle growth (47, 48). IGF1 positively regulates muscle mass through the PI3K and AKT pathway, whereas MSTN acts as a negative regulator by regulating the SMAD pathway. In our preliminary observation, the expression of these two genes was not significantly regulated by Rspo2 treatment in C2C12 cells. It is still possible that Rspo2 can modify IGF1 and/or MSTN signaling by modulating signaling components. Overall, our study clearly suggests that RSPOs may play a more positive role in skeletal myogenesis via the WNT/β-catenin pathway.

Acknowledgments—We thank Drs. Lucy Liaw and Doug Spicer for comments and suggestions and Norma Albrecht for proofreading the manuscript. We are grateful to Drs. Masanobu Kawai and Doug Spicer for providing reagents. We also thank Nancy Chandler-Conrey in Recombinant Viral Vector Core (supported by National Institutes of Health Grant P20 RR015555 to R. Friesel, Program Director) for producing Rspo1 and ΔNTCF4 retroviruses, and Bioinformatics core for qRT-PCR analyses (supported by P20 RR018789) at Maine Medical Center Research Institute.
