Regulation of the Follistatin Gene by RSPO-LGR4 Signaling via Activation of the WNT/ β -Catenin Pathway in Skeletal Myogenesis

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WNT signaling plays multiple roles in skeletal myogenesis during gestation and postnatal stages. The R-spondin (RSPO) family of secreted proteins and their cognate receptors, members of leucine-rich repeat-containing G protein-coupled receptor (LGR) family, have emerged as new regulatory components of the WNT signaling pathway. We previously showed that RSPO2 promoted myogenic differentiation via activation of WNT/ β -catenin signaling in mouse myoblast C2C12 cells *in vitro*. However, the molecular mechanism by which RSPO2 regulates myogenic differentiation is unknown. Herein, we show that depletion of the LGR4 receptor severely disrupts myogenic differentiation and significantly diminishes the response to RSPO2 in C2C12 cells, showing a requirement of LGR4 in RSPO signaling during myogenic differentiation. We identify the transforming growth factor β (TGF- β) antagonist *follistatin* (*Fst*) as a key mediator of RSPO-LGR4 signaling in myogenic differentiation. We further demonstrate that *Fst* is a direct target of the WNT/ β -catenin pathway. Activation and inactivation of β -catenin induced and inhibited *Fst* expression, respectively, in both C2C12 cells and mouse embryos. Specific TCF/LEF1 binding sites within the promoter and intron 1 region of the *Fst* gene were required for RSPO2 and WNT/ β -catenin-induced *Fst* expression. This study uncovers a molecular cross talk between WNT/ β -catenin and TGF- β signaling pivotal in myogenic differentiation.

WNT signals, which are transmitted via multiple intracellular signaling cascades, including the canonical WNT/ β -catenin pathway, play key roles in various aspects of skeletal myogenesis during gestation and postnatal stages (1, 2). During embryonic myogenesis, β -catenin function is essential for establishing the myogenic potential of the *Pax3*-positive progenitor cells within presomitic mesoderm cells and nascent somites (3). Different WNT proteins secreted from the surrounding tissue, such as dorsal neural tube and lateral ectoderm tissues, differentially regulate the myogenic program, especially the expression of myogenic determination factors, within somites (4–6). While activation of the WNT/ β -catenin pathway by dorsal neural tube-derived WNT1 and WNT3A proteins preferentially induces *Myf5* expression, demarcating epaxial muscle in somite explants, lateral mesoderm-derived WNT7A induces *Pax3* and *MyoD* via the noncanonical WNT pathway to specify hypaxial muscle (5, 7). In addition, the WNT11 ligand expressed in the myotome regulates directional elongation of myofibers within the myotome in a β -catenin-independent manner without affecting myogenic differentiation (8).

In adult skeletal muscle, WNT/ β -catenin signaling promotes myogenic differentiation of satellite cells (muscle-resident stem cells) by antagonizing mitogenic NOTCH signals through the inhibition of glycogen synthase kinase 3 β (9). However, specific WNT ligands responsible for this regulation have not been identified. Activation of the WNT/ β -catenin pathway is also associated with the CD45-positive stem cell population present in regenerating skeletal muscles (10). In addition, the WNT7A protein regulates self-renewal of satellite cells via the noncanonical WNT pathway in a fibronectin-dependent manner (11).

Members of the R-spondin (RSPO) family of secreted cysteine-rich proteins (RSPO1, -2, -3, and -4) activate the WNT/ β -catenin signaling pathway at the receptor level in various cellular contexts (12, 13). The RSPO family proteins share two furin-like cysteine-

rich (CR) domains followed by a single thrombospondin type I repeat (TSR) domain (12, 13). The CR domains are essential for activation of WNT/ β -catenin signaling (14, 15). Interestingly, the RSPO proteins potentiate the activities of the WNT proteins in WNT/ β -catenin signaling (14–16), indicating that RSPOs are important regulators of WNT/ β -catenin signaling.

Several cognate receptors for the RSPO proteins have been identified. The leucine-rich repeat-containing G protein-coupled receptors 4, 5, and 6 (LGR4/5/6), markers for the intestinal and hair follicle stem cells (17, 18), were identified as RSPO receptors (19–22). Crystal structure analysis showed that the CR2 domain of RSPO1 directly interacts with the ectodomain of the LGR4 family receptors (23–25). Depletion of the LGR4 and LGR5 receptors in HEK 293T cells disrupts activation of WNT/ β -catenin signaling by RSPOs and the synergy between RSPOs and WNTs, indicating that the LGR4 family receptors are active components of RSPO-induced WNT/ β -catenin activation (19–21). In addition, the RSPO proteins were reported to bind to the extracellular domain of the LRP6 receptor, a coreceptor for canonical WNT signaling, in some studies (15, 26, 27). However, other studies failed to demonstrate RSPO-LRP6 binding (14, 19, 20, 28), leaving the role of

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FIG 1 Inhibition of *Lgr4* expression by RNA interference disrupted myogenic differentiation in C2C12 cells. (A) Expression of the *Lgr4* family of genes in C2C12 cells determined by qRT-PCR. U, undifferentiated, exponentially growing cells; D1 to D3, C2C12 cells cultured in differentiation medium for 1 to 3 days, respectively. Gene expression level was determined and normalized to *Gapdh* expression in triplicate samples. (B) *Lgr4* expression in C2C12 cells transduced with lentiviruses bearing control *eGFP*- or *Lgr4*-specific shRNA determined by qRT-PCR. (C) Immunofluorescence staining for myogenic differentiation markers MYOG and MyHC in control, *Lgr4A*-KD, and *Lgr4B*-KD C2C12 cells. Cells were cultured in differentiation medium for 1 day or 4 days. Cells were stained with anti-MYOG and MyHC primary antibodies followed by Cy3-conjugated secondary antibodies. Cell nuclei were counterstained with DAPI. (D and E) MYOG-positive nuclei or the nuclei in MyHC-positive cells were counted and are presented as percentages of the total number of nuclei as a differentiation index. (F) Distribution of MyHC-positive cells for a cell fusion index. MyHC-positive cells and fibers containing a single nucleus (Mono), two to five nuclei, and more than five nuclei were counted. The percentage for each group is presented. Error bars indicate standard errors of the means (SEM). P values were calculated by Student's t test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significantly different.

LRP6 as an RSPO receptor inconclusive. The Frizzled receptors involved in both canonical and noncanonical WNT signaling do not directly bind the RSPO proteins (26, 29). Recently, RSPO1 was shown to inhibit the function of ZNRF3, a plasma membrane-bound E3 ubiquitin ligase which regulates the level of Frizzled and likely LRP6 receptors on the plasma membrane by ubiquitin-dependent degradation (30). RSPO1 binding to both LGR4 and ZNRF3 is suggested to induce a clearance of ZNRF3 on the plasma membrane (30), thereby resulting in an increase in the number of the available WNT receptors on the plasma membrane and a potentiation of WNT signaling activity. In addition to having a regulatory role in WNT/ β -catenin signaling, RSPOs play a role in noncanonical WNT signaling. In *Xenopus laevis* embryos, RSPO3 and WNT5A cooperatively activate the noncanonical WNT sig-

naling pathway through the Frizzled7 receptor (29). This activation depends on RSPO3 interaction with syndecan4 through the TSR domain of RSPO3. Taken together, the RSPO and LGR4 family proteins are a novel class of WNT signaling regulators that can activate the WNT pathway via mechanisms distinct from those of classical WNT proteins.

We previously showed that the RSPO2 protein enhances myogenic differentiation and myocyte fusion in a WNT/ β -catenin signaling-dependent manner in C2C12 myoblasts (31). In contrast, inhibition of both *Rspo2* and *Rspo3* gene expression by RNA interference (RNAi) significantly compromised myogenic differentiation (31). In this study, we investigate the molecular mechanism of RSPO function in promoting myogenic differentiation. We show that the LGR4 receptor plays an active role and mediates

RSPO2 function during myogenic differentiation in C2C12 myoblast cells. Furthermore, we provide evidence that the TGF- β antagonist follistatin (FST) is a crucial mediator of RSPO-LGR4 function in myogenesis and that *Fst* gene transcription is directly regulated by a β -catenin/TCF4 transcription factor complex activated by the RSPO-LGR4 signaling cascade *in vitro* and *in vivo*. This study proposes signaling cross talk in which FST induced by the RSPO2-LGR4- β -catenin signaling cascade leads to inhibition of the TGF- β signaling pathway during skeletal myogenesis.

MATERIALS AND METHODS

Mice. *Myf5*^{+/-cre} mice (32) were kindly provided by Thomas Gridley. Mice carrying conditional β -catenin loss-of-function (LOF) *Ctnnb1*^{flox} (33) and gain-of-function (GOF) *Ctnnb1*^{flox(Ex3)} (34) alleles were obtained from The Jackson Laboratory and Terry Yamaguchi, respectively. Mouse embryos were collected from pregnant, timed-mated female mice. The day of conception was designated embryonic day 0.5. Mice and embryos were genotyped using genomic DNA PCR as described elsewhere (32–34). 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.

Cell culture. The mouse myoblast cell line C2C12 and human embryonic kidney 293T (HEK 293T) cells were obtained from the American Type Culture Collection (ATCC), Manassas, VA, and maintained in growth medium (Dulbecco's modified Eagle's medium [DMEM] containing 10% fetal bovine serum [FBS] and 1% penicillin-streptomycin) under 5% CO₂ at 37°C. To induce myogenic differentiation, nearly confluent C2C12 cells were cultured in differentiation medium (DMEM containing 2% heat-inactivated horse serum and 1% penicillin-streptomycin) for up to 5 days. Fresh differentiation medium was replaced every 2 days. Recombinant FST, RSPO1, RSPO2, and WNT3A proteins were obtained from R&D Systems (Minneapolis, MN) and treated as indicated in the figure legends. 4-Hydroxytamoxifen (4-OHT; Sigma-Aldrich, St. Louis, MO) was added to the cells at a concentration of 1 μ M.

Viral vectors and transduction. Lentiviral vectors (pLKO.1 based) encoding short hairpin RNAs (shRNAs) specific to the mouse *Lgr4*, *Fst*, and control enhanced green fluorescent protein (*eGFP*) genes developed by the RNAi Consortium were obtained from Thermo Scientific (Lafayette, CO) and Sigma-Aldrich. cDNA encoding an active form of human β -catenin [β -cat(S33A)] fused to the ligand-binding domain of an estrogen receptor with a high affinity for tamoxifen (called ERT) was inserted into the pWPI lentiviral vector (obtained from Didier Trono). Lentiviruses were packaged in the recombinant viral vector facility at the MMCRI. C2C12 cells transduced with the shRNA viruses were selected in growth medium containing puromycin (2 μ g/ml) to establish cell pools stably expressing shRNA. C2C12 cells were transduced with the β -cat(S33A)ERT lentivirus to nearly 100% transduction efficiency as determined by lentiviral-vector-derived GFP expression.

DNA and siRNA transfection. Plasmid DNA transfection was performed using TransIt-LT1 reagent (Mirus Bio, Madison, WI) according to the manufacturer's instructions. Briefly, DNA TransIt mix was incubated with cells for 16 h, and the transfected cells were harvested at 2 days after transfection for luciferase assay. Small interfering RNAs (siRNAs) specific to the mouse *Lgr4*, *Fst*, and *Ctnnb1* genes and nontargeting siRNA were obtained from Thermo Scientific/Dharmacon. siRNA was transfected into C2C12 cells using Lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, CA) in a reverse-transfection format according to the manufacturer's protocol. Briefly, siRNA diluted in serum-free Opti-MEM I medium (Invitrogen) was mixed with the Lipofectamine reagent. The siRNA mixture was directly added to culture plates and incubated for 20 min at room temperature. C2C12 cells resuspended in growth medium without antibiotics were seeded into the culture plate containing the siRNA-Lipofectamine mixture (the final concentration of siRNA was 25 nM). Myogenic differentiation was induced at 2 days after transfection.

FIG 2 Depletion of *Lgr4* expression attenuated RSPO2-enhanced myogenic differentiation and WNT/ β -catenin signaling. (A) Immunofluorescence staining for the MyHC protein in differentiating control and *Lgr4* KD C2C12 cells treated with RSPO2 (200 ng/ml) for 4 days. (B) The myogenic differentiation index was calculated by dividing the number of nuclei in MyHC-positive cells by the total number of nuclei and expressing the result as a percentage. (C) The cell fusion index was presented as a distribution of MyHC-positive cells based on their nucleus number. (D) Activation of WNT/ β -catenin signaling was measured by the activity of the super *TopFlash* reporter. Control and *Lgr4*-KD C2C12 cells in 24-well plates were transfected with the *TopFlash* reporter (350 ng/well) and control *Renilla* luciferase reporter (150 ng/well) plasmid DNAs and stimulated with the RSPO2 (25 ng/ml) and WNT3A (50 ng/ml) proteins for 24 h. Luciferase activity was measured and normalized to the activity of control *Renilla* luciferase. Samples were prepared in triplicate. Error bars indicate SEM. *P* values were calculated by Student's *t* test. ***, *P* < 0.001; **, *P* < 0.01.

The knockdown efficiency was determined by quantitative real-time PCR (qRT-PCR) at 2 days after transfection.

Microarray transcriptome analysis and qRT-PCR. Total RNA was isolated from cultured cells using either an RNeasy plus kit (Qiagen, Carol Stream, IL) or TRIzol reagent (Invitrogen). For microarray analysis, RNA samples prepared in triplicate were analyzed using Affymetrix mouse gene ST1.0 arrays. Differential gene expression profiles were analyzed by the Bioconductor Program. Hybridization, scanning, and data processing were performed in the Genetics and Bioinformatics Core Facilities at the University of Vermont. For qRT-PCR, first-strand cDNA was synthesized using a ProtoScript first-strand cDNA synthesis kit (New England BioLabs, Ipswich, MA). Normally, 1 μ g of total RNA was used for cDNA synthesis. cDNA in an amount equivalent to 20 ng of total RNA was used for qRT-PCR and in a customized RT² Profiler PCR array (SA Biosciences, Valencia, CA). The PCR primer sequences and primer sets used in this study are listed in Tables S1 and S2 in the supplemental material, respectively.

FIG 3 Enhanced myogenic differentiation by RSPO2 during the early stage of myogenic differentiation. (A) Differentiating C2C12 cells were treated with the RSPO2 proteins (200 ng/ml) at the different time points indicated. Treatment conditions a to e correspond to image labels and bars a to e in panels B to D. GM, growth medium; DM, differentiation medium; IF, immunofluorescence. (B) Images of MyHC immunofluorescence staining of RSPO2-treated differentiating C2C12 cells. (C) Percentages of MyHC-positive nuclei in total nuclei. (D) Distribution of MyHC-positive cells containing a single nucleus, 2 to 5 nuclei, and more than 5 nuclei in differentiating C2C12 cells treated with RSPO2. Experiments were performed in duplicate, and more than 1,000 total nuclei were counted. Error bars indicate SEM. *P* values were calculated by Student's *t* test. **, *P* < 0.01.

Western blot analysis. Cells were lysed with lysis buffer (10 mM Tris-Cl, pH 7.2, 2 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, and 1% sodium deoxycholate) containing 50 mM sodium fluoride, 1 mM phenylmethanesulfonyl fluoride, 0.2 mM sodium vanadate, and protease inhibitor mixture (set V; EMD Chemicals, Gibbstown, NJ). The cell lysates (15 to 20 μ g) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. After the membranes were blocked with 5% skim milk in phosphate-buffered saline containing 0.1% Tween 20 for 1 h at room temperature, they were incubated with primary antibodies at 4°C overnight. Secondary antibodies conjugated with horseradish peroxidase (1:5,000 dilution; GE Healthcare/Life Sciences, Pittsburgh, PA) were applied to the membranes for 2 h at room temperature. The membranes were incubated with SuperSignal West Dura Luminol enhancer solution (Thermo Scientific/Pierce) and immediately exposed to X-ray film (Premium Autoradiography Film, Metuchen, NJ) for signal detection. Antibodies against β -catenin (1:500 dilution; BD Biosciences; San Jose, CA), FST (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and β -tubulin (1:1,000; Santa Cruz Biotechnology) were used.

Immunofluorescence staining. Immunofluorescence staining was performed as previously described (31). Primary antibodies against myogenin (MYOG) (F5D, 1:100 dilution; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), myosin heavy chain (MyHC) (MF20, 1:200 dilution; Developmental Studies Hybridoma Bank), and β -catenin (1:100 dilution; BD Biosciences) and secondary anti-mouse IgG antibody conjugated with Cy3 (1:800 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) were used. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Cell images were obtained using a Leica DFC340 FX digital camera attached to a Leica DM IRB inverted epifluorescence microscope.

Fst-luciferase reporters and luciferase assay. Mouse *Fst* genomic DNAs spanning from bp -1846 to bp +20, containing the promoter and a portion of exon 1, and from bp +128 to bp +2168, containing the remaining exon 1 and intron 1, were PCR amplified. To create the *Fst4-Luc* reporter construct, the promoter/exon 1 and exon 1/intron 1 fragments were inserted into sequences upstream and downstream of the *luciferase* gene in the pGL2-basic vector (Promega, Madison, WI), respectively. Other *Fst4-Luc* derivatives were constructed similarly using shorter PCR-amplified *Fst* genomic DNA fragments. The promoter/exon 1 DNA containing mutations at the T1 and T2 sites was generated by ligating two PCR-amplified DNA fragments upstream and downstream of the T1/T2 sites, flanked with the EcoRI restriction enzyme site, thereby disrupting the T1/T2 sites with the EcoRI binding sequence.

Luciferase assays were performed using the dual-luciferase assay kit (Promega) according to the manufacturer's procedures. *Renilla* luciferase-thymidine kinase DNA (*RL-TK*) was used as a transfection control.

ChIP. C2C12- β -cat(S33A)ERT cells (80% confluence) in two 60-mm dishes cultured in the absence or presence of 4-OHT overnight were fixed with 2% formaldehyde for 10 min at room temperature. Chromatin extraction and immunoprecipitation were performed according to the manufacturer's protocol using a ChIP-IT express chromatin immunoprecipitation (ChIP) kit (Active Motif, Carlsbad, CA). Anti- β -catenin antibody (Santa Cruz Biotechnology) and a negative-control anti-hemagglutinin (HA) mouse antibody (Sigma-Aldrich) were used for chromatin immunoprecipitation. The primer sequences used to amplify the *Fst* genomic DNA isolated from the immunoprecipitated chromatin are listed in Table S3 in the supplemental material.

In vitro protein translation and EMSA. The TNT T7 transcription/translation system with rabbit reticulocyte lysate (Promega) was used to

TABLE 1 Genes regulated by RSPO2 in C2C12 cells

Gene symbol	Gene product	Fold induction ^a			
		Microarray		qRT-PCR	
		U1	D1	U1	D1
<i>Axin2</i>	Axin2	2.502	3.912	37.372	21.363
<i>Car3</i>	Carbonic anhydrase 3	2.489	2.81	ND	ND
<i>Ch25h</i>	Cholesterol 25-hydroxylase	3.075	2.207	ND	ND
<i>Cxcr6</i>	Chemokine (C-X-C motif) receptor 6	2.572	2.33	5.616 ^{b,c}	6.755 ^{b,c}
<i>Enpp2</i>	Ectonucleotide pyrophosphatase/phosphodiesterase 2	2.919	7.945	7.931	3.258
<i>Fst</i>	Follistatin	2.986	4.786	10.549	10.342
<i>Il33</i>	Interleukin 33	2.869	2.388	5.484 ^b	4.303 ^b
<i>Lgr5</i>	Leucine-rich repeat containing G protein-coupled receptor 5	3.677	4.444	51.921 ^b	47.331 ^b
<i>Pfn2</i>	Profilin 2	2.387	4.677	ND	ND
<i>Qpct</i>	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	2.103	2.441	ND	ND
<i>Tgfb2</i>	Transforming growth factor beta 2	2.404	2.715	4.524 ^b	3.173 ^b
<i>Tspan8</i>	Tetraspanin 8	3.35	3.782	5.683	4.398
<i>Aspn</i>	Asporin	-2.447	-2.894	-2.583 ^b	-1.877 ^b
<i>Cxcl12</i>	Chemokine (C-X-C motif) ligand 12	-2.521	-2.254	-4.256 ^b	-2.242 ^b
<i>Cxcl5</i>	Chemokine (C-X-C motif) ligand 5	-2.414	-3.609	-14.507 ^b	-21.22 ^b
<i>Enpp1</i>	Ectonucleotide pyrophosphatase/phosphodiesterase 1	-2.531	-2.509	-2.699	-1.927
<i>Enpp3</i>	Ectonucleotide pyrophosphatase/phosphodiesterase 3	-2.241	-2.646	-2.061	-1.766
<i>F3</i>	Coagulation factor III	-2.736	-2.572	ND	ND
<i>Fgf7</i>	Fibroblast growth factor 7	-3.772	-3.745	-4.801 ^b	-6.012 ^b
<i>Hgf</i>	Hepatocyte growth factor	-2.784	-2.808	-2.916 ^{b,c}	-2.673 ^b
<i>Lphn2</i>	Latrophilin 2	-2.682	-2.367	ND	ND
<i>Ptn</i>	Pleiotrophin	-2.761	-3.871	-6.058 ^b	-1.561 ^{b,d}
<i>Rgs2</i>	Regulator of G protein signaling 2	-2.49	-4.442	-2.223	-1.7

^a P values of the fold induction of each gene are smaller than 0.01 except for the ones noted. U1, undifferentiated cells on day 1; D1, differentiated cells on day 1; ND, not determined.

^b qRT-PCR was performed using a customized RT² profiler PCR array from SA Biosciences. The names of primer sets are listed in Table S4 in the supplemental material.

^c $P < 0.05$.

^d Not statistically significant.

produce MYC-tagged human TCF4 protein. Synthesis of the TCF4 protein was confirmed by Western blotting using an anti-MYC antibody (1:1,000 dilution; Santa Cruz Biotechnology). Ten picomoles of synthetic double-stranded DNA oligonucleotide with a 5' overhang was labeled with [γ -³²P]dCTP (3,000 Ci/mmol; PerkinElmer, Waltham, MA) using the Klenow fragment of DNA polymerase I (New England BioLabs). For the electrophoretic mobility shift assays (EMSA), 2 μ l of a reticulocyte lysate containing TCF4 was mixed with 0.4 pmol of radiolabeled probes in binding buffer [20- μ l final volume, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 7% glycerol, 0.05% Nonidet P-40, 0.1 mg/ml poly(dI-dC), 70 mM KCl, 50 mM dithiothreitol, 1 mM MgCl₂]. For confirming the specificity of the DNA-TCF4 complex, a 50-fold excess of unlabeled double-stranded wild-type or mutant oligonucleotide or 2 μ g of anti-MYC (Developmental Studies Hybridoma Bank) or anti-HA (Sigma-Aldrich) mouse antibody was added into the reaction mixture 15 min prior to the addition of the labeled probe. After incubation for 30 min at room temperature, the DNA-TCF4 complex was separated on a 5% nondenaturing polyacrylamide gel in a half-concentration of Tris-borate-EDTA buffer. Signal was detected by exposing the dried gel to X-ray film. The oligonucleotide sequences used in EMSA are listed in Table S4 in the supplemental material.

Whole-mount *in situ* hybridization. Whole-mount *in situ* hybridization was performed on mouse embryos fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) as described previously (35). An antisense digoxigenin-incorporated RNA probe was synthesized from the plasmid containing an 850-bp cDNA containing the 5' untranslated region and a portion of the protein coding sequence of the *Fst* RNA. Stained embryos were photographed with a Zeiss AxioCam digital camera under a Zeiss Stemi-SV6 stereomicroscope.

RESULTS

Downregulation of the LGR4 receptor suppresses myogenic differentiation in C2C12 myoblast cells. To gain insight into the functional roles of the LGR4 family of RSPO receptors in skeletal myogenesis, we examined the RNA expression of the *Lgr4* family genes in C2C12 mouse myoblast cells by qRT-PCR (Fig. 1A). *Lgr4* RNA expression was mildly increased by day 1 of myogenic differentiation but decreased to a level similar to or slightly lower than that of undifferentiated cells by day 3 of differentiation. *Lgr6* RNA expression also transiently increased by day 1 of differentiation. In contrast, *Lgr5* expression continuously increased throughout differentiation. Compared to the ratio of housekeeping *Gapdh* RNA expression in exponentially growing, undifferentiated cells, *Lgr4* transcripts seem most abundantly expressed among the family members (data not shown).

To determine the functions of the LGR4 receptor during myogenic differentiation *in vitro*, *Lgr4* gene expression was inhibited by RNA interference in C2C12 cells. The knockdown (KD) efficiencies of the *Lgr4* gene by two specific shRNAs (*Lgr4A* and *Lgr4B*) stably expressed via lentiviral transduction were determined to be approximately 60% and 85%, respectively, as measured by qRT-PCR (Fig. 1B). When both stable *Lgr4*-KD cell pools were induced for myogenic differentiation, they exhibited severe deficits in myogenic differentiation. The number of myogenin (MYOG)-positive cells was significantly reduced in *Lgr4*-KD cells on differentiation day 1 compared to that in control C2C12 cells

FIG 4 Depletion of *Fst* compromised RSPO2-enhanced skeletal myogenesis in C2C12 cells. (A) *Fst* expression in undifferentiated (U) and differentiating (D1 and D3) C2C12 cells treated with control BSA (200 ng/ml) or RSPO2 (200 ng/ml) for 1 and 3 days was determined by qRT-PCR. *Fst* expression was normalized to *Gapdh* expression. Samples were prepared in triplicate. (B) Depletion of *Fst* expression by shRNA in C2C12 cells determined by qRT-PCR. *Gapdh* expression was used for normalization. Samples were tested in duplicate. (C to E) Immunofluorescence staining for MyHC expression in *Fst*-KD C2C12 cells and control eGFP-shRNA cells cultured in the presence of control BSA (200 ng/ml) and RSPO2 (200 ng/ml). Cells were harvested at differentiation day 5. (E) The percentages of nuclei in MyHC-positive cells relative to the total number of nuclei and percentages of MyHC-positive cells containing a single nucleus, 2 to 5 nuclei, 6 to 10 nuclei, and more than 10 nuclei were calculated. Experiments were performed in duplicate, and more than 1,000 total nuclei were counted. Error bars indicate SEM. *P* values were calculated by Student's *t* test. ***, *P* < 0.001; **, *P* < 0.01.

transduced with eGFP-specific shRNA (Fig. 1C and D). Immunostaining for the myosin heavy-chain (MyHC) protein on differentiation day 4 also showed that both myogenic differentiation and cell fusion were significantly compromised in *Lgr4*-KD cells compared with those in control cells (Fig. 1C, E, and F). Together, these results showed that *Lgr4* is essential for myogenic differentiation.

Depletion of the LGR4 receptor attenuates myogenesis promotion and WNT/ β -catenin activation induced by RSPO2. To determine whether the promyogenic activity of RSPO2 requires the LGR4 receptor, RSPO2 was added to control and *Lgr4*-KD cells under myogenic differentiation conditions. RSPO2 significantly promoted myogenic differentiation and cell fusion in control cells expressing eGFP shRNA, as we previously showed with naive C2C12 cells (Fig. 2A) (31). However, RSPO2 activity to enhance myogenic differentiation and myocyte fusion was significantly impaired in *Lgr4*-KD cells (Fig. 2B and C), suggesting that RSPO2 activity is mediated largely through the LGR4 receptor.

The promyogenic activity of RSPO2 depends on the activation of WNT/ β -catenin signaling in C2C12 cells (31). The LGR4 family receptors are crucial for mediating RSPO-induced WNT/ β -catenin signaling in various cell types (19–22). To determine whether activation of WNT/ β -catenin by RSPO2 is defective in *Lgr4*-KD cells, we performed the *TopFlash* reporter assay to assess activation of the WNT/ β -catenin pathway. While RSPO-mediated activation of the reporter gene was severely hampered in *Lgr4*-KD cells, WNT3A-mediated activation of the reporter was not affected (Fig. 2D). Thus, the LGR4 receptor is required specif-

ically for RSPO2-mediated, but not WNT3A-mediated, activation of β -catenin to promote myogenesis.

RSPO2 action is restricted to the early stage of myogenic differentiation in C2C12 cells. We previously showed that continuous treatment of the RSPO2 protein in differentiating C2C12 cells for up to 4 days significantly enhanced myogenic differentiation and myocyte fusion (31). However, it is not known whether RSPO2 activity mostly influences the early, late, or entire stage of myogenic differentiation. To determine which stage during myogenic differentiation is most sensitive to RSPO2 treatment, we treated C2C12 cells with the RSPO2 protein at different time points during myogenic differentiation (Fig. 3A). RSPO2 incubation during the first 24 h or 4 consecutive days resulted in equivalent myogenic effects (Fig. 3B to D). However, when RSPO2 was added at day 2 or later during differentiation, it did not produce any significant effect on myogenic differentiation and myocyte fusion (Fig. 3B to D). Therefore, RSPO2 exerts its effect during the first 24 h of the myogenic differentiation process in C2C12 cells.

Identification of genes modulated by RSPO2. To identify the molecular targets that may mediate RSPO2's effect on myogenic differentiation and myocyte fusion, we performed transcriptome analysis in differentiating C2C12 cells treated with RSPO2 or control bovine serum albumin (BSA) for 24 h. Additionally, we analyzed transcript expression in exponentially growing C2C12 cells cultured in the absence and presence of RSPO2 for 24 h.

In exponentially growing C2C12 cells, 15 upregulated and 18 downregulated genes were identified as having a >2-fold change with RSPO2 (see Table S5 in the supplemental material). In dif-

FIG 5 *Fst* rescued defective skeletal myogenesis in *Lgr4*-KD C2C12 cells. (A) *Fst* expression in undifferentiated *Lgr4*-KD C2C12 cells treated with control BSA (200 ng/ml) or RSPO2 (200 ng/ml) for 1 day was determined by qRT-PCR. *Fst* expression was normalized to *Gapdh* expression. Samples were prepared in triplicate. (B to D) Exogenous FST protein rescued myogenic defects in *Lgr4*-KD C2C12 cells. Immunofluorescence staining for MyHC expression in *Lgr4*-KD C2C12 cells and control eGFP-shRNA cells cultured in the presence of control BSA (500 ng/ml) and FST (500 ng/ml). Cells were harvested at differentiation day 5. (E) The percentages of nuclei in MyHC-positive cells relative to the total number of nuclei and percentages of MyHC-positive cells containing a single nucleus, 2 to 5 nuclei, 6 to 10 nuclei, and more than 10 nuclei were calculated. Experiments were performed in duplicate, and more than 1,000 total nuclei were counted. Error bars indicate SEM. *P* values were calculated by Student's *t* test. **, *P* < 0.01; *, *P* < 0.05.

differentiating C2C12 cells, we identified 57 genes that were upregulated and 41 genes that were downregulated by 2-fold or more after RSPO2 treatment (see Table S6 in the supplemental material). Among the differentially regulated genes, 12 upregulated and 11 downregulated genes were common in both exponentially growing and differentiating C2C12 cells (Table 1).

Expression of 17 commonly regulated genes was further validated by qRT-PCR analyses (Table 1). A well-established WNT/ β -catenin signaling target, *Axin2*, was induced approximately 37- and 21-fold by RSPO2 in undifferentiated and differentiating C2C12 cells, respectively, confirming that RSPO2 activates WNT/ β -catenin signaling in these cells. *Lgr5*, a member of the RSPO receptor gene family, was upregulated by RSPO2. *Lgr5* expression is positively regulated by WNT/ β -catenin activation in intestinal stem cells (17), further confirming the activation of WNT/ β -catenin signaling by RSPO2. Repression of the fibroblast growth factor 7 (*Fgf7*) and hepatocyte growth factor (*Hgf*) genes by RSPO2 suggests that RSPO2 inhibits the production of autocrine factors that promote proliferation of myogenic cells.

FST is a key mediator of RSPO2-LGR4 signaling. Among the RSPO2 downstream target genes that we identified, we decided to further analyze the functional interaction between RSPO2 and the *folliculin* (*Fst*) gene because the FST protein plays significant positive regulatory roles during myogenic differentiation and myoblast fusion *in vitro* (36, 37) and in muscle hypertrophy *in vivo* (38) through inhibition of TGF- β family signaling.

We first analyzed *Fst* expression by qRT-PCR in C2C12 cells treated with RSPO2 during myogenic differentiation. *Fst* expression dramatically increased between day 1 and day 3 of myogenic

differentiation in C2C12 cells (Fig. 4A). *Fst* transcript levels further increased approximately 8- to 10-fold in C2C12 cells treated with RSPO2 under both exponential-growth and differentiation conditions (Table 1 and Fig. 4A), consistent with the microarray data. RSPO2 treatment, however, did not cause any change in myostatin (*Mstn*) expression in the same samples (data not shown).

To investigate whether the *Fst* gene is a mediator of the myogenic activity of RSPO2 in C2C12 cells, we examined the effects of RSPO2 in C2C12 cells transduced with lentivirus encoding *Fst*-specific shRNA. We confirmed that *Fst* shRNA reduced endogenous *Fst* expression by approximately 80% compared to expression of control eGFP shRNA in C2C12 cells (Fig. 4B). Under myogenic differentiation conditions, *Fst*-KD cells displayed reduced myogenic differentiation and myocyte fusion (Fig. 4C to E). Furthermore, myogenic differentiation and myocyte fusion enhanced by RSPO2 were significantly compromised in *Fst*-KD cells (Fig. 4C to E).

To determine whether the LGR4 receptor is essential for RSPO2-induced *Fst* expression, *Fst* expression levels were analyzed in *Lgr4*-KD C2C12 cells cultured in the presence or absence of RSPO2. The *Fst* transcript level was reduced by approximately 70% in naive *Lgr4*-KD cells compared to control cells (Fig. 5A). When RSPO2 was added, *Fst* expression was significantly less effectively induced in *Lgr4*-KD cells than in control cells (Fig. 5A).

To determine whether exogenous FST can rescue myogenic-differentiation defects observed in *Lgr4*-KD C2C12 cells, *Lgr4*-KD cells were differentiated in the presence of the FST protein. FST treatment effectively rescued the differentiation defects almost to

FIG 6 The WNT/β-catenin pathway regulates *Fst* expression in C2C12 cells and mouse embryos. (A) C2C12 cells were transiently transfected with control nontargeting (nt) siRNA or siRNA specific to the β-catenin gene. Transfected cells were incubated with RSPO1 (100 ng/ml) or RSPO2 (200 ng/ml) in differentiation medium for 1 day and harvested for total RNA isolation and qRT-PCR. *Gapdh* expression was used for normalization. Samples were prepared in duplicate. Error bars indicate SEM. *P* values were calculated by Student's *t* test. ***, *P* < 0.001. (B) Endogenous FST protein level was analyzed by Western blotting in C2C12 cells transiently transfected with β-catenin gene-specific siRNAs 1 day after myogenic differentiation was induced by RSPO1 (100 ng/ml) and RSPO2 (200 ng/ml). (C and D) Whole-mount *in situ* hybridization of *Fst* gene expression in mouse embryos at embryonic day 10.5. Mouse embryos with β-catenin GOF in *Myf5*-positive myogenic cells (*Ctnnb1*^{flox/Ex3/+}; *Myf5*^{Cre/+}) and wild-type (wt) embryos (*Ctnnb1*^{+/+}; *Myf5*^{Cre/+}) were collected from matings between *Myf5*^{Cre/+} and *Ctnnb1*^{flox/Ex3/+} mice. Mouse embryos with β-catenin LOF in *Myf5*-positive cells (*Ctnnb1*^{flox/flox}; *Myf5*^{Cre/+}) and wild-type embryos (*Ctnnb1*^{flox/+}; *Myf5*^{Cre/+}) were collected from matings between *Myf5*^{Cre/+}; *Ctnnb1*^{flox/+} compound mice and *Ctnnb1*^{flox/flox} mice. Dotted lines indicate the following embryonic structures: forelimb bud (FLB), hind limb bud (HLB), somite (Sm), and tail bud (TB). Sm1, Sm4, and Sm6 are indicated by black and blue arrows. Red arrows indicate somites showing an *Fst* expression defect.

the level of control cells expressing *eGFP* shRNA (Fig. 5B to D). Taken together, these results strongly indicate that FST is a key downstream mediator of RSPO2-LGR4 signaling to promote myogenic differentiation and myocyte fusion.

Fst expression is regulated by the WNT/β-catenin pathway in C2C12 cells and mouse embryos. RSPO2-enhanced myogenic differentiation is dependent mainly on activation of WNT/β-catenin signaling (31); therefore, we examined whether *Fst* expression is also regulated through activation of WNT/β-catenin signaling. The siRNA-mediated knockdown of the *Ctnnb1* gene, encoding the β-catenin protein, significantly suppressed both the *Fst* transcript level and the protein expression induced by RSPOs in C2C12 cells (Fig. 6A and B). In addition, DKK1, a specific antagonist of WNT/β-catenin signaling, also effectively blocked RSPO-induced *Fst* expression (Fig. 6C).

To investigate whether β-catenin regulates *Fst* expression in mouse embryos, we conditionally inactivated or activated the β-catenin function in *Myf5*-positive embryonic myogenic cells *in vivo*. *Fst* expression in somites closely overlaps *Myf5* expression

(39, 40), and *Myf5* expression within somites is regulated by WNTs derived from the dorsal neural tube in cooperation with sonic hedgehog protein from the ventral neural tube and notochord (4–6, 41). Furthermore, the robust *Rspo1* and *Rspo3* expression in the dorsal neural tube overlaps *Wnt1* and *Wnt3a* expression (42), raising a strong possibility that the *Fst* gene in *Myf5*-positive cells is under the regulation of the WNT/β-catenin pathway, activated by both WNTs and RSPOs.

Activation of β-catenin in *Myf5*-positive myogenic cells increased *Fst* expression within somites (red arrows in two upper panels of Fig. 6D) and produced ectopic expression within the most rostral somites (red brackets in two upper panels of Fig. 6D). In contrast, inactivation of β-catenin in the same *Myf5*-positive cells significantly abolished *Fst* expression (Fig. 6D, lower panels). Interestingly, inhibition of *Fst* expression is not observed in nascent somites (somites 1 to 5) and become evident in more-mature somites (e.g., somite 6 and somites at the interlimb level, depicted by blue arrows and brackets, respectively, in the two lower panels in Fig. 6D). Thus, we conclude that activation of WNT/β-catenin

FIG 7 The *Fst* gene is a direct transcriptional target of WNT/β-catenin signaling. (A) Tamoxifen-dependent nuclear translocation of the β-cat(S33A)ERT protein. 293T cells transduced with a lentivirus encoding the β-cat(S33A)ERT construct were treated with 4-OHT (1 μM) for 1 h and immunostained for the β-catenin protein. Arrows indicate nuclear β-cat(S33A)ERT protein. (B) Gene expression in C2C12 cells stably transduced with a β-cat(S33A)ERT lentivirus, determined by qRT-PCR. Cells were treated with 4-OHT for 4 h and harvested for RNA isolation. RNA expression was normalized to *Gapdh* RNA expression. Samples were collected in duplicate. Error bars indicate SEM. *P* values were calculated by Student's *t* test. ***, *P* < 0.001. (C) Schematic structures of the mouse *Fst* gene and *Fst*-luciferase (*Luc*) reporter gene constructs. A 4.0-kb DNA sequence of the mouse *Fst* gene containing the promoter, exon 1, and intron 1 was analyzed for transcription factor binding sites using MatInspector 4.0, and a number of conserved TCF/LEF1-binding elements (TBEs) were identified. Exon 1 and potential TBEs (labeled from T1 to T11) are indicated. Confirmed TBEs (black lines) in regions labeled A and B are indicated. The luciferase gene was inserted immediately downstream of the start codon of the *Fst* gene. (D) Detection of β-catenin binding to the *Fst* gene by ChIP. C2C12-β-cat(S33A)ERT cells were treated with 4-OHT (1 μM) for 24 h to induce activation of β-catenin. Untreated cells were used as a negative control. Chromatin was immunoprecipitated with anti-β-catenin and control anti-HA antibodies (Ab), respectively. PCR was performed using the primer sets spanning the potential TBEs on genomic DNA extracted from the immunoprecipitated chromatin. Genomic DNA isolated from chromatin prior to immunoprecipitation

signaling is necessary and sufficient to activate *Fst* expression in myogenic cells *in vitro* and *in vivo*.

***Fst* is a direct transcription target of the β -catenin/TCF transcription factor complex.** To determine whether *Fst* is a direct target of the β -catenin/TCF transcription activator complex, we analyzed *Fst* expression in C2C12 cells expressing a tamoxifen-inducible form of the constitutively active β -catenin protein [C2C12- β -cat(S33A)ERT]. Treatment with 4-OHT, an active tamoxifen analog, induced immediate nuclear translocation of the overexpressed β -catenin protein within 1 h in cultured cells (Fig. 7A). *Fst* and *Axin2* transcripts were effectively induced after 4 h of treatment with 4-OHT, strongly suggesting that *Fst* may be a direct transcriptional target of the β -catenin/TCF complex (Fig. 7B).

Search for transcription factor binding sites resulted in 11 conserved TCF/LEF1 binding elements (TBEs) within the 4.0 kbp of the DNA sequence of the mouse *Fst* gene spanning from the 5' upstream region to the first-intron region (Fig. 7C). We performed ChIP assays using anti- β -catenin antibody in chromatin preparations from untreated and 4-OHT-treated C2C12- β -cat(S33A)ERT cells. Among 8 primer sets spanning one or two TBEs, we found that 5 primer sets produced robust PCR products from DNA isolated from the chromatin complex immunoprecipitated with the β -catenin antibody in a 4-OHT-dependent manner (Fig. 7D). The two tandem TBEs in the promoter region (T1 and T2) and five TBEs localized within intron 1 (T7 to T11) were identified as potential β -catenin/TCF binding sites.

To further examine whether the TCF4 protein binds these identified sites, we performed EMSA using double-stranded oligonucleotides containing each TBE. *In vitro*-translated TCF4-Myc proteins specifically bound to the T1/T2 and T8 sites with an affinity comparable to that of a known TBE derived from the *Axin2* gene promoter (Fig. 7E) (43). The specificity of binding was verified with competition with an excess amount of unlabeled double-stranded oligonucleotides specific to the wild-type or mutant TBE (Fig. 7E) and by disruption of the TCF4-DNA complex by preincubation with anti-MYC antibodies (data not shown). While we did not observe significant TCF4 binding to the T7 site, we observed weak but specific binding of TCF4 to the T9, T10, and T11 sites.

To determine the function of the identified TBEs in RSPO2-induced *Fst* expression, the 4.0 kbp of DNA containing all identified TBEs was linked to the luciferase reporter gene (named *Fst4-Luc* in Fig. 7C) and tested for its response to WNT/ β -catenin signaling activation. This reporter was effectively induced by RSPO2 or WNT3A treatment (Fig. 7F) and by coexpression of the TCF4 and β -catenin(S33A) proteins in C2C12 cells (Fig. 7G). When T1 and T2 elements in the *Fst* promoter region were either deleted (*Fst4 Δ A-Luc*) or mutated [*Fst4(mutT1/T2)-Luc*] in the *Fst4-Luc* reporter construct, approximately 70 to 90% of the reporter activity induced by RSPO2, WNT3A, or coexpression of

β -cat(S33A)/TCF4 disappeared, whereas deletion of the T7 to T11 elements in the first intron region of the *Fst* gene (*Fst4 Δ B-Luc*) generated an approximately 50-to-60% reduction of the reporter activity (Fig. 7F and G). The reporter lacking all five TBEs (*Fst4 Δ AB-Luc*) failed to be induced by RSPO2, WNT3A, and β -cat(S33A)/TCF4 (Fig. 7F and G). Taking these data together, we conclude that the identified TBEs within the *Fst* gene are the functional binding sites for WNT/ β -catenin signaling and that the *Fst* gene is a direct downstream target of WNT/ β -catenin signaling.

DISCUSSION

The *Lgr4* receptor mediates RSPO signals during myogenic differentiation. The RSPO proteins enhance myogenic differentiation and myocyte fusion in a WNT/ β -catenin-dependent manner in mouse C2C12 myoblast cells (31). The recent discovery that proteins of the LGR4 family are RSPO receptors (19–22) raises questions of whether the LGR4 family members mediate the myogenic activities of the RSPO proteins in C2C12 cells. Our gene expression analysis of the *Lgr4* family members identified that *Lgr4* is the most abundantly expressed gene in both undifferentiated and differentiating C2C12 cells. Depletion of *Lgr4* expression by RNA interference severely compromised the onset and progression of myogenic differentiation, suggesting that the LGR4 receptor plays a crucial role in the myogenic differentiation of C2C12 cells.

We previously reported that inhibition of the *Rspo2* and *Rspo3* genes by siRNA significantly compromised myogenic differentiation in C2C12 cells (31). Therefore, the differentiation defects observed in *Lgr4*-KD cells may be due to the defect in the mediation of endogenous RSPO signaling. In support of this notion, RSPO2 treatment of *Lgr4*-KD cells generated a significantly reduced myogenic response compared to that of control cells. Consistently, the WNT/ β -catenin signaling reporter assay confirmed that RSPO-induced activation of the WNT/ β -catenin pathway was largely defective in *Lgr4*-KD cells but that WNT/ β -catenin activation by WNT3A was basically intact. These results indicate that myogenic-differentiation defects associated with LGR4 depletion is linked to a failure of mediating endogenous RSPO signals.

Is the LGR4 receptor engaged only in RSPO-dependent signaling during myogenic differentiation? It is reported that loss of the *Lgr4* gene attenuates embryonic and postnatal bone formation by regulating the cyclic AMP (cAMP)-protein kinase A (PKA)-ATF4 pathway in osteoblasts (44). So far, no direct evidence indicates that the RSPO proteins regulate the cAMP-PKA-ATF4 pathway through the LGR4 receptor. Recently, it was also demonstrated that overexpression of the LGR5 receptor induced the G_{12/13}-Rho GTPase pathway independently of RSPOs (45). Therefore, future investigation of whether the deficits in myogenic differentiation observed in *Lgr4*-KD cells is also linked to the disruption of the RSPO-independent GPCR pathway is warranted.

(input) was used as a control for PCR efficiency and accuracy. (E) Binding of TCF4 to the TBEs of the *Fst* gene in EMSA. ³²P-labeled double-stranded oligonucleotides containing individual TBEs (T1 to T11 and T_{Axin2}) were incubated with *in vitro*-translated human TCF4 protein with an N-terminal MYC tag. For competition, a 50-fold excess amount of unlabeled double-stranded oligonucleotides was added into the binding reaction mixture. DNA-TCF4 complexes and unbound free probe are indicated with brackets, and a nonspecific band is marked with an asterisk. mut, mutant. (F) Luciferase reporter assay. C2C12 cells in 24-well plates were transfected with the *Fst-Luc* reporter constructs (200 ng/well) and treated with RSPO2 (50 ng/ml) and WNT3A conditional medium (10%, vol/vol) for 48 h. (G) C2C12 cells in 24-well plates were cotransfected with the *Fst-Luc* reporters (100 ng/well) and β -cat(S33A) and TCF4 expression plasmids (100 ng each/well). Luciferase activities in triplicate samples were normalized to the activity of the cotransfected *Renilla* luciferase reporter (50 ng/well). Error bars indicate SEM.

Fst is a direct target for WNT/ β -catenin signaling in myogenic cells. The FST protein, an antagonist of the TGF- β family proteins (46, 47), plays positive roles in skeletal myogenesis. The FST protein enhances myogenic differentiation and myocyte fusion *in vitro* (36, 37). Mice lacking the *Fst* gene show reduced intercostal and diaphragm muscles at birth (48), and *Fst* gene haploinsufficiency is associated with reduced muscle mass in adult mice (38). Furthermore, overexpression of the *Fst* gene in skeletal muscle in transgenic mice or administration of *Fst*-bearing recombinant adenoviruses results in a significant muscle mass increase (49–51). These FST functions are associated mainly with antagonizing myostatin (MSTN), a member of the TGF- β family and a strong inhibitor of skeletal muscle growth (52, 53), and activin (38). In addition, FST regulates the mTOR pathway in muscle hypertrophy independently of MSTN inhibition (54).

Despite *Fst*'s critical roles in skeletal muscle, the regulatory mechanism of its expression in skeletal myogenesis is not well known. Inhibition of histone deacetylase by trichostatin A induces *Fst* expression mediated by MYOD and CREB transcription factors in myogenic cells *in vitro* (36). While there are a few reports suggesting that *Fst* expression depends on WNT signaling in other biological contexts (55, 56), no concrete molecular connection between WNT/ β -catenin signaling and *Fst* expression has been established in skeletal myogenesis.

Our study provides compelling evidence that FST is a key mediator of myogenic activity of RSPO-LGR4 signaling via activation of WNT/ β -catenin signaling and that the mouse *Fst* gene is a direct target of β -catenin signaling in myoblasts *in vitro* and *in vivo*. Activation and blockade of β -catenin signaling resulted in induction and inhibition, respectively, of the *Fst* gene or FST protein expression in C2C12 cells and mouse embryos.

We identified functional TBEs in the promoter and the intron 1 region of the mouse *Fst* gene using a combination of ChIP, EMSA, and a *Fst* gene reporter assay. The tandemly positioned T1/T2 sites located in the 1.6-kbp region (region A) upstream from the transcription start site of the *Fst* gene are the most critical TBEs mediating RSPO2- or WNT-activated β -catenin signaling, which represents approximately 70% of the reporter activity. In EMSA, T1/T2 DNA formed a strong complex with TCF4. DNA sequences of both sites are identically conserved in human, rat, and mouse genomes, suggesting that T1/T2 TBEs are likely to play the same role in mammals. Interestingly, the T1/T2 DNA-TCF4 complex displayed a mobility similar to that of the complex of TCF4 and *Axin2* DNA, which contains only one TBE, suggesting that TCF4 likely binds either T1 or T2, not both.

Among the TBEs within the intron 1 region (region B) identified by ChIP assay, only the T8 site, which is highly conserved in human, rat, and mouse genomes, efficiently bound the TCF4 protein in EMSA. The other TBEs showed either poor or no binding by TCF4 *in vitro*. This discrepancy between *in vivo* ChIP and *in vitro* EMSA results may be explained by a few possibilities. First, multiple TCF4 proteins may bind the T8 site and other TBEs simultaneously in a cooperative manner in cells; however, this cooperative binding may not occur *in vitro*. Second, these weak or nonbinding sites may have a high affinity for other members of the TCF/LEF1 family, all of which can form a functional complex with the activated β -catenin protein in cells. Finally, because the average DNA size of the fragmented chromatin is about 500 bp and the distances between PCR amplification primer pairs spanning TBEs are less than 200 bp, it is possible that chromatin immunoprecipitated

via β -catenin binding to the T8 site contains neighboring TBE DNA sequences. It is noteworthy that the T8 site is located approximately midway between the T7 and T11 sites. The first two possibilities are unlikely, because the *Fst* luciferase reporter lacking region B (*Fst4 Δ B-Luc*) still yielded approximately 50% of the *Fst4-Luc* reporter, suggesting that the function of TBEs within region B is relatively minor and arguing against the possibility that the multiple TBEs within region B are all functional. Considering these findings together, we conclude that the T1/T2 sites within region A and the T8 site within region B are functional targets for WNT/ β -catenin signaling in muscle differentiation. Interestingly, a TBE within the rat *Fst* promoter identical to the nonfunctional T4 site of the mouse *Fst* promoter is determined to be a target for WNT/ β -catenin in human embryonic carcinoma cells (55), suggesting the possibility of a context-dependent TBE use.

Our study suggests the strong possibility of FST-mediated cross talk between the WNT/ β -catenin and TGF- β signaling pathways during myogenic differentiation. Considering the robust positive role of FST in adult muscle hypertrophy by antagonizing TGF- β signaling, it would be noteworthy to investigate whether RSPO-LGR4 signaling and activation of the WNT/ β -catenin pathway can also induce *Fst* expression in adult skeletal muscle and whether it regulates muscle hypertrophy; therefore, RSPO and LGR4 may provide new therapeutic targets for muscle-wasting diseases.

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