Functional characterization of *Cynoglossus semilaevis* R-spondin2 and its role in muscle development during embryogenesis

Bo Wang¹, Fan Yang¹, Rui Li¹, Xuemei Li¹, Xiaolong Wu¹, Zheng Sun¹, Jieming Zhai², Yan He¹ and Jie Qi^{1*}

¹Key Laboratory of Marine Genetics and Breeding, Ministry of Education, College of Marine Life Sciences, Ocean University of China, 5 Yushan Road, Qingdao 266003, China

²Laizhou Mingbo Aquatic Co., Ltd., Wujiazhuang Village, Sanshandao, Laizhou 261418, China

(Received 7 March 2018, accepted 8 July 2018; J-STAGE Advance published date: 18 October 2018)

R-spondin2 (Rspo2) is a member of the R-spondin family, which plays important roles in cell proliferation, cell fate determination and organogenesis. Rspo2 exhibits important functions during embryonic development and muscle maintenance in adult human, mouse and *Xenopus*. In the present study, the tongue sole *Cynoglossus semilaevis* Rspo2 (*CsRspo2*) gene was isolated and characterized, and its role in muscle development during embryogenesis was studied. Our results showed that *CsRspo2* expression was abundant during gastrulation and significantly high during somite formation, but then decreased markedly after hatching. *CsRspo2* expression was high in brain and gill, moderate in heart, ovary and testis, and almost undetectable in muscle and other tissues. Moreover, the potential involvement of Rspo2 in muscle development was investigated. We found that overexpression of *CsRspo2* mRNA in zebrafish embryos resulted in slow development and abnormal muscle formation at the embryonic stage. Our work provides a fundamental understanding of the structure and potential functions of *CsRspo2* during muscle development.

Key words: *R-spondin2*, *Cynoglossus semilaevis*, muscle development, Wnt signaling pathway

INTRODUCTION

R-spondin (Rspo) is a secreted protein that is transiently expressed during development of the central nervous system, specifically in the roof plate of the neural tube (Kamata et al., 2004; Lowther et al., 2005; Rong et al., 2014). The Rspo family comprises four members (Rspo1–Rspo4), which were discovered in mouse, human and *Xenopus*. The amino acid sequences of Rspo proteins are highly conserved, especially within vertebrate species. Several structurally distinct regions are recognized as follows: (i) a signal peptide sequence for secretion, (ii) two or more cysteine-rich furin-like (FU) domains, (iii) a thrombospondin type I repeat (TSR/TSP-1) domain, and (iv) a low-complexity C-terminal region (Yoon and Lee, 2012).

Many studies have explored the potential biological

functions of the Rspo family. For example, Rspo1 mutations disrupt sexual development in human (Tomaselli et al., 2008). In mouse, Rspo1 also participates in the formation of the mammary ductal system (Chadi et al., 2009). *Rspo3* mutant mice show abnormal growth during the formation of fetal blood vessels (Aoki et al., 2007). Rspo3 also binds to syndecan4 to regulate Wnt/planar cell polarity signaling (Ohkawara et al., 2011; Astudillo et al., 2014). *Rspo4* is involved in inherited anonychia (Bergmann et al., 2006; Brückle et al., 2008; Ishii et al., 2008; Khan et al., 2012). Besides, *Rspos* may also contribute to development of the dorsal neural tube under the regulation of Wnts in mouse (Kamata et al., 2004; Lowther et al., 2005; Rong et al., 2014). *Rspo1* participates in zebrafish gonad development and differentiation (Zhang et al., 2011). Full-length cDNAs of *Rspo1–3* were cloned from the gonads of medaka (*Oryzias latipes*) (Zhou et al., 2012). Several complete or partial sequences of *Rspo* cDNAs from other fish species, such as fugu (*Takifugu rubripes*), platyfish (*Xiphophorus maculatus*) and tilapia (*Oreochromis niloticus*), have been

Edited by Rimantas Kodzius

* Corresponding author. E-mail: qjje@ouc.edu.cn

DOI: <http://doi.org/10.1266/ggs.18-00009>

deposited in the GenBank database.

Previous studies have focused on the biological functions of *Rspo2*. In human, *Rspo2* gene fusion occurs in colon tumors, and a genome-wide association study suggested that the *Rspo2* locus is linked to genetic susceptibility to Dupuytren's disease (Dolmans et al., 2011). In mouse, in situ hybridization (ISH) of late embryonic and fetal tissues showed that *Rspo2* transcripts are present in dental mesenchyme at the bell stage and in dental papilla, hippocampus, ventral thalamus, intrinsic muscle of the tongue, and lung mesenchyme (Nam et al., 2007b). When the first morphological sexual differentiation occurs in the teleost medaka (*O. latipes*), *Rspo2* expression profiles show a female-specific increase; furthermore, ISH analysis revealed that *Rspo2* is predominantly expressed in germ cells and surrounding cells; however, steroid treatment significantly upregulates *Rspo2* expression in normal males relative to that in normal females (Zhou et al., 2012). Other studies have also investigated the participation of *Rspo* family members, including *Rspo2*, in the activation of the Wnt signaling pathway or in Wnt-mediated biological phenomena (Kamata et al., 2004; Kazanskaya et al., 2004; Bergmann et al., 2006; Lu et al., 2008; Friedman et al., 2009; Ohkawara et al., 2011).

The tongue sole *Cynoglossus semilaevis* is one of the most commercially important flatfish species, and, similar to many flatfishes, exhibits sexual growth dimorphism; in addition, females and males can have significantly different body weights (Liu et al., 2014). Muscle development is highly important for commercial fishes. Thus, in the present study, we explored the functions of *C. semilaevis* *Rspo2* (*CsRspo2*) during early muscle development. Quantitative real-time (qRT)-PCR was also performed to evaluate *CsRspo2* expression in different tissues and embryonic developmental stages. The effects of *CsRspo2* on myogenesis were determined by immunostaining whole-mount zebrafish embryos after *CsRspo2* mRNA injection and evaluating the expression pattern of the myogenic marker *MyoD* in *CsRspo2* mRNA-injected zebrafish embryos.

MATERIALS AND METHODS

Fish and embryo collection *Cynoglossus semilaevis* adults and embryos were obtained from Laizhou Mingbo Aquatic (Laizhou, Shandong, China). Tissue samples including heart, liver, spleen, kidney, brain, gill, muscle, intestine, testis and ovary were collected from six random healthy adults (three females and three males). Artificially fertilized eggs were incubated at 22 ± 0.5 °C in hatching tanks. Embryo samples were collected at different developmental stages (1-cell, 16-cell, multi-cell, morula, high blastula, low blastula, gastrula, 15-somite, 27-somite, heart-beating, hatching stage, 1 dph [day(s) post-hatching] and 3 dph). Each sample, which con-

tained 25 embryos, was collected in triplicate. All samples were immediately frozen using liquid nitrogen and stored at -80 °C for total RNA or genomic DNA preparation.

RNA extraction and cDNA synthesis Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol, treated with RNase-free DNase I (TaKaRa, Dalian, China) to degrade genomic DNA, and then frozen at -80 °C. cDNA was transcribed from 1 µg of total RNA using the reverse transcriptase M-MLV Kit (TaKaRa) and random primers (NNNNNN) according to the manufacturer's instructions. The quality and quantity of total RNA were evaluated through 1.5% (w/v) agarose gel electrophoresis and spectrophotometry using a NanoPhotometer Pearl (Implen, Munich, Germany).

Isolation of *C. semilaevis* *Rspo2* The core cDNA fragment of the *CsRspo2* gene was amplified by PCR with degenerate primers (*Rspo2*-core-Fw/Rv, Table 1) designed according to the highly conserved sequences of *Rspo2* homologs in other teleosts. Subsequently, 5' and 3' rapid amplification of cDNA ends (RACE) were performed to obtain the full-length cDNA of *CsRspo2* using the SMART RACE cDNA Amplification Kit (Clontech, San Jose, CA, USA) following the manufacturer's protocol. On the basis of the obtained *CsRspo2* cDNA fragment, gene-specific primers for 5'-RACE (*Rspo2*-5'Rv1 and *Rspo2*-5'Rv2) and 3'-RACE (*Rspo2*-3'Fw1 and *Rspo2*-3'Fw2) were designed. All amplified PCR products were separated by agarose gel electrophoresis. The target bands were purified and cloned in the pEASY-T5 vector in accordance with the protocol of the pEASY-T5 Zero Cloning Kit (TransGen Biotech, Beijing, China).

Bioinformatic and phylogenetic analyses Domains of the obtained protein were analyzed using the SMART program (<http://smart.embl-heidelberg.de/>). The molecular mass of the mature protein was determined using ProtParam (<http://www.expasy.ch/tools/protparam.html>). Homology searching in the GenBank database was carried out using the BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple sequence alignments of *CsRspo2* and other known vertebrate *Rspo2*s were performed by ClustalX 2.1 (Jeanmougin et al., 1998). The Ensembl ID accession numbers of sequences are given in Supplementary Table S1. A phylogenetic tree was constructed by the Bayesian method and maximum likelihood method with the GTR+I+G substitution model using phyMLv3.1, and the branching reliability was tested by bootstrap resampling with 1,000,000 replicates (Guindon et al., 2010). The *Rspo* family protein sequences were extracted from the online genome database Ensembl (<http://asia.ensembl.org/index.html>).

Table 1. Primers used in this study

Primer	Sequence (5'–3')	Usage
Rspo2-core-Fw	TCGMCTVTTCTCATTTGCSCTGAT	Core fragment PCR
Rspo2-core-Rv	CTCCACACTCCATGGTRTCKTCYA	Core fragment PCR
Rspo2-5'Rv1	CTTTTATTTCTCCTCCAGCG	5'-RACE
Rspo2-5'Rv2	GTTTACGCAGCCGTTGTCCT	5'-RACE
Rspo2-3'Fw1	TGCCAGCCCAAACCTCTTCCT	3'-RACE
Rspo2-3'Fw2	GCAGAATAGAGAACTGTGAGC	3'-RACE
CsRspo2-qPCR-Fw	CCTGCACCAGGAGAAACAAA	qRT-PCR
CsRspo2-qPCR-Rv	CAATGGTGGGACAGGGTATTG	qRT-PCR
18S-qPCR-Fw	GGTAACGGGAATCAGGGT	qRT-PCR
18S-qPCR-Rv	TGCCTTCCTTGGATGTGGT	qRT-PCR

Gene expression analysis by qRT-PCR Total RNA was extracted from different embryonic developmental stages and the 10 selected tissues from six healthy *C. semilaevis* adults (three females and three males). Afterward, cDNA synthesis was performed. The expression patterns of *Rspo2* were analyzed by qRT-PCR. Specific primers (CsRspo2-qPCR-Fw/Rv) for *CsRspo2* were designed according to the cloned sequences with the Integrated DNA Technologies website (<http://sg.idtdna.com/Primerquest/Home/Index>). RNA abundance was normalized using 18S rRNA (18S-qPCR-Fw/Rv) as the reference gene (Liu et al., 2014). qRT-PCR was performed on a Light-Cycler Roche 480 (Roche Applied Science, Mannheim, Germany) in a 20- μ l reaction volume containing 10 μ l of 2 $\times$ SYBR Premix Ex Taq II (TaKaRa), 0.4 μ l of each primer (10 μ M), 2 μ l of diluter cDNA (5 ng/ μ l) and 7.2 μ l of nuclease-free water. Melting curves were generated after amplification reaction completion to confirm the specificity of the amplicons. The same organs from three male or three female individuals were pooled as one sample for expression analysis. All qRT-PCR assays were performed in triplicate. Relative expression levels of the target gene were calculated by the $2^{-\Delta\Delta C_t}$ comparative Ct method.

***Rspo2* mRNA synthesis and microinjection into zebrafish embryos** Zebrafish adults were maintained at 27 °C under 14-h light and 10-h dark conditions. The pEASY-T5 Zero Cloning Vector containing the correct *CsRspo2* sequence (Supplementary Fig. S2) was linearized using SpeI, and *CsRspo2* mRNA was synthesized *in vitro* using the mMESSAGE mMACHINE T7 Kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). Because transgenic techniques have not yet been established in *C. semilaevis*, we injected *CsRspo2* mRNA into wild-type zebrafish (Hashimoto et al., 2007; Hu et al., 2015; Li et al., 2015). Each embryo was injected with 10 pg *CsRspo2* mRNA at the one-cell stage. Control groups were injected with RNA-free water. The embryos were then

collected at the 11–12-somite stages and also at the same time as the control samples for whole-mount ISH, and at 30 h post-fertilization (hpf) for muscle staining.

Immunostaining of whole-mount zebrafish embryos (muscle staining) Whole-mount F59 staining was carried out as previously described (Bernick et al., 2010; Li et al., 2011). Both the injected embryos and control samples were fixed with fresh 4% paraformaldehyde for 3–5 h at room temperature and then washed five times (5 min each) with 1 $\times$ PBS-Tween (PBS, 0.1% Tween-20). All samples were kept in cold 100% methanol for at least 1 h at –20 °C. Prior to addition of the primary antibody (1:100), the samples were blocked with blocking solution (1% BSA, 2% goat serum, 1% DMSO and 1 $\times$ PBS-Tween) for 1 h. Anti-MyHC for slow muscles (F59, Developmental Studies Hybridoma Bank, University of Iowa, USA) was used, and the secondary antibody (1:200) was FITC-conjugated anti-mouse antibody (Sigma, St. Louis, MO, USA). Fluorescence was observed under a Nikon Eclipse Ti-U microscope (Nikon, Tokyo, Japan).

Whole-mount in situ hybridization (WISH) of zebrafish *MyoD* gene A plasmid harboring zebrafish *MyoD* (donated by Dr. Michael Rebagliati) was linearized with NotI (New England Biolabs, Ipswich, MA, USA). The RNA probe was synthesized using a DIG RNA Labeling Kit (Roche Applied Science) in accordance with the manufacturer's instructions. WISH of zebrafish embryos was performed as previously described (Barth and Wilson, 1995).

Dual-luciferase reporter assay Luciferase activities were measured using a dual-luciferase assay to explore the potential function of *Rspo2* in the Wnt pathway. Topflash luciferase activity was normalized to that of Renilla as reported previously (Feng et al., 2012). Briefly, one-cell stage embryos were injected with *CsRspo2* mRNA (25, 50 or 100 pg) plus 100 pg Topflash DNA and 20 pg Renilla

plasmid DNA. Two hundred embryos were injected for each sample. After the injected embryos were raised to the shield stage at $28 \pm 0.5^\circ\text{C}$, three groups of 30 embryos for each sample were ground and lysed in 50 μl $1\times$ passive lysis buffer (Promega, Madison, WI, USA) at room temperature. After low-speed centrifugation, the supernatant was assayed using the Dual-Luciferase Reporter Assay kit (Promega) following the manufacturer's instructions.

Statistical analysis Data are expressed as mean $\pm$ standard error of the mean (SEM). qRT-PCR data were statistically analyzed using one-way analysis of variance (ANOVA) followed by the LSD test. Different groups of dual-luciferase reporter were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical analysis was performed by SPSS 17.0 (IBM, Armonk, NY, USA). P values < 0.05 indicate statistical significance.

A

```

ATGCAGTTTCGACTTCTCTCATTTCGCCCTGATCGTATTGAAGTGTATGGAGTACAGCAACTGTCCAGGCGCCGTGCGACCCGCTGGAGGAG
M Q F R L F S F A L I V L N C M E Y S N C Q A P S H R W R R
      signal peptide
AAATAAAGAGCTAGCTATGGAGCCTACCCCATCTGTAAAGGGTGTCTCGGTGTGCTCCAAAGGACAACGGCTGCGTAAACTGCCAGCCCA
N K R A S Y G A Y P I C K G C S V C S K D N G C V N C Q P
      FU1 region
AACTCTTCTTTTCTCGCAGTGTAGAGGATGAGGCAGTATGGGAGTGTCTTACGACTGTCCGGCCGGATATTACGGCATGCGCAGC
K L F L F L R R E R M R Q Y G E C L H D C P A G Y Y G M R S
      FU2 region
CCTGAACCTCAACATGTCTCCAGGTGCAGAATAGAGAACTGTGAGTCTCTTCAAGTAAAGACTTCTGCACCAAGTGCAAGTCAGGGTT
P E L N M C S R C R I E N C E S C F S K D F C T K C K S G F
      TSP-1 region
TTACCTACACAAAGGGCGCTGCTTTGACAAATGCCCTGAGGGCTTTTGTCTCTGGAAGATACCATGGAGTGTGGAGAGGGCTGTGAGG
Y L H K G R C F D K C P E G F A P L E D T M E C G E G C E
TGGGCCAGTGGAGCGAGTGGGGAGCCTGCACCAGGAGAAACAAACCTGTGGCTATAAGTGGGGTCTGGAAACCCGGACGCGGCACATT
V G Q W S E W G A C T R R N K T C G Y K W G L E T R T R H I
      low-complexity C-terminal region
GTAAGAAACCCCAAGGACACAATACCCCTGTCCACCATTTGCTGAGTCCAGGATGTGCAAAATGGCCATGAGGCACTGCAGGAGAGG
V K K P P K D T I P C P T I A E S R M C K M A M R H C R R G
CCCAGTGAAGCAGCCGACCCAAAGACCCCAAGGAAGCAAGCAAGGCTTGGGGCCAGAACCCAGCAGTACCACTTGG
P V K H R P K D P K K K N K Q R L R L R A Q N Q H S D H L
CCTCTGACGCTCCAGCAATGA
A S A R S S Q *

```

B

<i>H. sapiens</i>	MQFRLFSFALILNCMDYSHCOG---NRWRKSRASYNSNPICKGCLSKDNGCSRCKLFEFLRREMRQYGECLHSCPSGGYGHRAEDMNRRCRCR	97
<i>C. lupus</i>	MQFRLFSFALILNCMDYSHCOG---NRWRKSRASYNSNPICKGCLSKDNGCSRCKLFEFLRREMRQYGECLHSCPSGGYGHRAEDMNRRCRCR	97
<i>M. musculus</i>	MQFRLFSFALILNCMDYSHCOG---NRWRKSRASYNSNPICKGCLSKDNGCSRCKLFEFLRREMRQYGECLHSCPSGGYGHRAEDMNRRCRCR	97
<i>D. rerio</i>	MQFRLFSFALIVLNCMDYSHCOG---NRWRKSRASYNSNPICKGCLSKDNGCSRCKLFEFLRREMRQYGECLHSCPSGGYGHRAEDMNRRCRCR	100
<i>O. latipes</i>	MQFRLFSFALIVLNCMDYSHCOG---NRWRKSRASYNSNPICKGCLSKDNGCSRCKLFEFLRREMRQYGECLHSCPSGGYGHRAEDMNRRCRCR	99
<i>A. mexicanus</i>	MQFRLFSFALIVLNCMDYSHCOG---NRWRKSRASYNSNPICKGCLSKDNGCSRCKLFEFLRREMRQYGECLHSCPSGGYGHRAEDMNRRCRCR	98
<i>C. semilaevis</i>	MQFRLFSFALIVLNCMDYSHCOG---NRWRKSRASYNSNPICKGCLSKDNGCSRCKLFEFLRREMRQYGECLHSCPSGGYGHRAEDMNRRCRCR	99

<i>H. sapiens</i>	IENCSCFSKDFCTKCKVGYLHGRGCFDCEGFAPELDTMECEGCEVGHSEWGTCSRNNRTCGFKWGLETRIRIVKKPKDITPCPTIAESRCK	197
<i>C. lupus</i>	IENCSCFSKDFCTKCKVGYLHGRGCFDCEGFAPELDTMECEGCEVGHSEWGTCSRNNRTCGFKWGLETRIRIVKKPKDITPCPTIAESRCK	197
<i>M. musculus</i>	IENCSCFSKDFCTKCKVGYLHGRGCFDCEGFAPELDTMECEGCEVGHSEWGTCSRNNRTCGFKWGLETRIRIVKKPKDITPCPTIAESRCK	197
<i>D. rerio</i>	IDCEIIFSKDFCTKCKVGYLHGRGCFDCEGFAPELDTMECEGCEVGHSEWGTCSRNNRTCGFKWGLETRIRIVKKPKDITPCPTIAESRCK	200
<i>O. latipes</i>	IDNCSCFSKDFCTKCKVGYLHGRGCFDCEGFAPELDTMECEGCEVGHSEWGTCSRNNRTCGFKWGLETRIRIVKKPKDITPCPTIAESRCK	199
<i>A. mexicanus</i>	IDNCSCFSKDFCTKCKVGYLHGRGCFDCEGFAPELDTMECEGCEVGHSEWGTCSRNNRTCGFKWGLETRIRIVKKPKDITPCPTIAESRCK	198
<i>C. semilaevis</i>	IENCSCFSKDFCTKCKVGYLHGRGCFDCEGFAPELDTMECEGCEVGHSEWGTCSRNNRTCGFKWGLETRIRIVKKPKDITPCPTIAESRCK	199

<i>H. sapiens</i>	MMRHCRGGG---KRAFAKEKKNKKRRHLIERACEQHSVLADRANQ	244	73.1
<i>C. lupus</i>	MMRHCRGGG---KRAFAKEKKNKKRRHLIERACEQHSVLADRANQ	244	74.0
<i>M. musculus</i>	MMRHCRGGG---KRAFAKEKKNKKRRHLIERACEQHSVLADRANQ	244	72.7
<i>D. rerio</i>	MMRHCRGGSGK---HRSKDPKKK---NKQRLAFRAQNHSDHLASRSGG	249	88.2
<i>O. latipes</i>	MMRHCRGGSGK---HRSKDPKKK---NKQRLAFRAQNHSDHLASRSGG	246	92.2
<i>A. mexicanus</i>	MMRHCRGGSGR---HRSKDPKKK---NKQRLAFRAQNHSDHLASRSGG	245	85.2
<i>C. semilaevis</i>	MMRHCRGG---FVK---HRSKDPKKK---NKQRLAFRAQNHSDHLASRSGG	245	

Fig. 1. Structural domains and amino acid sequence of CsRspo2. (A) Nucleotide coding sequence (cds) of *Cynoglossus semilaevis R-spondin2* (*CsRspo2*). The deduced amino acid sequence is reported underneath the cds using single-letter code. Each conserved region is boxed with a different color: green represents the N-terminal signal peptide, red represents FU1, yellow represents FU2, purple represents TSP-1 and black represents the C-terminal region. (B) Multiple alignment of full-length CsRspo2 amino acid sequences with Rspo2 from six other species. Conserved residues are highlighted in black, and percent identities to CsRspo2 are shown to the right of the C-termini.

RESULTS

Cloning and sequence analysis of *CsRspo2* We cloned the full-length ORF of *Rspo2* from adult *C. semilaevis* ovary (GenBank accession number: KM361077). The ORF of *CsRspo2* is 735 bp, and encodes 244 amino acids (Fig. 1A); the estimated molecular mass is 28.34 kDa. Similar to previously identified Rspo2 orthologs in other species, *CsRspo2* also possesses conserved functional regions, including a signal peptide at the N-terminus, two FU regions, one TSP-1 region, and a low-complexity C-terminal region (Fig. 1A).

Homology and phylogenetic analysis The deduced full-length *CsRspo2* protein sequence was aligned with orthologous protein sequences from other vertebrates (Fig. 1B). This revealed that *CsRspo2* displayed high similarity to other vertebrate Rspo2s. The C-terminal sequence of the Rspo2 proteins was clearly less conserved than the other domains (Fig. 1B).

A phylogenetic tree was constructed by the Bayesian

Fig. 2. Phylogenetic tree of Rspo2 protein from *C. semilaevis* and Rspo proteins from other vertebrates. The tree was constructed by the Bayesian method with GTR+I+G, where MCMC=1,000,000. Numbers at the tree nodes are posterior probabilities. Rspo sequences were retrieved from Ensembl (see Materials and Methods). Cs: *Cynoglossus semilaevis*; Tr: *Takifugu rubripes*; Lo: *Lepisosteus oculatus*; Xt: *Xenopus tropicalis*; Gg: *Gallus gallus*; Ol: *Oryzias latipes*; Dr: *Danio rerio*; Hs: *Homo sapiens*; Mm: *Mus musculus*; Cl: *Canis lupus*; Am: *Astyanax mexicanus*; and Pm: *Petromyzon marinus*. The scale bar indicates substitutions per site.

method with the GTR+I+G model for all vertebrate Rspo isoforms to examine the *Rspo* gene family evolution. This clearly indicated that the *C. semilaevis* Rspo2 is an Rspo2 (Fig. 2). The *Petromyzon marinus* (sea lamprey) Rspo1 sequence was established as an outgroup. According to our identification, *CsRspo2* clustered with the fish Rspo2 homologs, and the relationships displayed in the phylogenetic tree are generally in accordance with classical taxonomy.

Expression profiles of *CsRspo2* during early embryogenesis The temporal expression pattern of *CsRspo2* during embryonic development at 14 development stages was determined by qRT-PCR. As shown in Fig. 3, the mRNA level of *CsRspo2* was low from the unfertilized egg stage to the low blastula stage. The transcript level increased remarkably during gastrulation and remained significantly high during somite formation, but then significantly decreased after hatching. In general, *CsRspo2* was highly expressed from the gastrulation stage to the hatching stage, but weakly expressed in the other examined stages.

Expression pattern of *CsRspo2* in different tissues Tissue-specific expression of *CsRspo2* in adult *C. semilaevis* was studied by qRT-PCR. The results revealed that the transcript level of *CsRspo2* was high in the brain and gill, moderate in the heart, ovary and testis, and almost undetectable in the intestine, kidney, liver, spleen and muscle (Fig. 4).

CsRspo2 regulates MyoD expression *CsRspo2* mRNA

Fig. 3. Relative expression levels of *CsRspo2* during embryonic developmental stages in *C. semilaevis*. Relative mRNA level is shown as mean $\pm$ SEM ($n = 3$). qRT-PCR data were statistically analyzed using one-way ANOVA followed by the LSD test using SPSS 17.0. Different letters (a–c) indicate significant differences between columns ($P < 0.05$). dph, day(s) post-hatching.

was synthesized *in vitro* to investigate the function of *Rspo2* during embryogenesis. Control samples and embryos injected with *CsRspo2* mRNA were collected at the 11–12 somite stages. Whole-mount ISH was used to analyze the expression pattern of *MyoD* during zebrafish embryogenesis. As shown in Fig. 5, compared with the control group (Fig. 5A-a and 5A-a1), the *CsRspo2*-mRNA-injected embryos showed abnormal short somites and downregulated *MyoD* expression on one or both sides

Fig. 4. Relative expression levels of *CsRspo2* in different tissues from *C. semilaevis*. Brain, gill, heart, intestine, kidney, liver, muscle, spleen, ovary and testis were isolated from six healthy adults randomly (three females and three males) for investigation. Data are shown as mean $\pm$ SEM. One-way ANOVA (SPSS 17.0) was performed to determine significant differences between means. Different letters (a–c) indicate significant differences between columns ($P < 0.05$).

(Fig. 5A-b and 5B-a–d).

CsRspo2 inhibits muscle development during embryogenesis The zebrafish embryos were stained with anti-MyHC antibody (F59) to further investigate the function of *CsRspo2* in muscle development; this antibody detects mostly slow myofibrils, although it also reacts weakly with fast myofibrils. Slow fibrils in control embryos were well organized with striation at 30 hpf (Fig. 6A). Overexpression of *CsRspo2* resulted in severe disruption of thick and thin filament organization in zebrafish embryos (Fig. 6B–6D).

CsRspo2 inhibits Wnt3a activity in the canonical Wnt/ β -catenin signaling pathway A dual-luciferase reporter assay was performed to determine whether *CsRspo2* regulated Wnt/ β -catenin signaling (Fig. 7). The results showed that co-injection of *Wnt3a* mRNA with Topflash and Renilla plasmid DNAs elicited strong signals in the reporter activity. This induction was significantly and dose-dependently inhibited by co-injection with several doses of *CsRspo2* mRNA. This result indicated that *CsRspo2* overexpression inhibited Wnt3a activity.

DISCUSSION

Rspo2 was isolated and characterized from the tongue sole *C. semilaevis*. This study is the first to identify and characterize *Rspo2* in this species. Sequence comparisons with other species revealed that the predicted *CsRspo2* protein was homologous to mammalian *Rspo2*: an N-terminal signal peptide, two FU regions, a TSP-1 region and a low-complexity C-terminal region are con-

Fig. 5. Whole-mount in situ hybridization (WISH) of the zebrafish muscle-specific gene *MyoD* after injection of embryos with *CsRspo2* mRNA. (A) Positive signals from antisense probe hybridization are purple or blue. (a, a1) Control group with normally developed embryos at the 11–12-somite stages, (b) embryo injected with *CsRspo2* mRNA, and (c) sense probe ISH. (B) Abnormal embryos showing slow development after injection. *MyoD* mRNA-positive signals are indicated by black arrows. Scale bars = 50 μ m.

Fig. 6. Immunostaining of whole-mount zebrafish embryos by anti-MyHC for slow muscles (F59). Embryos were injected with RNA-free water or 200 pg *CsRspo2* mRNA. (A) Slow fibrils in control embryos. (B, C, D) Organization of filaments in slow muscles of *CsRspo2* mRNA-injected zebrafish embryos was severely defective (arrows). Scale bars = 50 μ m.

Fig. 7. *Rspo2* regulates Wnt3a activity. One-cell stage embryos were injected with 20 pg of *Wnt3a* mRNA alone or in combination with 25 (+), 50 (++) or 100 (+++) pg of *CsRspo2* mRNA. Topflash/Renilla luciferase reporter DNA (T+R) was injected in all groups. The embryos were collected at the shield stage, and luciferase activity was determined. Values are mean $\pm$ SE. *** $P < 0.001$.

served in these species (Chen et al., 2002; de Lau et al., 2012). These results indicate that the Rspo2 protein has been structurally conserved during vertebrate evolution. Moreover, on a phylogenetic tree, *CsRspo2* clus-

tered with other fish Rspo2 proteins, coinciding with the separation between fish and tetrapod lineages. Each R-spondin paralog clustered with those of other vertebrates. Rspo1 and Rspo3 are more closely related to each other, as are Rspo2 and Rspo4. Based on branch lengths, it appears that Rspo4 is the most divergent within vertebrates, whereas Rspo2 is the most highly conserved. In fact, more than 70% of Rspo2 residues are completely conserved among vertebrates. The existence of four paralogs is consistent with ancestral whole-genome duplications. However, it is noteworthy that there is no evidence in fish for additional paralogs arising from teleost-specific whole-genome duplication for this gene family. In addition, the predicted 3D model structures of Rspo2 proteins from *O. latipes*, *D. rerio* and *C. semilaevis* showed that these proteins contain the same number of β -sheets and possess no α -helix (Supplementary Fig. S1). All these findings support a high degree of conservation of Rspo proteins among species (Kamata et al., 2004; de Lau et al., 2012).

We mapped the spatial and temporal expression patterns of *Rspo2* in *C. semilaevis* and determined its function during embryogenesis. qRT-PCR revealed that *CsRspo2* was barely transcribed before gastrulation, which is consistent with the pattern in zebrafish (Tatsumi et al., 2014), where *Rspo2* mRNA was not detected until zygotic expression begins at the 75% epiboly stage. Moreover, the *CsRspo2*

expression pattern in different tissues showed similarities with that in mouse and zebrafish (Nam et al., 2007b; Aoki et al., 2008; Tatsumi et al., 2014), which suggests a common function for *Rspo2* in diverse species. High expression levels of *CsRspo2* in the gill, brain and gonads may be related to its functions in dorsal neural tube development (Kamata et al., 2004; Nam et al., 2007b) and gonad differentiation (Zhou et al., 2012).

However, in contrast to the comparable *Rspo2* expression level in both genders of *C. semilaevis*, *Rspo2* of medaka was abundantly expressed in the XX gonads, but barely in the XY male gonads (Zhou et al., 2012). This phenomenon may be attributed to a number of differences between XX/XY (i.e., *O. latipes*) and ZZ/ZW (i.e., *C. semilaevis*) sex determination mechanisms among species or to other unknown reasons. These observations also imply that *CsRspo2* has multiple functions, which are largely unknown in fish and warrant further study.

Given that transgenic methodology has not yet been established for *C. semilaevis*, in the present study *CsRspo2* mRNA was injected into zebrafish embryos at the one-cell stage to determine its function. *CsRspo2* acted as a negative regulator in early myogenesis during zebrafish embryogenesis (Fig. 5). Further, our experiments with F59 staining showed that the organization of filaments in slow muscles in *CsRspo2* mRNA-injected zebrafish embryos was severely disrupted (Fig. 6). Previous work has shown that Wnt signaling is required for early embryonic myogenesis (Buckingham, 2001), and Wnt signaling participates in embryonic muscle development and skeletal muscle maintenance; dysregulation of Wnt signaling can lead to severe developmental defects and perturbation during muscle development and other processes (Cossu and Borello, 1999; Clevers, 2006; MacDonald et al., 2009; von Maltzahn et al., 2012).

According to previous studies, *Rspo* family proteins regulate the Wnt signaling pathway (Kazanskaya et al., 2004; Kim et al., 2006; Astudillo et al., 2014). In particular, *Rspo2* either enhances or antagonizes Wnt signaling in specific organisms or cells/tissues. In most cases, *Rspo2* enhances Wnt signaling (Kazanskaya et al., 2004; Kim et al., 2008; Jin et al., 2011); the phenotypes arising from the deletion of *Rspo2* and addition of *Rspo2* recombinant protein are the opposite of those achieved by activation and inhibition of Wnt signaling (Nam et al., 2007a). For example, *Rspo2* is a secreted activator of Wnt/ β -catenin signaling in *Xenopus* embryos (Kazanskaya et al., 2004). In contrast, *Rspo2* also inhibits Wnt signaling. *Rspo2* exerts no positive influence on Wnt signaling during cochlea development in mice (Mulvaney et al., 2013). In addition, in human colorectal cancer, *Rspo2* enables a transient activation of the Wnt pathway and a pronounced upregulation of *LGR5* expression. *LGR5* in turn interacts with *Rspo2* to stabilize membrane-associated ZNRF3, and promotes

LRP6 degradation. The negative feedback loop of the Wnt β -catenin pathway is then induced by *Rspo2*, and attenuates β -catenin accumulation and Wnt target gene expression (Wu et al., 2014). In this study, a dual-luciferase reporter assay showed that *CsRspo2* regulated Wnt3a activity in the Wnt/ β -catenin signaling pathway (Fig. 7). Together with the relationship between muscle development and Wnt signaling reported in previous studies, these findings suggest that *CsRspo2* inhibits the Wnt/ β -catenin signaling pathway to regulate muscle development during embryogenesis. Thus, we speculate that *CsRspo2* eventually exerts a net growth-suppressive effect on muscle development, perhaps due to negative feedback of *CsRspo2* to the Wnt signaling pathway.

This result was similar to the function of a known Wnt antagonist family, namely the secreted frizzled-related proteins (Suzuki et al., 2004). Myogenesis in the mesoderm and somites is inhibited by Wnt antagonists (Borello et al., 1999). Moreover, β -catenin is sufficient to induce skeletal muscle development, which suggests that Wnt signaling acts through the canonical pathway to promote myogenesis (Mulvaney et al., 2013). Activation of the Wnt signaling pathway leads to the transformation of non-myogenic cells into the myogenic lineage (Poleskaya et al., 2003; Otto et al., 2008). Additionally, *CsRspo2* may regulate the Wnt pathway and other important pathways, such as Nodal and Tgf- β , to affect early embryonic development (Baker et al., 1999). Therefore, the molecular mechanisms underlying the inhibitory role of *Rspo2* in regulating Wnt/ β -catenin signaling and its essential function during embryonic development should be investigated.

CONCLUSIONS

We have identified and characterized the structure and expression profiles of *Rspo2* in *C. semilaevis*. Evidence from gene structure and phylogenetic position demonstrated that *CsRspo2* is a homolog of mammalian *Rspo2*. *CsRspo2* was hardly expressed during early developmental stages. Moreover, we provided evidence that *CsRspo2* plays a negative role in muscle development. Future studies should elucidate the molecular mechanisms underlying the observed inhibitory role of *Rspo2* in regulating Wnt/ β -catenin signaling, and thus provide novel insights into Wnt/ β -catenin signaling in vertebrates.

We thank Mr. Wenhui Ma and Ms. Xiaomei Wang, of Laizhou Mingbo Aquatic Co., Ltd., for kindly providing the tongue sole samples. This study was supported by Fundamental Research Funds for the Central Universities (No. 201822026) and the Natural Science Foundation of Shandong Province (No. ZR2018MC029).

REFERENCES

- Aoki, M., Kiyonari, H., Nakamura, H., and Okamoto, H. (2008) R-spondin2 expression in the apical ectodermal ridge is essential for outgrowth and patterning in mouse limb development. *Dev. Growth Differ.* **50**, 85–95.
- Aoki, M., Mieda, M., Ikeda, T., Hamada, Y., Nakamura, H., and Okamoto, H. (2007) R-spondin3 is required for mouse placental development. *Dev. Biol.* **301**, 218–226.
- Astudillo, P., Carrasco, H., and Larraín, J. (2014) Syndecan-4 inhibits Wnt/ β -catenin signaling through regulation of low-density-lipoprotein receptor-related protein (LRP6) and R-spondin 3. *Int. J. Biochem. Cell Biol.* **46**, 103–112.
- Baker, J. C., Beddington, R. S., and Harland, R. M. (1999) Wnt signaling in *Xenopus* embryos inhibits *Bmp4* expression and activates neural development. *Genes Dev.* **13**, 3149–3159.
- Barth, K. A., and Wilson, S. W. (1995) Expression of zebrafish *nk2.2* is influenced by *sonic hedgehog/vertebrate hedgehog-1* and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **121**, 1755–1768.
- Bergmann, C., Senderek, J., Anhu, D., Thiel, C. T., Ekici, A. B., Poblete-Gutiérrez, P., van Steensel, M., Seelow, D., Nürnberg, G., Schild, H. H., et al. (2006) Mutations in the gene encoding the Wnt-signaling component R-spondin 4 (*RSPO4*) cause autosomal recessive anonychia. *Am. J. Hum. Genet.* **79**, 1105–1109.
- Bernick, E. P., Zhang, P. J., and Du, S. (2010) Knockdown and overexpression of *Unc-45b* result in defective myofibril organization in skeletal muscles of zebrafish embryos. *BMC Cell Biol.* **11**, 70.
- Borello, U., Coletta, M., Tajbakhsh, S., Leyns, L., De Robertis, E. M., Buckingham, M., and Cossu, G. (1999) Transplacental delivery of the Wnt antagonist *Frzb1* inhibits development of caudal paraxial mesoderm and skeletal myogenesis in mouse embryos. *Development* **126**, 4247–4255.
- Brüchle, N. O., Frank, J., Frank, V., Senderek, J., Akar, A., Koc, E., Rigopoulos, D., van Steensel, M., Zerres, K., and Bergmann, C. (2008) *RSPO4* is the major gene in autosomal-recessive anonychia and mutations cluster in the furin-like cysteine-rich domains of the Wnt signaling ligand R-spondin 4. *J. Invest. Dermatol.* **128**, 791–796.
- Buckingham, M. (2001) Skeletal muscle formation in vertebrates. *Curr. Opin. Genet. Dev.* **11**, 440–448.
- Chadi, S., Buscara, L., Pechoux, C., Costa, J., Laubier, J., Chaboissier, M. C., Pailhoux, E., Vilotte, J. L., Chanut, E., and Le Provost, F. (2009) R-spondin1 is required for normal epithelial morphogenesis during mammary gland development. *Biochem. Biophys. Res. Commun.* **390**, 1040–1043.
- Chen, J.-Z., Wang, S., Tang, R., Yang, Q.-S., Zhao, E., Chao, Y., Ying, K., Xie, Y., and Mao, Y.-M. (2002) Cloning and identification of a cDNA that encodes a novel human protein with thrombospondin type I repeat domain, *hPWTSR*. *Mol. Biol. Rep.* **29**, 287–292.
- Clevers, H. (2006) Wnt/ β -catenin signaling in development and disease. *Cell* **127**, 469–480.
- Cossu, G., and Borello, U. (1999) Wnt signaling and the activation of myogenesis in mammals. *EMBO J.* **18**, 6867–6872.
- de Lau, W. B., Snel, B., and Clevers, H. C. (2012) The R-spondin protein family. *Genome Biol.* **13**, 242.
- Dolmans, G. H., Werker, P. M., Hennies, H. C., Furniss, D., Festen, E. A., Franke, L., Becker, K., van der Vlies, P., Wolffenbuttel, B. H., Tinschert, S., et al. (2011) Wnt signaling and Dupuytren's disease. *N. Engl. J. Med.* **365**, 307–317.
- Feng, Q., Zou, X., Lu, L., Li, Y., Liu, Y., Zhou, J., and Duan, C. (2012) The stress-response gene *redd1* regulates dorsoventral patterning by antagonizing Wnt/ β -catenin activity in zebrafish. *PLoS One* **7**, e2674.
- Friedman, M. S., Oyserman, S. M., and Hankenson, K. D. (2009) Wnt11 promotes osteoblast maturation and mineralization through R-spondin 2. *J. Biol. Chem.* **284**, 14117–14125.
- Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* **59**, 307–321.
- Hashimoto, H., Uji, S., Kurokawa, T., Washio, Y., and Suzuki, T. (2007) Flounder and fugu have a single *lefty* gene that covers the functions of *lefty1* and *lefty2* of zebrafish during L–R patterning. *Gene* **387**, 126–132.
- Hu, H., Xin, N., Liu, J., Liu, M., Wang, Z., Wang, W., Zhang, Q., and Qi, J. (2015) Characterization of F-spondin in Japanese flounder (*Paralichthys olivaceus*) and its role in the nervous system development of teleosts. *Gene* **575**, 623–631.
- Ishii, Y., Wajid, M., Bazzi, H., Fantauzzo, K. A., Barber, A. G., Blaydon, D. C., Nam, J. S., Yoon, J. K., Kelsell, D. P., and Christiano, A. M. (2008) Mutations in R-spondin 4 (*RSPO4*) underlie inherited anonychia. *J. Invest. Dermatol.* **128**, 867–870.
- Jeanmougin, F., Thompson, J. D., Gouy, M., Higgins, D. G., and Gibson, T. J. (1998) Multiple sequence alignment with Clustal X. *Trends Biochem. Sci.* **23**, 403–405.
- Jin, Y. R., Turcotte, T. J., Crocker, A. L., Han, X. H., and Yoon, J. K. (2011) The canonical Wnt signaling activator, R-spondin2, regulates craniofacial patterning and morphogenesis within the branchial arch through ectodermal-mesenchymal interaction. *Dev. Biol.* **352**, 1–13.
- Kamata, T., Katsube, K., Michikawa, M., Yamada, M., Takada, S., and Mizusawa, H. (2004) *R-spondin*, a novel gene with thrombospondin type 1 domain, was expressed in the dorsal neural tube and affected in *Wnts* mutants. *Biochim. Biophys. Acta* **1676**, 51–62.
- Kazanskaya, O., Glinka, A., del Barco Barrantes, I., Stanek, P., Niehrs, C., and Wu, W. (2004) R-Spondin2 is a secreted activator of Wnt/ β -catenin signaling and is required for *Xenopus* myogenesis. *Dev. Cell* **7**, 525–534.
- Khan, T. N., Klar, J., Nawaz, S., Jameel, M., Tariq, M., Malik, N. A., Baig, S. M., and Dahl, N. (2012) Novel missense mutation in the *RSPO4* gene in congenital hyponychia and evidence for a polymorphic initiation codon (p.M1I). *BMC Med. Genet.* **13**, 120.
- Kim, K. A., Waggle, M., Tran, K., Zhan, X., Dixon, M. A., Liu, S., Gros, D., Korver, W., Yonkovich, S., Tomasevic, N., et al. (2008) R-Spondin Family Members Regulate the Wnt Pathway by a Common Mechanism. *Mol. Biol. Cell* **19**, 2588–2596.
- Kim, K. A., Zhao, J., Andarmani, S., Kakitani, M., Oshima, T., Binnerts, M. E., Abo, A., Tomizuka, K., and Funk, W. D. (2006) R-Spondin proteins: a novel link to β -catenin activation. *Cell Cycle* **5**, 23–26.
- Li, H., Xu, J., Bian, Y. H., Rotllant, P., Shen, T., Chu, W., Zhang, J., Schneider, M., and Du, S. J. (2011) *Smyd1b.tv1*, a key regulator of sarcomere assembly, is localized on the M-Line of skeletal muscle fibers. *PLoS One* **6**, e28524.
- Li, M., Tan, X., Jiao, S., Wang, Q., Wu, Z., You, F., and Zou, Y. (2015) A new pattern of primordial germ cell migration in olive flounder (*Paralichthys olivaceus*) identified using nanos3. *Dev. Genes Evol.* **225**, 195–206.
- Liu, C., Xin, N., Zhai, Y., Jiang, L., Zhai, J., Zhang, Q., and Qi, J. (2014) Reference gene selection for quantitative real-time RT-PCR normalization in the half-smooth tongue sole (*Cynoglossus semilaevis*) at different developmental stages,

- in various tissue types and on exposure to chemicals. *PLoS One* **9**, e91715.
- Lowther, W., Wiley, K., Smith, G. H., and Callahan, R. (2005) A new common integration site, *Int7*, for the mouse mammary tumor virus in mouse mammary tumors identifies a gene whose product has furin-like and thrombospondin-like sequences. *J. Virol.* **79**, 10093–10096.
- Lu, W., Kim, K. A., Liu, J., Abo, A., Feng, X., Cao, X., and Li, Y. (2008) R-spondin1 synergizes with Wnt3A in inducing osteoblast differentiation and osteoprotegerin expression. *FEBS Lett.* **582**, 643–650.
- MacDonald, B. T., Tamai, K., and He, X. (2009) Wnt/ β -catenin signaling: components, mechanisms, and diseases. *Dev. Cell* **17**, 9–26.
- Mulvaney, J. F., Yatteau, A., Sun, W. W., Jacques, B., Takubo, K., Suda, T., Yamada, W., and Dabdoub, A. (2013) Secreted Factor R-Spondin 2 is Involved in Refinement of Patterning of the Mammalian Cochlea. *Dev. Dyn.* **242**, 179–188.
- Nam, J. S., Park, E., Turcotte, T. J., Palencia, S., Zhan, X., Lee, J., Yun, K., Funk, W. D., and Yoon, J. K. (2007a) Mouse *R-spondin2* is required for apical ectodermal ridge maintenance in the hindlimb. *Dev. Biol.* **311**, 124–135.
- Nam, J. S., Turcotte, T. J., and Yoon, J. K. (2007b) Dynamic expression of *R-spondin* family genes in mouse development. *Gene Expr. Patterns* **7**, 306–312.
- Ohkawara, B., Glinka, A., and Niehrs, C. (2011) *Rspo3* binds syndecan 4 and induces Wnt/PCP signaling via clathrin-mediated endocytosis to promote morphogenesis. *Dev. Cell* **20**, 303–314.
- Otto, A., Schmidt, C., Luke, G., Allen, S., Valasek, P., Muntoni, F., Lawrence-Watt, D., and Patel, K. (2008) Canonical Wnt signalling induces satellite-cell proliferation during adult skeletal muscle regeneration. *J. Cell Sci.* **121**, 2939–2950.
- Polesskaya, A., Seale, P., and Rudnicki, M. A. (2003) Wnt signaling induces the myogenic specification of resident CD45⁺ adult stem cells during muscle regeneration. *Cell* **113**, 841–852.
- Rong, X., Chen, C., Zhou, P., Zhou, Y., Li, Y., Lu, L., Liu, Y., Zhou, J., and Duan, C. (2014) R-Spondin 3 regulates dorsoventral and anteroposterior patterning by antagonizing Wnt/ β -catenin signaling in zebrafish embryos. *PLoS One* **9**, e99514.
- Suzuki, H., Watkins, D. N., Jair, K. W., Schuebel, K. E., Markowitz, S. D., Chen, W. D., Pretlow, T. P., Yang, B., Akiyama, Y., van Engeland, M., et al. (2004) Epigenetic inactivation of *SFRP* genes allows constitutive WNT signaling in colorectal cancer. *Nat. Genet.* **36**, 417–422.
- Tatsumi, Y., Takeda, M., Matsuda, M., Suzuki, T., and Yokoi, H. (2014) TALEN-mediated mutagenesis in zebrafish reveals a role for *r-spondin 2* in fin ray and vertebral development. *FEBS Lett.* **588**, 4543–4550.
- Tomaselli, S., Megiorni, F., De Bernardo, C., Felici, A., Marrocco, G., Maggiulli, G., Grammatico, B., Remotti, D., Saccucci, P., Valentini, F., et al. (2008) Syndromic true hermaphroditism due to an R-spondin1 (*RSPO1*) homozygous mutation. *Hum. Mutat.* **29**, 220–226.
- von Maltzahn, J., Chang, N. C., Bentzinger, C. F., and Rudnicki, M. A. (2012) Wnt signaling in myogenesis. *Trends Cell Biol.* **22**, 602–609.
- Wu, C., Qiu, S., Lu, L., Zou, J., Li, W. F., Wang, O., Zhao, H., Wang, H., Tang, J., Chen, L., et al. (2014) RSPO2-LGR5 signaling has tumour-suppressive activity in colorectal cancer. *Nat. Commun.* **5**, 3149.
- Yoon, J. K., and Lee, J.-S. (2012) Cellular signaling and biological functions of R-spondins. *Cell. Signal.* **24**, 369–377.
- Zhang, Y., Li, F., Sun, D., Liu, J., Liu, N., and Yu, Q. (2011) Molecular analysis shows differential expression of *R-spondin1* in zebrafish (*Danio rerio*) gonads. *Mol. Biol. Rep.* **38**, 275–282.
- Zhou, L., Charkraborty, T., Yu, X., Wu, L., Liu, G., Mohapatra, S., Wang, D., and Nagahama, Y. (2012) R-spondins are involved in the ovarian differentiation in a teleost, medaka (*Oryzias latipes*). *BMC Dev. Biol.* **12**, 36.