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; Brüchle 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...
deposited in the GenBank database. 

Previous studies have focused on the biological functions of Rsps2. In human, Rsps2 gene fusion occurs in colon tumors, and a genome-wide association study suggested that the Rsps2 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 Rsps2 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), Rsps2 expression profiles show a female-specific increase; furthermore, ISH analysis revealed that Rsps2 is predominantly expressed in germ cells and surrounding cells; however, steroid treatment significantly upregulates Rsps2 expression in normal males relative to that in normal females (Zhou et al., 2012). Other studies have also investigated the participation of Rsps family members, including Rsps2, 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 Rsps2 (CsRsps2) during early muscle development. Quantitative real-time (qRT)-PCR was also performed to evaluate CsRsps2 expression in different tissues and embryonic developmental stages. The effects of CsRsps2 on myogenesis were determined by immunostaining whole-mount zebrafish embryos after CsRsps2 mRNA injection and evaluating the expression pattern of the myogenic marker MyoD in CsRsps2 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 contained 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 Rsps2 The core cDNA fragment of the CsRsps2 gene was amplified by PCR with degenerate primers (Rsps2-core-Fw/Rv, Table 1) designed according to the highly conserved sequences of Rsps2 homologs in other teleosts. Subsequently, 5’ and 3’ rapid amplification of cDNA ends (RACE) were performed to obtain the full-length cDNA of CsRsps2 using the SMART RACE cDNA Amplification Kit (Clontech, San Jose, CA, USA) following the manufacturer’s protocol. On the basis of the obtained CsRsps2 cDNA fragment, gene-specific primers for 5’-RACE (Rsps2-5’Rv1 and Rsps2-5’Rv2) and 3’-RACE (Rsps2-3’Fw1 and Rsps2-3’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 CsRsps2 and other known vertebrate Rsps2s 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 Rsps family protein sequences were extracted from the online genome database Ensembl (http://asia.ensembl.org/index.html).
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-μl reaction volume containing 10 μl of 2× SYBR Premix Ex Taq II (TaKaRa), 0.4 μl of each primer (10 μM), 2 μl of diluter cDNA (5 ng/μl) and 7.2 μl 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−ΔΔCt 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× 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× 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

| Primer          | Sequence (5’–3’)                      | Usage         |
|-----------------|---------------------------------------|---------------|
| Rspo2-core-Fw   | TCGMCTVTTCCTCATTTTGCSCCTGAT           | Core fragment PCR |
| Rspo2-core-Rv   | CTCCACACTCATGGTTRTCTCCTYA             | Core fragment PCR |
| Rspo2-5'Rv1     | CTTTTATTTTCTCTCCACCC                | 5’-RACE       |
| Rspo2-5'Rv2     | GTTTACGACCGCCCTTGTCTCT              | 5’-RACE       |
| Rspo2-3'Fw1     | TGGCCACCCCAACTCTCTCT                | 3’-RACE       |
| Rspo2-3'Fw2     | GCAGATAAGAGACTGTGAGC                | 3’-RACE       |
| CsRspo2-qPCR-Fw | CCTGCACACAGGAGAAACAAAA              | qRT-PCR       |
| CsRspo2-qPCR-Rv | CAATGTCGAAGCAGGTATTG                | qRT-PCR       |
| 18S-qPCR-Fw     | GGTAAACGGGGAATACGGGT                | qRT-PCR       |
| 18S-qPCR-Rv     | TGCCCTCCTTTGGATGTTG                 | qRT-PCR       |
plasmid DNA. Two hundred embryos were injected for each sample. After the injected embryos were raised to the shield stage at 28 ± 0.5 °C, three groups of 30 embryos for each sample were ground and lysed in 50 μl 1× 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 ± 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.

---

![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 Repo2 from six other species. Conserved residues are highlighted in black, and percent identities to CsRspo2 are shown to the right of the C-termini.](image-url)
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 CsRapo2 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 method with 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 mRNAs are highly expressed in the brain and gill during early stages of development, and weakly expressed in other tissues. The expression levels of CsRspo2 are shown in Fig. 4, with significant differences observed between columns (P < 0.05). dph, day(s) post-hatching.
was synthesized *in vitro* to investigate the function of Rsopo2 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. 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**

Rsopo2 was isolated and characterized from the tongue sole C. semilaevis. This study is the first to identify and characterize Rsopo2 in this species. Sequence comparisons with other species revealed that the predicted CsRsopo2 protein was homologous to mammalian Rsopo2: an N-terminal signal peptide, two FU regions, a TSP-1 region and a low-complexity C-terminal region are con-
Sole Rsps2 and its role in muscle development

Served in these species (Chen et al., 2002; de Lau et al., 2012). These results indicate that the Rsps2 protein has been structurally conserved during vertebrate evolution. Moreover, on a phylogenetic tree, CsRsps2 clustered with other fish Rsps2 proteins, coinciding with the separation between fish and tetrapod lineages. Each R-spondin paralog clustered with those of other vertebrates. Rsps1 and Rsps3 are more closely related to each other, as are Rsps2 and Rsps4. Based on branch lengths, it appears that Rsps4 is the most divergent within vertebrates, whereas Rsps2 is the most highly conserved. In fact, more than 70% of Rsps2 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 Rsps2 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 Rsps2 in C. semilaevis and determined its function during embryogenesis. qRT-PCR revealed that CsRsps2 was barely transcribed before gastrulation, which is consistent with the pattern in zebrafish (Tatsumi et al., 2014), where Rsps2 mRNA was not detected until zygotic expression begins at the 75% epiboly stage. Moreover, the CsRsps2

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 CsRsps2 mRNA. (A) Slow fibrils in control embryos. (B, C, D) Organization of filaments in slow muscles of CsRsps2 mRNA-injected zebrafish embryos was severely defective (arrows). Scale bars = 50 µm.

Fig. 7. Rsps2 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 CsRsps2 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 ± SE. *** P < 0.001.
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 XXXY (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, 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 organs 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 (Polesskaya 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 hPWTSR thrombospondin type I repeat domain, was expressed in the dorsal mesoderm of skeletal muscles of zebrafish embryos. BMC Cell Biol. 11, 70.

Borello, U., Coletta, M., Tjahbakhsh, S., Leysn, L., De Robertis, E. M., Buckingham, E., and Cossu, G. (1999) Transplacental delivery of the Wnt antagonist Fzrb1 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 Steenzel, M., Zerres, K., and Bergmann, C. (2008) RSP04 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. Opini. Genet. Dev. 11, 440–448.

Chadi, S., Buscara, L., Pechoux, C., Costa, J., Laubier, J., Chaboissier, M. C., Pailhoux, E., Vilotte, J. L., Chanat, E., and Le Provost, F. (2009) R-spondin1 is required for normal axis formation and demarcates a zone of neuronal differentiation in the embryonic forebrain. Development 126, 1755–1768.

Bergmann, C., Senderek, J., Anhuf, D., Thiel, C. T., Ekici, A. B., Poblete-Gutiérrez, P., van Steenzel, 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 orga-

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 pla-
cental 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 ac-

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 pla-
cental 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 ac-

tral patterning by antagonizing Wnt/β-catenin activity in zebrafish. PloS One 7, e52674.

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 cov-
ers 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, Z., 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.

Katama, 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., Stennek, P., Niehrs, C., and Wu, W. (2004) R-Spondin2 is a secreted activator of Wnt/beta-catenin signaling and is required for Xenopus myogenesis. Dev. Cell 7, 525–534.

Khan, T. N., Klar, J., Nawaz, S., Jamiel, 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., Wagle, 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 beta-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) Smyd1Bulletin, is 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, C., Mohapatra, S., Wang, D., and Nagashama, Y. (2012) R-spondins are involved in the ovarian differentiation in a teleost, medaka (Oryzias latipes). BMC Dev. Biol. 12, 36.