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Research paper

Expression pattern and functional analysis of *R-spondin1* in tongue sole *Cynoglossus semilaevis*

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ABSTRACT

R-spondin 1 (*Rspo1*) is a potential female-determining gene in mammals that could regulate the Wnt/β-catenin signaling pathway. The deletion of *Rspo1* causes sex reversal in females. To investigate sexual determination and differentiation, we cloned and analyzed the *Rspo1* gene in *Cynoglossus semilaevis*. Phylogenetic and gene structure analyses revealed that *Rspo1* gene exhibited high sequence conservation and contained an N-terminal signal peptide, two furin-like cysteine-rich domains (FU1 and FU2), a thrombospondin type 1 repeat, and a C-terminal region enriched with basic charged amino acids. qRT-PCR revealed that *Rspo1* expressed sexual dimorphism in gonad, with higher expression levels in the ovary than in the testis, thus, suggesting the involvement of *Rspo1* in gonad differentiation. In situ hybridization results demonstrated that *Rspo1* was expressed in premature germ cells, including spermatogonia and spermatocytes in the testis and stage II and stage III oocytes in the ovary. The methylation levels in two CpG sites of *Rspo1* promoter significantly differed among females, males, and pseudomales. After 30 days of exposure to high temperature, the expression of *Rspo1* significantly decreased in female individuals, some of which were prone to males. However, no difference of *Rspo1* gene expression was observed between the control group and high-temperature group in males. These preliminary findings suggested that *Rspo1* played a crucial role in sex determination and development. This study laid the groundwork for further sex control breeding techniques in *C. semilaevis*.

1. Introduction

The R-Spondin family is a group of four secreted proteins (Rspo1–4) known to be potent agonists of Wnt signaling with important functions in development, stem cell survival and differentiation, and oncogenesis. The four Rspo family members contain an N-terminal signal peptide, two furin-type cysteine-rich domains (FU1 and FU2), one thrombospondin-type 1 domain (TSP1), a basic amino acid-rich domain (BR), and a C-terminal region rich with positively charged amino acids (Kamata et al., 2004; de Lau et al., 2012). Rspo1 binds to leucine-rich repeat-containing G protein-coupled receptor, which is synergized with soluble Wnt3a to induce LRP6 phosphorylation of β-catenin signaling pathway (Binnerts et al., 2007; Wei et al., 2007; Carmon et al., 2011). Rspo1-activating Wnt/β-catenin signaling pathway and Fox2-leading pathway act independently and complementary to each other to promote ovarian development (Kocer et al., 2008; Garcia-Ortiz et al., 2009).

Rspo1 plays a critical role in the reproductive system via the Rspo1-activating Wnt/β-catenin signaling pathway, especially in early sex determination and differentiation (Nam et al., 2007; Tomaselli et al., 2011). In vertebrates, *Rspo1* displays a conserved and female-specific increase in expression. Mutations of *Rspo1* in humans induce testis formation and male development in XX individuals, causing complete female-to-male sex reversals (Parma et al., 2006). In $Rspo1^{-/-}$ XX mouse, germ cell proliferation was severely impaired. This yielded female-to-male sex reversal in individuals and promoted *Sox9* up-regulation, which was correlated with differentiation of Sertoli cells and formation of masculinized gonad (Chassot et al., 2008a; Chassot et al., 2011). Similarly, testis development also occurred in XY $Rspo1^{KO}Sox9^{CKO}$ mice, which indicated that *Rspo1* is an essential activator in ovarian development in normal and sex reversal situations ($Sox9^{CKO}$) (Lavery et al., 2012). Studies on how the *Rspo1* executes

Abbreviations: FU, furin-like cysteine-rich domain; TSP1, thrombospondin-type 1 domain; BR, basic amino acid-rich domain; RACE, rapid-amplification of cDNA ends; UTR, untranslated region; ORF, open reading frame; ISH, in situ hybridization; DIG, digoxigenin; SD, Standard Deviation

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functions were carried out by deleting the domains. Evidences revealed that the two FU domains were primarily responsible for mediating the activation of the Wnt/β-catenin signaling pathway. Deletion of all or part of the FU domain abolished the ability to activate canonical Wnt signaling (Nam et al., 2006; Kim et al., 2008), whereas the lack of TSP1 and BR domains activated canonical Wnt signaling less effectively (Kim et al., 2008).

Wnt signaling is one of the key signal pathways that control cell proliferation, differentiation, and morphogenesis during embryogenesis and in adults. The Wnt ligands activate two major intracellular pathways known as the canonical (or β-catenin-dependent) and non-canonical (β-catenin-independent) pathways (Wodarz and Nusse, 1998; Polakis, 2000). Rspos serve as activators for canonical Wnt signaling pathway, which interact with cell-surface receptors FZD and LRP5/6 to activate Wnt/β-catenin signaling pathway. Then, β-catenin forms a complex with TCF/LEF transcription factors to regulate target gene transcription (Kazanskaya et al., 2004; Li et al., 2009). Several studies have shown that female differentiation requires the activation of the canonical Wnt/*β*-catenin signaling pathway, which is controlled by *Rspo1* (Kim et al., 2005; Chassot et al., 2008a; Tomizuka et al., 2008). The over-expression of goat *Rspo1* could rescue sex-reversal in *Rspo1*-knockout XX mice but could not perturb testis differentiation in XY or sex-reversed XX mice (Buscara et al., 2009). Besides, *Rspo1* could regulate sexual differentiation in chicken and red-eared slider turtle (Smith et al., 2008). In teleost, it has been reported that Wnt/*β*-catenin signaling pathway played an essential role in ovarian differentiation, and these studies have been implemented in *Acanthopagrus schlegeli*, *Oryzias latipes*, *Oncorhynchus mykiss* and *Danio rerio* (Nicol and Guiguen, 2011; Wu and Chang, 2009; Zhang et al., 2010; Zhou et al., 2012). In recent study, it was found that *Rspo1* signaling pathway might be involved in both ovarian and testicular development by TALEN in *Oreochromis niloticus* (Wu et al., 2016). *Rspo1* is an important regulator that activates female determination and development via the canonical Wnt/*β*-catenin signaling pathway and has been recognized by many scholars.

C. semilaevis is an economically important marine fish in China with a typical feature, that is, the female individual is significantly greater than the male. Sex-reversed individuals of *C. semilaevis* have been identified from cultured groups and share similar characteristics to males, which is not beneficial for the fishery culture. Therefore, it is essential to explore the mechanism of sex determination and gonadal development. Studies in other species reveal that *Rspo1* plays a key role in sex determination and differentiation, but specific functions of *Rspo1* need to be investigated. In this study, we assessed the temporal and spatial expression profiles of *Rspo1* in different tissues and embryo development stages. The expression profiles by temperature treatment were also examined. These results will facilitate further studies on sex control breeding in this species.

2. Materials and methods

2.1. Samples

The *C. semilaevis* specimens were purchased from Yellow Sea Aquatic Product Co. Ltd. Tissues and organs, including brain, gill, heart, intestine, kidney, liver, muscle, spleen and gonad were collected from three one-year-old male and female individuals. Samples were snap-frozen in liquid nitrogen and stored at − 80 °C. Simultaneously, unfertilized eggs and embryos at different developmental stages were collected, 30 embryos (or unfertilized eggs) were collected per developmental stage.

2.2. Temperature treatment

Embryos were incubated at 20 °C, the natural temperature for *C. semilaevis* spawning and fertilization. For this study, a batch of embryos collected from three pairs of parents was used. The fish (total length (TL) = 13 ± 2 mm) were separated into two groups after 25 days post fertilization (dpf). One group was reared at 20 °C throughout the thermosensitive period (TSP) named control group. The other group was reared at 28 °C during the entire TSP and was designated as the high-temperature group (heating group). Then, the two groups were exposed to natural temperature (20 °C) after 100 dpf. During the heating process, 100 individuals (TL = 45 ± 3 mm) were chosen randomly from the two groups after heating for 30 days. The individuals were separated into genotypic females and males using female-specific markers (Wang et al., 2013). Ten genotypic males and thirty genotypic females were chosen from each group to collect the gonads. Isolating gonads from juveniles with TL < 60 mm is difficult, so we collected the whole abdomen that contained the gonadal anlagen by removing the head along with most of the muscular dorsal and ventral parts of the fish. Further, RNA was extracted from collected samples, and reverse-transcribed to cDNA. qRT-PCR was performed to detect the expression of *Rspo1*.

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted using Trizol Reagent (Invitrogen, USA) in accordance with the manufacturer's protocol. Reverse transcription and cDNA synthesis were performed using the Reverse Transcriptase M-MLV Kit (TaKaRa, China) in accordance with the manufacturer's instructions. Quality and quantity were evaluated via 1.5% agarose gel electrophoresis and spectrophotometry using NanoPhotometer Pearl.

2.4. Molecular clone of Rspo1 gene

The core cDNA fragment of *Rspo1* was amplified with a pair of degenerate primers (*Rspon1*-Fw/Rv, Table 1), and the primers were designed using Primer Premier 5.0. 5′ and 3′ RACE were performed using SMART RACE cDNA Amplification Kit (Clontech, USA). All amplified PCR products were separated by 1.5% agarose gel electrophoresis, purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, USA), cloned using pMD18-T Vector, and sequenced.

2.5. Bioinformatics analysis and phylogenetic tree construction

Homologous nucleotide and protein sequences were confirmed through the BLAST search at NCBI. Multiple sequence alignments were conducted using ClustalX 2.1, and a phylogenetic tree was constructed using MrBayes.

2.6. Quantitative real-time PCR (qRT-PCR)

A specific primer pair (*Rspo1*-RT-PCR-Fw/Rv, Table 1) was designed based on the characteristics of *Rspo1*. *18S rRNA* and *GAPDH* were used as reference gene at the different developmental stages of the embryo and tissues, respectively. In addition, temperature treatment samples were also used for qRT-PCR, and *GAPDH* was selected as reference gene. Three biological replicates of each sample were analyzed, and each sample was run in triplicates. qRT-PCR was performed in a 20 μg solution containing 10 ng of template cDNA and SYBR Premix Ex Taq II (TaKaRa, China). The relative expression of *Rspo1* gene was calculated using the 2-ΔΔCt comparative Ct method.

2.7. In situ hybridization (ISH)

Gonad samples used for *ISH* were immediately fixed in 4% PFA overnight at 4 °C, dehydrated in gradients of increasing methanol, and stored in 100% methanol at − 20 °C. The *ISH* of *Rspo1* expression in the gonads was performed using a probe (Table 1) spanning the 5′ untranslated region (UTR) of Rspo1 cDNA. DIG-labeled RNA sense and antisense probes were synthesized using the DIG RNA Labeling Kit (SP6/T7) (Roche, Germany) in accordance with the manufacturer's