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Research paper

Characterization of F-spondin in Japanese flounder (*Paralichthys olivaceus*) and its role in the nervous system development of teleosts

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ABSTRACT

F-spondin was originally isolated from the developing embryonic floor plate of vertebrates, secreting numerous kinds of neuron-related molecules. The protein performs a positive function in nervous system development, which is attributed to the high conservation of F-spondin protein, an extracellular matrix (ECM) protein in several species. However, its precise function remains unknown, especially in marine fish. In this study, the *F-spondin* of Japanese flounder (*Paralichthys olivaceus*).
was cloned, and its expression pattern and structural characteristics were analyzed. The 2421 bp-long cDNA ORF of *PoF-spondin* was obtained and divided into 14 exons spread over 61,496 bp of the genomic sequence. Phylogenetic analysis showed that *PoF-spondin* was actually the ortholog of the human *spon1* gene and shared high identities with other teleost *spon1a* genes. Quantitative RT-PCR analysis showed that *PoF-spondin* was maternally expressed, and transcripts were present from one-cell stage to hatching stage, peaking at tailbud stage. Tissue distribution analysis indicated that *PoF-spondin* was detectable mainly in the gonads (especially in the ovary) and the brain. Whole mount in situ hybridization analysis revealed that the *PoF-spondin* transcription distributed throughout the cleavage of the ball in the early stage and expressed at a high level in the floor plate of the trunk at tailbud and pre-hatching stages. Furthermore, the expression of genes related to nervous system development (*spon1b*, *foxo3b*, and *foxj1a*) was significantly increased after the injection of *PoF-spondin* into the embryos of wild-type zebrafish. Furthermore, *PoF-spondin* significantly suppressed the expression of the chordamesoderm marker gene *ntl*, increased the expression of *otx2*/*krox20*, ectoderm mark genes, and left the expression of dorsal mesodermal marker gene *gsc* unaffected at 50% epiboly stage in zebrafish. In short, our results suggest that *PoF-spondin* functions in the development of the teleost nervous system.

1. Introduction

The immense diversity of neural cells and the specific connections in different neurons are crucial for the formation and function of neural circuits. The floor plate is one of the first differentiation cells in the embryonic nervous system, which is located at the ventral midline of the neural tube; it is implicated in the control of neural cell pattern and axonal growth in developing vertebrate nervous systems (Jessell and Dodd 1990; Schoenwolf and Smith 1990). In addition, these cells secrete numerous kinds of neuron-related molecules, such as Sonic hedgehog (Tanabe and Jessell 1996), Netrin1 (Kennedy and Tessier-Lavigne 1995), and F-spondin (Klar et al. 1992).

F-spondin was originally found in developing floor plates (Klar et al. 1992), coding the extracellular matrix (ECM) protein called F-spondin with high conservation in evolution. The F-spondin protein belongs to the spondin family (Feinstein and Klar 2004) and widely exists in numerous species, such as frogs, chicks, mice, and zebrafish (Burstyn-Cohen et al. 1999; Higashijima et al. 1997; i Altaba et al. 1993; Klar et al. 1992). In general, the F-spondin protein includes the Reelin domain at its N-terminal, a spondin domain, and six C-terminal thrombospondin repeats (TSRs) (Burstyn-Cohen et al. 1999). *In vivo*, the proteolytic process occurs in the F-spondin protein between the Reelin/spondin and TSR domains, and then the TSR domains begin plasmin cleavage between repeats 1 to 4 and 5 and 6 (Tzarfaty-Majar et al. 2001). These structural characters of *F-spondin* are designated various functions in many processes during development and adult stages. *F-spondin* primarily contributes to the outgrowth of commissural neuron growth (Zisman et al. 2007). In recent years, the F-spondin protein has been reported to be a putative ligand for amyloid precursor protein (APP) related to Alzheimer's disease (AD) (Ho and Südhof 2004; Hoe and Rebeck 2008). In mice, *F-spondin* has been proven to improve memory performance and reduce amyloid-β levels (Hafez et al. 2012). In addition, *F-spondin* performs a negative function in differentiation and migration of osteoclastic precursors (Oka et al. 2011), and

Abbreviations: ECM, extracellular matrix; TSRs, thrombospondin repeats; APP, amyloid precursor protein; AD, Alzheimer disease; ISH, in situ hybridization; DIG, digoxigenin; SEM, standard error of the mean; ORF, open reading frame; TSR1, thrombospondin type 1 repeat.

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contributes to chondrocyte terminal differentiation and mineralization (Palmer et al. 2010). However, the function of *F-spondin* is limited to certain factors, such as its concentration, and substrate-attached and soluble form (Schubert et al. 2006). Therefore, the extraction and release site of *F-spondin* may determine its availability and efficacy.

Although understanding of *F-spondin* has been obtained to a certain extent, its *in vivo* physiological function remains unknown. To our knowledge, the *F-spondin* in marine fish has been rarely reported, much less its construction and function. In our study, the *PoF-spondin* of Japanese flounder (*Paralichthys olivaceus*) was cloned, and the expression pattern and structure characteristics were analyzed. Moreover, the preliminary function of *PoF-spondin in vivo* was investigated via zebrafish (*Danio rerio*), an ideal model for vertebrate development and gene function study (Udvadia and Linney 2003). The results suggest that *PoF-spondin* functions in the development of the teleost nervous system.

2. Materials and methods

2.1. Sample preparation of Japanese flounder and zebrafish

Japanese flounder were raised at 17 °C in a commercial hatchery industry of Haiyang City, China. Artificially fertilized eggs were incubated in a hatching tank with seawater and continuous aeration. Embryos during different development stages, including one-cell, eight-cell, blastula, gastrula, neurula, somites, tailbud, heart-beating, and hatching, were collected. Six healthy Japanese flounder adults (three females and three males) were selected for collecting the brain, heart, gill, kidney, liver, muscle, and gonads (ovary and testis). Samples were frozen immediately in liquid nitrogen and then stored at −80 °C until use for RT-PCR. The samples of different stages, including morula, gastrula, tailbud and pre-hatching, were collected and fixed in 4% PFA overnight, dehydrated in a gradient increasing quality fraction methanol (30%, 50%, and 70% methanol), and then stored in 100% methanol at 4 °C.

Zebrafish were maintained in standard fish facility conditions with a 14 h:10 h light/dark cycle and fed living brine shrimp twice per day. Water temperature was maintained at 28 °C. Embryos at 50% epiboly stage were immediately fixed in 4% paraformaldehyde–PBS (4% PFA) or frozen into liquid nitrogen.

2.2. Isolation of total RNA and cDNA synthesis

Total RNA was extracted from samples using Trizol Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. A total of 1 μg RNA from each sample was reverse transcribed according to the instructions of the PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Dalian, China). The final volume was 20 μL.

2.3. Molecular cloning of PoF-spondin gene

A pair of primers, Po-FSP-cf-fw/rv, was designed (Table 1) for core fragment PCR amplification. 5′ and 3′ RACE were employed to gain the full-length cDNA of *PoF-spondin* by Smart™ RACE cDNA amplification kit (Clontech, CA, USA) according to the manufacturer's instruction. Based on the central fragment, Po-FSP3′-fw1, Po-FSP3′-fw2, Po-FSP5′-rv1, and Po-FSP5′-rv2 (Table 1) were designed for 5′ and 3′ RACE amplification by using Touchdown and Nest PCR. PCR products were identified by agarose gel electrophoresis and purified with the Gel DNA Recovery Kit (Zymoclean, CA, USA) for sequencing.

2.4. Sequence analysis

Sequence data were analyzed using the Lasergene program (version 7.0) and DNAMAN. The phylogenetic tree was constructed by MEGA (version 5.0) using Poisson correction distance based on neighbor-joining method (Saitou and Nei 1987) with 1000 bootstrap replicates

Table 1
Primers used in this study.

Primers	Sequence (5′–3′)	Usage	Annealing temperature (°C)
Po-FSP-cf-fw	GGAGTGTGGTTGGTCCCAGAAGG	Core fragment	56
Po-FSP-cf-rv	TTCCTTGCGTCGTTTCTTCTCGTC	Core fragment	
Po-FSP3′-fw1	AAGGGGCTGAGGACGAGACA	3′RACE	62
Po-FSP3′-fw2	GTCTTGTGGGAAGGGTCATAC	3′RACE	56
Po-FSP5′-rv1	CAATGCGTTCTACCACCAC	5′RACE	54
Po-FSP5′-rv2	TGGAAGAATCCTCATCACTACAT	5′RACE	56
Po-FSP-RT-fw	GGTTGGTCCCAGAAGGT	qRT-PCR	60
Po-FSP-RT-rv	AGGATCATAAAACGGGCT	qRT-PCR	
Po-18s-RT-fw	GGTAACGGGGAATCAGGGT	qRT-PCR	60
Po-18s-RT-rv	TGCCTTCCTTGGATGTGGT	qRT-PCR	
Zf-FSP1b-RT-fw	CACTCCAGAGACGTGCATTTA	qRT-PCR	60
Zf-FSP1b-RT-rv	TTTAGCATCCTCTGCCTCATC	qRT-PCR	
Zf-FSP1a-RT-fw	GGTGACCAAGAGGAGGATTATG	qRT-PCR	60
Zf-FSP1a-RT-rv	GTGGAGTGCTTTCTGTCACT	qRT-PCR	
Zf-Foxo3b-fw	TTCAAAGACAAAGGCGACAG	qRT-PCR	60
Zf-Foxo3b-fw	CGTGCAGTCTTGGTGTATTT	qRT-PCR	
Zf-Foxj1a-fw	GGAGACCTTAAACCGAGTCCC	qRT-PCR	60
Zf-Foxj1a-fw	TGGTCCTGACAAATCCTGTCC	qRT-PCR	
Zf-Gsc-fw	GAGACGACACCGAACCATTT	qRT-PCR	60
Zf-Gsc-rv	CCAAACCTCTACCTTCTCTTCAC	qRT-PCR	
Zf-Ntl-fw	GTGAAAGTCGGTGGGATTCA	qRT-PCR	60
Zf-Ntl-rv	CTTCCTTGTGGTCACTTCTCTC	qRT-PCR	
Zf-Otx2-fw	TAAAGGTGCAGGGAGTCAAAG	qRT-PCR	60
Zf-Otx2-rv	TGAAATAGGTCGGCGTTCTG	qRT-PCR	
Zf-Krox20-fw	CAGAACCAAGCAGACATGGA	qRT-PCR	60
Zf-Krox20-rv	GTTTGGCTTTGGAGAGGAGTAG	qRT-PCR	
Zf-Sox17-fw	CTCAGTACTGCGAGAACCATAC	qRT-PCR	60
Zf-Sox17-rv	GCTGGTGATGGTAGCTGAAT	qRT-PCR	
Zf-ACTB-RT-fw	GCTCCGGTATGTGCAAAGCC	qRT-PCR	60
Zf-ACTB-RT-rv	CAACCATCACTCCCTGATGTCT	qRT-PCR	
Otx2-ISH-fw	CCTGCCATCCTTCCAATAACA	ISH-probe	62
Otx2-ISH-rv	CACTCGCACACATCCTCTCTA	ISH-probe	