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Functional conservation and divergence of duplicated the suppressor of cytokine signaling 1 in blunt snout bream (*Megalobrama amblycephala*)

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ABSTRACT

The suppressor of cytokine signaling 1 (SOCS1) is an essential feedback regulator extensively involved in many different cytokine signaling pathways, such as regulation of the immune system and growth of organism. However, the molecular and functional information on *socs1* genes in freshwater fish is unclear. In the present paper, we identified and characterized the full-length closely related but distinct *socs1* genes (*socs 1a* and *−1b*) in blunt snout bream (*Megalobrama amblycephala*). The bioinformatic analysis results showed that duplicated *socs1s* shared majority conserved motifs with other vertebrates. Both *socs1a* and *−1b* mRNAs were detected throughout embryogenesis, and gradually increase and then constantly expressed after 16 hpf. Whole-mount in situ hybridization demonstrated that *socs1a* and *socs1b* mRNAs were detected in the brain at 12hpf and 24hpf, and in the notochord and brain at 36hpf. In adult fish, the *socs1a* mRNA were strongly expressed in the heart, eye, kidney, spleen and gonad, but were found to be relatively low in the intestine and liver. On the other hand, the expression of *socs1b* mRNA was significantly high in the muscle, eye and spleen, and relatively low in the intestine, liver, skin and heart. The results of hGH treatment experiment showed that *socs1a* and *1b* mRNAs were upregulated markedly in the kidney, muscle and liver. Overexpression of *socs1s* significantly inhibit the GH and JAK/STAT factor *stat3* and the inhibitory effect of SOCS1s on GH may be involved in JAK-STAT signaling pathway. These results indicate that SOCS1 plays an important role in regulating growth and development.

1. Introduction

The suppressors of cytokine signaling (SOCS) is a pleiotropic molecule, which is a key regulator of the immune system, growth, vertebrate homeostasis and development of organism (Jin et al., 2008). SOCS1 was initially found by three independent laboratories, and it is known to function as janus kinase- (Jak-) binding protein, a signal transducer and activator of transcription (stat-)inducible stat inhibitor (Endo et al., 1997; Naka et al., 1997; Ohya et al., 1997). SOCS1 proteins contain a central SH2 domain, a C-terminal SOCS box and a variable N-terminus extended SH2 subdomain (ESS), which is essential for a high-affinity receptor of the SH2 domain to the object phosphopeptides (Dalpke et al., 2008). It is essential for the binding of SOCS1 to JAK2 that the SRC homology 2 (SH2) domain and the kinase inhibitory region (KIR), while SOCS1 is as well known as pseudo-substrate, which not required for the binding (Kamizono et al., 2001; Kamura et al., 2004; Vesterlund et al., 2011).

The suppressor of cytokine signaling (SOCS) family is known as an significant feedback inhibitor of cytokine receptor signaling, and its mechanism is the same as that of other cytokines. Most cytokine receptors transduce signals by Janus kinase (JAK) family by non-covalently binding to membrane adjacent regions of receptor chains (Heim, 1999; Zhang et al., 2010). By activating JAKs in turn, SOCS1 serve as a phosphorylate signal transducer and activator of transcription (STAT) proteins (Dalpke et al., 2008; Wang and Secombes, 2008). The dimers of phosphorylated STATs subsequently translocate to the nucleus and activate gene expression (Alexander, 2002; Zhong et al., 2005). The *socs1* gene expression is able to inhibit JAK2 activity, STAT5 phosphorylation, and GH-responsive promoters (Ahmed and Farquharson, 2010). SOCS1 exerts an extensive negative regulation of the associated signal pathway through the JAK-STAT, including JNK, NF-κB and p38 (He et al., 2006; Ryo et al., 2003).

Blunt snout bream (*Megalobrama amblycephala*) is a common freshwater species in the Chinese fish polyculture systems, which yields

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more than 0.83 million tons in 2016 (Zheng et al, 2017). In this study, the full-length of duplicated *socs1* was identified in blunt snout bream, and their expression patterns were then characterized in the mature tissues and different embryonic stages. We also analyzed the effects of these two genes on GH and JAK-STAT pathway in the blunt snout bream, and explore the mechanism of GH regulation by SOCS1s.

2. Materials and methods

2.1. Experimental fish

Zygotes of the blunt snout bream were generated via artificial insemination and obtained from Genetics and Breeding Center for Blunt Snout Bream, Shanghai Ocean University, China. Fertilized eggs (250–350) were nursed in glass douche at room temperature 22 °C. Embryos were stored (at 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44 hpf) after fertilization by immersing in RNA Store (Tiangen, Shanghai, China) and stored at −80 °C until needed for gene expression experiment pattern in embryos. Adult fish (3+, ~800 g) was supplied and kept in circulating water at 24–26 °C and fed twice daily on commercial pellets for one week before experiment. These fish were anaesthetized, and tissue samples were immediately dissected, frozen in liquid nitrogen and stored at −80 °C.

2.2. GH treatments

For GH treatment, thirty juvenile fishes (1+, ~80 g) were transferred into two 50-l indoor tanks. During 3 days without feeding, these fish in two tanks were anesthetized in MS222 and then given an intraperitoneal injection of phosphate-buffered saline (PBS, control) or 0.1 mg/ml of recombinant human GH (hGH, Shanghai United Cell Biotechnology Company, China) per gram body weight in a volume of 100 µl. Their liver, kidney and muscle were immediately excised at a time of 0, 1, 3, 6, 12 h after stimulation, frozed in liquid nitrogen, and stored at −80 °C until use.

2.3. Molecular cloning of blunt snout bream socs1a and socs1b cDNAs

Total RNA was isolated from blunt snout bream using a TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. To amplify cDNA fragment of *socs1*, reverse transcription of total RNA was conducted. The primer pairs were *socs1a*-PS-F/-R for *socs1a* and *socs1b*-PS-F/-R for *socs1b* (Table 1). They were designed based on available *socs1* gene sequence from teleost fishes (grass carp, zebrafish, rainbow trout and Atlantic croaker). To obtain the 5′ and 3′ ends of sequence of *socs1*, SMART RACE cDNA amplification kit (Clontech, USA) was used following the manufacturer's protocol. The primer pairs of *socs1a*-5RACE-I/O, *socs1b*-5RACE-I/O (Table 1) were used for 5′RACE analysis. The primer pairs of *socs1a*-3RACE-I/O, *socs1b*-3RACE-I/O (Table 1) were used for 3′RACE analysis. After purification, PCR products were ligated into the cloning vector pGEM-T (Promega, USA), and transformed into *Escherichia coli* DH5α competent cells. Positive clones were sent for sequencing.

2.4. Phylogenetic analysis

The putative sequences of *socs1a* and *−1b* nucleotide which were obtained from the National Center for Biotechnology Information BLASTP search program, were compared between different species. Nucleotide sequences of *socs1s* were analyzed using BioEdit 7.0.0.1. Phylogenetic analysis was performed using coding sequences with the neighbor-joining method in MEGA 7. Gap sites in the alignment were used for the phylogenetic reconstruction, and reliability of the estimated tree was evaluated using the bootstrap method with 1000 pseudo-replications.

2.5. Whole-mount in situ hybridization

Embryos harvested used for in situ hybridization analysis were fixed at 4 °C in 4% phosphate-buffered paraformaldehyde overnight and stored at −20 °C in methanol. Fixed embryos were washed carefully with phosphate-buffered saline (PBS) containing 0.1% Tween-20, transferred to 100% methanol and stored at −20 °C for a minimum of 24 h. The preparation of RNA probes: The cDNAs amplified by the primer pairs *socs1a*-situ-F/-R for *socs1a* and *socs1b*-situ-F/-R for *socs1b* were cloned into vectors (Tiangen, Beijing). Whole-mount in situ hybridization using digoxigenin (DIG)-labeled RNA riboprobe was performed as reported previously with modification (Thisse and Thisse, 2014; Zou et al., 2009). Embryos were added to the prepared digoxin FLASH mRNA probes, were hybridized with appropriate revolutions at 60 °C hybridization oven, and excess probes were washed off with different concentrations of SSCT and incubated with anti-DIG antibodies conjugated with alkaline phosphatase (AP) insoluble precipitates. Photographs were taken with a Nikon SMZ1500 fluorescence microscope.

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

Total RNAs were isolated from embryos of different stages of development or mature tissue using TRIzol reagent (Invitrogen, USA). After DNase treatment, 700 ng of total RNA was reverse transcribed to cDNA using a Prime Script RT reagent kit (Takara, Japan). Quantitative real-time PCR (qPCR) was performed on a CFX96 Touch™ realtime PCR Detection System (BioRad, Hercules, CA, USA). After screening the three internal reference genes ofβ-actin, GAPDH and 18S, we found that 18S was the most stable expression in different developmental stages and tissues of blunt snout bream. Therefore, 18S was used to analyze the internal reference gene of SOCS1 gene expression profile. Amplification of 18S gene was carried out by 18S-qRT-F/-R (Table 1) and used as control. Primer pairs were *socs1a*-qRT-F/-R and *socs1b*-qRT-F/-R (Table 1). The qRT-PCR was implemented using SYBR Green Premix Ex Taq (TaKaRa, Japan). The qRT-PCR program includes predenaturation at 94 °C for 5 min, 40 cycles of amplification at 94 °C for 5 s, and 58 °C for 30 s (to detect fluorescence). Each experiment was repeated in triplicates. Data from the qRT-PCR are expressed as means ± SE. Statistical significance for different tissues/embryo at different stage of expression were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's post-hoc tests, and for GH treatment were analyzed using *t* test. Statistical significance was accepted at the level of $P < 0.01$.

2.7. 2.7. Construct the overexpressed vectors for SOCS1a and SOCS1b and AG490 treatment

To construct the overexpressed vectors for SOCS1a and SOCS1b, the ORF of *socs1a* was amplified using primers SOCS1a-op-F1-BamHI /SOCS1a-op -R1-XhoI and the ORF of *socs1b* was amplified using primers SOCS1b-op-F1- BamHI /SOCS1b-op-R1-XhoI. Then, the PCR products and pEGFP-N1 were treated with the corresponding *Bam*HI and *Xho*I restriction enzyme, T4 ligase was used to connect the vector to the target fragment, transformed into *Escherichia coli DH5α* competent cells. The recombinant plasmid was sent for sequencing.

The caudal fin cells of blunt snout bream (WCF) cells were transferred to six-well plates before the experiment. At approximately 80% confluence, cells were washed with PBS and incubated with antibiotic-free nutrient solution. According to the manufacturer's instructions, the overexpressed plasmid (pEGFP-OPSOCS1a and pEGFP-OPSOCS1b) was transfected with Lipofectamine 2000 Reagent, the control group was treated with the same amount of empty plasmid (pegfp-n1). After 6 h later, the cells were cultured in 10% fetal bovine serum medium. After 24 h, 50 mol/L AG490 was added to the half of cells, while the untreated AG490 cells were used as the control group. After culturing 12 h

Table 1
Primer sequences used in this study.

Prime name	Sequence (5′–3′)	Assay technique
socs1a-PS-F	CAGTGGAAGGGAATCAAGGC ATAGCGGATGAGGGAAGGC	Fragment PCR
socs1a-PS-R		
socs1b-PS-F	AACACACTTTCACCCCTTCC	Fragment PCR
socs1b-PS-R	CTCACAGGCAGTTGTTGAATAC	
socs1a-5RACE-I	AGCATTGAGGTGGTCTTCGC	socs1a 5′RACE
socs1a-5RACE-O	TCGCTCTGCCGACTGTCC	
socs1a-3RACE-I	CCAATCCTCAAAGACTTCCTCCTGTGCCGCAGACGGATTAT	socs1a 3′RACE
socs1a-3RACE-O		
socs1b-5RACE-I	CCCAAGAGGGAGTTCGGC	socs1b 5′RACE
socs1b-5RACE-O	GAGCCCGATAGCTGAGAGTGA	
socs1b-3RACE-I	GTATGCCGTATCGGGGAGA	socs1b 3′RACE
socs1b-3RACE-O	GAGGATTCAGTTTAGCGG	
socs1a-situ-F	CAGGCAGCCATTTTACAGACAC	WISH-socs1a
socs1a-situ- R	CAGTATGATTCCTGCGTCCTAA	
socs1b-situ-F	TCGGGACCAGCAGGAATGT	WISH-socs1b
socs1b-situ-R	TGTTCCAGTTGGGTCAATGAAG	
socs1a-qRT-F	TGACCTTCCTGGTACGGT	qRT-PCR
socs1a-qRT-R	GGGTTCTGTGCTCACTTGGG	
socs1b-qRT-F	ATGGAGGAATGGTATGAGTGGG	qRT-PCR
socs1b-qRT-R	TCAAGTAATTCACAGCCTGGGT	
SOCS1a-op-F1-BamHI	CGCGGATCCTAGGATGGTGGCGCACAGTAC	Recombinant protein vector construction
SOCS1a-op-R1-XhoI	CCGCTCGAGTGAGTATCGTCTTCATAGCGG	Recombinant protein vector construction
SOCS1b-op-F1-BamHI	CGCGGATCCGGAGGAATGGTATGAGTGGG	Recombinant protein vector construction
SOCS1b-op-R1-XhoI	CCGCTCGAGCCAGTTGGGTCAATGAAGA	Recombinant protein vector construction
stat3-qRT-F	CGCATCACTGTTGGTTCGG	qRT-PCR
stat3-qRT-R	GCAGCCATTAGAGGTTCACG	
18S-qRT-F	ACCGCAGCTAGGAATAATGG	qRT-PCR
18S-qRT-R	GGTCGGAACTACGACGGTAT	qRT-PCR
β-actin-F	ACCCACACCGTGCCCATCTA	qRT-PCR
β-actin-R	CGGACAATTTCTCTTTCGGCTG	qRT-PCR
GAPH-F	YGCYGGCATCTCCCTCAA	
GAPH-R	TCAGCAACAGRTGGCTGTAG	
GH-F	TGTCGGTGGTGCTGGTT	
GH-R	CGCCTCAATGGAGTCAGAGT	

under 28 °C, collecting cells for follow-up study. Each experiment was performed in triplicate.

2.8. Effects of SOCS1a and SOCS1b overexpression on GH and JAK-stat pathway

After 24 h, overexpression of the plasmids in WCF cells (refer section 2.7), the experimental group was treated with AG490 for 12 h, and the cells were then collected for follow-up study. The blunt snout bream-specific GH antibody was prepared by Nanjing Jinsirui Biotechnology Co., Ltd. The principle of GH measurement was based on the sandwich technology of biotin double antibody. The standard curve was drawn with the concentration of the standard substance as the abscissa and the value of D_{450} as the ordinate, according to the regression equation of standard curve, the sample concentration was calculated. The expressions of JAK-STAT pathway factor STAT3 and GH were examined by real-time PCR. The primers are listed in Table 1. The experiment was performed in triplicate.

3. Results

3.1. Identification of blunt snout bream duplicate socs1cDNA

Using 3′- and 5′-RACE, the full-length cDNA sequences of *socs1a* (GenBank Acc. No. KX902196) and *socs1b* (GenBank Acc. No. KX902197) genes were obtained in blunt snout bream. The *socs1a* encodes 201-aa residues and *socs1b* encodes 197-aa residues (Fig. 1). Sequence analysis revealed that the peptides SOCS1a protein of blunt snout bream shares high sequence identity with those of grass carp (97%) and zebrafish (85%), respectively. The SOCS1b protein of blunt snout bream also share high identity with zebrafish (81%). Multiple alignments suggested the high evolutionary conservation in their coding sequences. The deduced protein sequences of blunt snout bream SOCS1a and SOCS1b were discovered to be of high similarity to the respective targets in other vertebrates, particularly in SH2 domain and SOCS box (Fig. 1).

The relative molecular mass of SOCS1a was about 23000 and PI was 9.37 by ExPASy prediction. Similarly, the relative molecular mass of SOCS1b was about 24300 and PI was 10.98. Protein modeling also showed that the spatial arrangement of α-helical and β-sheet structures in blunt snout bream SOCS1s, which forms the basis of SH2 domain and SOCS box, were highly comparable to their human counterparts (Fig. 2). Moreover, phylogenetic analysis demonstrated that both teleost *socs1a* and *socs1b* grouped well with high bootstrap support (Fig. 3). These results suggest that *socs1a* and *socs1b* maybe derived from fish-specific genome duplication.

3.2. Expression patterns of socs1a and socs1b mRNAs

Both *socs1a* and *−1b* mRNAs were detected in most adult tissues in blunt snout bream. As shown in Fig. 4, *socs1a* mRNAs were highly expressed in the heart, eye, kidney, spleen and gonad, while scarcely expressed in the intestine and liver. The expression level of *socs1b* was high in the muscle, eye and spleen, and relatively low in the intestine, liver, skin and heart. The temporal and spatial expression patterns of blunt snout bream *socs1a* and *−1b* mRNA were examined during embryogenesis.

Both *socs1a* and *−1b* mRNAs were detected throughout

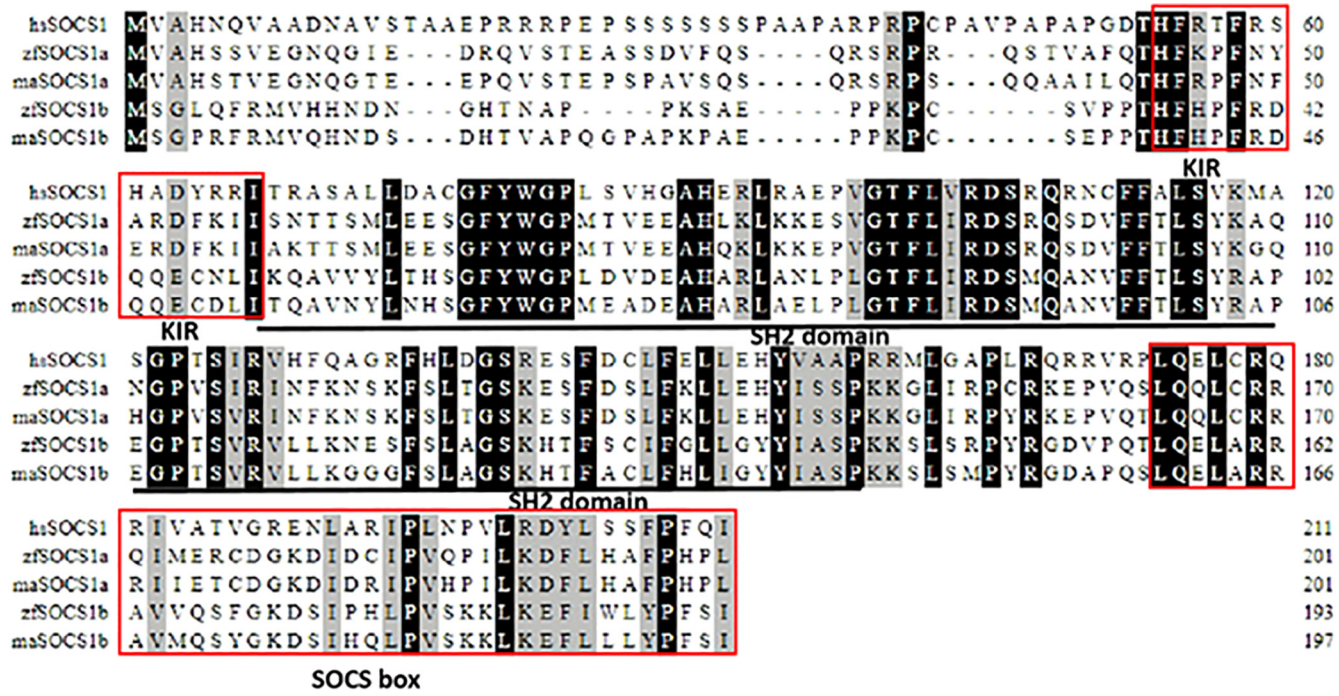

Fig. 1. Alignment of deduced blunt snout bream (ma) SOCS1a and SOCS1b amino acid sequences with zebrafish (zf) and human (hs) homologs. Amino acids are designated using single-letter codes. The SH2 domain is indicated by a solid black line, and the SOCS1s signature (SOCS box and KIR) is indicated by red frame.

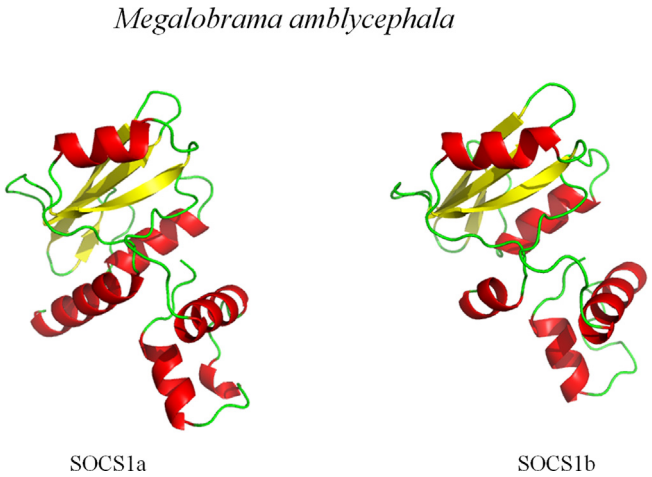

Fig. 2. Protein modeling of blunt snout bream SOCS1s are predicted by the Phyre2. There are α-helixes (in red) and β-sheets (in yellow) forming the SH2 domain and SOCS box within the SOCS1s protein in blunt snout bream.

embryogenesis (Fig. 4). The expression level of *socs1a* was initially low but increased from zygotes to 4 hpf, with a sudden build up to a moderate level at 5–15 hpf, steady fluctuated between 16hpf to 24hpf, and finally increased to a maximum level. The expression of *socs1b* has a similar trend with *socs1a* during embryogenesis (Fig. 4). Compared with the controls, whole-mount in situ hybridization results showed that *socs1a* and *socs1b* mRNAs were detected in the brain at 12hpf and 24hpf (Fig. 5d, e, g, h), notochord and brain at 36hpf (Fig. 5f, i).

3.3. Transcriptional responses of socs1a and socs1b in juvenile fish under GH treatment

To determine the divergent expression patterns of duplicated *socs1* genes response to GH, juvenile blunt snout bream was given an injection of recombinant hGH. The relative amounts of *socs1a* and *−1b* mRNAs in the liver, kidney, and muscle were estimated by quantitative real-time PCR. The *socs1a* and *socs1b* mRNAs were transiently up-regulated by recombinant GH treatments until 3 h (Fig. 6) in the liver, then decreased to original value. The mRNAs show the same trend in the kidney and muscle. Overall the mRNA level increased by about 10-fold, except for the SOCS1b mRNA in the kidney which showed only 5-fold increase.

3.4. Efficiency of the overexpressed plasmids after transfection and their influence on GH expression

To evaluate the effects of overexpressed SOCS1a and SOCS1b on related GH and JAK-stat pathway, we used the real-time PCR results to establish the expression profiles. The overexpression efficiency showed that the expression levels of *socs1a* and *socs1b* were significantly up-regulated in the different experimental groups ($p < 0.01$), the expression of these were increased about 300 folds (Fig. 7). To investigate the effect of SOCS1a and SOCS1b overexpression on GH, we detected GH synthesis level in cells by RT-qPCR and GH ELISA kit. Our results showed that GH expression was significantly inhibited after SOCS1s overexpressed compared with transfected the empty vector (Fig. 8), whether protein level or molecular level. Interestingly, using AG490 to stimulate cells can also inhibit GH expression. When AG490 acted on WCF cells overexpressing SOCS1s for 12 h, the GH mRNA and peptide were significantly reduced, even resulting in a superposition effect (Fig. 8).

3.5. Effects of SOCS1a and SOCS1b overexpression on JAK-STAT factor stat3

To further detect the role of SOCS1 in JAK-STAT, the absolute amounts of stat3 mRNA in cell were estimated by quantitative real-time PCR. Our results showed that the stat3 mRNA was also inhibited strongly by SOCS1a and SOCS1b, as similar with AG490 (Fig. 9). After AG490 acted on WCF cells overexpressing SOCS1s for12h, the expression level of stat3 mRNA was significantly lower than that of the control group. Compared with AG490 and the transfected empty plasmid group, the expression level of stat3 mRNA was

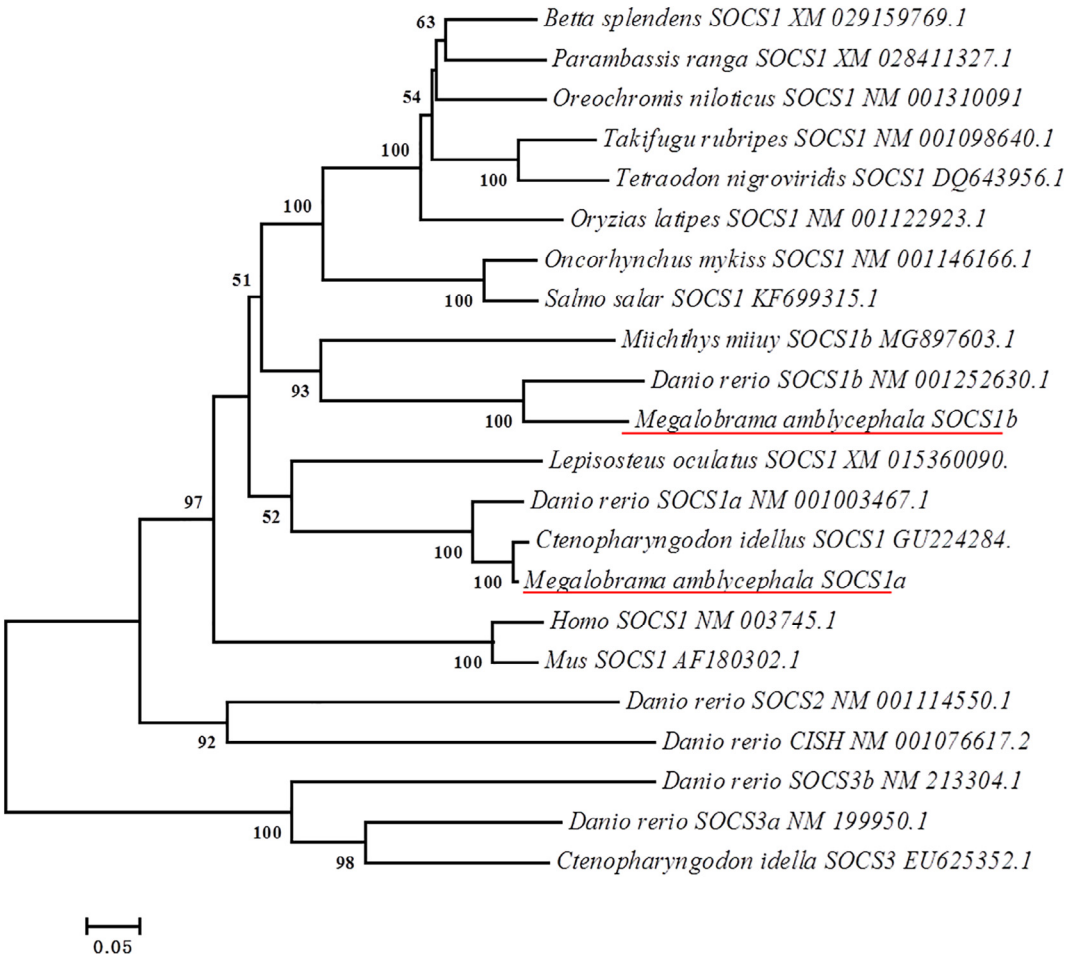

Fig 3. Phylogenetic analysis of vertebrate SOCS1 putative peptide. Accession numbers of sequences retrieved from GenBank and European Molecular Biology Laboratory are shown. The tree was constructed by the neighbor-joining method using MEGA 7 software. Objective gene has been highlighted in red line.

significantly lower (Fig. 9).

4. Discussion

In this study, we isolated and characterized two distinct *socs1* genes in blunt snout bream, a commercially highly-productive aquaculture fish species in China. The overall sequence identity between blunt snout bream SOCS1a and SOCS1b mature peptides was only 49%, and they are located in different chromosomes after blast to the draft genome of blunt snout bream (Liu et al., 2017). Additionally, the two teleost SOCS1s cluster well with their orthologs in other teleost species, suggesting that they are encoded by homologous genes. These results suggest that *socs1a* and *socs1b* should be derived from fish-specific genome duplication (Jin et al., 2007).

The bioinformatic analysis showed that SOCS1s in blunt snout bream have the same domains with SOCS1 from other species. The mature peptides of both SOCS1s homologs of blunt snout bream is composed of a central SH2-domain, which is involved in substrate binding through the recognition of cognate phosphotyrosine motifs, additional functional motifs KIR and ESS in the N-terminal region, and a conserved C-terminal domain known as the SOCS box (Delgado-Ortega et al., 2013; Piessevaux et al., 2008). A unique amino acid pattern is found at or near the KIR domain of fish SOCS1 molecules, which is considered to be a significant difference between the mammals and fish sequences (Wang and Secombes, 2008). This insertion is also existed in the blunt snout bream SOCS1. However, the impact of the fish-specific insertion is unclear and further investigation is needed to unearth its function.

During embryogenesis, both *socs1a* and −*1b* mRNAs of blunt snout bream showed increased expression from the zygote to 44 hpf larval stage. Whole-mount in situ hybridization showed that SOCS1s were mainly transcribed in the brain at different embryonic development stages, however, *socs1a* and −*1b* were detected in the notochord at 36 hpf. Zebrafish *socs1a* mRNAs are transcribed in the somites during 14–19 somites, and in the myotome during 20–25 somites (Thisse and Thisse, 2014). There is hardly any report that describe the transcriptional or translational information of *socs1b* at embryonic stages in other fish species. Moreover, analysis of SOCS1 null mice has shown that this protein is indispensable for normal postnatal development. At birth, there was no obvious difference between *socs1*-deficient mice and their normal littermates, but within 10 days the SOCS1 null mice exhibited stunted growth and died within the first 3 weeks of life (Alexander et al., 1999). It is suggested that the difference of embryonic expressions of *socs1s* between blunt snout bream and zebrafish is due to physiological adaptation in different teleost species. Our results suggest that *socs1a* and *socs1b* genes in blunt snout bream may play overlapping biological roles in the development of cephalic nervous system at transcriptional level.

Blunt snout bream socs1a and −1b mRNA are also ubiquitously detected in numerous tissues, as similarly observed in mammals and other teleost fish species (Jiang et al., 2016; Yao et al., 2015). Tetraodon socs1 is highly expressed in the liver, gonad, and spleen tissues, and moderately expressed in the intestine, gills, and kidney, whilst in trout, socs1 is most highly expressed in the intestine followed by spleen, head, kidney, and gills (Wang and Secombes, 2008). The highest transcription level of socs1a mRNA was detected in the heart, eye,

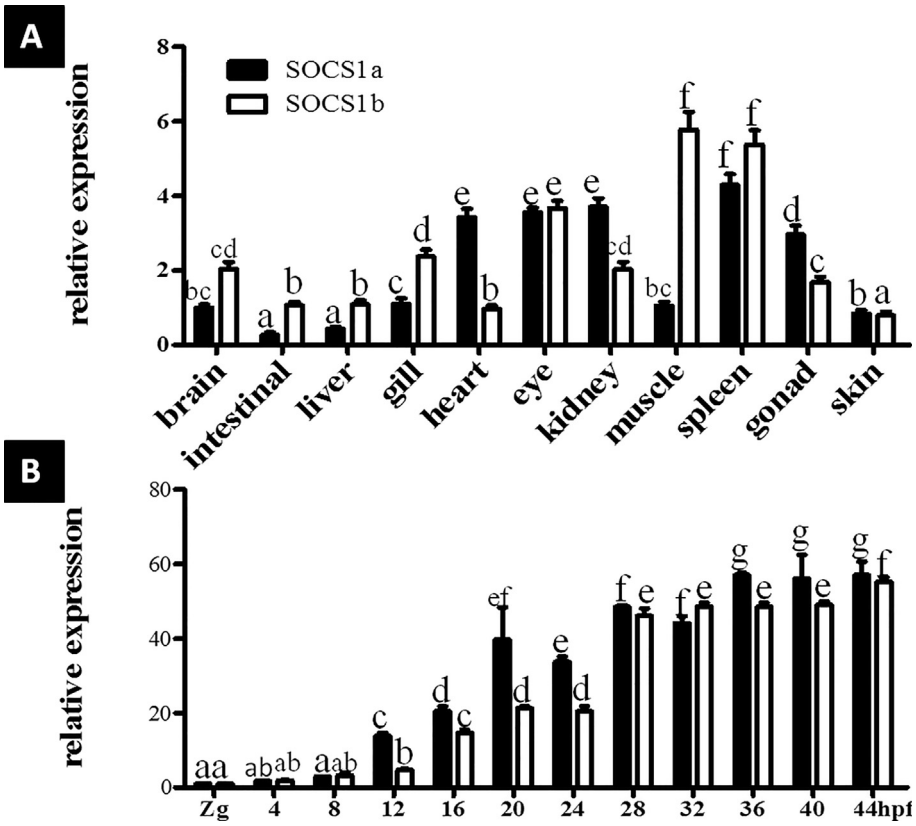

Fig. 4. Expression of *socs1a /-1b* mRNAs in embryos and adult tissues of blunt snout bream. The relative expression was analyzed by *q*RT-PCR. The relative expression of each particular gene transcript was calculated based on the standard curve, the 18S gene expression served as the reference genes. The results are given as mean ± SE for separate fish (n = 5). Differences among groups were analyzed using ANOVA followed by post hov test. Columns marked with different letters are significantly different $P < 0.01$.Zg, zygote; hpf, hours post-fertilization.

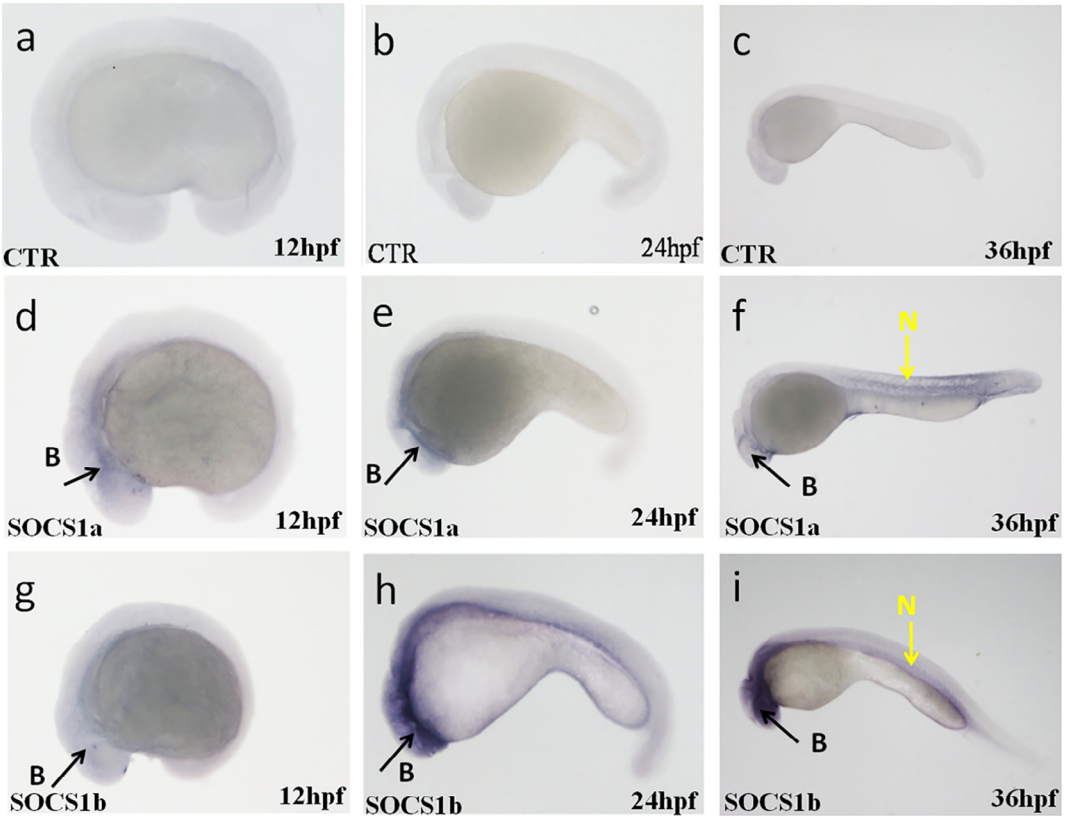

Fig. 5. Whole-mount embryo in situ hybridization analysis at different embryonic stages of *socs1a/−1b* mRNA. SOCS1a sense probe (a, b and c), *socs1a* antisense probe (d, e and f), and *socs1b* sense probe (g, h and i) were used. All embryos are viewed laterally with the head to the left. B, brain; N, notochord. Scale bar = 500 μm.

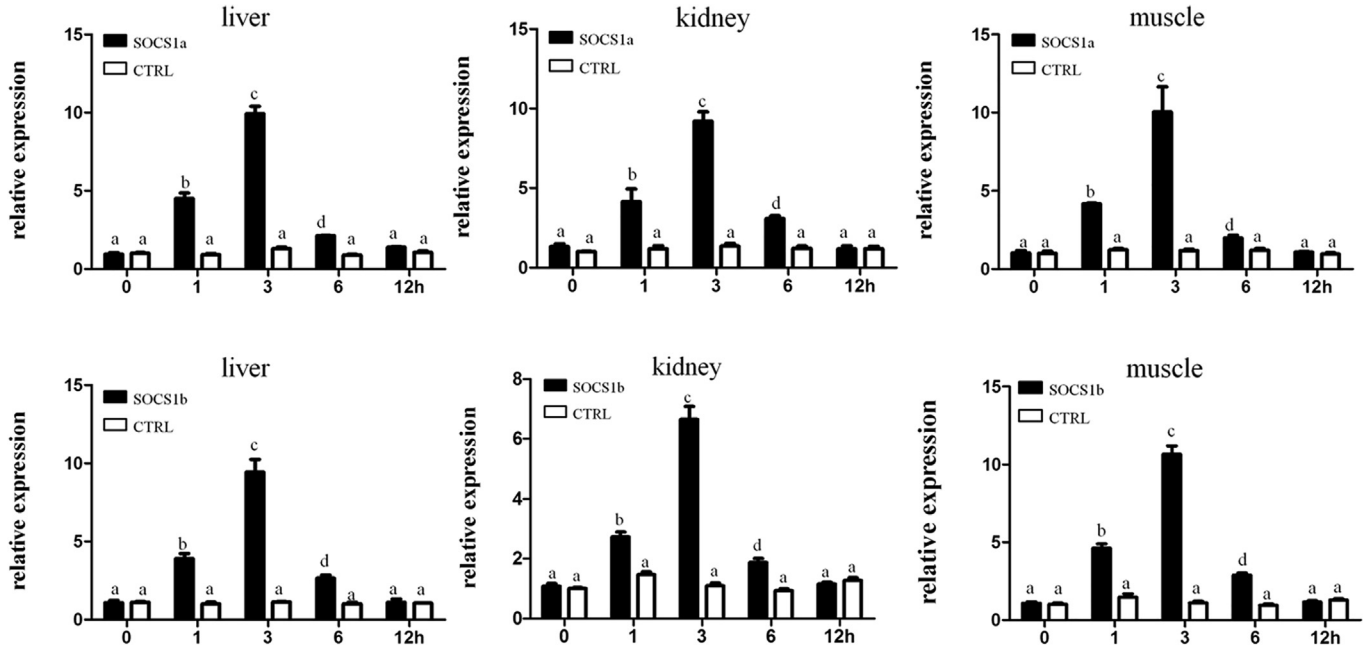

Fig. 6. Regulation of duplicated *socs1a /-1b* mRNAs in liver, kidney, muscle of juvenile blunt snout bream by hGH treatment. Fish were injected with 0.1 mg/ml of recombinant human GH per gram body weight in a volume of 100 μl or with PBS as control (CTR). 18S gene expression served as the reference genes. The results are given as mean ± SE for separate fish (n = 3). Columns marked with different letters are significantly different $P < 0.01$.

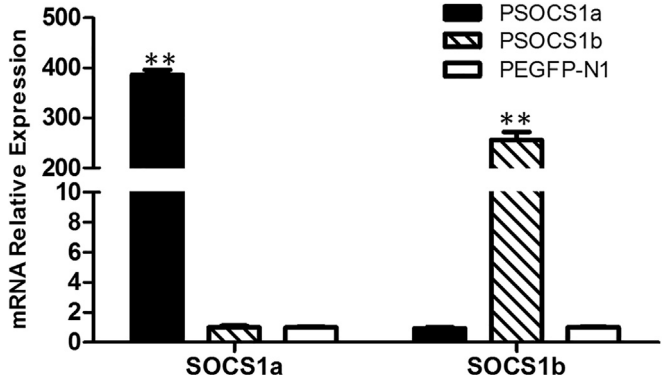

Fig. 7. Quantitative profiles of SOCS1a and SOCS1b overexpression efficiency in WCF cells. The levels of the overexpressed plasmids in different experimental groups were detected. Data represent mean ± SE (n = 3). Asterisk indicates a significant difference. * $P < 0.05$ and ** $P < 0.01$.

kidney, spleen and gonad blunt snout bream, while scarcely expressed in the intestine and liver. Meanwhile, socs1b mRNA was highly expressed in the muscle, eye and spleen, and relatively low in the intestine, liver, skin and heart. The socs1s high expression and low expression levels in different tissues may hint at the functional differences between the two paralogs. Moreover, the gene expression analysis in various tissues by RT-qPCR showed that blunt snout bream SOCS1s were highly expressed in immune relevant tissues such as spleen and kidney. These expression characteristics indicate that the *socs1* gene may have a role in host immune responses of teleost.

Growth hormone (GH) can induce cells to produce SOCS1 protein, and SOCS1 signaling inhibits downstream signaling of GHR activation in mammal (Pass et al., 2009). Generally, SOCS protein is maintained at low levels *in vivo*, but their expression can be immediately induced by treatment with various cytokines or growth factors, including GH (Fujimoto and Naka, 2003). Blunt snout bream *socs1s* mRNA were strongly induced in the liver, kidney and muscle by hGH. Consistent studies have found that signal transduction of GH induced SOCS1 mRNA expression in carp hepatocytes, with parallel time,

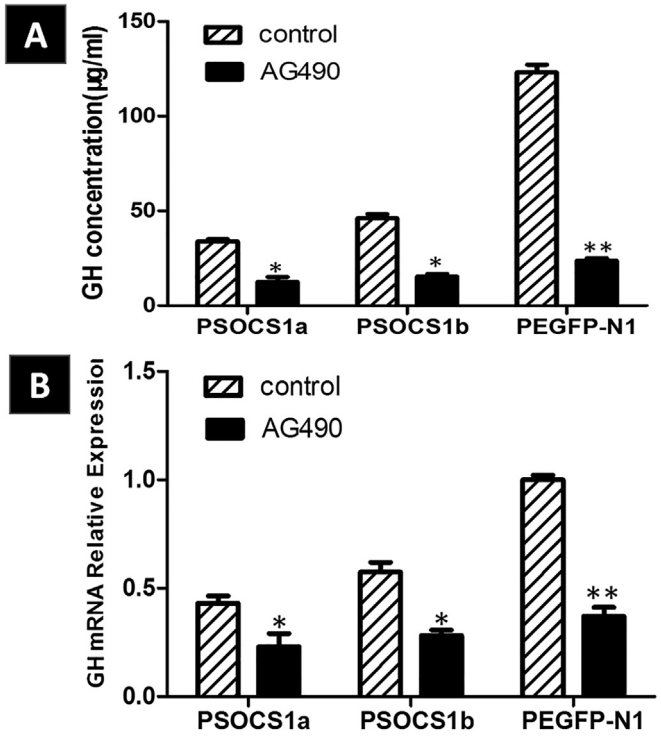

Fig. 8. Effects of SOCS1a and SOCS1b overexpression on GH expression level in WCF cells. The WCF cells were transfected with pEGFP-OPSOCS1a, pEGFP-OP SOCS1b or pEGFP-N1. After 24 h, WCF cells were stimulated with 50 mol/L AG490, while the untreated AG490 cells were used as the control group. The relative expression of GH was analyzed by qRT-PCR. The GH protein content was measured by ELISA method. Asterisk indicates a significant difference. Data represent mean ± SE (n = 3).* P < 0.05 and ** P < 0.01.

phosphorylation of JAK2, STATs, MAPK, PI3K, and protein kinase B (Akt) increase likewise (Jiang et al., 2016). In addition, our result show that SOCS1a and SOCS1b could inhibit GH. Previous study also showed that GH signaling is reduced by the action of SOCS1 proteins

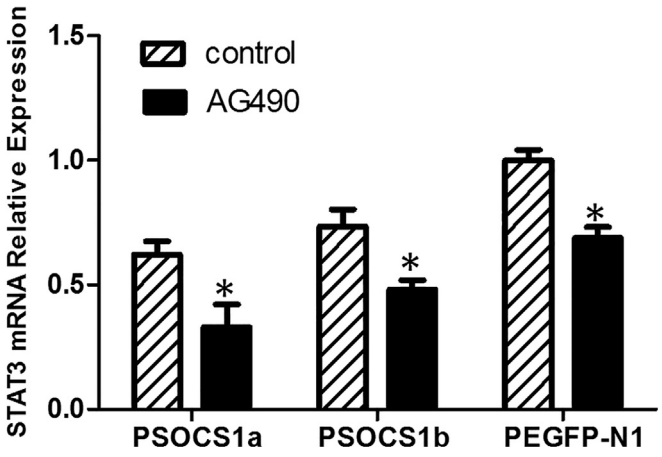

Fig. 9. Effects of SOCS1a and SOCS1b overexpression on stat3 mRNA. Data represent mean ± SE (n = 3). Cultured WCF cells were transfected with pEGFP-OPSOCS1a, pEGFP-OP SOCS1b or pEGFP-N1,and expression of stat3 was compared with that in the AG490 groups. Asterisk indicates a significant difference. * $P < 0.05$.

in homozygotic GH-transgenic zebrafish. These results showed that the action mode of growth hormone seemed to be mediated by blunt snout bream SOCS1s signal, reversely, SOCS1s could inhibit the transmission of GH signal.

To explore the mechanism of GH signal mediated by SOCS1s, we used AG490 to treat overexpressed cells as experimental group. Our results show that AG490 can also inhibit GH and produce superposition effect with overexpression of SOCS1s. It is well-known that AG490, as a synthetic lipid derivative, can specifically inhibit the activity of Januskinase 2 (JAK2), thereby blocking signal transduction. It is an artificial synthesis of specific signal transduction blocker by JAK-STATs pathway (Now and Yoo, 2016). As a member of SOCS family, SOCS1 is an important regulator to regulate JAK-STAT pathway. SOCS1 bind directly to JAK and inhibit its kinetic activity, while other members of this family compete with STATs for the anchorage sites present in the receptor (Kopchick and Andry, 2000). Our result also show that SOCS1 can inhibit the JAK-STAT pathway in blunt snout bream. We assume that the inhibitory effect of SOCS1s on GH may be involved in JAK-STAT signaling pathway.

In summary, we isolated duplicated *socs1* genes from blunt snout bream and the bioinformatics were analyzed in detail. Although these genes encode peptides that share a high degree of sequence similarity, they were expressed in multiple tissues or during different embryonic stages with different expression patterns. Both *socs1a* and *−1b* were up-regulated by hGH in the kidney, liver and muscle of juvenile fish. Inhibition of GH and JAK-STAT pathway by SOCS1a and SOCS1b were identified using overexpression experiments, meanwhile, the inhibitory effect of SOCS1s on GH may be involved in JAK-STAT signaling pathway. These findings suggest that the two *socs1s* play important roles in regulating embryonic development and tissue growth in blunt snout bream, but additional studies are needed to elucidate the underlying mechanisms.

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