

Type II SOCS as a feedback repressor for GH-induced Igf1 expression in carp hepatocytes

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Abstract

Type II suppressor of cytokine signaling (SOCS) serve as feedback repressors for cytokines and are known to inhibit growth hormone (GH) actions. However, direct evidence for SOCS modulation of GH-induced insulin-like growth factor 1 (Igf1) expression is lacking, and the post-receptor signaling for SOCS expression at the hepatic level is still unclear. To shed light on the comparative aspects of SOCS in GH functions, grass carp was used as a model to study the role of type II SOCS in GH-induced Igf1 expression. Structural identity of type II SOCS, Socs1–3 and cytokine-inducible SH2-containing protein (Cish), was established in grass carp by 5'/3'-RACE, and their expression at both transcript and protein levels were confirmed in the liver by RT-PCR and LC/MS/MS respectively. In carp hepatocytes, GH treatment induced rapid phosphorylation of JAK₂, STATs, MAPK, PI3K, and protein kinase B (Akt) with parallel rises in *socs1–3* and *cish* mRNA levels, and these stimulatory effects on type II SOCS were shown to occur before the gradual loss of *igf1* gene expression caused by prolonged exposure of GH. Furthermore, GH-induced type II SOCS gene expression could be negated by inhibiting JAK₂, STATs, MEK_{1/2}, P₃₈^{MAPK}, PI3K, and/or Akt respectively. In CHO cells transfected with carp GH receptor, over-expression of these newly cloned type II SOCS not only suppressed JAK₂/STAT₅ signaling with GH treatment but also inhibited GH-induced grass carp Igf1 promoter activity. These results, taken together, suggest that type II SOCS could be induced by GH in the carp liver via JAK₂/STATs, MAPK, and PI3K/Akt cascades and serve as feedback repressors for GH signaling and induction of *igf1* gene expression.

Key Words

- ▶ suppressor of cytokine signaling
- ▶ growth hormone
- ▶ insulin-like growth factor
- ▶ intracellular feedback
- ▶ signal transduction
- ▶ hepatocytes
- ▶ grass carp

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Introduction

The members of suppressor of cytokine signaling (SOCS) are key regulators of innate (Strebosky *et al.* 2012) and adaptive immune systems (Tamiya *et al.* 2011). In mammals, at least eight members of SOCS family, including Socs1–7 and cytokine-inducible SH2-containing protein (Cish), have been identified (Jin *et al.* 2008). All of them share the common features with a SH2 domain in the central core followed by a

highly conserved SOCS box in the C-terminus. Socs1 and Socs3 also have the additional kinase inhibitory region (KIR) and extended SH2 motif (ESS) upstream of SH2 domain, which are absent in other SOCS members (Piessevaux *et al.* 2008b). SOCS proteins are widely expressed at tissue level (Delgado-Ortega *et al.* 2011), and their production can be induced by microbial/viral infection or by cytokine treatment (Hebenstreit

et al. 2003, Cheng *et al.* 2009). At cellular level, SOCS expression is well documented to serve as the feedback repressor for cytokine signaling via inhibition of JAK/STAT pathway (Crocker *et al.* 2008) and plays a protective role in preventing hyperactivation of immune system, which can lead to autoimmune/inflammatory disorders (Liang *et al.* 2014). In mammals, SOCS proteins can also act as negative modulators for growth hormone (GH) actions (Birzniece *et al.* 2009) and contribute to the cross talk between immune system and somatotrophic axis (Ahmed & Farquharson 2010). In rodents, GH treatment is known to induce Socs2, Socs3, and Cish expression in the liver, both *in vivo* and *in vitro* (Adams *et al.* 1998, Tollet-Egnell *et al.* 1999). Given that (i) JAK₂/STAT₅ pathway is a key component of GH signaling (Vijayakumar *et al.* 2010); (ii) Socs1–3 and Cish expression can inhibit JAK₂ activity, STAT₅ phosphorylation, and GH-responsive promoters (Greenhalgh & Alexander 2004, Greenhalgh *et al.* 2005); and (iii) Socs2 knockout can lead to gigantism in mouse model (Metcalf *et al.* 2000) and the growth enhancement is dependent on STAT₅ (Greenhalgh *et al.* 2002a), SOCS proteins, especially Socs2, are proposed to be the feedback repressors for GH signaling, which may contribute to the pulsatile actions of GH on Igf1 production at the hepatic level (Choi & Waxman 2000).

Of note, conflicting results against the role of SOCS as a 'signal terminator' for GH actions have also been reported. In mice, SOCS2 over-expression could induce gigantism with accelerated growth as in the case of Socs2 knockout (Greenhalgh *et al.* 2002b). Besides, serum levels and hepatic content of Igf1 in Socs2-null mice were found to be similar to those of wild-type littermates (Metcalf *et al.* 2000). In the same study, the role of SOCS2 in GH-induced IGF1 production and body growth was also questioned, as most of the organs in Socs2-null mice were enlarged without parallel rise in IGF1 expression. In transfection studies with cell lines (e.g., HEK293 cells), SOCS2 over-expression could induce dual effects on GH signaling, being inhibitory at low doses but stimulatory at high doses (Favre *et al.* 1999). In 3T3-F442A adipocytes, however, SOCS2 and CISH expressions had no effects on GH-induced promoter activity in GH-responsive genes (e.g., Spi-2.1) (Adams *et al.* 1998). Although the mechanisms for SOCS inhibition of JAK/STAT pathway through KIR inhibition of Janus kinase (JAK) activity, blocking signal transducer and activator of transcription (STAT) recruitment by SH2 domain, and decaying activated receptor complex via SOCS box have been documented (Greenhalgh & Alexander 2004, Greenhalgh *et al.* 2005), except for a single report in C2C12 myoblasts, suggesting

that mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinase (PI3K) activation may inhibit GH-induced SOCS2 and CISH expression (Sadowski *et al.* 2001), not much is known for the post-receptor signaling for GH-induced SOCS expression, especially in the liver with relevance to IGF1 production. To date, the direct evidence for SOCS modulation of IGF1 expression induced by GH is still lacking.

In recent years, SOCS proteins have been cloned in fish models, e.g., trout (Wang *et al.* 2010, Maehr *et al.* 2014), salmon (Skjesol *et al.* 2014), catfish (Yao *et al.* 2015), turbot (Zhang *et al.* 2011), and zebrafish (Wang *et al.* 2011). Data mining of genome databases not only reveals the orthologues of mammalian SOCS but also reveals new paralogues of Cish, Socs3, and Socs5 unique to fish species (Jin *et al.* 2008, Wang *et al.* 2011), which are presumably the result of fish-specific 3R genome duplication (Kassahn *et al.* 2009). Based on the phylogenetic analysis of the new sequences with those reported in invertebrates, two families of SOCS, type I and type II SOCS, have been proposed (Jin *et al.* 2008). Type I SOCS (including Socs4–7, Socs5b, and Socs9 found in both vertebrates and invertebrates) represents the lineage closely related to the ancestral SOCS, whereas type II SOCS (including Socs1–5, Cish, and the CISH homologue Socs9) can be identified only in vertebrates and believed to be derived from type I SOCS by gene duplication (Jin *et al.* 2007, 2008). Functional studies in fish models also reveal that type II SOCS (e.g., Socs1–3 and Cish) are highly responsive to microbial infection or exposure to endotoxin/cytokines, whereas the corresponding responses for type I SOCS are marginal/undetectable (Wang *et al.* 2010, 2011). For the role of SOCS in GH signaling, notable rises of *socs1* and *socs3* mRNA could be found in the liver of zebrafish with GH transgene. However, Igf1 expression was reduced with no effects on body growth or JAK₂/STAT₅ signaling components (Studzinski *et al.* 2009). Recently, elevations of *socs1* and *socs2* transcripts have also been reported in trout liver during cortisol inhibition of GH-induced Igf1 expression (Philip *et al.* 2015), suggesting that SOCS proteins may play a role in Igf1 regulation by GH in fish model.

To shed light on the role of SOCS in GH functions in fish model, type II SOCS, including Socs1–3 and Cish, were cloned in grass carp and their gene copy number and tissue expression, especially in the liver, were determined. Using carp hepatocytes, the possible involvement of JAK/STAT, MAPK, and PI3K/Akt cascades in GH-induced type II SOCS expression was examined. Using transfection

studies in CHO cells, the effects of over-expression of grass carp type II SOCS on (i) JAK₂/STAT₅ signaling coupled to carp GH receptor (GHR) and (ii) GH-induced grass carp *Igf1* promoter activity were also investigated. Our studies for the first time elucidate the post-receptor signaling for GH-induced type II SOCS expression at the hepatic level and provide evidence that *Socs1–3* and *Cish* can act as feedback repressors for GH signaling and induction of *Igf1* gene transcription in the carp liver.

Materials and methods

Animal and test substances

One-year-old grass carp (*Ctenopharyngodon idellus*) with body weight 2.0–2.5 kg were obtained from local markets, housed in 250L aquaria at 20°C under 12h light:12h darkness photoperiod, and fed to satiation twice daily for at least 7 days before experimentation. As the fish at this stage were pre-pubertal and sexual dimorphism was not apparent, grass carp of mixed sexes were used for tissue sampling and hepatocyte preparation. During the process, the fish was killed by MS222 anesthesia (0.05%; Sigma) followed by spinosectomy according to the protocol approved for animal use at the University of Hong Kong. Test substances, including porcine GH, IL-1 β , TNF α , lipopolysaccharide (LPS), JAK₂ inhibitor 1,2,3,4,5,6-hexabromocyclohexane (HEX), STAT1 inhibitor fludarabine (FA), STAT₃ inhibitor ethyl-1-(cyano-2,3,5,6-tetrafluorophenyl)-6,7,8-trifluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (ETDDC), STAT₅

inhibitor N1-(11H-Indolo[3,2-c]quinolin-6-yl)-N2,N2-dimethylethane-1,2-diamine (IQDMA), P₃₈^{MAPK} inhibitor PD169316, MEK_{1/2} inhibitor U0126, PI3K inhibitor LY294002, and protein kinase B (Akt) inhibitor 1L6-hydroxymethyl-chiro-inositol-2-O-methyl-3-O-octadecyl-*sn*-glycero carbonate (HIMOC), were obtained from Calbiochem. For transfection studies, the JAK₂/STAT₅-responsive 8 \times GHRE-LUC reporter and STAT₅ expression vector STAT₅.pcDNA were generous gifts from Peter S Rotwein (Oregon Health & Science University, Portland, OR, USA).

Molecular cloning, copy number, and tissue expression of *Socs1–3* and *Cish*

For molecular cloning of type II SOCS, total RNA was extracted from the carp liver and subjected to 5'/3'-RACE using primers designed based on zebrafish *Socs1–3* and *Cish*. Sequence alignment, 3D protein modeling, and phylogenetic analysis were conducted using CLUSTAL-W, SWISS-MODEL, and MEGA 6.0 respectively. To deduce gene copy number of *Socs1–3* and *Cish*, Southern blot was performed in genomic DNA isolated from carp blood (Lin *et al.* 2015). For tissue expression of type II SOCS, northern blot was conducted in total RNA prepared from the carp liver and pituitary, whereas RT-PCR was examined in selected tissues/brain areas using primers for respective gene targets with PCR conditions described in Table 1. Using LC/MS/MS, protein expression of *Socs1–3* and *Cish* was also evaluated in the carp liver using a SCIEX TripleTOF-5600 system (AB SCIEX, Concord, ON, Canada) as described previously (Wong *et al.* 2013).

Table 1 Primer sequences and PCR conditions for RT-PCR of type II SOCS.

Gene target/accession No Primer sequence	PCR condition				Product size
	Denaturing	Annealing	Extension	Cycle no.	
<i>socs1</i> /GU224284 F: 5'-CAGCAGGACCCATTT-3' R: 5'-GACCGTGTTCCTTTGT-3'	94°C 30s	49°C 30s	72°C 30s	35	232bp
<i>socs2</i> /GQ478990 F: 5'-CGTCGGAGGGCACATTTTC-3' R: 5'-CGGAGTCGTGAGCAGCAG-3'	94°C 30s	55°C 30s	72°C 30s	35	280bp
<i>socs3</i> /EU625352 F: 5'-GGTCCG AGATATGTTGAATGA-3' R: 5'-CAGTGTCTTGATGACAAGGTGA-3'	94°C 30s	55°C 30s	72°C 30s	35	240bp
<i>cish</i> /GU384205 F: 5'-AGAGCTCATTTGCTGATACCT-3' R: 5'-GGAAACGTCCGAGACTGTACT-3'	94°C 30s	58°C 30s	72°C 30s	35	328bp
β -actin/M25013 F: 5'-CTGGTATCGTGATGGACTCT-3' R: 5'-AGCTCATAGC TCTTCTCCAG-3'	94°C 30s	56°C 30s	72°C 30s	30	285bp

Table 2 Primer sequences and PCR conditions for real-time PCR of target transcripts.

Gene target Primer sequence	PCR condition					Product size and T _m value
	Denature	Anneal	Extension	Detection	Cycle no.	
<i>socs1</i> F: 5'-CAGCAGGCAGCCATTT-3'; R: 5'-GACCGTGTTCCTTTGT-3'	94°C 30s	49°C 30s	72°C 30s	80°C 20s	35	232 bp and 83.1°C
<i>socs2</i> F: 5'-CGTCGGAGGGCACATTC-3' R: 5'-CGGAGTCGTGAGCAGCAG-3'	94°C 30s	55°C 30s	72°C 30s	82°C 20s	35	280 bp and 86.1°C
<i>socs3</i> F: 5'-GGTCCG AGATATGTTGAATGA-3' R: 5'-CAGTGTCTTGATGACAAGGTG-3'	94°C 30s	55°C 30s	72°C 30s	82°C 20s	35	240 bp and 84.8°C
<i>cish</i> F: 5'-AGAGCTCATTGCTGATACT-3' R: 5'-GGAAACGTCCGAGACTGACT-3'	94°C 30s	58°C 30s	72°C 30s	84°C 20s	35	328 bp and 85.2°C
<i>igf1</i> F: 5'-TCTCACTGGTGCTGTGCGTCCT-3' R: 5'-GCTCTGAAAGCAGCATTGTC-3'	94°C 30s	65°C 30s	72°C 30s	84°C 20s	40	203 bp and 87.5°C
<i>18S</i> F: 5'-AGCAACTTTAGTATACGCTATT-3' R: 5'-CTGAGAAACGGCTACCACATC-3'	94°C 30s	64°C 30s	72°C 30s	79°C 20s	40	210 bp and 85.0°C

Measurement of *socs1–3* and *cish* mRNA expression in carp hepatocytes

Carp hepatocytes (with >95% viability) were prepared by collagenase digestion (Reindl *et al.* 2011), seeded in 24-well plates at $\sim 0.7 \times 10^6$ cells/mL/well at 28°C, and challenged with test substances for the duration as indicated. Individual experiments were repeated with hepatocytes obtained from three to four cell preparation and each of them was conducted with quadruplicates in the same study. After treatment, total RNA (4 µg/well, OD_{260/280} of ~ 2.0) was isolated, digested with DNase I, and subjected to real-time PCR according to the conditions described in Table 2 using a RotorGene-Q System (Qiagen). Serial dilutions of plasmid DNA with ORF of target genes were used as the standards for standard curve construction for data calibration using RotorGene Q-Rex software and parallel measurement of *18S* RNA was used as an internal control (for the details of cell preparation and experiments, see Supplemental Fig. 1, see section on supplementary data given at the end of this article).

Western blot of post-receptor signaling targets in carp hepatocytes

To examine GH action on JAK/STAT, MAPK, and PI3K/Akt cascades, Western blot was performed in carp hepatocytes after GH treatment as described previously (Jiang & Wong 2013) with antibodies for the phosphorylated form and total protein of MEK_{1/2} (1:1000), ERK_{1/2} (1:5000), P₃₈^{MAPK} (1:1000), Akt (1:1000), JAK₂ (1:1000), STAT₁ (1:1000), STAT₃

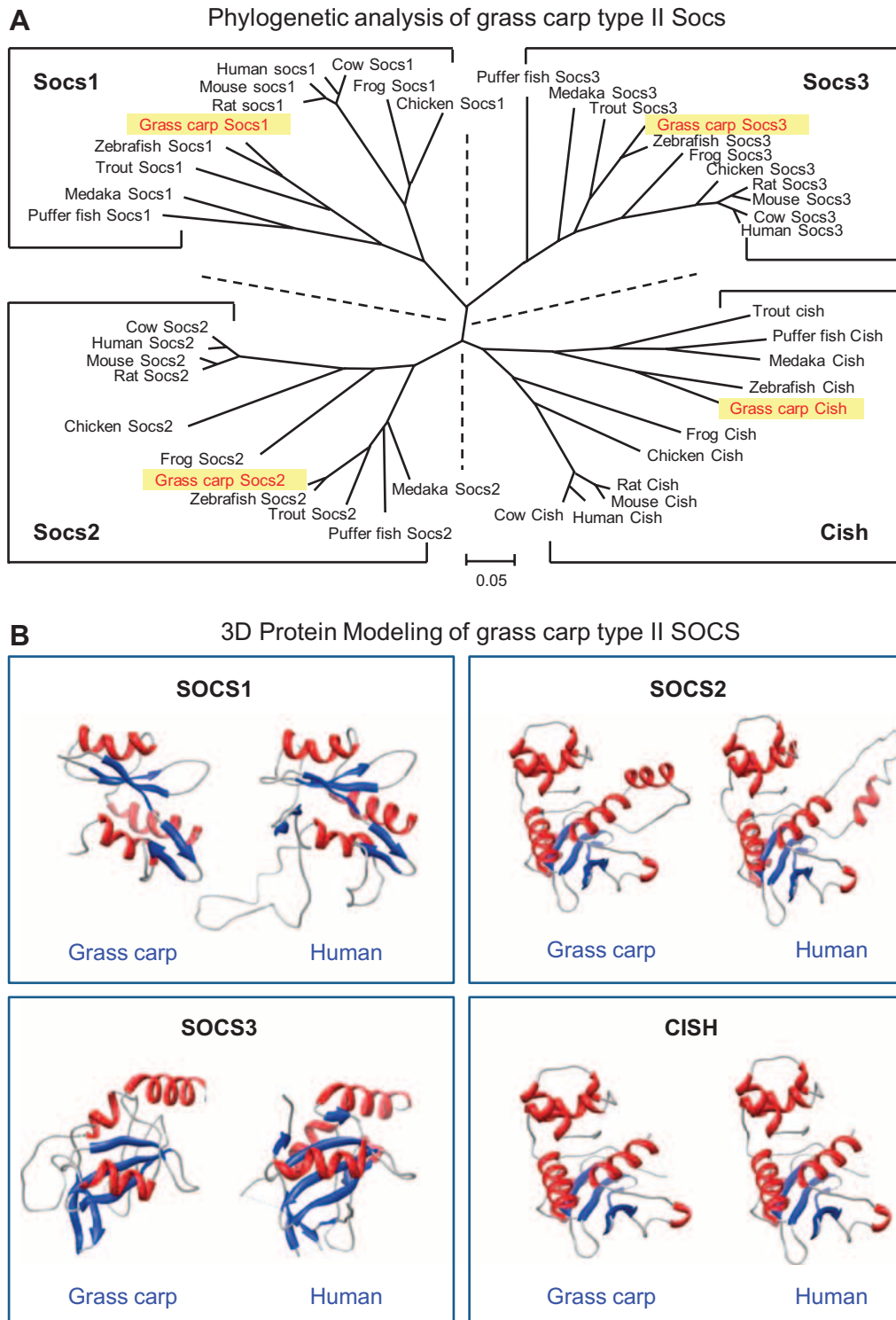
(1:1000), and STAT₅ (1:1000) respectively (Cell Signaling). Parallel blotting of β-actin was used as the loading control.

Functional expression of *Socs1–3* and *Cish* in CHO cells

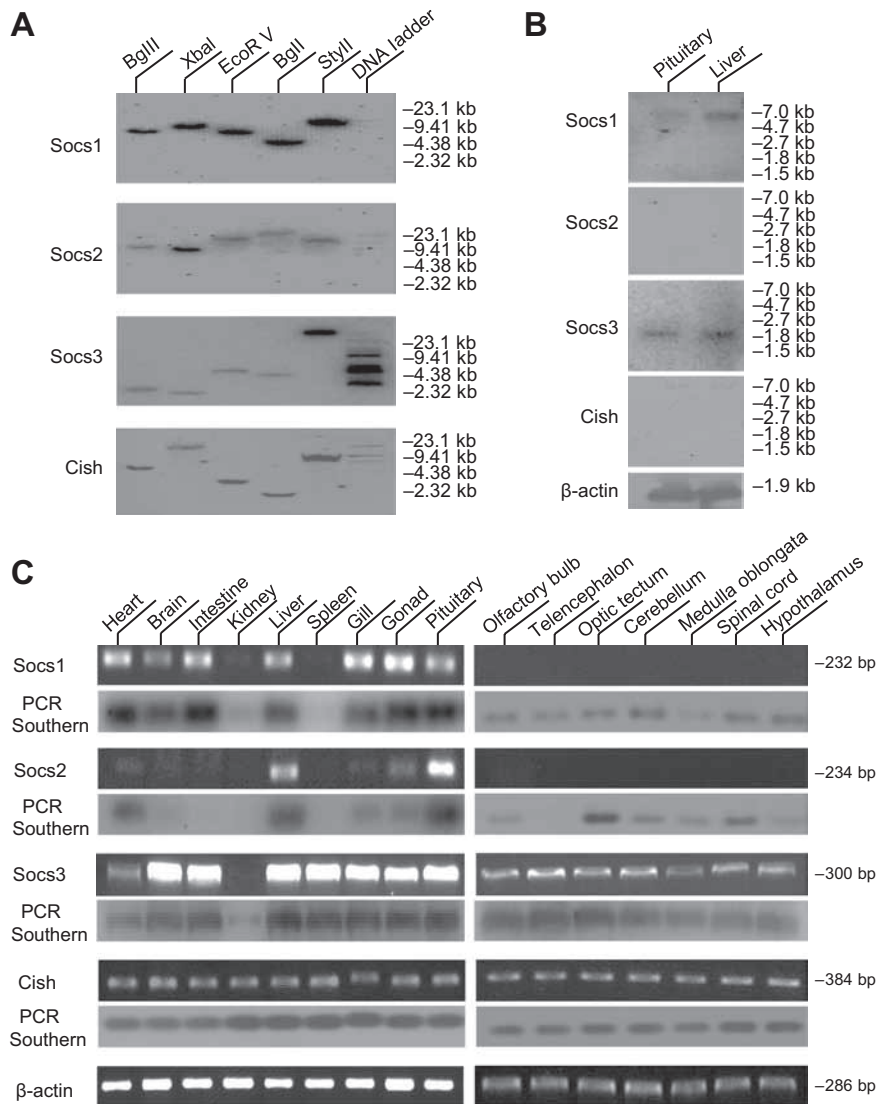
To characterize the functionality of grass carp type II SOCS, the ORF of the respective gene targets was subcloned into pcDNA3.1 (Invitrogen) to generate the expression vectors for *Socs1–3* and *Cish* respectively. To study the effects of type II SOCS on GH signaling, CHO cells with stable expression of grass carp GHR were used as the host cells for transient transfection of 8×GHRE.LUC, TK-Renilla (as internal control), and expression vectors for the respective type II SOCS with either GH treatment or co-transfection with STAT₅.pcDNA by the protocol as described previously (Sun *et al.* 2014). In parallel studies, a 1.07 kb grass carp *Igf1* promoter was cloned into the LUC reporter pGL3.basic and substituted for 8×GHRE.LUC in transfection study with CHO cells with GH treatment in the presence of JAK₂/STAT₅ inhibitors or co-transfection with the expression vectors for type II SOCS. After that, firefly and renilla luciferase activities expressed in CHO cells were measured using a Dual-Glo Luciferase Assay Kit (Promega).

Data transformation and statistics

Transcript expression of *igf1*, *socs1–3*, and *cish* was measured in terms of femtomole transcript detected/10⁶ cells/well. As *18S* RNA did not exhibit notable changes

**Figure 1**

Phylogenetic analysis and *in silico* protein modeling of grass carp Socs1–3 and Cish. (A) Unrooted analysis of the phylogenetic relationship of carp Socs1–3 and Cish with the corresponding sequences identified in other vertebrates using neighbor-joining method by MEGA 6.0. The scale bar represents the evolutionary distance, and bootstrap values for individual nodes in the guide tree (in the range of 76–100%) have been omitted for clarity. (B) Protein modeling of carp SOCS1–3 and CISH by SWISS-MODEL using crystal structures of their human counterparts as the templates. The α -helices (in red) and β -sheets (in blue) forming the SH2 domain and SOX box within the respective type II SOCS in grass carp were mapped according to the corresponding structures in human SOCS1–3 and CISH, respectively.

**Figure 2**

Gene copy number and tissue expression of Socs1–3 and Cish in grass carp. (A) Southern blot of Socs1–3 and Cish using genomic DNA isolated from grass carp with digestion of restriction enzymes followed by hybridization with DIG-labeled probes for the respective targets of type II SOCS. (B) Northern blot of Socs1–3 and Cish using total RNA prepared from the carp pituitary and liver. Hybridization was performed with the probes used for Southern blot and parallel blotting of β -actin expression was used as the internal control. (C) Tissue expression profiling of Socs1–3 and Cish in grass carp by RT-PCR. Total RNA was prepared from selected tissues and brain areas and used for RT-PCR with primers specific for the respective gene targets. The authenticity of PCR products was confirmed by PCR Southern and RT-PCR for β -actin was used as the internal control.

in our experiments, the raw data for target mRNA were simply transformed as a percentage of the mean value in control group (as '%Ctrl'). For Western blot, signals detected by X-ray film were quantified by densitometry scanning and the ratio of phosphorylated form and total protein of respective kinases (as 'p:t ratio') was used as an index for their activation. For transfection studies, the raw data for firefly luciferase (in ALU) was normalized as a ratio of renilla luciferase data detected in the same sample. The normalized data were then expressed as fold increase compared with the mean value in the control group (as 'fold induction'). Data presented (mean \pm S.E.M.) were pooled from four experiments and analyzed by Student's *t*-test or ANOVA followed by Newman–Keuls test with difference considered as significant at $P < 0.05$.

Results

Molecular cloning, copy number, and tissue expression of Socs1–3 and Cish

To establish the structural identity of type II SOCS in grass carp, full-length cDNAs for *socs1–3* and *cish* were cloned in the carp liver (Supplemental Fig. 2), and phylogenetic analysis by neighbor-joining method revealed that these grass carp cDNAs could be grouped in the respective clades of type II SOCS with very close evolutionary relationship to the corresponding zebrafish sequences (Fig. 1A). The deduced protein sequences of grass carp Socs1–3 and Cish were found to be highly homologous to the respective targets in other vertebrates, especially in SH2 domain and SOCS box (also for KID & ESS motifs in

Socs1 and Socs3, Supplemental Fig. 3). Protein modeling also revealed that the spatial arrangement of α -helical and β -sheet structures in the newly cloned Socs1–3 and Cish, which form the basis of SH2 domain and SOCS box, was highly comparable to their human counterparts (Fig. 1B).

As shown in Fig. 2A, Southern blot with carp genomic DNA digested with BgIII, XbaI, BgII, StyII, and EcoR V, respectively, consistently produced a single band after hybridization with the probes for the respective type II SOCS, implying that Socs1–3 and Cish are single-copy genes in carp genome. For tissue expression of type II SOCS, northern blot was performed in the carp liver and pituitary (Fig. 2B). In the two tissues examined, a single transcript of 7.0 and 1.8 kb in size were detected with the probes for Socs1 and Socs3, respectively; however, hybridization signals were not apparent for Socs2 and Cish. Using RT-PCR, Socs3 and Cish signals were located ubiquitously in tissues including the gills, liver, kidney, gonad, intestine, spleen, pituitary, and heart as well as in brain areas including the olfactory bulbs, telencephalon, optic tectum, hypothalamus, cerebellum, medulla oblongata, and spinal cord (Fig. 2C). Similar findings were also noted for Socs1 and Socs2, except that Socs1 was not detected in the spleen and Socs2 signals could not be identified in the kidney, intestine, spleen, and telencephalon.

To confirm that type II SOCS are also expressed at the protein level, LC/MS/MS was performed in trypsin-digested lysate prepared from the carp liver (Supplemental Fig. 4A, B, C and D). Proteomic analysis of mass spectra of peptide fragments produced after trypsin digestion has

revealed the presence of peptide products of grass carp type II SOCS (with 19 peptides for Socs1, 14 peptides for Socs2, 28 peptides for Socs3, and 23 peptides for Cish with confident level for target identification at 99% and protein coverage of respective type II SOCS ranging from 61.8 to 88.9%), indicating that the *socs1–3* and *cish* transcripts detected can be translated into respective proteins in the carp liver.

Signal transduction for GH-induced type II SOCS mRNA expression in carp hepatocytes

To examine type II SOCS expression at hepatic level, carp hepatocytes were used as a cell model, and treatment with IL-1 β and TNF α or the endotoxin LPS was effective in elevating the mRNA levels of *socs1–3* and *cish* in this cell culture system (Fig. 3A). In carp hepatocytes, GH treatment also induced *igf1* transcript expression with notable loss of Igf1 responsiveness at high dose (1 μ g/mL)/prolonged exposure (≥ 24 h) to GH (Fig. 3B and C). As shown in Fig. 4A, GH could also up-regulate type II SOCS expression with different kinetics, with (i) transient rises in *socs1* and *socs3* mRNA levels peaked at 1 h after GH induction and (ii) gradual elevation of *socs2* and *cish* mRNA levels up to 6 h or longer with GH exposure. By fixing the duration of GH treatment at 1 h, increasing doses of GH (1–1000 ng/mL) were also found to stimulate *socs1–3* and *cish* mRNA expression in a concentration-related fashion (Fig. 4B).

To elucidate the signal transduction mediating GH-induced type II SOCS expression, Western blot

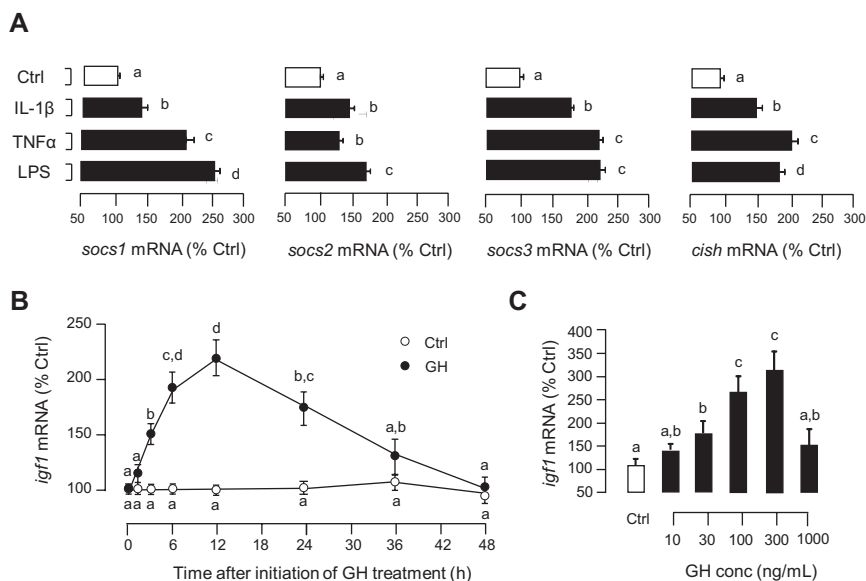


Figure 3

Target gene expression in carp hepatocytes with immune challenge and GH treatment. (A) Effects of immunological stimulants on type II SOCS mRNA expression in carp hepatocytes. In this experiment, hepatocytes were incubated for 6 h with IL-1 β (0.1 μ g/mL), TNF α (0.1 μ g/mL), and LPS (1 μ g/mL) respectively. (B) Time course and (C) dose dependence of *igf1* mRNA expression in carp hepatocytes with GH stimulation. The dose of GH treatment was fixed at 500 ng/mL for time-course study, whereas the duration of drug testing was fixed at 12 h for dose-dependence experiment. In these studies, static incubation of hepatocytes was performed with/without test substances. After that, total RNA was isolated and used for real-time PCR measurement of respective gene targets. Experimental groups denoted by different letters represent a significant difference at $P < 0.05$ (ANOVA followed by Newman–Keuls test).

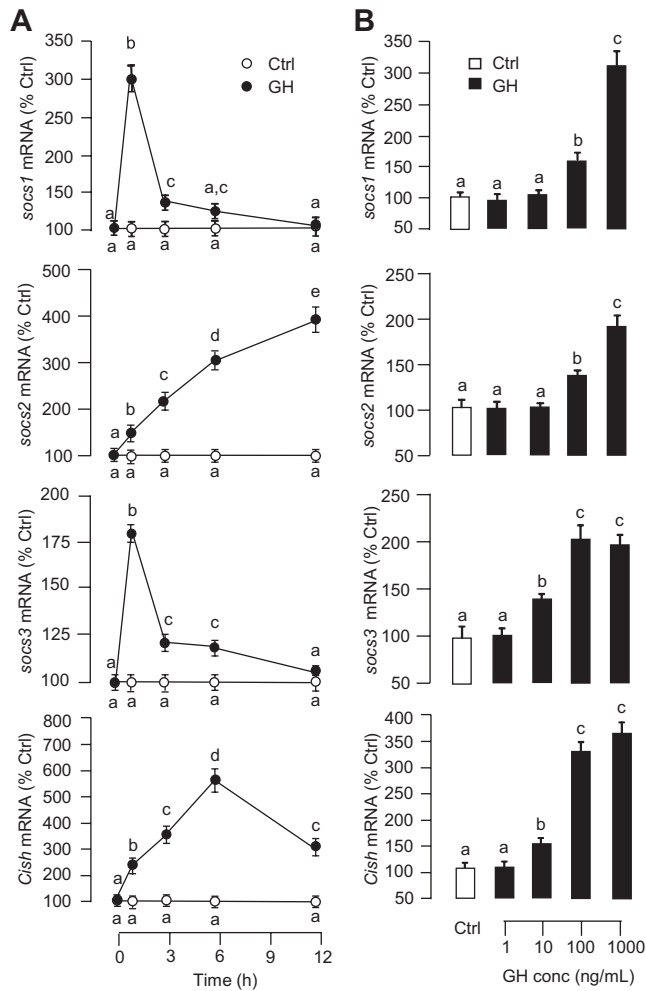


Figure 4 GH stimulation of type II SOCS mRNA expression in carp hepatocytes. (A) Time course and (B) dose dependence of GH treatment on *Socs1–3* and *cish* mRNA expression in carp hepatocytes. The dose of GH was used at 500 ng/mL for the time course, whereas the duration of drug treatment was fixed at 1 h for dose-dependent study. After treatment, total RNA was extracted and subjected to real-time PCR for *socs1–3* and *cish* mRNA measurement. Groups denoted by different letters represent a significant difference at $P < 0.05$ (ANOVA followed by Newman–Keuls test).

was conducted in carp hepatocytes to test whether GH stimulation could activate the JAK/STAT pathway. As shown in Fig. 5A, GH treatment induced rapid phosphorylation of JAK₂, STAT₁, STAT₃, and STAT₅ with the highest responses observed for STAT₅ (close to 40-fold increase). In parallel studies, hepatocytes were challenged with GH with simultaneous treatment of the JAK₂ inhibitor HEX, STAT₁ inhibitor FA, STAT₃ inhibitor ETDDC, or STAT₅ inhibitor IQDMA (Fig. 5B). In these cases, GH-induced *socs1–3* and *cish* mRNA expression was either attenuated or negated by the inhibitors targeting JAK₂/STATs pathway. In carp hepatocytes, a 10-min exposure

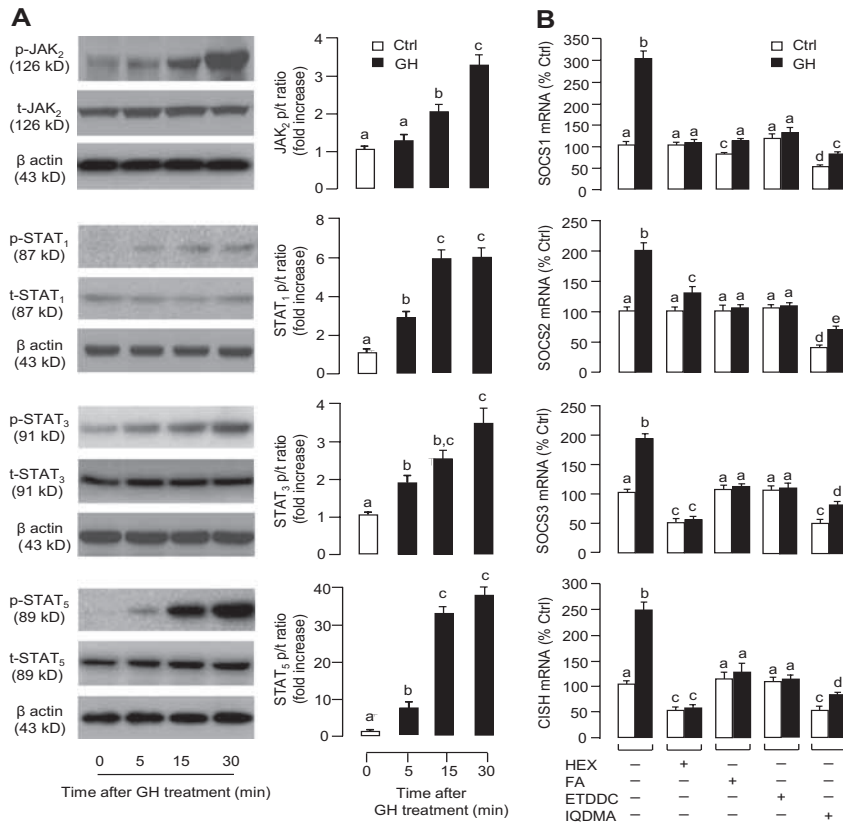
to GH was found to induce MEK_{1/2}, ERK_{1/2}, P₃₈^{MAPK}, and Akt phosphorylation (Fig. 6A). Co-treatment with the MEK_{1/2} inhibitor U0126 or P₃₈^{MAPK} inhibitor PD169316 also reduced or abolished GH-induced *socs1–3* and *cish* mRNA expression. Treatment with the PI3K inhibitor Ly294002 or Akt inhibitor HIMOC, interestingly, could inhibit GH-induced *socs3* mRNA expression but had no effects on the corresponding responses of other type II SOCS (Fig. 6B).

Socs1–3 and Cish expression on GHR signaling via JAK₂/STAT₅ pathway

As the pharmacological tools for SOCS are not yet available, a molecular approach was used to examine *Socs1–3* and *Cish* expression on GHR signaling in grass carp. In CHO cells with grass carp GHR expression and transfection with the JAK₂/STAT₅-responsive reporter 8×GHRE.LUC, GH treatment could induce luciferase activity expression and this effect was suppressed by co-treatment with the JAK₂ inhibitor HEX or STAT₅ inhibitor IQDMA (Fig. 7). In this cell model, transfection with increasing levels of the respective expression vectors for carp *Socs1–3* and *Cish* also induced a dose-dependent inhibition on basal as well as GH-induced luciferase activity expression (Fig. 8A). To test whether the effects of type II SOCS were mediated by targeting STAT₅ signaling, STAT₅ over-expression by transfection with STAT₅.pcDNA was performed (Fig. 8B). In this case, a significant rise in luciferase activity expression was noted with STAT₅.pcDNA transfection and this stimulatory action was reduced dose dependently by over-expression of carp *Socs1–3* and *Cish* respectively. Consistent with the role of JAK₂/STAT₅ in GHR signaling, the effect of STAT₅ over-expression on luciferase activity was markedly enhanced by GH, and again, this potentiating effect was also sensitive to the inhibition by over-expression of type II SOCS (Fig. 8C).

Socs1–3 and Cish expression on GH-induced Igf1 promoter activity

In CHO cells with GHR expression, Igf1 promoter activity was examined by transfection with the pGL3.basic reporter carrying a 1.07 kb grass carp Igf1 promoter. In this case, luciferase activity mediated by Igf1 promoter could be induced by GH treatment, and this stimulation was blocked by co-treatment with the JAK₂ inhibitor HEX (Fig. 9A) or STAT₅ inhibitor IQDMA (Fig. 9B). Similar to the preceding studies with the JAK₂/STAT₅-responsive

**Figure 5**

JAK/STAT pathway in GH-induced type II SOCS mRNA expression in carp hepatocytes. (A) GH stimulation of JAK₂, STAT₁, STAT₃, and STAT₅ phosphorylation. Cell lysate was harvested from hepatocytes after GH treatment (500 ng/mL) up to 30 min and used for Western blot for phosphorylated form ('p-') and total protein ('t-') of the respective signaling targets. Parallel blotting of β -actin was used as internal control and activation status of signaling target was quantified as the ratio of p- and t- form of the same protein (as 'p:t ratio'). (B) Blocking JAK/STAT pathway on GH-induced *socs1-3* and *cish* mRNA expression. Hepatocytes were incubated with GH (500 ng/mL) for 1 h with/without co-treatment of JAK₂ inhibitor HEX (50 μ M), STAT₁ inhibitor FA (50 μ M), STAT₃ inhibitor ETDDC (300 nM), and STAT₅ inhibitor IQDMA (50 μ M) respectively. After that, total RNA was isolated and used for real-time PCR measurement of *socs1-3* and *cish* mRNA expression.

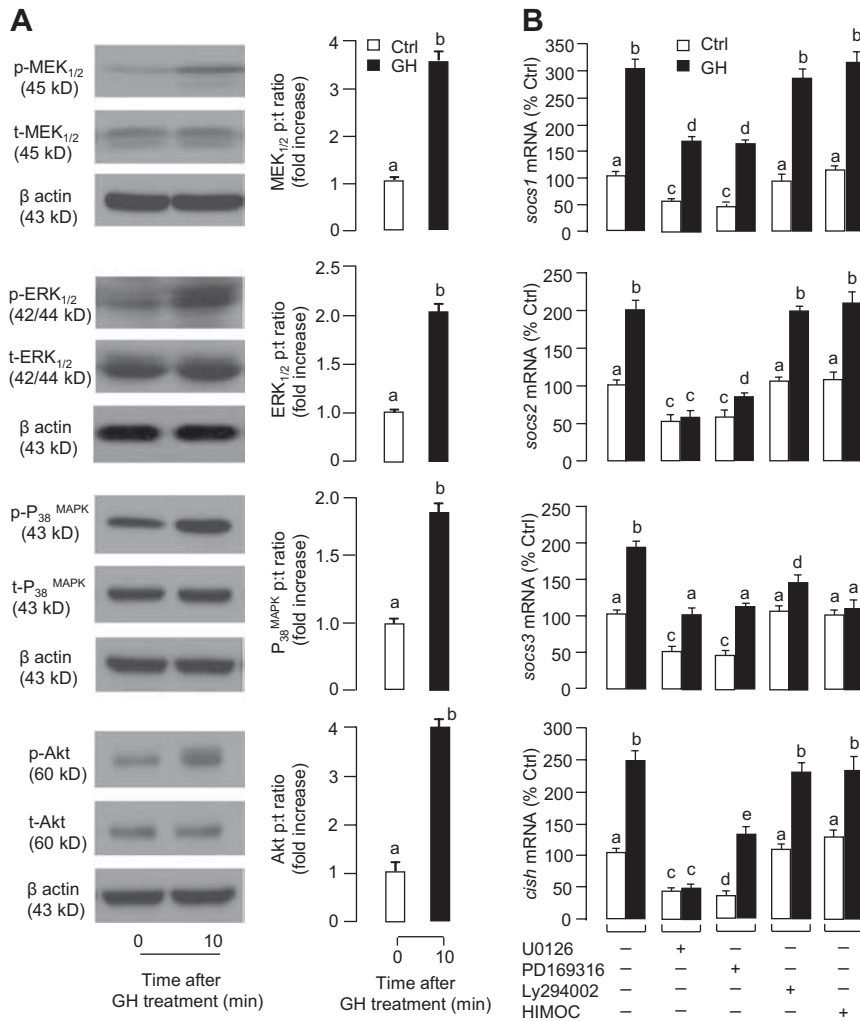
reporter 8 \times GHRE.LUC, both basal and GH-induced luciferase activity expression mediated by Igf1 promoter could be reduced or negated in a dose-related fashion by co-transfection with increasing levels of the expression vectors for grass carp type II SOCS (Fig. 9C, D, E and F).

Discussion

As a first step to study the role of type II SOCS in GH functions in carp model, *Socs1-3* and *Cish* were cloned in grass carp, and phylogenetic analysis of their cDNA sequences has confirmed that they could be clustered in the clades of respective subfamilies of type II SOCS. As revealed by sequence alignment and protein modeling, the protein structures of *Socs1-3* and *Cish* were found to be highly conserved, especially in the SH2 domain and SOCS box. The spatial arrangement of β -sheets and α -helices forming the two domains in carp *Socs1-3* and *Cish* is highly comparable with their human counterparts. In mammals, SH2 domain is a target recognition motif commonly found in tyrosine kinases and involved in protein-protein interaction with phosphotyrosine residues (Machida & Mayer 2005). Previous studies (e.g., in hepatocytes and lymphocytes) have shown that SH2 domain in SOCS proteins is essential

for their association with JAK₂ (Machida & Mayer 2005, Liu *et al.* 2006) and can compete with STAT₅ binding for cytokine receptors to inhibit JAK₂/STAT₅ signaling (Hilton 1999). Similar to SH2 domain, SOCS box also plays a role in SOCS inhibition of cytokine signaling as it can interact with elongin B/C, culin B, and Ring-box 2 to form a complex with E3 ubiquitin ligase activity (Yoshimura *et al.* 2007) and trigger proteasomal degradation of JAK₂ by SOCS binding (Piessevaux *et al.* 2008a). The presence of structural motifs in grass carp *Socs1-3* and *Cish* for signal termination of JAK/STAT pathway raises the possibility that the type II SOCS in fish model may also participate in the down-regulation of cytokine signaling in a way similar to mammals.

As revealed by genomic Southern blot, the newly cloned type II SOCS are confirmed to be single-copy genes in the carp genome. Expression profiling using RT-PCR also reveals that *Socs1-3* and *Cish* are widely expressed at tissue level as well as in various brain areas in grass carp. Although the four type II SOCS could all be located in the liver and pituitary by RT-PCR, northern blot signals for the respective transcripts were detected only for *Socs1* (7.0 kb) and *Socs3* (1.8 kb) but not *Socs2* and *Cish*, suggesting that *Socs1* and *Socs3* are the major forms of type II SOCS expressed in these two tissues. Recently, tissue expression

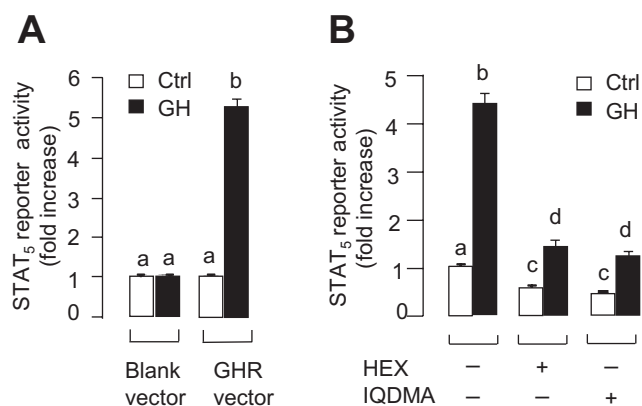
**Figure 6**

MAPK and PI3K/Akt pathways in GH-induced type II SOCS mRNA expression in carp hepatocytes. (A) GH stimulation of MEK_{1/2}, ERK_{1/2}, P₃₈^{MAPK}, and Akt phosphorylation. Cell lysate was harvested from hepatocytes after 10-min treatment of GH (500 ng/mL) and used for Western blot for phosphorylated form ('p-') and total protein ('t-') of the respective signaling targets. Parallel blotting of β-actin was used as internal control and activation status of signaling target was quantified as the ratio of p- and t- form of the same protein (as 'p:t ratio'). (B) Blocking MAPK and PI3K/Akt pathways on GH-induced socs1–3 and *cish* mRNA expression. Hepatocytes were challenged with GH (500 ng/mL) for 1 h with/without co-treatment of the MEK_{1/2} inhibitor U0126 (10 μM), P₃₈^{MAPK} inhibitor PD169316 (10 μM), PI3K inhibitor Ly294002 (10 μM), and Akt inhibitor HIMOC (20 μM) respectively. After that, total RNA was isolated and subjected to real-time PCR for quantitation of the respective transcripts of type II SOCS.

of SOCS has also been reported in fish models, including salmon (Skjesol *et al.* 2014), yellow perch (Shepherd *et al.* 2012), rainbow trout (Wang & Secombes 2008, Wang *et al.* 2010), puffer fish (Jin *et al.* 2007), and turbot (Zhang *et al.* 2011). Similar to grass carp, SOCS expression was found to be widely expressed, e.g., in the gills, spleen, gut, liver, kidney, muscle, and brain. However, species-specific variations could still be noted. For examples, in rainbow trout, notable levels of SOCS and *Cish* gene expression could be located in the spleen and kidney with little/low levels of signal found in the liver (Wang & Secombes 2008, Wang *et al.* 2010). This is different from grass carp, in which Socs1–3 and *Cish* were readily detectable in the liver; however, Socs1 and Socs2 signals in the spleen and kidney were low/undetectable. The different patterns of type II SOCS expression observed suggest that the functional dominance of individual SOCS may vary at the tissue level among different species. In mammals, hepatic expression of SOCS has also been reported and found to have tumor

suppressor activity (Miyoshi *et al.* 2005) and modulatory effects on liver regeneration (Brand *et al.* 2007). However, the biological actions of SOCS in the liver of fish species have yet to be characterized.

In mammals, up-regulation of SOCS expression is well documented after microbial/viral infection or cytokine treatment (Strebosky *et al.* 2012), and these SOCS responses constitute an intracellular feedback to turn off/tune down cytokine signaling via the JAK/STAT pathway and prevent overshoot of proinflammatory activities (Tamiya *et al.* 2011). Cytokine induction of SOCS can not only be found in immune tissues but also be found in the liver (Tollet-Egnell *et al.* 1999). The liver not only represents a key component of the somatotrophic axis but also an immunological organ for antigen presentation, NK/T cell priming, and production of complement factors (Racanelli & Rehmann 2006). In this study, Socs1–3 and *Cish* expression, both for transcripts (by RT-PCR & real-time PCR) and proteins (by

**Figure 7**

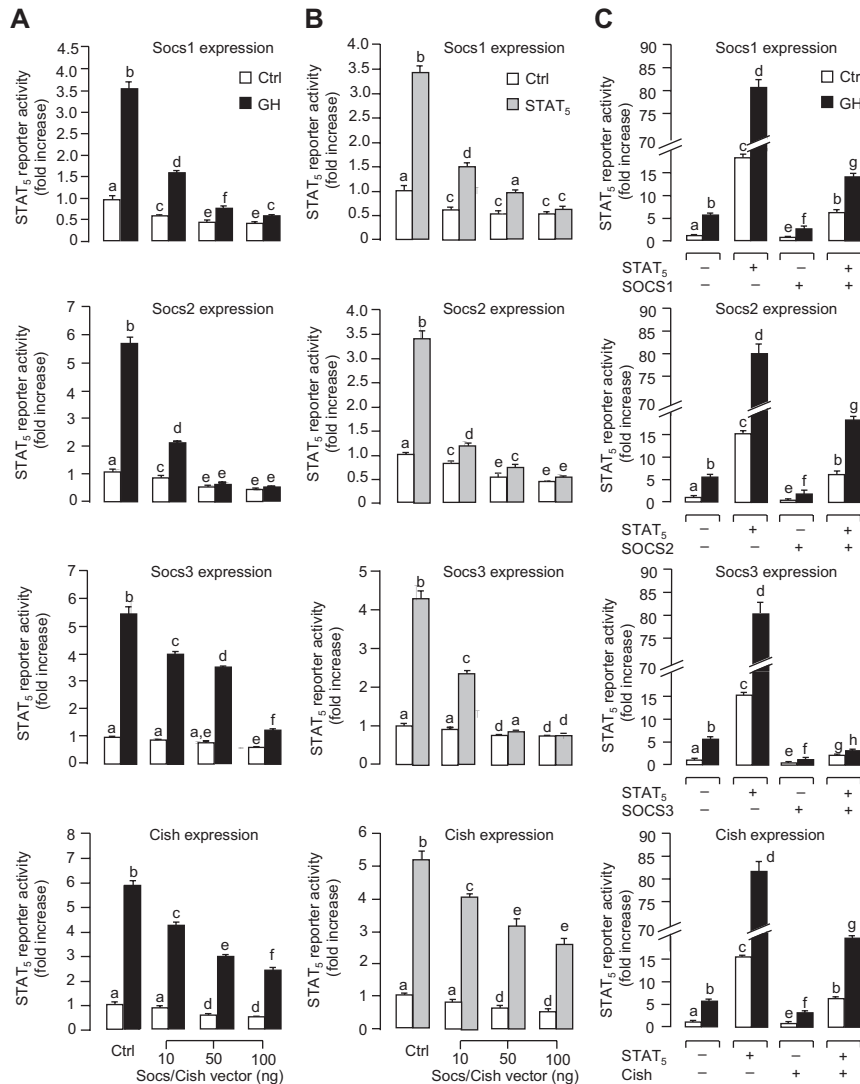
JAK₂/STAT₅ pathway coupled to carp GHR. (A) Functional expression of carp GHR in CHO cells. Stable transfection of CHO cells with the expression vector for grass carp GHR was performed (with parallel transfection of the blank vector as a control). After the line of CHO cells with GHR expression was selected, transient transfection with the JAK₂/STAT₅-responsive reporter 8×GHRE. LUC was conducted and GHR activation was induced by GH treatment (500 ng/mL) for 24 h. (B) Blocking JAK₂/STAT₅ pathway in STAT₅ signaling via GHR expressed in CHO cells. In CHO cells with GHR expression, transient transfection with the STAT₅-responsive LUC reporter was performed followed by GH induction (500 ng/mL) for 24 h with co-treatment of JAK₂ inhibitor HEX (50 μM) or STAT₅ inhibitor IQDMA (50 μM). In these studies, cell lysate was prepared after drug treatment and used for luciferase activity measurement as described in 'Materials and methods' section.

LC/MS/MS), have been confirmed in the carp liver, and mRNA levels of these type II SOCS could be increased in carp hepatocytes by LPS exposure or treatment with IL-1β and TNFα. These findings indicate that hepatic expression of type II SOCS could be induced by endotoxin and cytokines, which may serve as feedback repressors for immune responses occurring in the carp liver. In fish models, LPS/cytokine-induced *socs1–3* gene expressions have been reported in the head kidney of puffer fish (Jin *et al.* 2007) and trout monocytes/macrophages (Wang & Secombes 2008), corroborating with the idea that the role of SOCS as signal terminators for cytokine actions is well conserved in vertebrate evolution (Wang *et al.* 2011). In carp hepatocytes, GH consistently induced *igf1* mRNA expression in a time- and dose-dependent manner. Of note, down-regulation of Igf1 responses was also observed with high concentration/prolonged exposure to GH, suggesting the presence of intrinsic mechanisms to turn off/tune down GH signaling. As *socs1–3* and *cish* mRNA expression could be elevated by GH treatment in carp hepatocytes and these stimulatory effects occurred before/during Igf1 down-regulation with prolonged incubation with GH, the possible involvement of type II SOCS in signal termination for GH-induced Igf1 expression cannot be excluded. In rodents, hepatic expression of Socs2–3

and Cish can be induced by GH both *in vivo* and *in vitro* (Tollet-Egnell *et al.* 1999, Greenhalgh & Alexander 2004) and deregulation of GH signaling by Socs1–3 has been reported in cell lines, e.g., F442A fibroblasts (Greenhalgh & Alexander 2004). However, direct evidence showing SOCS inhibition of GH-induced Igf1 expression has not been documented.

Although the signal transduction for GH-induced SOCS/CISH expression at the hepatic level is still unknown, Socs2 interaction with GHR (Greenhalgh *et al.* 2002a) and Socs1 and Socs3 inhibition of JAK₂ activation (Hansen *et al.* 1999) and GH-responsive promoters (Adams *et al.* 1998) have been reported in various cell models. Recently, down-regulation of GH signaling by SOCS2 via SOCS box-dependent ubiquitination and proteasomal degradation of GHR has been demonstrated in mouse liver (Vesterlund *et al.* 2011). In our studies, GH treatment induced rapid phosphorylation of JAK₂, STAT₁, STAT₃, STAT₅, MEK_{1/2}, ERK_{1/2}, and P₃₈^{MAPK} in carp hepatocytes. Furthermore, GH-induced *socs1–3* and *cish* mRNA expression was also sensitive to the blockade of JAK₂ (by HEX), STAT₁ (by FA), STAT₃ (by ETDDC), STAT₅ (by IQDMA), MEK_{1/2} (by U0126), and P₃₈^{MAPK} (by PD169316), implying that GH-induced type II SOCS expression in the carp liver is mediated by JAK₂/STATs and MAPK cascades. Although Akt phosphorylation was also noted after GH induction, except for the drop in transcript level for *socs3*, GH-induced *socs1–2*, and *cish* mRNA expression were not affected by blocking PI3K (by Ly294002) or Akt (by HIMOC). Apparently, the PI3K/Akt pathway is involved in GH-induced *socs3*, but not *socs1–2* and *cish* gene expression. Our results are consistent with GHR coupling to JAK₂/STATs, MAPK, and PI3K/Akt signaling reported in mammals (Miquet *et al.* 2010). Recently, GH activation of STAT₅, ERK_{1/2}, and Akt and their involvement in GH-induced Igf1 expression have also been demonstrated in trout hepatocytes (Reindl *et al.* 2011). To our knowledge, our study represents the first report to elucidate the post-receptor signaling for GH-induced type II SOCS expression at the hepatic level.

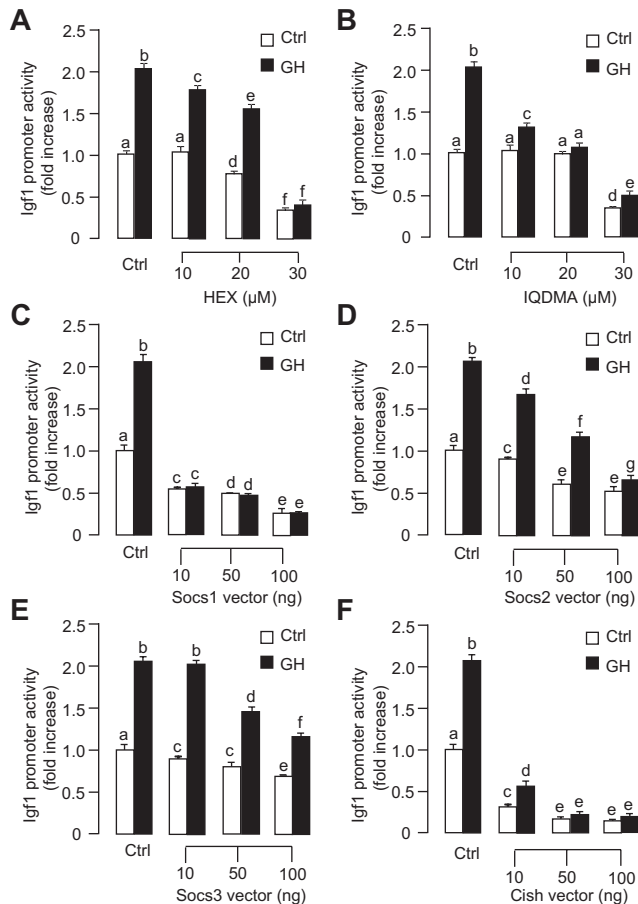
In mammals, SOCS expression induced by cytokines is known to inhibit STAT₅ activation as well as transcription of STAT₅-responsive genes, e.g., ALS and Spi-2.1 genes (Adams *et al.* 1998, Boisclair *et al.* 2000). As the JAK₂/STAT₅ pathway is also a key component of the post-receptor signaling for GH-induced Igf1 expression at the hepatic level (Choi & Waxman 2000, Davey *et al.* 2001), it would be logical to assume that SOCS expression could also down-regulate GH-induced *igf1* gene transcription via interference of JAK₂/STAT₅ signaling. In CHO cells

**Figure 8**

Type II SOCS expression on JAK₂/STAT₅ signaling via carp GHR. CHO cells with GHR expression were transfected with JAK₂/STAT₅-responsive reporter 8×GHRE.LUC with/without co-transfection of increasing amount of the expression vectors for carp Socs1–3 and Cish respectively. Treatment was initiated for 24 h with (A) static incubation with GH (500 ng/mL), (B) over-expression of STAT₅ by co-transfection with STAT₅ expression vector, and (C) STAT₅ over-expression with GH treatment (500 ng/mL). After that, cell lysate was prepared from CHO cells and assayed for luciferase activity as an index for JAK₂/STAT₅ reporter activation as described in 'Materials and methods' section.

with the expression of carp GHR, GH treatment or STAT₅ over-expression was effective in activating a JAK₂/STAT₅-responsive reporter with GHR-responsive elements in its 5' promoter and the stimulatory effect by GH could be markedly enhanced by STAT₅ over-expression. These stimulatory actions, however, could be dose dependently suppressed by over-expression of grass carp Socs1–3 and Cish. As GH activation of the JAK₂/STAT₅-responsive reporter could be consistently inhibited by blocking JAK₂ (by HEX) and STAT₅ (by IQDMA) in our system, our results suggest that type II SOCS expression in carp model can inhibit JAK₂/STAT₅ signaling coupled to GHR. Our findings are comparable to the previous studies in 3T3-F442A cells, in which GH-induced activation of STAT₅-responsive Spi-2.1 promoter could be negated by Socs1 and Socs3 over-expression (Adams *et al.* 1998). To examine the role of type II SOCS on GH-induced Igf1 expression in fish

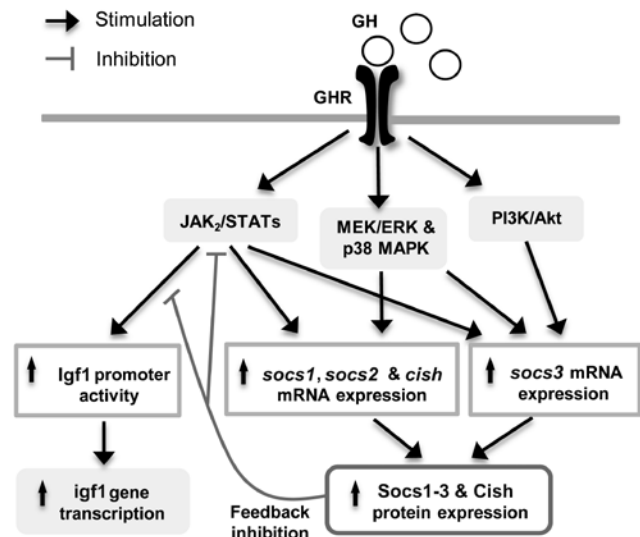
model, the full gene of grass carp *igf1* was cloned and a well-conserved STAT₅ binding site was mapped in close proximity to a HNF1α binding site located in its proximal promoter region (A O L Wong & W W Wong, unpublished data). Similar bipartite pattern of STAT₅ and HNF1α binding sites has also been reported in Igf1 promoter of common carp (Vong *et al.* 2003) and salmon (Moghadam *et al.* 2007), implying that gene transcription of *igf1* in fish models is also responsive to STAT₅ signaling. This idea is also consistent with our studies in CHO cells with GHR expression transfected with a luciferase-expressing reporter carrying the 1.07 kb grass carp Igf1 promoter. In this case, GH-induced Igf1 promoter activity could be reduced or negated by inhibiting JAK₂ (by HEX) or STAT₅ (by IQDMA), implying that JAK₂/STAT₅ pathway is involved in GH-induced *igf1* gene transcription in carp model. Similar to our studies with JAK₂/STAT₅ reporter,

**Figure 9**

Type II SOCS expression on *Igf1* promoter activation via $JAK_2/STAT_5$ signaling coupled to GHR. CHO cells with GHR expression were transfected with pGL3.basic carrying the 5' promoter of grass carp *igf1* gene. The cells were then challenged with GH (500 ng/mL) in the presence of (A) the JAK_2 inhibitor HEX, (B) the $STAT_5$ inhibitor IQDMA, or (C, D, E and F) with co-transfection of increasing amount of the expression vectors for carp Socs1–3 and Cish respectively. After 24-h treatment, lysate was prepared from CHO cells and assayed for luciferase activity as an index for *Igf1* promoter activation as described in 'Materials and methods' section.

both basal and GH-induced *Igf1* promoter activity could be suppressed in a dose-related manner by over-expression of Socs1–3 and Cish. These results, as a whole, provide evidence for the first time that type II SOCS expression was effective in blocking GH-induced *igf1* gene transcription via inhibition of $JAK_2/STAT_5$ signaling.

In summary, we have cloned the type II SOCS, including Socs1–3 and Cish, in grass carp and characterized their gene copy number and tissue expression profiles. In carp hepatocytes, GH treatment could induce transcript expression of these type II SOCS via $JAK_2/STAT_5$, MAPK, and/or PI3K/Akt cascades, and these effects were shown to occur before/during *Igf1* down-regulation induced

Working model in carp hepatocytes**Figure 10**

Working model for GH-induced type II SOCS expression and their effects on $JAK_2/STAT_5$ signaling and *igf1* gene transcription in carp hepatocytes. In carp hepatocytes, GH stimulation can induce gene expression of type II SOCS including SOCS1–3 and CISH via $JAK_2/STAT_5$, MAPK and/or PI3K/Akt cascades functionally coupled to GHR. The $JAK_2/STAT_5$ pathway is also a major component in the post-receptor signal transduction for GH-induced *Igf1* promoter activation and gene expression. Subsequent protein translation following the rises of type II SOCS mRNA induced by GH could elevate the levels of corresponding SOCS proteins in carp hepatocytes, which may serve as the intracellular feedback repressors to inhibit $JAK_2/STAT_5$ pathway and lead to the down-regulation of *Igf1* promoter activity and *igf1* gene transcription occurred during the prolonged period of GHR activation. This intracellular feedback may constitute the intrinsic mechanisms acting at the hepatic level for signal termination of GH in the somatotrophic axis in carp species.

by prolonged exposure to GH. In CHO cells with GHR expression, GH could not only activate $JAK_2/STAT_5$ signaling but also up-regulate grass carp *Igf1* promoter activity. These stimulatory actions, however, could be suppressed by over-expression of carp Socs1–3 and Cish. These findings, taken together, suggest that type II SOCS may serve as feedback repressors for GH activation of $JAK_2/STAT_5$ signaling and *igf1* gene transcription in the carp liver (Fig. 10). Probably, GHR activation could induce *igf1* and type II SOCS gene expression via overlapping signal transduction pathways, and the subsequent rises in SOCS proteins might act as the negative feedback signals to inhibit GH-induced *Igf1* expression at hepatic level. Whether type II SOCS can also contribute to the pulsatile pattern of *Igf1* expression induced by GH (e.g., in rodents, Choi & Waxman 2000) is still unclear; however, our studies do provide (i) the evidence for the presence of an intracellular feedback for signal termination of GH in the carp liver and (ii) a new insight into the possible cross talk

between immune system and somatotrophic axis in fish model. As GH resistance caused by bacterial/viral infection is known to compromise the overall productivity of fish farming, our findings may have potential application in developing new strategy targeting type II SOCS expression for growth promotion in commercial fish.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-15-0423>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

X J and J X were responsible for molecular cloning, gene copy number determination, tissue distribution, and transcript expression for grass carp type II SOCS; A M was in charge of hepatocyte preparation and GH-induced *igf1* gene expression; M H took care of LC/MS/MS for protein expression of type II SOCS and Western blot for signaling kinases; A O L W was the PI and grant holder, and worked with X J on manuscript preparation and revision.

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