

SHORT COMMUNICATION

IGF-1 induces SOCS-2 but not SOCS-1 and SOCS-3 transcription in juvenile Nile tilapia (*Oreochromis niloticus*)

Cai-Zhi Liu¹, Yuan Luo¹, Samwel Mchele Limbu^{1,2}, Li-Qiao Chen^{1,*} and Zhen-Yu Du^{1,*}**ABSTRACT**

Insulin-like growth factor-1 (IGF-1) plays a crucial role in regulating growth in vertebrates whereas suppressors of cytokine signaling (SOCS) act as feedback inhibitors of the GH/IGF-1 axis. Although SOCS-2 binds the IGF-1 receptor and inhibits IGF-1-induced STAT3 activation, presently there is no clear evidence as to whether IGF-1 could induce SOCS gene expression. The current study aimed to determine whether IGF-1 could induce the transcription of SOCS in juvenile Nile tilapia (*Oreochromis niloticus*). We show that there is a common positive relationship between the mRNA expression of *IGF-1* and *SOCS-2* under different nutritional statuses and stimulants, but not the mRNA expression of *SOCS-1* and *SOCS-3*. Furthermore, rhIGF-1 treatment and transcriptional activity assay confirmed the hypothesis that IGF-1 could induce *SOCS-2* expression, whereas it had no effect or even decreased the expression of *SOCS-1* and *SOCS-3*. Overall, we obtained evidence that the transcription of *SOCS-2*, but not *SOCS-1* or *SOCS-3*, could be induced by IGF signaling, suggesting that *SOCS-2* serves as a feedback suppressor of the IGF-1 axis in juvenile Nile tilapia.

KEY WORDS: Nile tilapia, Insulin-like growth factor, Suppressor of cytokine signaling, Modulation

INTRODUCTION

Insulin-like growth factor-1 (IGF-1) has extensive functions in differentiation, development, reproduction, growth and aging in vertebrates (Adashi, 1998; Cohen et al., 2009; Gougeon, 1996; Laron, 2001; Rommel et al., 2001). IGF-1 is primarily transcribed, synthesized and secreted in the liver (Daughaday and Rotwein, 1989; Yakar et al., 1999). Generally, the synthesis of IGF-1 is induced by growth hormone (GH) through the JAK2/STAT5 pathway (Teglund et al., 1998). However, GH is not the only regulator of IGF-1 and its activation can occur independent of GH during early development (Nakae et al., 2001).

The suppressor of cytokine signaling (SOCS) family of proteins was originally described as one of the most important feedback inhibitors of JAK/STAT signaling (Crocker et al., 2008). *SOCS-1* and *SOCS-3*, via inhibition of JAK/STAT signaling and competition for the binding of IRS1, cause induced insulin resistance in obesity and diabetes models (Howard and Flier, 2006; Yoshimura et al., 2007). GH treatment induces *SOCS-2* and *SOCS-3* expression in the liver, both *in vivo* and *in vitro* (Adams

et al., 1998; Tollet-Egnell et al., 1999). Moreover, *SOCS-2* is proposed to be a feedback inhibitor of the GH/IGF-1 axis, which is reflected by the overgrowth phenotype of *SOCS-2*-deficient mice (Metcalf et al., 2000). Although *SOCS-2* binds the IGF-1 receptor and inhibits IGF-1-induced STAT3 activation (Dey et al., 1998; Zong et al., 2000), presently there is no clear evidence as to whether IGF-1 could induce *SOCS* gene expression.

In teleost fishes, IGF-1 is an important regulator of somatic growth and is deemed as a potential endocrine biomarker that can both reflect and predict growth rates in fish (Picha et al., 2008). Recently, a comprehensive study indicated that type II SOCS could be induced by GH and inhibit GH-induced IGF-1 expression in carp hepatocytes, suggesting that SOCS presumably has a feedback regulation role in GH signaling in fish (Jiang et al., 2016). However, knowledge about the feedback regulation of the IGF-1 signaling system is still limited. Our previous study suggested that juvenile Nile tilapia *SOCS-1* and *SOCS-3* are mainly involved in innate immune response regulation, whereas *SOCS-2* functions are primarily in metabolic regulation (Liu et al., 2016). In the present study, we assessed the simultaneous expression of *IGF-1* and *SOCS-1*, *-2* and *-3* under treatments with different nutritional statuses and stimulants. Furthermore, we tested the hypothesis that IGF-1 could induce *SOCS-2* expression in juvenile Nile tilapia. This hypothesis was confirmed using rhIGF-1 treatment and a transcriptional activity assay. Our results demonstrate a common positive relationship between the mRNA expression of *IGF-1* and *SOCS-2* under different treatments. Furthermore, IGF-1 can induce *SOCS-2* expression, whereas it has no effect or even decreases the expression of *SOCS-1* and *SOCS-3* in juvenile Nile tilapia.

MATERIALS AND METHODS

This research project was approved by the Animal Ethics Committee of East China Normal University and all experiments were conducted according to the principles and procedures of the Laboratory Animal Management Ordinance of China.

Experimental animals

Juvenile male Nile tilapia (approximately 2.0 g) were obtained from a commercial farm in Qingyuan (Guangdong Province, China) and acclimated in 300-liter opaque polyethylene tanks. Fish were fed with a commercial diet (Tongwei, Chengdu, Sichuan province, China) prior to experiments. Water temperature was maintained at 28±2°C with a 12 h:12 h light:dark photoperiod, and one-third by volume of the water was changed every day.

Experimental design and sampling

After 1 week of acclimation, the fish were divided into two sets of experiments. In one set, 80 visually healthy juvenile Nile tilapia with relatively similar masses (3.0±0.5 g) were randomly distributed into four 140-liter opaque polyethylene tanks (20 fish each tank) in order to study the simultaneous expression of *IGF-1*

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and *SOCS-1*, -2 and -3 under different nutritional statuses. The four tanks were divided into two dietary groups (two tanks per group): a control diet group (CD) and a high-fat diet group (HFD). The ingredients and formulation of the CD and HFD are shown in Table S1. The fish were hand-fed twice daily at 4% average body mass per day for 4 weeks. During the trial, fish were raised under the same conditions as those used during the acclimation period as described above. At the end of the trial, fish were starved for 24 h and three fish per tank were dissected for liver sample collection. The remaining fish in the CD group were used for a short-term fasting trial. Fish in one tank were continually fed (fed group) while in another tank they were fasted (fasted group) for 3 days. Liver samples from the fed and fasted groups were collected 1 h after the last meal for quantification of mRNA expression of *IGF-1* and *SOCS-1*, -2 and -3.

In the second set of experiments, Nile tilapia were fed with a commercial diet for 5 weeks until their mass reached approximately 15±2 g. These fish were then used for insulin stimulation, bacterial challenge and recombinant human IGF-1 (rhIGF-1) stimulation experiments in order to determine the regulation of target gene expression by hormonal factors, pathogenic factors and regulation of *SOCS-1*, -2 and -3 expression by IGF-1, respectively. For insulin intraperitoneal (i.p.) injection, 40 fish were placed in two separate groups (20 fish per group). In the first group, fish were injected with bovine insulin (5 µg g⁻¹ body mass) (Sigma-Aldrich, St Louis, MO, USA) after overnight fasting, as previously described (Pierce et al., 2011); in the second group, fish were injected with saline water as a control. Liver samples were collected after 1, 6 and 12 h treatment.

The bacteria, *Streptococcus agalactiae*, used in the present study were obtained from the Aquatic Disease Control Laboratory (College of Marine Sciences, Hainan University). They were cultured in brain heart infusion broth (BHI) (Haling, Shanghai, China) medium at 37°C until their concentration reached 10⁶ CFU ml⁻¹. The bacterial cells were collected by centrifugation and washed twice, and subsequently resuspended in 0.85% NaCl. Six fish were i.p. injected with the bacterial suspension (2 µl g⁻¹ body mass, 10⁶ CFU ml⁻¹) after being fasted overnight, and the control group was similarly injected with sterilized 0.85% NaCl (Mian et al., 2009; Poochai et al., 2014). Liver, gill and head kidney (HDK) tissues were collected after 24 h post-injection. For rhIGF-1 i.p. injection, 24 fish were fasted for 12 h, then injected with rhIGF-1 (100 or 300 ng g⁻¹ body mass) (Abcam, Cambridge, UK). For the control group, a similar number of fish was injected with saline. Liver samples were collected 6 h post-injection for further analysis.

All fish were anesthetized with MS-222 (100 mg l⁻¹) before sampling. The samples collected were immediately frozen in liquid nitrogen, followed by storage at -80°C until they were used for analysis.

RNA isolation and cDNA synthesis

Total RNA was isolated using TransZol™ (TransGen, Beijing, China). The quality and quantity of the total RNA was determined using a NanoDrop 1000 spectrophotometer (Hach, Loveland, CO, USA). Approximately 800 ng total RNA of each sample was used to synthesize complementary DNA (cDNA) using a PrimeScript™ RT Master Mix Kit with a gDNA Eraser (Perfect Real Time) (Takara Bio, Kusatsu, Shiga, Japan) for quantitative real-time PCR (qRT-PCR).

qRT-PCR analysis

qRT-PCR was carried out in a CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The amplification was

performed in a total volume of 20 µl, containing 400 ng of cDNA as template, 10 µl of SYBR Mix (KW BIO, Beijing, China), 2.0 µl of each primer (5 µmol l⁻¹) (Table S2) and nuclease-free water. The qRT-PCR amplification began with 10 min at 95°C, followed by 40 cycles of 5 s at 95°C and 20 s at 60°C. A melting curve (0.5°C increments every 5 s from 65°C to 95°C) was performed after the amplification phase for confirmation. Elongation factor 1 alpha (*EF1a*) was used as reference gene (He et al., 2015). The comparative C_T method (2^{-ΔΔC_T} method), as described previously (Livak and Schmittgen, 2001), was used for mRNA expression.

Plasmids construction

Genomic DNA was isolated from the liver tissue of Nile tilapia using the phenol/chloroform method. Based on the Nile tilapia genome database and a promoter-finding program (Neural Network Promoter Prediction, NNPP; http://www.fruitfly.org/seq_tools/promoter.html) (Reese, 2001), the putative promoter sequences (1000–2000 bp upstream of the translation start site) of Nile tilapia *SOCS-1*, -2 and -3 were amplified using specific primer pairs with the MluI/BglII site (Table S2). The obtained PCR fragments were inserted into the MluI/BglII site of the pGL3 basic vector (Promega, Madison, WI, USA) and verified by DNA sequencing. For expression plasmids, the IGF-1 open reading frame (GenBank accession no. NM_001279503.1) fragment was amplified using specific primers (Table S2), and subcloned into the HindIII/BamHI site of the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) to construct IGF-1-pcDNA3.1. Plasmids for transfection were prepared from overnight bacterial culture using the EndoFree Plasmid Mini Kit (CWBio, Beijing, China) according to the manufacturer's instructions.

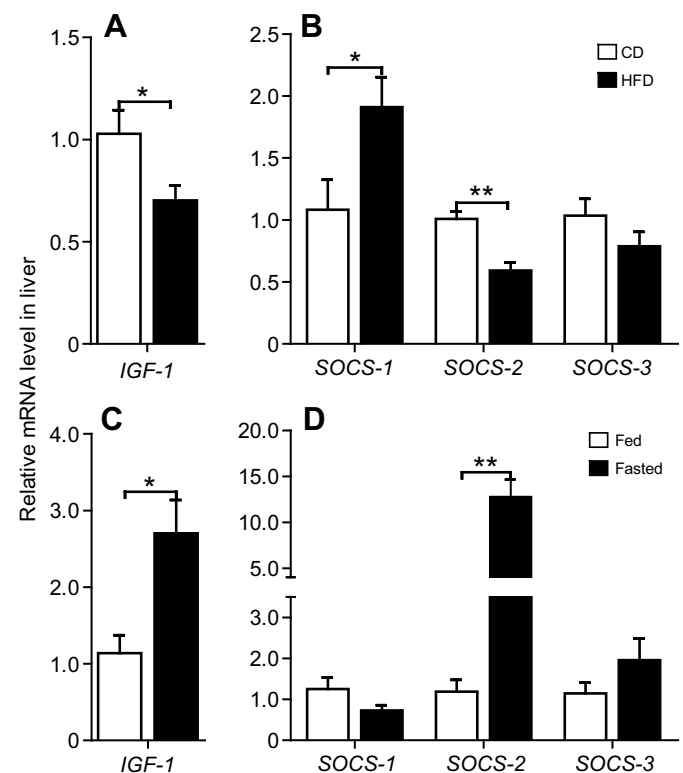


Fig. 1. The effect of different nutritional statuses on *IGF-1* and *SOCS-1*, -2 and -3 expression. (A,B) The effect of over-nutrition on *IGF-1* and *SOCS-1*, -2 and -3 mRNA levels in liver ($n=6$). CD, control diet; HFD, high-fat diet. (C,D) The effect of short-term fasting on *IGF-1* and *SOCS-1*, -2 and -3 mRNA levels in liver ($n=6$). * $P<0.05$; ** $P<0.01$. Results are shown as means±s.e.m.

Cell culture, transfection and luciferase assay

Human embryonic kidney 293 cells (HEK293T) were kindly provided by Miss Yali Chen from Shanghai Children's Medical Center (Shanghai, China). They were cultured in Dulbecco's modified eagle medium (DMEM) mixture (10% FBS, 1% penicillin-streptomycin; Gibco, Waltham, MA, USA) at 37°C in a humidified incubator with 5% carbon dioxide. One day prior to transfection, HEK293T cells were placed in 48-well culture dishes (Falcon, Tewksbury, MA, USA) and cultured in the DMEM mixture (0.2 ml per well) overnight until the cells were grown to 80–90% confluence.

Cell transfection and a dual-luciferase reporter assay were performed as previously reported in our laboratory (He et al., 2015), with some minor modifications. Briefly, each well was co-transfected (Lipofectamine 3000; Invitrogen) with 200 ng plasmids mixture, including a positive control vector (pEGFP), a normalized vector (pRL-TK), reporter vectors (pSOCS-1-pGL3, pSOCS-2-pGL3 and pSOCS-3-pGL3) and an expression vector (IGF-1-pcDNA3.1). The suitable mole ratio was 1:10:15:15 based on our preliminary experiments. The control wells were transfected with the pGL3-basic plasmid and empty pcDNA3.1 as reporter and expression vectors, respectively. After 48 h of transfection, firefly luciferase and *Renilla* luciferase activities were determined using the TransDetect™ Double-Luciferase Reporter Assay Kit (TransGen) according to the manufacturer's instructions. Briefly, the cells were washed with phosphate buffered saline (PBS) and lysed in 1× cell lysis buffer at room temperature for 10 min. The cell lysate (20 µl) was transferred into a plate with 100 µl luciferase assay reagent, mixed and firefly luciferase activity was measured using FLUOstar Omega (BMG Labtech, Ortenberg, Germany). Afterwards, luciferase assay reagent II (100 µl) was added to measure the *Renilla* luciferase activity. The firefly luciferase activity

was normalized relative to the *Renilla* luciferase activity. Each assay was performed in triplicate.

Statistical analysis

Results are presented as means±s.e.m. Data were tested for normality using the Shapiro–Wilk test and homogeneity of variance using Levene's test. All data were normally distributed and exhibited homogeneity of variance except *SOCS-2* in the fed and fasted groups, i.p. insulin injection after 1 and 6 h, bacteria challenge in the liver and dual luciferase; *SOCS-3* in the fed and fasted groups and i.p. rhIGF-1 stimulation; and the *IGF-1* group after 1 h i.p. injection. However, these data were all normally distributed after log transformation. Therefore, statistical differences between each of the two groups for every experiment were assessed using a two-tailed independent *t*-test. A *P*-value of less 0.05 was chosen as the level of significance. One-way ANOVA was used to determine statistical differences in the transcriptional activity assay followed by Tukey's multiple-range test. All statistical tests were conducted using SPSS 17.0 software for Windows (IBM, Armonk, NY, USA).

RESULTS AND DISCUSSION

Expression analysis of *IGF-1* and *SOCS-1*, -2 and -3 under different nutritional statuses

The expression of *IGF-1* is strongly associated with nutritional status (Picha et al., 2008) because it is a potential growth indicator. To determine the simultaneous expression of *IGF-1* and *SOCS-1*, -2 and -3 under different nutritional statuses, a feeding trial (CD or HFD) and a short-term fasting trial (fed or fasted) were performed. The results showed that, after 4 weeks, HFD decreased the mRNA expression of *IGF-1* and *SOCS-2* in the liver and increased the mRNA expression of *SOCS-1* (Fig. 1A,B). Furthermore, 3 days fasting increased the mRNA expression of *IGF-1* and *SOCS-2* in the

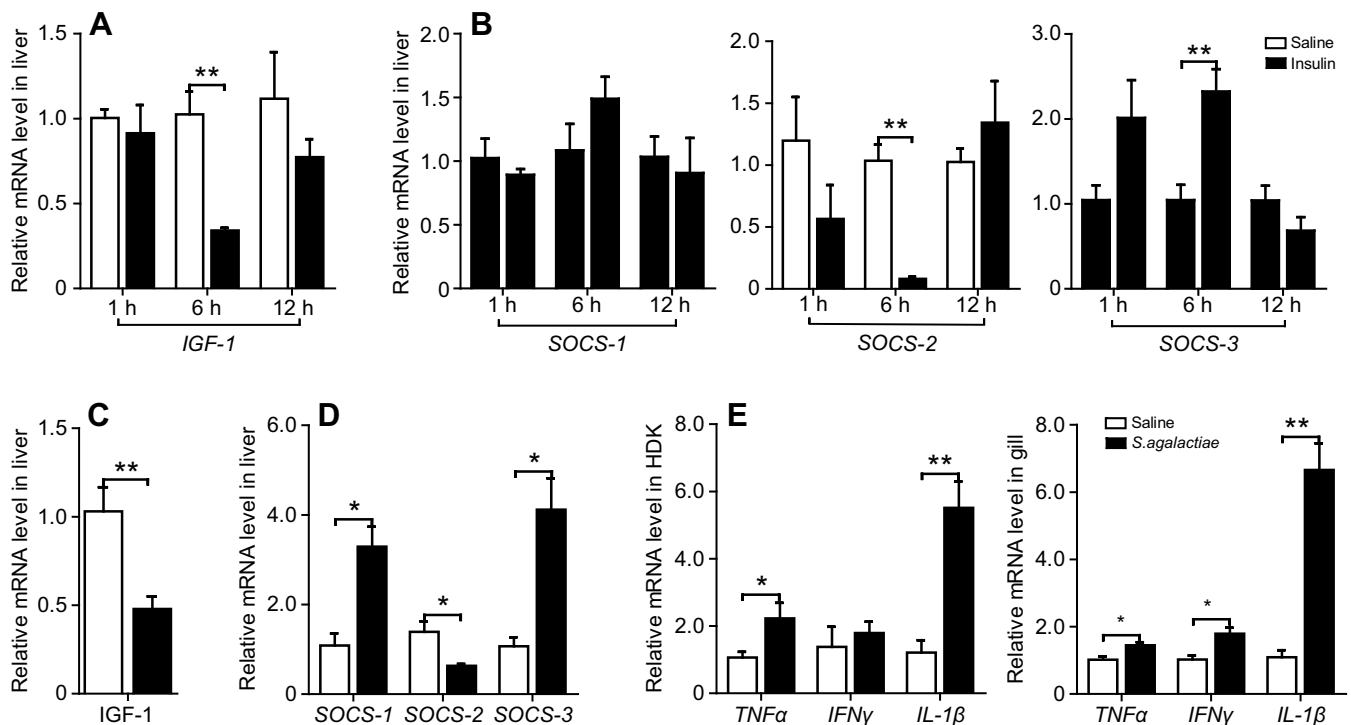


Fig. 2. The effect of different stimulants on *IGF-1* and *SOCS-1*, -2 and -3 expression. (A) *IGF-1* and (B) *SOCS-1*, -2 and -3 mRNA levels after 1, 6 and 12 h insulin treatment ($n=6$). (C) *IGF-1* and (D) *SOCS-1*, -2 and -3 mRNA levels in liver after 24 h infection with *Streptococcus agalactiae* ($n=6$). (E) *TNFα*, *IFNγ* and *IL-1β* mRNA levels in head kidney (HDK) and gill after 24 h infection with *Streptococcus agalactiae* ($n=6$). * $P < 0.05$; ** $P < 0.01$. Results are shown as means±s.e.m.

liver, but no changes in *SOCS-1* and *SOCS-3* mRNA levels were recorded (Fig. 1C,D). In mammals, a decreased level of *IGF-1* is commonly accompanied with obesity (Lewitt, 2017). In this study, Nile tilapia fed on the HFD for 4 weeks had decreased *IGF-1* mRNA expression, increased weight gain and visceral fat deposition (Table S3), which induced obesity, consistent with previous findings in mammals. However, HFD increased the mRNA expression of *IGF-1* in Senegalese sole, but did not induce obesity and even was associated with the lowest growth rate compared with low-fat diets (Campos et al., 2010). It has been shown that prolonged starvation generally results in suppression of *IGF-1* expression in fish (Wood et al., 2005). However, short-term fasting increased the *IGF-1* expression in rabbitfish (Ayson et al., 2007), as well as in the present study. In addition, we found that the expression of *SOCS-2* also varied with nutritional status and had a positive transcriptional relationship with *IGF-1*. These results suggest that *SOCS-2* as a feedback suppressor may be involved in regulation of the *IGF-1* signaling system.

Expression responses of *IGF-1* and *SOCS-1*, -2 and -3 to stimulants

To determine the regulation of target gene expression by pathogenic and hormonal factors, juvenile Nile tilapia were i.p. injected with insulin and *S. agalactiae*. The results showed that the mRNA expression of *IGF-1* and *SOCS-2* were decreased in the liver, whereas the mRNA expression of *SOCS-3* was upregulated after 6 h post insulin treatment (Fig. 2A,B). However, no change was found in *SOCS-1* mRNA expression (Fig. 2B). The i.p. injection of *S.*

agalactiae significantly decreased the mRNA expression of *IGF-1* in the liver (Fig. 2C). *SOCS-1* and *SOCS-3* as feedback inhibitors have been reported to limit the inflammatory responses in mammals (Dalpke et al., 2001). This is in line with our previous results (Liu et al., 2016) that immune challenge increased the mRNA expression for *SOCS-1* and *SOCS-3* and decreased the mRNA expression of *SOCS-2* in the liver (Fig. 2D). In addition, the high expression of immune-related genes in HDK and gill further confirmed the immune responses induced by bacterial stress (Fig. 2E). Similar results were found in sea bream, whereby pathogenic infection decreased the *IGF-1* mRNA expression (Deane and Woo, 2005; Sitja-Bobadilla et al., 2008). In this regard, our findings demonstrate explicitly a positive simultaneous expression connection between *IGF-1* and *SOCS-2* in juvenile Nile tilapia.

The effect of *IGF-1* on *SOCS-1*, -2 and -3 transcription

In mammals, it has been confirmed that *SOCS-2* limits *IGF-1* action (Dey et al., 1998; Michaylira et al., 2006). However, the expression of *SOCS-1*, -2 and -3 was not altered in response to *IGF-1* in primary chondrocytes (Pass et al., 2012). To examine the regulation of *SOCS-1*, -2 and -3 expression by *IGF-1* in fish, we injected juvenile Nile tilapia with rh*IGF-1*. Results indicated that a low dose of rh*IGF-1* (100 ng g⁻¹ body mass) induced *SOCS-2* mRNA expression, whereas a high dose (300 ng g⁻¹ body mass) caused a loss of *SOCS-2* responsiveness (Fig. 3A). Both *SOCS-1* and *SOCS-3* mRNA levels were decreased with rh*IGF-1* treatment (Fig. 3A). Furthermore, a dual-luciferase reporter assay was conducted to investigate the effect of *IGF-1* on *SOCS* promoter activity. After

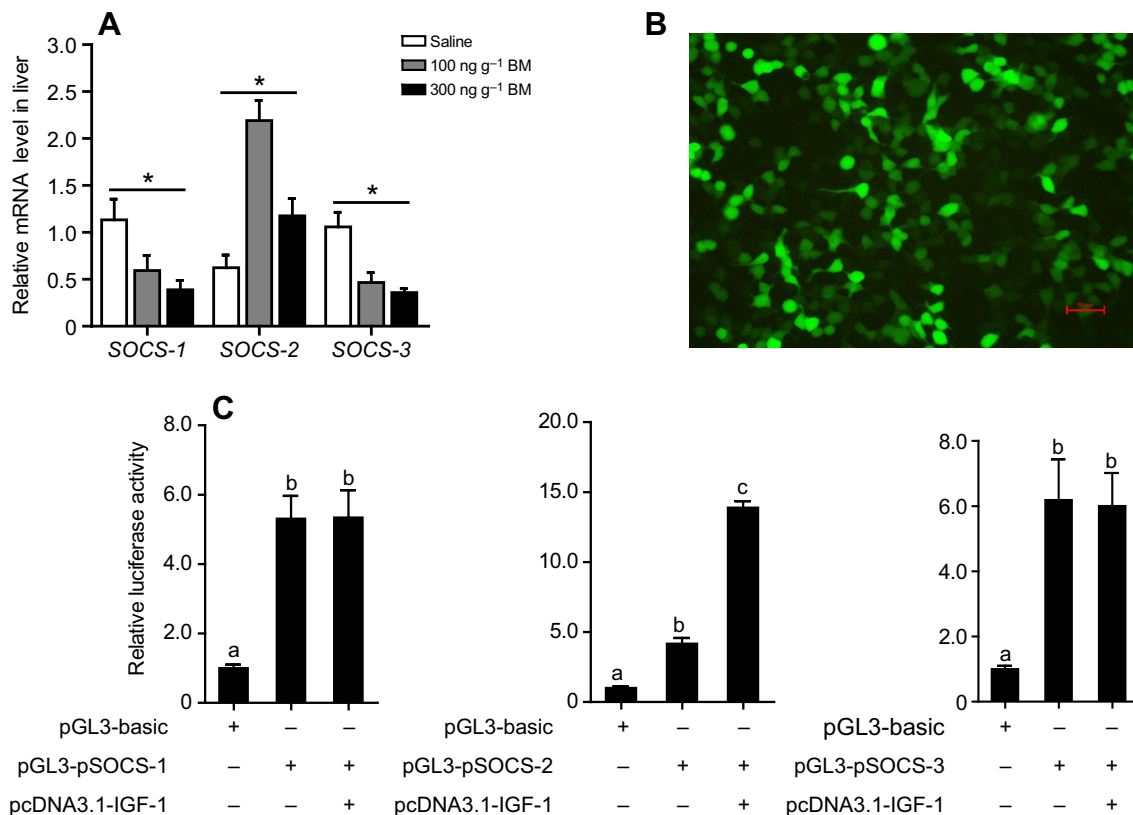


Fig. 3. The effect of *IGF-1* on *SOCS-1*, -2 and -3 transcription. (A) The mRNA expression of *SOCS-1*, -2 and -3 in liver after 6 h rh*IGF-1* injection ($n=6$). * $P<0.05$. (B,C) Transcriptional activity assay in the HEK293T cell line seeded on a 48-well plate. (B) pEGFP was transfected to determine the transfection efficiency. The high and ubiquitous signals of GFP after 48 h of transfection indicate high transfection efficiency. Scale bar, 50 μ m. (C) Relative dual-luciferase activity analysis of *IGF-1* in the *SOCS-1*, -2 and -3 promoters (left to right) in HEK293T cells. pGL3-basic was used as a control. Different letters indicate a significant difference from the control. Results are shown as means \pm s.e.m. ($n=3$).

48 h of transfection, there were high and ubiquitous signals of green fluorescent protein, indicating high transfection efficiency (Fig. 3B). The activity of luciferase driven by the *SOCS-1*, *-2* and *-3* promoters (Fig. 3C, second column in each graph) was significantly higher than the activity driven by the empty pGL3 basic vector (Fig. 3C, first column in each graph), indicating that the putative promoter sequence contained exactly the promoter. In this case, luciferase activity mediated by the *SOCS-2* promoter could be enhanced by overexpression of *IGF-1* (Fig. 3C, third column of the second graph) and has no effects on *SOCS-1* and *SOCS-3* promoters (Fig. 3C, third column of the first and third graphs). A previous study showed that GH can induce the expression of both *IGF-1* and *SOCS-2*, with the latter inhibiting the GH signaling transduction in carp hepatocytes (Jiang et al., 2016). Our results indicate that IGF-1 can induce the transcriptional expression of *SOCS-2*, whereas it has no effect on the promoter activity of *SOCS-1* and *SOCS-3* and even inhibits the mRNA levels of *SOCS-1* and *SOCS-3*.

Conclusions

Taken together, our results indicate that the expression of *IGF-1* in juvenile Nile tilapia can be affected by nutritional status, hormonal induction and pathogenic factor. In addition, *SOCS-2*, but not *SOCS-1* or *SOCS-3*, showed a positive simultaneous expression in relation to *IGF-1*. The treatments using rhIGF-1 and dual-luciferase reporter assay provide evidence that IGF-1 could induce the transcriptional expression of *SOCS-2*. These findings propose new insights that boost our understanding of the IGF signaling system.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.L., Z.D.; Methodology: C.L., Y.L., Z.D.; Validation: C.L., Y.L., S.M.L.; Formal analysis: C.L., Y.L.; Investigation: C.L., Y.L.; Resources: C.L., Y.L.; Data curation: C.L., S.M.L.; Writing - original draft: C.L., S.M.L., L.C., Z.D.; Writing - review & editing: Z.D.; Visualization: C.L., Z.D.; Supervision: L.C., Z.D.; Project administration: L.C., Z.D.; Funding acquisition: L.C., Z.D.

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Supplementary information

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