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Ctenopharyngodon Idella STAT3 alleviates autophagy by up-regulating *BCL-2* expression

Xingxing Chen[a], Kun Han[a], Gang Lin[a], Changxin Liu[a], Shanghong Wang[a], Xiao Shi[a], Zhizhen Hu[a], Chuxin Wu[b], Xiaowen Xu[a], Chengyu Hu[a,*]

[a] Department of Bioscience, College of Life Science, Nanchang University, Nanchang, 330031, China
[b] Yuzhang Normal University, Nanchang, 330103, China

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ABSTRACT

In mammals, STAT3 (Signal transducer and activator of transcription 3) plays an absolutely vital role in response to cytokines and growth factors. In mammals, IL-6/JAK/STAT3 pathway is closely linked to immune response and promotes cell proliferation, survival and metastasis. Some recent studies have already demonstrated that STAT3 regulates autophagy. As a downstream target gene of STAT3, Bcl-2 (B-cell lymphoma 2) not only participates in regulating apoptosis, but also responds to autophagy. STAT3 regulates autophagy through Bcl-2. In general, the generation of autophagy is always accompanied by the change of apoptosis, and the occurrence of apoptosis is often accompanied by the decreased of cell viability. In grass carp (*Ctenopharyngodon idella*), LPS-induced autophagy is involved in the release of pro-inflammatory cytokines. However, only the relationship between autophagy and cytokines was illustrated, in which the signaling pathways were not discussed. In the present study, we found that the autophagy inducer, Tunicamycin (Tm), can induce *C.Idella* Kidney cells (CIK) autophagy. When the cells were incubated with the recombinant human IL-6 (rIL-6) for a short period of times, the mRNA expression level of *C.Idella IL-6R* and *STAT3* were increased. At the same time, the number of GFP-LC3 puncta and the ratio of LC3-II/LC3-I were both decreased obviously in cells. It indicated that the rIL-6 can significantly alleviate autophagy induced by Tm. We speculated that CiSTAT3 may play a key role in the process. To confirm this hypothesis, we performed a rIL-6 activating CiSTAT3 assay. The result demonstrated that rIL-6 can induce CiSTAT3 to form homologous dimmer. The activated CiSTAT3 regulated the transcription activity of *CiBcl-2*, finally led to a decrease of autophagy. In addition, when cells were in the state of autophagy, apoptosis was increased and cell viability was decreased. When CiSTAT3 was activated, cell apoptosis weakened and cell viability was increased. The results suggest that CiSTAT3 plays an important role in maintaining the normal physiological process of cells.

1. Introduction

Autophagy is a process by which cells capture intracellular proteins, lipids, organelles and deliver them to the lysosomal compartment, where they are degraded and could be reused [1]. The resulting breakdown products, such as amino acid, nucleosides, carbohydrates and fatty acid, provide substrates for both biosynthesis and energy generation, thus maintaining cellular metabolism [2]. Autophagy not only guards cellular homeostasis in condition of endogenous distress [3] but also plays a primordial role in controlling intracellular pathogens in evolutionarily distant species, ranging from unicellular organisms to human [4]. Thus, together with the endoplasmic reticulum (ER) stress response, autophagy represents one of the most primitive examples of innate immune responses. In animals, this cell-autonomous defense mechanism also facilitates the recognition of infected cells by innate immune effectors, especially when infection leads to cell death [5] and sets off an elaborate inflammatory or immune response.

IL-6 is a pleiotropic cytokine that plays a major role in the response to injury and infection [6]. Elevated expression of IL-6 is implicated in diverse human diseases including inflammatory and autoimmune disorders such as rheumatoid arthritis, Crohn's disease, systemic lupus erythematosus, Castleman's disease, Behcet's disease, systemic juvenile idiopathic arthritis as well as in coronary artery, meurologic disease and in neoplasms [7–9]. Recently, it has been reported that IL-6 regulates the autophagic process through both inhibitory and stimulating effects [10–12].

* Corresponding author.
E-mail address: hucy2008@163.com (C. Hu).

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Table 1
Primer sequences and their applications in this study.

Primer name	Primer sequence (5′-3′)	Application
Bcl-2-pro-F	GCAAGATAATTAACACTTTCAGAT	Promoter cloning
Bcl-2-pro-R	CGTTATTAAACAGGATAAAAACGA	
STAT3-ORF-F	ATGGCCCAGTGGAATCAGCTA	ORF cloning
STAT3-ORF-R	TCAGTAGTGGCTACATCCCTG	
Bcl-2-ORF-F	ATGGCCAACGAAATTCGCTATG	
Bcl-2-ORF-R	TCACTTCTGAGCAAAAAAGGCG	
LC3-ORF-F	ATGCCTTCGGAAAAGACATTTAAAC	
LC3-ORF-R	TTACTGAGGACACGCAGTTCC	
STAT3-ORF-FLAG-HindIII-F	CCCAAGCTTATGGCCCAGTGGAATCAGCTA	Recombinant plasmid
STAT3-ORF-FLAG-XbaI-R	GCTCTAGATCAGTAGTGGCTACATCCCTG	
STAT3-ORF-GFP-HindIII-F	CCCAAGCTTGCATGGCCCAGTGGAATCAGCTA	
STAT3-ORF-GFP-XbaI-R	GCTCTAGATCAGTAGTGGCTACATCCCTG	
STAT3-ORF-PCDNA3.1-KpnI-F	CGGGGTACCGCCACCATGGCCCAGTGGAATCAGCTA	Real-Time-PCR
STAT3-ORF-PCDNA3.1-BamHI-R	CGCGGATCCTCAGTAGTGGCTACATCCCTG	
Bcl-2-ORF-pGL-KpnI-F	GGGGTACCGGCAAGATAATTAACACTTTCAGAT	
Bcl-2-ORF-pGL-XhoI-R	CCGCTCGACCGTTATTAAACAGGATAAAAACGA	
LC3-ORF-GFP-XhoI-F	CCGCTCGAGACATGCCTTCGGAAAAGACATTTAAAC	
LC3-ORF-GFP-EcoRI-R	CCGGAATTCTTACTGAGGACACGCAGTTCC	
IL-6R-RT-F	GACGGTCACTATGAAACCC	
IL-6R-RT-R	TTGATGCCTCGTGTAACC	
STAT3-RT-F	GAGTGCTGGAGATTGTCCC	
STAT3-RT-R	TTCCAGTCGGCCAGTTCTTC	
Bcl-2-RT-F	GACTCCTCTCCAAACTCTGAC	
Bcl-2-RT-R	TCCTTTCTATCTCGTCTCCAG	
β-Actin-F	CACTGTGCCCATCTACGAG	
β-Actin-R	CCATCTCCTGCTCGAAGTC	

Signal transducers and activators of transcription (STAT) belong to a classified DNA binding protein family comprising the seven sub-family members [13]. All of these proteins can bind to Janus kinase and initiate the classical JAK/STAT signal transduction pathway, which is closely associated with growth, survival, differentiation, and pathogen resistance of cells [14.15]. STAT3 is a member of STAT protein family [16], which was originally identified as a transcription factor activated by cytokine IL-6 and participates in inflammation, tumorigenesis and metabolic disorders [17–19]. In response to the cytokine stimulation, STAT3 is phosphorylated on tyrosine 705 by the JAK2/TYK2 kinase, results in its dissociation from the cytoplasmic tail of cytokine receptors and to form a dimer. Then the dimer transfers into the nucleus, binds to the promoter elements and gives rise to downstream gene expression [20.21].

Recently, a succession of reports indicated that miRNA enhances autophagy by targeting STAT3 [22]. Another interesting experiment showed that cells treated with STAT3 inhibitors can also enhance autophagy [22–24]. Furthermore, Yokoyama et al. (2007) showed that the inhibition of p-STAT3 can induce autophagy [25]. On the contrary, cytoplasmic non-phosphorylated STAT3 represses autophagy [26]. In other words, STAT3 appears to be intimately involved in the autophagic process.

Although accumulating evidences showed that autophagy is closely related to STAT3, the specific mechanisms remain unexplored in fish. Grass carp (*Ctenopharyngodon Idella*), a fish species of the largest production in the world, is one of the most important freshwater aquatic animals in China and a significant economical species extensively farmed in many Asian countries [27]. In this paper, we demonstrated that rIL-6 induced CiSTAT3 dimerization and activation. The activated CiSTAT3 regulated *CiBcl-2* transcription and then led to the decrease of autophagy.

2. Materials and methods

2.1. Vectors, strain and cell lines

The vector p3 × FLAG-myc-CMV-24, pEGFP-C1, pCDNA3.1, pGL3 and pEASY-T1 were bought from Sigma, BD-Biotechnology, Invitrogen, Promega and Transgen, respectively. Competent cell *Escherichia coli (E. coli)* DH5α strain was purchased from Promega. *C. Idella* kidney (CIK) cells acquired from *C. Idella* kidney tissue were kept in our lab. HEK-293T cells were kindly provided by Professor Pin Nie (Institute of Hydrobiology, Chinese Academy of Science).

2.2. Reagents and antibodies

Recombinant Human Interleukin-6 (rIL-6), Tunicamycin and Rapamycin were purchased from Sangon Biotech (Shanghai, China). Protein A/G agarose came from Santa Cruz Biotechnology. Transdetect cell counting kit (CCK) and One Step TUNEL Apoptosis Assay Kit were purchased from TransGen Biotech, Beyotime, respectively. Anti-FLAG antibody and anti-GFP antibody were purchased from Sigma and Abmart, respectively. Rabbit anti-CiLC3 and CiGAPDH antibodies were saved in our lab. The goat anti-mouse and anti-rabbit antibodies were bought from ZSGB-BIO (Beijing, China).

2.3. Cell culture and treatment

CIK cells were maintained in M199 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Before use, cells were differentiated for 12–18 h. To induce autophagy, cells were incubated with Tunicamycin (or Rapamycin) for 22 h, and then treated with rIL-6 at the indicated concentrations for 2 h. HEK-293T cells were grown in Dulbecco modified Eagle medium (DMEM; Life Technologies Inc.) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, cultured at 37 °C in an incubators containing 5% CO_2.

2.4. Plasmid construction

Total RNA was acquired from *C. Idella* kidney tissue by using RNA simple total RNA kit (TIANGEN). cDNA was obtained from Prime Script™ RT reagent Kit with gDNA Eraser (TAKARA). Then, PCR was performed to amplify *CiLC3* and *CiSTAT3* sequences with specific primers. The data of *CiSTAT3* (JX976548.1) and *CiLC3* (KC765139.1) were obtained from NCBI (https://www.ncbi.nlm.nih.gov/). The full-

length cDNA sequences of *CiBcl-2* were cloned and kept in our lab. Then, the plasmid FLAG-tagged *CiSTAT3*, GFP-tagged *CiSTAT3*, GFP-tagged *CiLC3*, pCDNA3.1-*CiSTAT3* and pGL3-*CiBcl-2* were obtained. The primers used in the present study were listed in Table 1.

2.5. Quantitative real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) was performed to detect the relative mRNA expression with *β-actin* as an internal reference gene on CFX Connect TM Real-Time System (Bio-Rad, Hercules, USA). Amplification reactions were performed in triplicate in 20 μl containing 2.0 μl cDNA samples, 10 μl 2 × SYBR premix Ex Taq (TAKARA), 0.4 μl of each primer and 7.2 μl ddH2O. RT-PCR was conducted under following condition: 1 cycle of 95 °C/5 min; 40 cycles of 95 °C/30 s; 55 C/30 s, and 72 °C/30 s. The result was analyzed by using the $2^{-\Delta\Delta CT}$ method. All experiment was repeated three times. All group data were given in terms of relative mRNA expressed as the mean (n = 3) ± SD, and then subject to Student's *t*-test. Differences were considered as significant at $P < 0.05$, and highly significant at $P < 0.01$.

Cells were seeded in 6-well plates and grown at 28 °C to reach approximately 80% confluence, then the cells were stimulated by Tunicamycin (5 μl, 2.5 mg/ml) or Rapamycin (10 μl, 1 mg/ml). Afterwards, we extracted samples to study the expression of *CiSTAT3* at 0, 6, 12, 24, 48 and 72 h post-stimulation. Each group was repeated in triplicate in the same way. In order to verify whether rIL-6 can affect CIK cells, the CIK cells stimulated by tunicamycin for 22 h were selected and treated with rIL-6 for 2 h. We extracted samples to study expression of *CiIL-6r*; *CiBCL-2* and *CiSTAT3* respectively.

2.6. Fluorescence microscope assays

CIK cells were seeded in a glass bottom cells culture dish (NEST). When cells grown reached approximately 70% confluence, they were transfected with GFP-CiLC3. 12 h later, Tunicamycin (or Rapamycin) was added to medium. 22 h later, rIL-6 was incubated to medium for 2 h. Then, cells were washed in PBS three times. After fixed with 4% paraformaldehyde, the cells were washed with PBS three times once again and dyed with DAPI. The fluorescence of GFP-*CiLC3* puncta was observed under a fluorescence microscope. The number of puncta was counted in 6 independent visual fields. The measured data was presented as the means ± SD from three separate experiments.

2.7. Immunoblotting assays

CIK cells were seeded in 6-well plates to reach 70–80% confluences, then they were treated with Tunicamycin and rIL-6 respectively. Thereafter, cells were lysed by 100 μl NP-40 lysis buffer (1% PMSF, 1‰ leupeptin and trasylol) and incubated at 4 °C for 30 min on a rocker platform. Cell lysates were centrifuged at 13500 g for 15 min at 4 °C to discard cell debris. 80 μl cells lysates separately mixed with 20 μl 5 × SDS sample loading buffer and boiled for 10 min at 95 °C. 10 μl cell lysates were measured the concentration of lysates by Enhanced BCA Protein Assay Kit (Beyotime). LC3 and GAPDH were detected by immunoblotting. GAPDH level was monitored as a loading control.

Processed cell extracts were separated by 12% or 15% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad). The membrane was blocked for 1 h at room temperature in TBST buffer (25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.5) containing 5% non-fat milk and probed with appropriate primary ABs at an appropriate dilution overnight at 4 °C. The membrane was washed three times by TBST, and then incubated with secondary ABs for 1 h at room temperature. The membrane was washed in TBST three times once again. At last, the membrane was stained with EasySee Western Blot Kit and detected using a chemiluminescence imaging system ChemiScope Mini. Finally, Image J software was utilized to analyze the gray value of the protein strip.

2.8. Co-immunoprecipitation assays

Co-immunoprecipitation (Co-IP) assay was used to study whether CiSTAT3 forms dimer *in vivo*. HEK 293T cells grown in complete DMEM medium in 10 cm^2 culture plates for 12–18 h were co-transfected with plasmids pCMV-CiSTAT3-ORF-FLAG and CiSTAT3-ORF-GFP by using Calcium Phosphate Cell Transfection Kit (Beyotime, China).

After 12 h, the fresh medium was replaced and the culture was continued for 24 h. Carefully remove the medium, wash the cells three times with 2 ml PBS. Then the cells were lysed in 1 ml RIPA lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Sodium orthovana-date (Na_3VO_4), 1 mM phenylmethylsulfonyl fluoride (PMSF)) containing a protease inhibitor cocktail (Sigma-Aldrich) at 4 °C for 30 min on a rocker platform. Cell lysates were centrifuged at 13500 g for 15 min at 4 °C to discard cell debris. For immunoprecipitation of the FLAG-tagged CiSTAT3, GFP-tagged CiSTAT3, 80 μl cell lysates were separately added with 20 μl 5 × Sample loading buffer and boiled for 10 min at 95 °C, finally were stored at − 20 °C as an input.

1 ml of the cell extractions were incubated with 40 μl Anti-FLAG M2 Affinity Gel (Sigma) overnight at 4 °C. As a negative control, 1 ml of the cell extractions were incubated with 5 μl normal mouse IgG (Beyotime) or 5 μl anti-GFP at 4 °C for 4 h, respectively. After incubation, cell extractions were added to 40 μl of protein A/G agarose (Santa Cruz) and incubated overnight at 4 °C. Beads were collected by centrifugation at 2000*g* for 2 min at 4 °C and washed thrice with Co-IP buffer, respectively, then suspended in 40 μl of 2 × SDS sample loading buffer and boiled for 10 min at 95 °C. The equal loading of sample was analyzed by Western blot with anti-FLAG and anti-GFP.

2.9. Dual-luciferase reporter assays

The activities of firefly and renilla luciferase in cell lysates were measured using Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instruction. CIK cells were seeded in 24-well plates to reach 70–80% confluence, then pCDNA3.1-ORF-CiSTAT3 (or pCDNA3.1-Basic) and pGL3-CiBcl-2 plasmids were co-transfected into cells by using FuGENE 6 Transfection Reagent (Promega). Cells were washed twice with 200 μl PBS, lysed with 100 μl 1 × passive lysis buffer at 4 °C for 15 min, and transferred to a new 1.5 ml centrifuge tube. The vortex shocked it for 10 s, then cell lysates were collected by centrifugation at 12000*g* for 2 min at 4 °C. Cell lysates (20 μl) was transferred to a plate and 100 μl luciferase assay reagent II and 1 × stop & glo reagent were added in sequence, then firefly and renilla luciferase activities were measured by GLOMAX96 Microplate Luminometer (Promega, USA), respectively.

2.10. Cell viability assays and TUNEL assays

The rIL-6-inducted cell viability was evaluated using CCK reduction assay. Approximately 2×10^3 CIK cells were plated in 96-well plate and cultured at 28 °C for 12–18 h, followed by tunicamycin stimulating cells for 22 h, at which time rIL-6 was added for further culture for 2 h. After that, each well was added 10 μl Trans-Detect Cell Counting Kit (CCK) (Transgen, China), and then continued to incubate for 2 h at 28 °C. The plates were measured absorbancy at wavelength 450 nm by using METTLER TOLEDO (ThermoFisher). Data were presented as means of at least three independent experiments.

The cell viability was also evaluated by One Step TUNEL Apoptosis Assay Kit. CIK cells were seeded in glass bottom cell culture dish (NEST). When cells grown reached approximately 70% confluence they were added to tunicamycin. 22 hr later, rIL-6 was incubated to medium for 2 h. Cells were washed in PBS just once time. After being fixed with 4% paraformaldehyde for 30 min, they were washed with PBS once time and incubated with PBS with 0.3% TritonX-100 for 5 min. Wash cells with PBS twice times, and add 50 μl TUNEL at 37 °C for 1 h. At last,

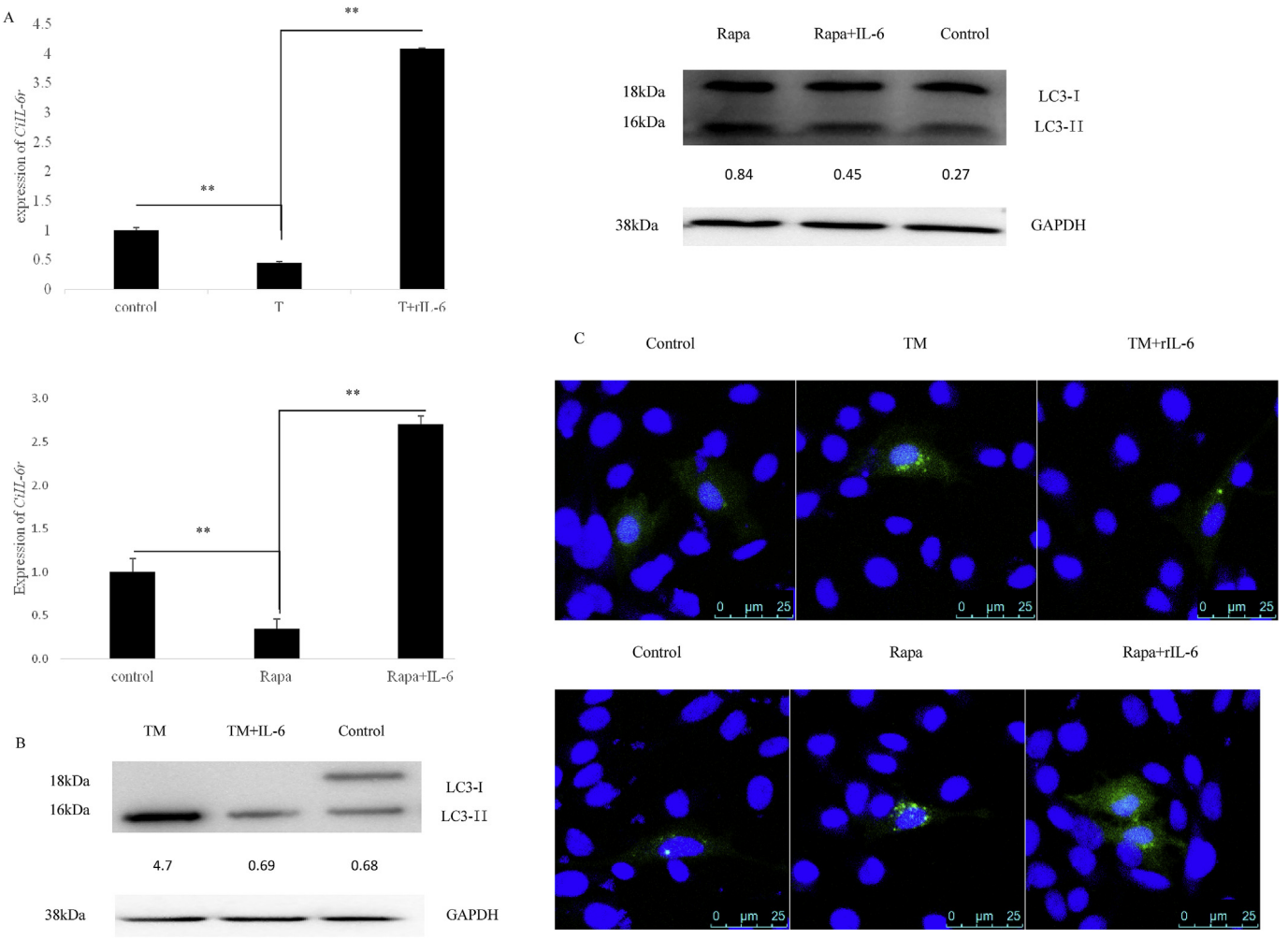

Fig. 1. rIL-6 alleviates autophagy. (A) The expression of *IL-6r* mRNA relative to *β-actin* was quantified by RT-PCR. Results were presented as the means ± SD from three separate experiments. (B) Western blot analysis of protein levels of LC3. (C) The image of CIK cells stable expression GFP-LC3 upon exposure to Tunicamycin for 24 h. (D) The endogenous LC3 puncta per cell were quantified by immunofluorescence method. Results were presented as the means ± SD from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

wash with PBS three times. The green fluorescence was observed under a fluorescence microscope and counted in 5 independent visual fields. number of puncta was counted in 6 independent visual fields from three separate experiments (Fig. 1D).

3. Results

3.1. rIL-6 alleviates autophagy

The expression pattern of *CiIL-6R* transcript in CIK cells was observed by real-time PCR. Tunicamycin (or Rapamycin) reduced *CiIL-6R* expression to less than half of the control. Similarly, when Tm (or Rapa) was used to stimulate CIK cells, the ratio of LC3-II/LC3-I was significantly increased. After rIL-6 was incubated with cells for 2 h, the expression of *CiIL-6R* was increased more than double of the control (Fig. 1A). The LC3-II/LC3-I ratio was decreased along with rIL-6 treatment, i.e. the ratio in Tm group and Tm + rIL-6 group was 4.7 and 0.69 respectively; the ratio in Rapa group and Tm + rIL-6 group was 0.84 and 0.45 respectively (Fig. 1B). It was almost exactly the same as the control group. The results indicated the inhibition of autophagy by rIL-6.

The level of LC3 puncta immunofluorescence is regarded as a way to seek changes in autophagosomes of living cells. Furthermore, when the autophagy inducer treated cells, lots of LC3 puncta were showed. When rIL-6 was incubated in autophagy cells for short incubation time, the LC3 puncta was decreased almost as the same as control (Fig. 1C). The

3.2. CiSTAT3 is involved in the inhibition of autophagy

To determinate whether CiSTAT3 participates in autophagy, we used Tunicamycin or Rapamycin to stimulate cells. qRT-PCR showed that the relative *CiSTAT3* mRNA expression was down-regulated after Tunicamycin (or Rapamycin) treatment. The lowest mRNA expression was at 24 h post-treatment (Fig. 2A and B). However, the expression of *CiIL-6r* and *CiSTAT3* were significantly up-regulated after treatment with rIL-6 (Figs. 1A and 2C).

3.3. CiSTAT3 upregulates the transcription level of CiBcl-2

To determinate if CiSTAT3 up-regulates the transcriptional level of *CiBcl-2*, we co-transfected the plasmid pGL3-*CiBcl-2* with pcDNA3.1-*CiSTAT3* into CIK cells. Luciferase activity was detected in cells lysates at 24 h post-transfection. The result showed that CiSTAT3 was able to regulate *CiBcl-2* promoter activity significantly (Fig. 3A). Furthermore, *CiBcl-2* mRNA expression level was significantly up-regulated after incubation of cells with rIL-6 (Fig. 3B). This result further supported the conclusion that CiSTAT3 up-regulated the expression of *CiBCL-2*.

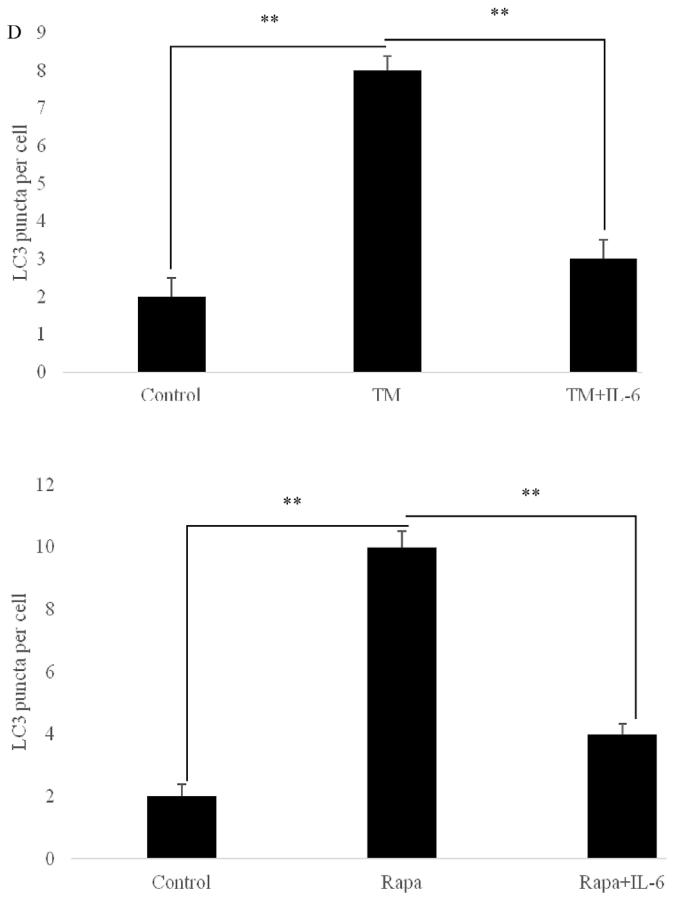

Fig. 1. (*continued*)

3.4. rIL-6 can induce homologous dimerization of CiSTAT3

Co-immunoprecipitation experiment was employed to investigate whether the CiSTAT3 dimerization occurs. The FLAG-tagged STAT3-ORF and GFP-tagged STAT3-ORF were co-transfected into HEK 293T cells. When the cell lysates were incubated with anti-FLAG affinity gel, the results of WB showed that FLAG-tagged STAT3-ORF can be detected but GFP-tagged STAT3-ORF cannot be. When the cell lysates were incubated with anti-GFP affinity gel, the results of WB showed that GFP-tagged STAT3-ORF can be detected but FLAG-tagged STAT3-ORF cannot be. These data indicated that FLAG-tagged STAT3-ORF cannot interact with GFP-tagged STAT3-ORF (Fig. 4A). Interestingly, when the cells were incubated with rIL-6, GFP-tagged STAT3-ORF can be detected in anti-FLAG affinity gel by WB, and FLAG-tagged STAT3-OFR can also be detected in anti-GFP affinity gel by WB. These results verified that IL-6 can induce homologous dimerization of CiSTAT3 (Fig. 4B). That is, rIL-6 can activate CiSTAT3.

3.5. CiSTAT3 enhances cell viability and inhibits cell apoptosis

CCK experiments indicated that Tm can lead to the decrease of cell viability. When rIL-6 was incubated with the cells for a short time, the viability of cells treated with Tm showed an upward trend (Fig. 5A). TUNEL experiments indicated that Tm can lead to enhance apoptosis, when rIL-6 was incubated with the cells for a short time, the apoptosis was reduced (Fig. 5B).

4. Discussion

The IL-6 receptor (IL-6r) is a heterodimer, consisting of an 80 kDa alpha subunit (IL-6r-alpha) and glycoprotein 130 (gp130). IL-6

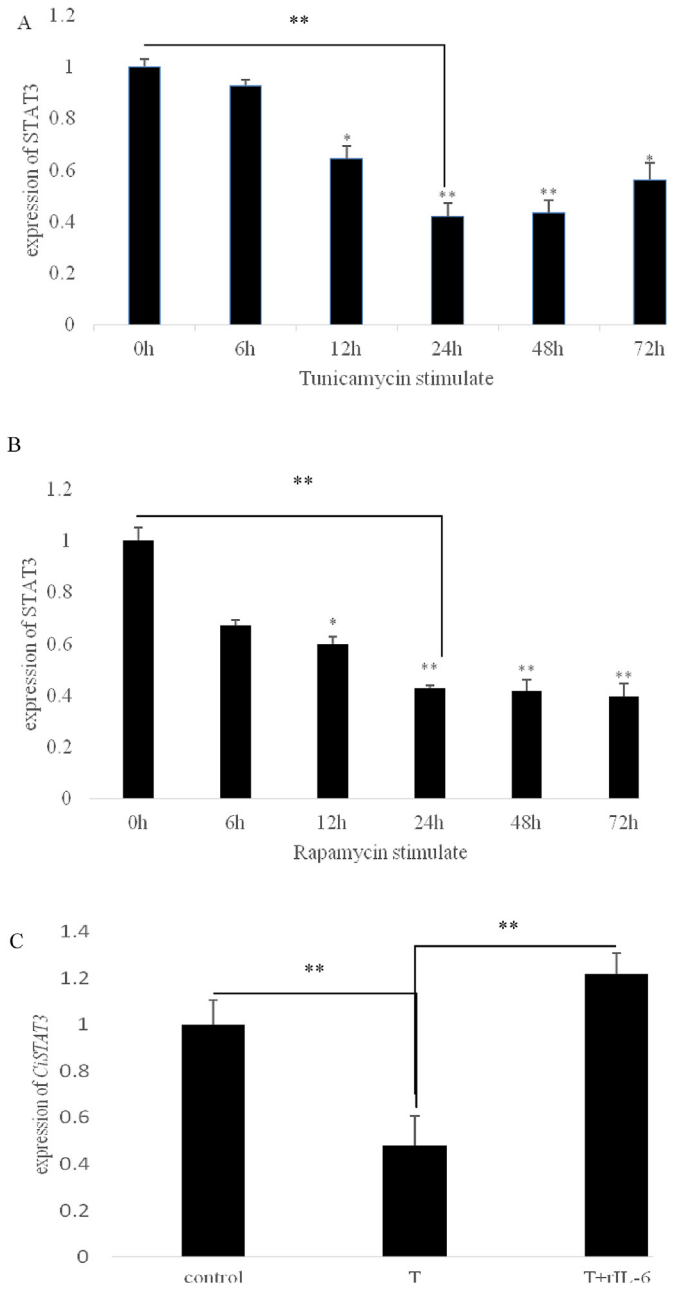

Fig. 2. CiSTAT3 is involved in the inhibition of autophagy. (A) Expression analysis of *CiSTAT3* mRNA in CIK cells stimulated by tunicamycin. (B) The expression analysis of *CiSTAT3* mRNA in CIK cells stimulated by rapamycin. (C) Expression analysis of *STAT3* mRNA in CIK cells under Tm stimulation and incubation with rIL-6 for 2 h. The measured data was presented as the means ± SD from three separate experiments.

interacts with the accessory transmembrane protein IL-6-alpha and binds to the signal-transducing gp130 subunit with high affinity to form the IL-6/IL-6r-alpha/gp130 ternary complex, which activates downstream signal transduction pathway [6]. STAT3 is an important downstream signaling protein of IL-6/IL-6r-alpha/gp130 signaling pathway.

Some studies have pointed out that IL-6 can inhibit autophagy [28.29]. LC3 is a specific protein in the initial stages of autophagy. LC3-I converts to LC3-II in the process. So, LC3-II/LC3-I ratio is chosen as a token of autophagy [30]. In this study, we also demonstrated that tunicamycin (or rapamycin) can induce autophagy in fish (Fig. 1B). Autophagy was reduced along with the incubation of rIL-6 (Fig. 1C and D).

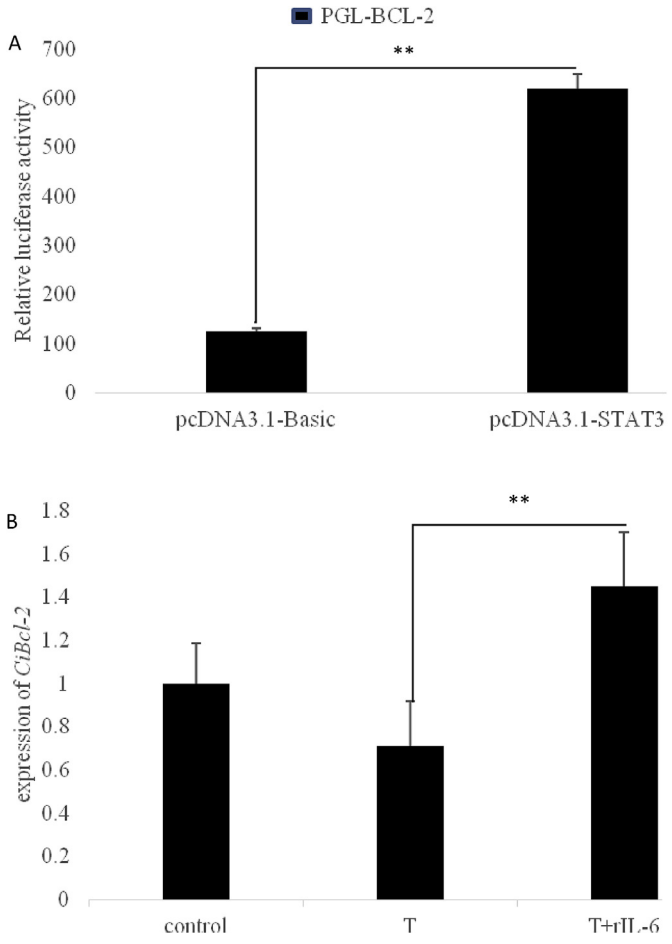

Fig. 3. CiSTAT3 upregulates the transcription level of *CiBcl-2*. (A) The effect of CiSTAT3 on the activity of *Bcl-2* promoter. (B) Expression analysis of *Bcl-2* mRNA in CIK cells under Tm stimulation and then incubation with rIL-6 for 2 h. The measured data was presented as the means ± SD from three separate experiments. $^{*}P < 0.05$.

We wanted to know how rIL-6 can inhibit autophagy in fish.

STAT3 was first recognized as a transcriptional enhancer of acute phase genes activated by IL-6 [31]. Many studies have confirmed that IL-6/JAK/STAT3 signaling pathway plays a key role in the growth and development of human cancers [32–34]. In this paper, we showed that *CiSTAT3* responded to Tunicamycin (or Rapamycin) stimulation

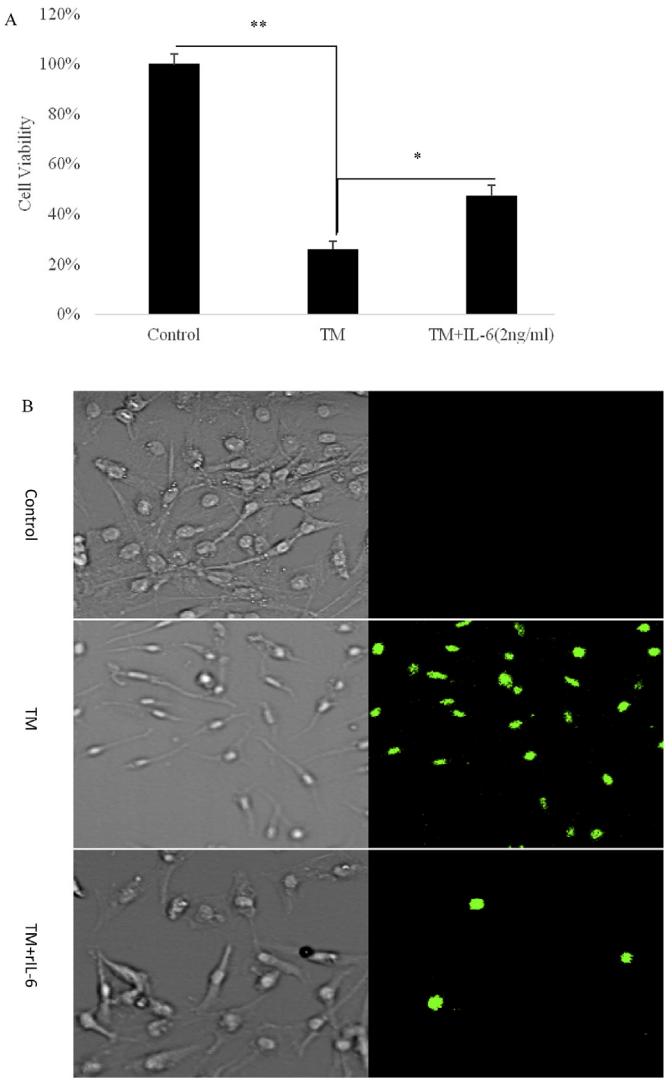

Fig. 5. Activated CiSTAT3 enhances cell viability and inhibits cell death. CIK cells were stimulated with TM for 22 h, after incubating rIL-6 for 2 h. (A) Cell viability was evaluated using CCK8 assay. (B) Cell apoptosis was observed by using One Step TUNEL Apoptosis Assay Kit.

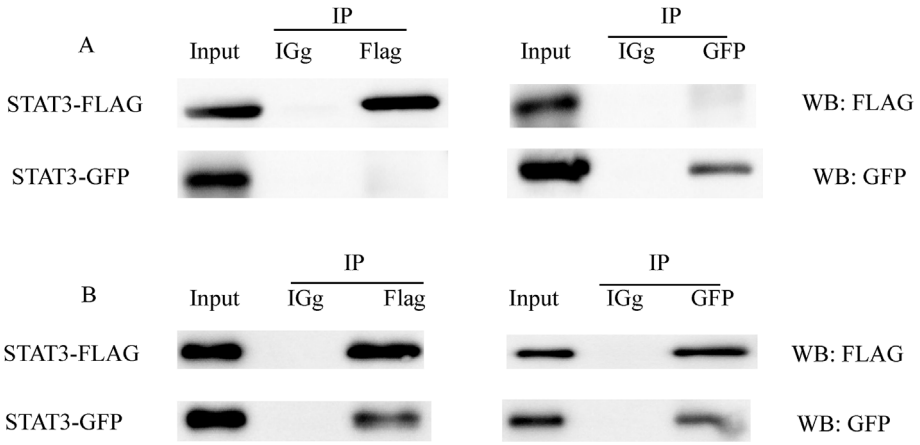

Fig. 4. rIL-6 induces CiSTAT3 to form homologous dimer. STAT3-ORF-FLAG and STAT3-ORF-GFP were co-transfected into HEK 293T cells. (A) GFP-tagged STAT3-ORF can't combine with STAT3-ORF-FLAG. (B) GFP-tagged STAT3-ORF can combine with FLAG-tagged STAT3-ORF in the presence of rIL-6.

(Fig. 2A and B). rIL-6 caused the increase of *CiIL-6* receptor (*CiIL-6r*) and *CiSTAT3* expression in grass carp cells (Figs. 1A and 2C). The above results indicated that IL-6/IL-6R/STAT3 signaling pathway also exists in fish.

In mammalian, STAT3 exists both in the nucleus and cytoplasm. Phosphorylation of STAT3 forms a dimer that avidly binds a consensus target site in the promoters of regulated genes [35]. So, the dimerization is one of the markers of STAT3 activation. In this paper, we also found CiSTAT3 dimerization in the presence of rIL-6 stimulation (Fig. 4).

STAT3 has been reported to be a transcriptional activator of *BCL-2* [36.37]. STAT3 transfers into the nucleus to activate *Bcl-2* and induces its expression [38.39]. On the contrary, the downregulation of STAT3 activity and Bcl-2 expression can induce autophagy [40]. Bcl-2 has been identified as an anti-autophagic effector protein and it interacts directly through its BH3 domain with Beclin1, the key activator of autophagy [41]. Miao et al. (2014) showed that Becn1 may also be a direct transcriptional target of STAT3 because cells transfected with constitutively activated STAT3 and a domain-interfering STAT3 mutant ($STAT3^{Y705F}$) increase Becn1 both at mRNA and protein level [42]. In this paper, we proved that CiSTAT3 upregulated the transcription level of *CiBcl-2* (Fig. 3A). Of course, the relationships among STAT3, Bcl-2 and Becn1 in fish are worthy of further investigation.

Autophagy and apoptosis play a significant role in regulating cell homeostasis and survival [43.44]. Quercetin can induce ER stress, autophagy and apoptosis [45–47]. On the contrary, the remission of ER stress will inhibit autophagy and apoptosis [48]. Thus, autophagy and apoptosis are positively correlated to some extent in cells under stress.

However, the relationship between autophagy and apoptosis is complex. Autophagy can induce apoptosis, but in some cases inhibits apoptosis [49], suggesting that STAT3 plays a regulator role. In this study, *CiSTAT3* expression and cell viability were decreased along with the increase of cell apoptosis under ER stress. When CiSTAT3 was activated, cell viability was increased (Fig. 5A), apoptosis and autophagy were decreased (Fig. 5B; Fig. 1B and C). This implies that fish STAT3 plays an extremely important role in regulating autophagy and apoptosis.

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