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Short communication

Functional characterization of grass carp runt-related transcription factor 3: Involvement in TGF-β1-mediated c-Myc transcription in fish cells

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ABSTRACT

In mammals, both runt-related transcription factor 3 (RUNX3) and c-Myc are the downstream effectors of transforming growth factor-β1 (TGF-β1) signaling to mediate various cellular responses. However, information of their interaction especially in fish is lacking. In the present study, grass carp (*Ctenopharyngodon idella*) *runx3* (*gcrunx3*) cDNA was cloned and identified. Interestingly, opposing effects of recombinant grass carp TGF-β1 (rgcTGF-β1) on *c-myc* and *runx3* mRNA expression were observed in grass carp periphery blood lymphocytes (PBLs). Parallelly, Runx3 protein levels were enhanced by rgcTGF-β1 in the cells. These findings prompted us to examine whether Runx3 can mediate the inhibition of TGF-β1 on *c-myc* expression in fish cells. In line with this, overexpression of grass carp Runx3 and Runx3 DN (a dominant-negative form of Runx3) in grass carp kidney cell line (CIK) cells decreased and increased *c-myc* transcript levels, respectively. Particularly, the regulation of Runx3 and Runx3 DN on *c-myc* mRNA expression was direct since they were presented in the nucleus without any stimulation. In addition, rgcTGF-β1 alone suppressed *c-myc* mRNA expression in CIK cells as in PBLs. Moreover, this inhibitory effect was also observed when grass carp Runx3 and Runx3 DN were overexpressed. These results strengthened the role of TGF-β1 signaling in controlling *c-myc* transcription. Taken together, TGF-β1-mediated *c-myc* expression was affected at least in part by Runx3, thereby firstly exploring the functional role of Runx3 in TGF-β1 down-regulation on *c-myc* mRNA expression in fish.

1. Introduction

Runt-related transcription factors (RUNX) are a family of proteins with an evolutionarily conserved Runt domain (RD), which play crucial roles in the tissue development and immune regulation [1,2]. Three members (RUNX1, RUNX2 and RUNX3) of this family have been characterized and their same or distinct roles in development of some tissues and cells have been revealed [3–5]. Among these members, RUNX3 has captured more attention in view of its involvement in T cell development and tumor pathogenesis induced by transforming growth factor-β1 (TGF-β1) [6–9]. In fact, TGF-β1 can bind to its transmembrane serine/threonine kinase receptors and it leads to the phosphorylation of Smads [10]. These phosphorylated receptor-regulated Smad proteins (R-Smads) can interact with RUNX3 to initiate transcription of some target genes functioning in cell development, growth and differentiation [11–13].

Transcription factor c-Myc is a central regulator of cell growth and proliferation [14] and a key target of TGF-β1 signaling [15,16]. As an example, TGF-β1 secreted by T lymphocytes can inhibit proliferation of various cells through directly affecting cell cycle regulator, c-Myc [16]. Besides, suppression on NF-κB/Rel by TGF-β1 decreases c-Myc expression and leads to apoptosis of immature B cells [17]. These findings highlight the role of c-Myc in TGF-β1-mediated proliferation and apoptosis of lymphocyte [18], but the precise mechanisms by which TGF-β1 regulates c-Myc expression remain unknown. It has been suggested that RUNX3 may inhibit cell cycle by stimulating inhibitors of cyclin/cyclin-dependent kinases [18]. However, it is unclear whether RUNX3 is involved in the regulation of TGF-β1 on c-Myc expression.

Runx3 genes have been identified in zebrafish, fugu and rainbow trout [19–24]. The inducible expression profiles of Runx3 in fish immune response remain elusive, and the functional evidence for Runx3 as a transcription factor in fish is lacking. Additionally, the role of c-Myc in apoptosis and cell proliferation *in vivo* has been investigated in a transgenic medaka, showing similar functions as human c-Myc [25]. Nevertheless, whether TGF-β1 can regulate c-myc expression in fish is still unclear.

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In the present study, we studied the role of Runx3 in TGF-β1-mediated *c-myc* mRNA expression in grass carp (*Ctenopharyngodon idella*) cells. In view of the high levels of grass carp *runx3* (*gcrunx3*) mRNA in periphery blood lymphocytes (PBLs) and the importance of periphery blood immune cells in fish immunology research and vaccine development [26], PBLs were used as cell model. The cells were treated with recombinant grass carp TGF-β1 (rgcTGF-β1) and *gcrunx3* mRNA expression was detected. Results showed that rgcTGF-β1 significantly down-regulated *c-myc* and up-regulated *gcrunx3* mRNA expression in the cells. Moreover, rgcTGF-β1 markedly increased Runx3 protein levels in PBLs. These findings prompted us to examine whether Runx3 can affect *c-myc* mRNA expression in fish cells. To address this hypothesis, overexpression of grass carp Runx3 or Runx3 DN (a dominant-negative form of Runx3) was performed in grass carp kidney cell line (CIK). Results showed overexpression of Runx3 and Runx3 DN had inhibitory and stimulatory effects on *c-myc* mRNA expression, respectively. In accordance with their direct transcriptional activities, both Runx3 and Runx3 DN showed nuclear localization under non-stimulation conditions. These data support the notion that Runx3 may function as an effector of TGF-β1 and be involved in the inhibition of TGF-β1 on *c-myc* mRNA expression in fish cells.

2. Materials and methods

2.1. Animals

Healthy grass carp weighing from 0.75 to 1 kg were purchased from Chengdu Tongwei Aquatic Science and Technology Company (Chengdu, China). Before experiments, the fish were acclimated to the laboratory environment (1000-L aquaria) for at least 2 weeks. During the procedures of cell preparation, the fish were sacrificed after anesthesia in 0.05% MS222 (Sigma-Aldrich, St. Louis, MO) and whole blood was collected. All animal experiments are in compliance with the Regulation of Animal Experimentation of Sichuan province, China and approved by the ethics committee of the University of Electronic Science and Technology of China (Permission number: 09-201501-07).

2.2. Cloning of gcrunx3 cDNA

Total RNA was isolated from grass carp thymus by using TRIzol Reagent (Invitrogen, Carlsbad, CA). About 2 μg of total RNA was subjected to reverse transcription by using Oligo $(dT)_{18}$ as the primer with SuperScript II Reverse Transcriptase (Invitrogen). Partial sequence of *gcrunx3* was amplified by the degenerate primers (Supplemental Table 1) which were designed based on the conserved regions of its counterparts in zebrafish (GenBank: NM_131604.2) and fugu (GenBank: NM_001098651.1). A fragment of *gcrunx3* cDNA was obtained by PCR and its full-length cDNA sequence was obtained using 3′- and 5′- Rapid Amplification of cDNA Ends (RACE, Invitrogen) by using the primers listed in Supplemental Table 1. Finally, the assembled full-length sequence of *gcrunx3* was amplified with Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) by using the primers for sequence validation in Supplemental Table 1.

2.3. Sequence analysis of gcrunx3

The open reading frame and deduced amino acid sequence of gcRunx3 were identified by ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/). Amino acid sequences were aligned with DNAMAN software (Lynnon Biosoft, Quebec, Canada). Characteristic domains were predicted by online SMART software (http://smart.Embl-heidelberg.De/). Phylogenetic tree was constructed by using MEGA 4 (http://www.megasoftware.net/) based on the Neighbor-Joining method with the bootstrap of 1000 repetitions.

2.4. Tissue distribution of gcrunx3 transcripts in grass carp

About 2 μg of total RNA from each selected tissue (spleen, intestine, gill, thymus, muscle, kidney, liver and head kidney) and PBLs was digested with RNase-free DNase I (Promega, Madison, WI) and then subjected to reverse transcription as described in section 2.2. Levels of *gcrunx3* and *β-actin* mRNA were quantified by real time quantitative PCR (RT-qPCR) using the primers listed in Supplemental Table 1. RT-qPCR was performed on a Bio-Rad CFX96 Real-time detection system (Bio-Rad Laboratories, Inc., Hercules, CA) in a final volume of 20 μl containing 10 μl of RealMasterMix (Tiangen, Beijing, China), 2 μl of the cDNA, 0.5 μl of each of forward and reverse primer (10 μM) and 7 μl of PCR water. The amplification program was 94 °C for 2 min, followed by 35 cycles of 94 °C for 20 s, 59 °C (*β-actin*) or 58.2 °C (*gcrunx3*) for 20 s and 66 °C for 20 s. After the amplification phase, a melting analysis (65–95 °C with a heating rate of 0.5 °C per second and a continuous fluorescence measurement) was routinely carried out to confirm the PCR product. For data calibration, a standard curve was generated for *gcrunx3* or *β-actin* gene from 10-fold serial dilutions (10^{-1}-10^{-6} femtomole/2 μl) of the plasmids containing the target gene. The PCR efficiency was calculated according to the equation $E = 10^{(-1/\text{slope})}$. In these experiments, *β-actin* was amplified as the internal control. Data were analyzed using the CFX manager (Bio-Rad) and normalized to *β-actin* after correcting for differences in amplification efficiency.

2.5. Expression analysis of gcrunx3 and c-myc in PBLs upon TGF-β1 stimulation

Grass carp PBLs were isolated by density gradient centrifugation as described previously [27]. The PBLs were seeded in 24-well plate (BD Biosciences, San Jose, CA) with the density of 6×10^5 cells/well. After overnight incubation at 28 °C, the cells were exposed to 100 ng/ml of rgcTGF-β1 [28] or medium as control for different times (1–24 h). After that, total RNA was extracted from the PBLs and RT-qPCR was performed to evaluate the mRNA levels of *gcrunx3* or grass carp *c-myc* (GenBank: EF194850.1) by using the primers listed in Supplemental Table 1.

2.6. Construction of expression plasmids

Based on the cDNA sequence of *gcrunx3* and *gcrunx3 DN* (1–180 aa) [29], the primers with restriction endonuclease cut sites were designed (Supplemental Table 1) and used to amply their sequences by using Phusion High-Fidelity DNA Polymerase. PCR product was sequenced and then ligated into pEGFP-N1 (GFP, Clontech, Palo Alto, USA) or pcDNA3.1/*myc*-His (−) A (Invitrogen) to generate pEGFP-Runx3 (Runx3), pEGFP-Runx3 DN (Runx3 DN) and Runx3-pcDNA3.1 expression plasmids.

2.7. Western blotting (WB) analysis

The anti-gcRunx3 polyclonal antibody (anti-gcRunx3 Ab) was a custom product from Abmart (Shanghai, China) and its specificity was validated by WB analysis. In brief, pcDNA3.1 plasmids or Runx3-pcDNA3.1 plasmids were transfected into HEK-293 cells. After 24 h transfection, HEK-293 cells and grass carp PBLs treated with or without 100 ng/ml of rgcTGF-β1 for different times were collected and lyzed by RIPA lysis buffer supplemented with complete EDTA-free Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland). Total protein concentration in the cell lysate was determined by Bradford protein assay (Bio-Rad). The protein samples were separated on 12% SDS-PAGE gel and electrophoretically transferred to a PVDF membrane (Millipore, Bedford, USA) by wet electro-blotting (Bio-Rad). The membranes were blocked in TBS/T buffer (25 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20, pH 7.4) with 5% (w/v) nonfat dry milk for 4 h at room temperature and incubated with the appropriate primary antibodies