PGC-1α is associated with Schizothorax prenanti myoblast differentiation

Yanying Zhaoa*, Ruiwen Lib*, Yaqiu Lina and Fan Luoab

aCollege of Life Science and Technology, Southwest University for Nationalities, Chengdu, People’s Republic of China; bReproductive Laboratory, Chengdu Woman Children Central Hospital, Chengdu, People’s Republic of China

ABSTRACT
PGC-1α has been considered as an important mediator of functional capacity of muscle. Our previous study indicated that the mRNA level of PGC-1α increased in muscle during Schizothorax prenanti (S. prenanti) growth. To understand the biological significance of PGC-1α up-regulation, S. prenanti myosatellite cells were isolated and the function of PGC-1α in myoblast differentiation was further investigated. The results indicated that PGC-1α over-expressing transfectants fused to form myotubes with higher mRNA level of myosin heavy chain isoform I (MyHCI). No obvious differentiation was observed in PGC-1α-targeted shRNA-transfected cells with a marked decrement of MyHCI expression. Furthermore, S. prenanti PGC-1α increased the expression of MyoD and MyoG, which controlled the commitment of precursor cells to myotubes. In contrast, the levels of MyoD and MyoG mRNA were down-regulated with shRNA-targeting PGC-1α transfection. These investigations indicate that PGC-1α is associated with myoblast differentiation and it elevates MyoD and MyoG expression levels in S. prenanti myoblast cells.

Introduction
Muscle tissue is made up of muscle cells. Muscle cell differentiation is a highly regulated process. After a series of histological and molecular changes, muscle cells differentiate into myoblasts, then myoblasts fuse to form myotubes, and eventually, myotubes mature to form muscle fibres (Shi and Garry, 2006; Shadrach and Wagers, 2011). Muscle-specific genes such as myogenic differentiation antigen (MyoD) and myogenin (MyoG) are expressed during muscle cell differentiation (Buckingham, 1992; Gurung and Parmaik, 2012). MyoD regulates the commitment of muscle precursor cells to myoblasts (Buck, 2001), while MyoG controls the differentiation of myoblasts to myotubes (Brzóska et al., 2002). Additionally, myosin heavy chain isoform I (MyHCI), also known as slow-twitch oxidative type I, is considered as one of the most appropriate markers for fibre type delineation, which metabolizes lipids as fuel (Zhu et al., 2013).

Peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α), firstly was characterized as a functional activator of peroxisome proliferator-activated receptor γ in brown adipose tissue (Puigserver et al., 1999). The PGC-1α protein contains four main functional domains i.e. the activation domain, myocyte-specific enhancer factor 2C binding domain, nuclear respiratory factor-1 binding domain, and the RNA binding domain (LeMoine et al., 2010). Furthermore, PGC-1α interacts with various nuclear receptor super-families, including peroxisome proliferator-activated receptors, hepatocyte nuclear factor 4α, retinoid X receptor α, as well as many transcription factors such as Forkhead box 1, sirtuin 1 and myocyte enhancer factors 2 to activate transcription of specific target genes (Handschin, 2010). As a key regulator of mitochondrial content and oxidative metabolism, PGC-1α is critical in the maintenance of glucose, lipid, and energy homeostasis in muscle and other tissues (Ventura-Clapier et al., 2008). In muscle, it transforms type IIB muscle fibres into a more oxidative phenotype and thereby PGC-1α influences meat quality such as colour, juiciness, and taste (Lin et al., 2002; Ueda et al., 2005; Yamaguchi et al., 2010). We previously cloned PGC-1α gene from Schizothorax prenanti (S. prenanti), a well-known commercial cold-water fish species distributed in the upper reaches of the Yangtze River and its tributaries in China, and found that the mRNA level of PGC-1α in muscle increased during S. prenanti growth (Li et al., 2012). And now we investigated the function of PGC-1α in S. prenanti myoblast differentiation.

Materials and methods
Viral vector production
The FUGW-PGC-1α-RFP vector was constructed by subcloning S. prenanti PGC-1α cDNA (Genbank. accession number JN195738) into FUGW at the sites of Hpal and Xbal with primers shown in Table 1. Insertion of cDNA was verified by DNA sequencing. In addition, a gene specific shRNA (table 1) for S. prenanti PGC-1α was designed online (https://rnaidesigner. invitrogen.com/mairexpress/) and synthesized according to the S. prenanti PGC-1α cDNA sequence information. The shRNA was cloned into vector pSicoR-GFP at the sites of Hpal and Xbol. Negative control RNA (table 1) shares no sequence similarities with any reported S. prenanti gene

CONTACT Yaqiu Lin yaqin@163.com College of Life Science and Technology, Southwest University for Nationalities, Chengdu 610041, People’s Republic of China
*These authors contributed equally to this paper.

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sequences. Recombinant vectors werealso verified by DNA sequencing. 293 T cells were grown on 10 cm plates to 70–80% confluence and co-transfected with these core plasmids and packaging plasmids pSPAX2, pMD2G, respectively (core plasmid: pSPAX2: pMD2G = 4:3:2). 48 h after transduction, RFP or GFP positive fluorescent cells were detected using an epifluorescent microscope (Nikon eclipse E600, Japan). The cell supernatants were collected and concentrated by ultracentrifugation.

**Isolation of *S. prenanti* myosatellite cells**

*S. prenanti* myosatellite cells were isolated as described by Fauconneau and Paboeuf (Fauconneau and Paboeuf, 2000). In brief, young *S. prenanti* (about 10 g) was anesthetized, slaughtered. Animal studies were approved by the Southwest University for Nationalities Institutional Committee for the Care and Use of Animals. The white myotomal muscle was excised under sterile conditions and minced. Fragments were centrifuged at 300 g for 5 min and washed twice in PBS supplemented with 1% penicillin/streptomycin. Enzymatic digestion was performed with 0.2% Type Ia collagenase (Sigma) in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, NY, USA) at 18 °C for 1 h with gentle agitation. The suspension was centrifuged at 750 g for 5 min, and the pellet was washed with DMEM, then given a second similar collagenase digestion, and centrifugation, the fragments were re-suspended in a trypsin solution (Sigma, 0.1% final concentration in DMEM) at 18 °C for 50 min with gentle agitation, then transferred to 2 volume of DMEM supplemented with 10% fetal bovine serum to block trypsin activity. Thereafter, the suspension was filtered on a 100 μm nylon cell strainer and centrifuged at 750 g for 2 min. The cells were re-suspended in DMEM supplemented with 10% fetal bovine serum and diluted to a final concentration of 10⁶ cells/mL.

**Cell culture, differentiation and transduction**

5×10⁵ cells/mL *S. prenanti* myosatellite cells were seeded and cultured at 18 °C in a humidified atmosphere containing 5% CO₂. When the cells were about 70% confluence, they were transfected with serially diluted and concentrated viral vector stocks. For each transfection, 10 μg/mL of polybrene (Sigma) was used in the transducing inoculum. When the cells were grown to 90% confluence, the medium was switched to differentiation media containing 2% horse serum to induce myogenic differentiation.

**Real-time RT–PCR**

Total RNAs were extracted from myoblast cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNA concentration was determined with a spectrophotometer (Biowave, Biocrom, UK). cDNA was synthesized by reverse transcription (Livak and Schmittgen, 2001). Statistical analysis was performed in a fluorescence temperature cycler (Bio-Rad, Hercules, CA, USA) with the primers shown in Table 1 under the following conditions: an initial denaturation at 95 °C for 1 min followed by 40 cycles of 30 s denaturation at 95 °C, 30 s annealing at optimal primer temperature (Table 1) and 30 s extension at 72 °C. *S. prenanti* β-actin was used as a loading control for normalization. The threshold cycle (CT) resulting from real-time PCR was analysed with a 2^ΔΔCt method (Livak and Schmittgen, 2001).

**Statistical analysis**

Data obtained from real-time RT–PCR wereanalysed statistically with SPSS Statistics V17.0 software and expressed as means ± SEM. The significance of the differences was determined by student’s t-test. Values of *p* < 0.05 were considered as statistical significance.

**Results**

**PGC-1α over-expression and interference**

To assess the biological significance of PGC-1α up-regulation during *S. prenanti* growth, *S. prenanti* myosatellite cells were isolated and two myoblast cell lines were established. One over-expressed PGC-1α via transfection of PGC-1α gene into *S. prenanti* myoblast cells, and the other decreased the constitutive expression of PGC-1α via transfection of the vector containing shRNA-targeting PGC-1α. 72 h after transfection, the mRNA
level of PGC-1α in *S. prenanti* myoblast cells was detected by real-time RT–PCR as shown in Figure 1. The results revealed that the content of PGC-1α increased to 12.67 ± 0.79 fold in myoblast cells FUGW-PGC-1α compared with the cells transfected with FUGW (*p* = .0047, Figure 1(A)). While the level of PGC-1α decreased to 0.11 ± 0.01 fold with pSicoR–shPGC1α viral infection (*p* = .0001, Figure 1(B)).

**PGC-1α induces myoblast differentiation**

Muscle differentiation is characterized by the alignment of the myoblasts into the myotubes (Phelps et al., 1998). The *S. prenanti* myoblast cells transfected with FUGW-PGC-1α differentiated to form myotube structures when cultured in differentiating medium on the 3rd day. The over-expressed PGC-1α cells formed typical myotubes with an elongated morphology compared with that observed in cells transfected with FUGW. In contrast, there was no obvious myotube formation in cells with decreased constitutive expression of PGC-1α via transfection of shRNA-targeting PGC-1α compared with those corresponding control cells. Therefore, PGC-1α regulated myogenic differentiation of *S. prenanti* myoblast cells.

**Effect of PGC-1α increases on MyoD, MyoG and MyHCI expression**

Specification of the myogenic lineage and differentiation of muscle cells are critically dependent on the basic helix–loop–helix transcription factors, MyoD and MyoG (Buckingham, 1992; Gurung and Parnaik, 2012). Furthermore, to understand the mechanism underlying the influence of PGC-1α on myoblast differentiation, the effect of PGC-1α on MyoD and MyoG expression was detected. As summarized in Figure 3, the expression level of MyoD and MyoG were up-regulated in *S. prenanti* myoblast cells with FUGW-PGC-1α transfection. In contrast, the levels of MyoD and MyoG mRNA were down-regulated with shRNA-targeting PGC-1α transfection. These results indicated that PGC-1α enhanced expression of MyoD and MyoG in *S. prenanti* myoblast cells, which was paralleled with the activation of the muscle differentiation programme. Additionally, with the transfection of PGC-1α, the MyHCI gene expression level significantly increased, while shRNA-targeting PGC-1α decreased MyHCI compared with the corresponding control cells (Figure 3). These results suggested that PGC-1α induced type I muscle fibre formation.

**Discussion**

In the past decade, some studies have focused on the function of PGC-1α in metabolic regulation of mitochondria, antioxidant defense and inflammatory response in muscle (Zhu et al., 2009; LeMoine et al., 2010; Summermatter et al., 2011), while the role of PGC-1α in regulating meat quality has been poorly documented. Our previous study indicated PGC-1α expression up-regulation in muscle during *S. prenanti* growth. In this study, we further investigated the function of PGC-1α in myoblast differentiation with over-expression and shRNA suppression strategies.

Firstly, *S. prenanti* myosatellite cells were isolated using type I collagenase and trypsin other than pronase digestion employed in isolation of pig myogenic satellite cells (Doumit and Merkel, 1992). In fact, a small quantity of *S. prenanti* myosatellite cells was obtained with pronase digestion in our study (data not shown).

**Figure 1.** PGC-1α over-expression and interference.

Notes: *S. prenanti* myosatellite cells were transfected with FUGW-PGC-1α, FUGW, PGC-1α targeted pSicoR-PGC-1α or pSicoR. 72 h later, the level of PGC-1α mRNA was tested by real-time RT–PCR in the cells transfected with FUGW-PGC-1α (A, **p** < .01 vs. that transfected with FUGW) and pSicoR–PGC-1α (B, ***p*** < .001 vs. that transfected with pSicoR).

**Figure 2.** Role of PGC-1α in *S. prenanti* myoblast differentiation.

Notes: When induced by 2% horse serum, *S. prenanti* myogenic differentiation was observed on the 3th day. FUGW denoted FUGW transfectants, FUGW-PGC-1α denoted FUGW-PGC-1α transfectants, pSicoR denoted negative control shRNA transfectants, pSicoR–PGC-1α denoted PGC-1α targeted shRNA transfectants.
shown). When PGC-1α transfected S. prenanti myosatellite cells were cultured in differentiating medium, the cells differentiated into typical myotubes. While the decreased constitutive expression of PGC-1α via transfection of shRNA-targeting PGC-1α led to no obvious myotube formation. The observation was parallel with our previous report that PGC-1α promoted typical myotube formation in murine C2C12 myoblast (Lin et al., 2014). These results suggested that PGC-1α was associated with myoblast differentiation.

Furthermore, the influence of PGC-1α on MyoD and MyoG, which controlled myoblast and myotube formation, were explored. PGC-1α increased both MyoD and MyoG expression. The up-regulation may accelerate myoblast differentiation. MyHCl, a marker for oxidative type I muscle fibre was also analysed and PGC-1α enhanced MyHCl expression. The detection indicated that PGC-1α induced type I muscle fibre formation, which was consistent with the report that PGC-1α can transform type IIb muscle fibres into a more oxidative phenotype (Lin et al., 2002). Our results revealed that PGC-1α was associated with S. prenanti myogenic differentiation. Since PGC-1α is a master regulator of mitochondrial biogenesis, the crosstalk between PGC-1α and myoblast differentiation may be related to mitochondria development.

Taken together, our study indicated that PGC-1α is closely associated with S. prenanti myogenic differentiation. The mechanisms may be attributed to increased expression of MyoD, MyoG and MyHCl.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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