Biochemical and Biophysical Research Communications xxx (2016) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Pou1f1, the key transcription factor related to somatic growth in tilapia (*Orechromis niloticus*), is regulated by two independent post-transcriptional regulation mechanisms

Dongfang Wang, Jingkai Qin, Jirong Jia, Peipei Yan, Wensheng Li*

State Key Laboratory of Biocontrol, Institute of Aquatic Economic Animals and Guangdong Province Key Laboratory for Aquatic Economic Animals, South China Sea Bio-Resource Exploitation and Collaborative Innovation Center, Research Institute of Sun Yat-Sen University in Shen Zhen, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510006, PR China

ARTICLE INFO

Article history:
Received 4 December 2016
Accepted 16 December 2016
Available online xxx

Keywords:
Pou1f1
miR-223-3p
AU-Rich element
Post-transcription regulation
Nile tilapia

ABSTRACT

This study aims to determine the post-transcriptional regulation mechanism of the transcription factor *pou1f1* (*pou class 1 homeobox 1*), which is the key gene for pituitary development, somatic growth in vertebrates, and transcription of several hormone genes in teleost fish. MicroRNA *miR-223-3p* was identified as a *bona fide* target of *pou1f*; overexpression of *miR-223-3p* in primary pituitary cells led to the down-regulation of *pou1f1* and downstream genes, and inhibition of *miR-223-3p* led to the up-regulation of *pou1f1* in Nile tilapia dispersed primary pituitary cells. An adenylate-uridylate-rich element (AU-Rich element) was found in the 3′UTR of *pou1f1* mRNA, and deletion of the AU-Rich element led to slower mRNA decay and therefore more protein output. A potential mutual relationship between *miR-223-3p* and the AU-rich element was also investigated, and the results demonstrated that with or without the AU-Rich element, *miR-223-3p* induced the up-regulation of a reporter system under serum starvation conditions, indicating that *miR-223-3p* and the AU-Rich element function independent of each other. This study is the first to investigate the post-transcriptional mechanism of *pou1f1*, which revealed that *miR-223-3p* down-regulated *pou1f1* and downstream gene expressions, and the AU-Rich element led to rapid decay of *pou1f1* mRNA. MicroRNA *miR-223-3p* and the AU-Rich element co-regulated the post-transcriptional expression of *pou1f1* independently in Nile tilapia, demonstrating that *pou1f1* is under the control of a dual post-transcription regulation mechanism.

1. Introduction

Pou1f1 is a Pou-homeodomain transcription factor that binds to the promoter region and transactivates the expression of *gh* (*growth hormone*), *prl* (*prolactin*), and *somatolactin* in teleost fish. Pou1f1 is necessary for normal development of the pituitary gland, facilitating the differentiation of lactotropes, somatotropes, and thyrotropes. Pou1f1 knock-out results in severe dwarfism and deficiency of these cell types in zebrafish [1]. The genes *gh*, *prl*, and *somatolactin* belong to the *gh/prl* superfamily; *somatolactin* is only expressed in the fish species but no other vertebrates [2], the *gh/prl* superfamily share the common transcription factor Pou1f1. Somatic growth is one of the major mechanisms most studied in economic fish species. The GH-IGF (insulin-like growth factor) axis, also known as the hypothalamus-pituitary-liver axis, is the core regulation system responsible for vertebrate growth [3]. To date, no research has been reported on the post-transcription regulation of *pou1f1*.

MicroRNAs are small non-coding RNAs of approximately 22 nucleotides that are involved in diverse physiological processes, such as development, growth, reproduction, immunity, and metabolism [4]. MicroRNAs inhibit target gene expression by promoting mRNA degradation, suppressing translation, and facilitating deacetylation by binding directly to microRNA recognition elements (MREs) in the 3'UTR of mRNA [5]. Multiple reports show that microRNAs are actively involved in the regulation of somatic growth. The muscle-specific microRNA *miR-206*, by inhibiting *myostatin*, contributes to muscular hypertrophy in Texel sheep (*Ovis aries*), which are renowned for their exceptional meatiness [6]. *Igf1* is regulated by *miR-206* in tilapia (*O.niloticus*), and specific

* Corresponding author.
E-mail address: lsslws@mail.sysu.edu.cn (W. Li).

http://dx.doi.org/10.1016/j.bbrc.2016.12.106
0006-291X/

knockdown of *miR-206* using antagormir results in significant weight gain [7]. In two tilapia strains with different growth traits, discrepant microRNA expression profiles are displayed, indicating that microRNAs may play an essential role in muscle growth [8].

The AU-Rich element is a regulatory element located in the 3'UTR of mRNAs that code for proto-oncogenes, nuclear transcription factors, and cytokines. Usually, the AU-Rich element determines the stability of mRNAs and causes mRNA rapid degradation [9]. The AU-Rich element has also been shown to activate *tnfα* (*tumor necrosis factor α*) translation in unstimulated macrophages [10]. Deletion of TNFα AU-rich elements from the mouse genome results in the enhanced stability of *tnfα* and contributes to the pathogenesis of chronic inflammatory arthritis and Crohn's-like inflammatory bowel disease [11,12]. The AU-Rich element in goldfish (*Carassius auratus auratus*) *tnfα* contributes to a shortened mRNA half-life and regulates the pathogenesis of many inflammatory diseases caused by bacterial endotoxins [13].

Co-existence of the AU-Rich element and the MRE occurs in the *tnfα* mRNA 3′UTR [14], and the interaction between microRNAs and AU-Rich element molecular components has been reported. For example, microRNA *miR-16* is necessary for AU-Rich element mediated mRNA degradation [14]; upon cell cycle arrest induced by serum starvation, *let-7,* in collaboration with an AU-Rich element, enhances target mRNA translation efficiency [15].

Nile tilapia (*O.niloticus*), with desirable traits of fast growth rate, strong fertility, omnivorousness, stress tolerance, and disease resistance, is an important species with economic value and provides a major source of premium protein. In this study, we demonstrate that microRNA-mediated gene silencing and another post-transcriptional mechanism independently co-regulate *pou1f1* expression in the tilapia.

2. Materials and methods

2.1. Experimental animals

Tilapia bought from Guangdong Tilapia Breeding farm, were cultured in circulating water in indoor tanks with the room temperature at 28 °C and a light-dark period of 16 h-8 h, and fed to satiety daily. Before experiments, tilapia were acclimated for at least one week. Before sacrificing, tilapia were narcotized with eugenol (Jingxin Bio, Guangzhou, China). All animal experiments were performed with the approval of the Sun Yat-Sen University Animal Care and Use Committee and in full compliance with its ethical guidelines.

2.2. Recombinant plasmid construction and candidate microRNAs prediction

For *pou1f1* 3′UTR cloning, KOD neo plus (TOYOBO, Osaka, Japan) was used to generate PCR (polymerase chain reaction) amplicons according to the manufacturer's protocol. For site-directed mutagenesis, primers were designed using PrimerX (http://www.bioinformatics.org/primerx/index.htm). Mutant plasmids were generated using high fidelity PfuUltra II Fusion HS DNA Polymerase (Agilent, Santa Clara, USA), and the template was digested by Dpn I (NEB, Ipswich, USA). Candidate microRNAs and MREs were predicted using PITA [16]. The minimum seed size was set at 8 and single G:U pairings and single mismatches within seed sequences were allowed. All primer sequences are listed in Table s1.

2.3. RNA extraction, first strand cDNA synthesis and relative quantitative real-time PCR

Tissues and organs harvested from tilapia or primary pituitary cells were prepared. Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, USA) according to the manufacturer's and laboratory protocol [17]. RNA concentration was determined using a Nanodrop200C spectrophotometer (Thermo Scientific, Waltham, USA), and RNA integrity was detected by 1% agarose gel electrophoresis and staining with ethidium bromide (Biovision, San Francisco, USA).

One microgram of total RNA was incubated with DNase I (NEB, Ipswich, USA) to eliminate contaminant DNA at 37 °C for 5 min and later supplemented with EDTA (5 mM) and heated at 75 °C for 5 min. For microRNA detection, specific reverse-transcription primers were added for first strand cDNA synthesis using M-MLV Reverse Transcriptase (Life Technology, Carlsbad, USA) according to the manufacturer's protocol. Tilapia *u6* snoRNA was used for the internal control. For the *pou1f1* reporter mRNA degradation assay in HEK293 cells, only random hexamer primers were used for reverse transcription, and human *18s* rRNA was used as an internal control.

The relative gene expression was detected by relative quantitative real-time polymerase chain reaction (PCR) using Thunderbird SYBR Green qPCR Mix (TOYOBO, Osaka, Japan). The quantitative PCR was performed using the CFX-96 Touch™ Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, USA), following the manufacturer's instructions. The conditions for the reaction were as follows: initial denaturation at 94 °C for 1 min, followed by 40 cycles starting with denaturation at 94 °C for 10 s, annealing at 58 °C for 10 s, and extension for 20 s. The fold change of gene expression was determined using the $2^{-\Delta\Delta CT}$ method. All primers sequences are listed in Table s1.

2.4. Immunoblotting

Considering that there are not commercial antibodies suitable for this research, an anti-rabbit polyclonal antibody to Pou1f1 was developed and utilized in the study. For protein extraction, 100 μl cell lysis reagent radio immunoprecipitation assay buffer (Byotime, Nantong, China) was supplemented with 1 mM PMSF (phenylmethanesulfonyl fluoride) (Beyotime, Nantong, China) before use. Briefly, the protein concentration was determined, and samples were prepared, and 100 μg sample was loaded, separated by Tricine-SDS-PAGE and transferred to a nitrocellulose membrane (0.2 μm, Pall Corporation, New York, USA). Membranes were blocked with 5% non-fat milk and incubated with specific antibodies at 4 °C overnight. β-actin monoclonal antibody (Sagene, Guangzhou, China) was used as an internal control. The next day, the membranes were washed 3 times (10 min each) with PBS (phosphate buffered saline), and then incubated with the HRP-conjugated goat anti-rabbit or anti-mouse IgG antibody (Boster, Wuhan, China) in PBS solution containing 5% (w/v) non-fat dry milk for 1 h at room temperature. Immunoreactivity was determined using an enhanced chemiluminescence ECL detection kit (Amersham, Buckinghamshire, UK), and gray intensity analysis was performed using IMAGE J 1.45 (NIH, Bethesda, USA).

2.5. Primary tilapia pituitary cells culture and transient transfection

The primary pituitary cell culture was conducted as previously described [17]. Briefly, intact pituitary glands were harvested from sacrificed tilapia and sliced (0.5 mm) using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co., Ltd, UK). Samples were treated sequentially with trypsin (25 mg/10 ml) shaking for 40 min at 27 °C, then with trypsin inhibitor (25 mg/10 mL), DNAse I (0.1 mg/10 mL), 2 mM EDTA, and 1 mM EDTA for 5 min each at room temperature. Then, the fragments were mechanically dispersed and filtered through a sterile nylon 200-mesh sieve and centrifuged at 200 g at 4 °C for 10 min. The dispersed cells were seeded into 24-

Please cite this article in press as: D. Wang, et al., *Pou1f1,* the key transcription factor related to somatic growth in tilapia (*Orechromis niloticus*), is regulated by two independent post-transcriptional regulation mechanisms, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.12.106