***Pou1f1*, the Key Transcription Factor Related to Somatic Growth in
Tilapia (*Oreochromis niloticus*), Is Regulated by Two Independent
Post-Transcriptional Regulation Mechanisms**

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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.

Keywords: *pou1f1*; miR-223-3p, AU-Rich element, post-transcription regulation, Nile tilapia

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 knockdown of *miR-206* using antagomir 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 *tnfa* (*tumor necrosis factor α*) translation in unstimulated macrophages [10]. Deletion of TNF α AU-rich elements from the mouse genome results in the

enhanced stability of *tnfa* 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*) *tnfa* 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 *tnfa* 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 1.

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. Immunoactivity 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-well plates (Corning, NY, USA) coated with poly-D-lysine (Sigma-Aldrich, St. Louise, US) at a density of 3×10^5 cells per well. Cells were incubated with 0.5 ml culture medium (Leibovitz's L-15

medium (Gibco, Carlsbad, USA) supplemented with 1.19 g/L HEPES, 2.66 g/L sodium chloride, 1 g/L bovine serum albumin (BSA), 100,000 U/L penicillin, and 100 mg/L streptomycin, pH 7.2.) at 27°C with saturated humidity. After 2 h, 10% FBS (fetal bovine serum) (Gibco) was added to the wells, and cells were cultured overnight.

The pituitary cells were then transfected using lipofectamine2000 (Life Technologies, Carlsbad, USA) by microRNA mimic (with or without FITC-label, synthesized by Riobio, Guangzhou, China) at the concentration of 10 nM and incubated for 24 h. The scrambled RNA duplex was nonhomologous to the tilapia genome and was used as a negative control. Transfection efficiency was detected using the inverted fluorescent microscope ECLIPSE Ti-E (Nikon, Tokyo, Japan) or flow cytometer FACS Calibur (BD Biosciences, Franklin Lakes, USA). Chemically modified antisense oligonucleotides (antagomir) were ordered (synthesized by Riobio, Guangzhou, China), *miR-223-3p* inhibitor was used in the experimental group and the negative control antagomir was used in the control group. Antagomir was transfected into the dispersed cells at 100 nM and incubated for 24 h, then cells were harvested for RNA extraction.

2.6. HEK293 cell culture, transient transfection, dual luciferase assay

HEK293 (ATCC) cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS, 2 mM glutamine, penicillin G sodium (100 U/ml), and streptomycin sulfate (100 U/ml). The incubation criteria was set as saturated humidity, 5% CO₂, and thermostatic 37°C.

The recombinant plasmid psiCHECK2 (200 ng/ml) and microRNA mimic (10 nM) were dissolved in MEM (minimum essential medium) and transfected using lipofectamine2000. Cells were cultured in MEM for 4 h, then the media was replaced with DMEM supplemented with FBS. After 24 h, the cells were harvested and lysed for the dual luciferase assay (Promega, Madison, USA). For detection, 20 µl cell lysate was detected using 20 µl of LAR II and then Stop & Glo, which were prepared according to the manufacturer's protocol. For dual luciferase assays, the relative renilla/firefly luciferase ratio was considered as the *pou1f1* reporter activity.

Actinomycin D (actD) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) to inhibit RNA transcription and determine the *pou1f1* mRNA decay rate, and 5 µg/ml actD was used to treat the HEK293 cells.

Serum starvation was performed for some experiments. After transfection for 24 h, the culture medium was removed and cells were gently washed with

37°C serum free culture medium three times, then incubated in serum free culture medium. Cells were harvested at 0 h, 1 h and 2 h.

2.7. Statistical analysis

All data were presented as the mean $\pm$ SEM, and the significance was determined by Student's t test or one-way ANOVA followed by Duncan's multiple range test using GraphPad Prism. A p value of $p < 0.05$ was considered to be statistically significant. $p < 0.05$ was noted with *, $p < 0.001$ with **, and $p < 0.0001$ with ***.

3. Results

3.1. *MiR-223-3p* was identified as a candidate target microRNA of *pou1f1*

The Nile tilapia microRNA transcriptome was obtained by next-generation sequencing and bioinformatics analysis. We identified 2,347 conserved and 1,143 novel microRNAs (data s1-2). To screen microRNAs targeting *pou1f1* mRNA, *pou1f1* 3'UTRs were obtained and cloned into the psiCHECK2 reporter plasmid. Primers were designed according to the NCBI nucleotide database, and the sequences were identified by sequencing multiple clones (data s3). Candidate target microRNAs were acquired by analyzing *pou1f1* 3'UTR sequences and the tilapia microRNA transcriptome data using the software PITA; the 40 top-rated candidate microRNAs are shown in Table s2. Dual

luciferase assays showed that the *pou1f1* reporter system was down-regulated by *miR-214*, *miR-124*, *miR-223-3p*, *miR-132*, *miR-31*, *miR-219*, and *miR-9* (Fig. 1). Overexpression of *miR-223-3p* and *miR-219* led to a decrease in the Renilla luciferase /Firefly luciferase ratio (Rluc/Fluc) by more than 50%.

The hormone *gh* is one of the genes transcribed by *Pou1f1* and plays a major role in regulating somatic growth. To explore the relationship between *gh* and microRNAs, *gh*, *miR-223-3p* and *miR-219* expression levels were investigated in tilapia of different weights. In fish weighing approximately 8 g, the expression level of *miR-223-3p* was approximately 35-fold greater than that of *gh* (Fig. s1A). In fish weighing approximately 50 g, the expression level of *miR-223-3p* was approximately 2-fold greater than that of *gh* (Fig. s1B). In fish weighing approximately 130 g, the expression of *gh* mRNA was the highest and was approximately 15-fold greater than that of *miR-223-3p* (Fig. s1C). In summary, the expression level of *gh* is negatively correlated with the expression level of *miR-223-3p*. Considering that *miR-219* is strictly expressed in the central nervous system (data not shown), *miR-223-3p* was chosen for subsequent research.

3.2. *MiR-223-3p* down-regulated *pou1f1* and downstream genes *in vitro*

The MRE of *miR-223-3p* in the *pou1f1* 3'UTR was predicted as the seed sequence of *miR-223-3p* because it was complementary to the 3'UTR. To determine whether *miR-223-3p* binds directly to the 3'UTR, site-directed mutagenesis was performed to convert nucleotides ACA to TGT (Fig. 2A). Dual luciferase assay results showed that mutagenesis of the *miR-223-3p* recognition element led to derepression of *miR-223-3p*-induced suppression (Fig. 2B).

FITC-labeled microRNA mimic was transfected transiently into tilapia primary pituitary cells. Cells were observed using a fluorescence microscope (Fig. s2), and transfection efficiency was determined by flow cytometry. Approximately half of the dispersed pituitary cells were transfected with the FITC-labeled microRNA mimic. To detect Pou1f1 protein level changes, we developed a Pou1f1 anti-rabbit polyclonal antibody (Fig. s3) in house. Overexpression of *miR-223-3p* led to Pou1f1 down-regulation at the protein level (Fig. 2C-D), and knockdown of *miR-223-3p* resulted in *pou1f1* mRNA up-regulation (Fig. 2E).

After *miR-223-3p* was confirmed as the *bona fide* target microRNA of *pou1f1*, we hypothesized that *miR-223-3p* may inhibit *pou1f1* and downstream genes. *MiR-223-3p* overexpression down-regulated the mRNA levels of *pou1f1*, *gh*, *somatolactin*, *prolactin1/2*, and *tsh β* (Fig. 3A-F).

3.3. AU-Rich element acted as a determinant of *pou1f1* mRNA stability

Regulatory elements were explored using software RegRNA2.0 and putative AU-Rich elements were predicted. The core (underlined) and adjacent sequence was as follows: AUAUAUAUCUAUGUGUGUGUUUUUUAUUUAAAA
UUGCAAUUUUAAA. The AU-Rich element and adjacent sequence were predicted to form a hairpin secondary structure by internal paring bonding (Fig. 4A-B).

The AU-Rich element is often found in the mRNA 3'UTR of transcription factors, proto-oncogenes, and cytokines and affect the stability and translation efficiency of mRNAs. To explore the possible role that the AU-Rich element may play, the AU-Rich element was deleted from the reporter plasmid. Wild-type and mutant mRNAs were expressed in HEK293 cells and actD was used to determine the stability of the different mRNAs. The AU-Rich deletion mRNA degraded at a slower rate while the wild-type mRNA decayed rapidly (Fig. 4C). The AU-Rich element deletion led to more protein output compared to the wild-type (Fig. 4D), indicating that the AU-Rich element mainly controls *pou1f1* mRNA stability and not translation efficiency.

3.4. MicroRNA *miR-223-3p* and the AU-Rich element regulated the post-transcriptional expression of *pou1f1* independently

Several reports showed that the AU-Rich element is necessary for microRNA mediated gene silencing. Since the AU-Rich element was found in the 3'UTR of *pou1f1* mRNA, we then determined whether the AU-Rich element is necessary for *miR-223-3p* inhibition of *pou1f1* mRNA translation. Fig. 4E shows that *miR-223-3p* suppressed the wild-type reporter gene expression; despite the AU-Rich element deletion, *miR-223-3p* still inhibited the reporter system.

A previous report showed that the AU-Rich element activated translation dependent on microRNAs under serum starvation [15]. We also adopted serum starvation to explore whether *miR-223-3p* and the AU-Rich element exert an influence on each other. After serum starvation for 1 or 2 hours, groups transfected with *miR-223-3p* exhibited a significant up-regulation of our reporter system with or without the AU-Rich element (Fig. 4F-G).

4. Discussion

In this paper, we found that the pituitary specific transcription factor *pou1f1* is post-transcriptionally regulated by two distinct pathways mediated by microRNA *miR-223-3p* and the AU-Rich element. Pou1f1 is a key transcription factor responsible for differentiation of cells secreting *gh*, *somatolactin*, *prolactin*, and *tsh β* [1]. Multiple microRNAs were demonstrated to down-regulate the *pou1f1* reporter system (Fig. 1), which shows that the

pou1f1 mRNA may harbor multiple MREs and be regulated by several microRNAs. This finding is consistent with the fact that mRNAs may be post-transcriptionally repressed by multiple microRNAs [18]. Although only *miR-223-3p* was investigated in this study, other microRNAs might also play a role in the post-transcriptional regulation of *pou1f1*.

Among the microRNAs analyzed, the expression level of *miR-223-3p* was demonstrated to be negatively correlated with the expression level of *gh* (Fig. s1). This finding indicates that *miR-223-3p* may play a role in somatic growth. The MRE of *miR-223-3p* in the *pou1f1* mRNA was identified, and *miR-223-3p* was demonstrated to be a *bona fide* regulatory molecule of *pou1f1* (Fig. 2). The MRE of *miR-223-3p* is not conserved among teleost fish, implying that *miR-223-3p* regulation of *pou1f1* might be unique in tilapia. Reporter assays and overexpression studies have inherent weaknesses due to uncertainty about the effective concentration and the bioavailability of other cellular factors that might be important in the process. Therefore, overexpression and knockdown of *miR-223-3p* were performed in dispersed primary tilapia cells *in vitro*. Overexpression of *miR-223-3p* led to down-regulation of *pou1f1* and downstream genes (Fig. 3), indicating that *miR-223-3p* might play a role in *pou1f1* regulation *in vivo*.

The AU-Rich element was found in the 3'UTR of *pou1f1* mRNA (Fig. 4A-B) and was shown to regulate *pou1f1* mRNA stability (Fig. 4C). Different types of AU-Rich elements vary in both length and nucleotide composition and are sorted into three categories. The core motif of Class I is a dispersed AUUUA and a U-rich region, the core motif of Class II is WWWWAUUUAWWWW, and the core motif of Class III is U-rich, GUUUG repeats [23,24]. The AU-Rich element in the 3'UTR of *pou1f1* mRNA belongs to Class II according to the core sequences. Typical Class II AU-Rich elements regulate mRNA stability. For example, the Class II AU-Rich element in the 3'UTR of *tnfa* leads to rapid mRNA decay [25], and AU-Rich element impairment leads to stability of *tnfa* mRNA [11]. Furthermore, mice carrying an endogenous deletion of the AU-Rich region of the *tnfa* gene suffer from inflammation and develop a Crohn's disease-like phenotype [12]. The function of the Class II AU-Rich element is conserved through eukaryotes [26], indicating that the AU-Rich element may play an important role in differentiation of lactotropes, somatotropes, and thyrotropes, somatic growth, and osmotic stress resistance. Alignments of sequences from the *pou1f1* mRNA 3'UTR in multiple teleost fish species showed that the AU-Rich element of *pou1f1* is distinctive in Nile tilapia.

Multiple reports show that MRE and AU-Rich elements co-exist in target mRNA 3'UTRs and co-regulate the post-transcriptional expression interdependently.

Under serum starvation, *let-7* up-regulates *tnfa* gene expression via a mechanism where the AU-Rich element recruits AGO and FXR1 (fragile X mental retardation-related protein) [15] to activate *tnfa* mRNA translation; miR-16 causes *tnfa* mRNA decay dependent on the existence of the AU-Rich element, and the AU-Rich element deteriorates the *tnfa* mRNA by recruiting an effector molecule TTP (tristetraprolin) [28]. This shows that under certain circumstances, microRNAs and AU-Rich elements share the same components to degrade mRNA [29]. However, our data showed that under normal circumstances, *miR-223-3p* suppressed the *pou1f1* reporter system with or without the AU-Rich element (Fig. 4E). Additionally, under serum starvation, *miR-223-3p* also up-regulated the *pou1f1* reporter system activity with or without the AU-Rich element (Fig. 4F-G). Therefore, *miR-223-3p* functions under normal or serum deprivation condition independent of the AU-Rich element. For the first time in fish, we show that MREs and AU-Rich elements may co-exist in the same 3'UTR and function independently of each other. Another report in mice and *Drosophila* shows that AU-Rich mediated mRNA degradation occurs independently from the microRNA machinery system [30], which indicates that independence of the AU-Rich element and microRNA machinery system could be a universal phenomenon in diverse

species. It should be noticed that the *in vivo* roles of *miR-233-3p* and the AU-Rich element need further investigation.

In this study, *miR-223-3p* was determined to be a *bona fide* target of *pou1f1* mRNA, and overexpression of *miR-223-3p* caused the down-regulation of *pou1f1*, *gh*, *somatolactin*, *prolactin 1/2*, and *tsh β* . Another post-transcriptional regulatory component, the AU-Rich element, was found in the *pou1f1* mRNA 3'UTR and the AU-Rich element affected *pou1f1* mRNA stability in a rapidly deteriorating manner. Two different regulatory elements, MRE and AU-Rich element, co-exist in the *pou1f1* 3'UTR and function independently of each other in the *pou1f1* 3'UTR.

Acknowledgment

This work was supported by the China Agriculture Research System (CARS-49), the National Science Foundation of China (31272639 and 31472259), the Guangdong Provincial Science and Technology Program (2015A020216006), Guangdong Oceanic & Fishery Program (A201601C02), the Developmental Funds for Strategic Emerging Industries of Shen Zhen (NYSW20140401010064) and the Modern Agriculture Talents Support Program (2016-2020) to Dr. Wensheng Li.

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Figure legends

Fig. 1. **Screening candidate microRNAs of *pou1f1* mRNA using a dual luciferase reporter system.** Recombinant *pou1f1*-psiCHECK2 plasmid (200 ng/ml) and microRNA mimic (10 nM) were transfected into HEK293 cells. After 24 h, cells were harvested and the relative Renilla/Firefly luciferase activity (relative RLuc/FLuc) ratio was measured, n=4. Data are presented as the mean $\pm$ SEM.

Fig. 2. ***MiR-223-3p* was identified as a *bona fide* target of *pou1f1* and regulated *pou1f1* expression *in vitro*.** (A) Nucleotide pairing of *miR-223-3p* and the predicted MRE was shown, and the mfe (minimum free energy) was -19.1 kcal/mol. (B) Site-directed mutagenesis in the MRE led to the derepression of the *miR-223-3p/pou1f1* reporter system, n=4. (C-D) Overexpression of *miR-223-3p* led to Pou1f1 down-regulation at the protein level in Nile tilapia dispersed primary pituitary cells, n=3. (E) Specific knock-down of *miR-223-3p* using antagormir in dispersed primary pituitary cells led to *pou1f1* mRNA up-regulation, n=4. All of the data are presented as the mean $\pm$ SEM, * $p < 0.05$, *** $p < 0.001$.

Fig. 3. **Overexpression of *miR-223-3p* led to the suppression of *pou1f1* and downstream gene expression.** (A-F) *MiR-223-3p* microRNA mimic

transfected into dispersed primary pituitary cells resulted in down-regulation of *pou1f1*, *somatolactin*, *gh*, *prolactin 1/2*, and *tsh β* at the mRNA level, n=4. All the data were represented as the mean $\pm$ SEM, * p <0.05, ** p <0.01.

Fig. 4. Post-transcriptional regulation of *pou1f1* by *miR-223-3p* and the AU-Rich element. (A) Graph of predicted AU-Rich element secondary structure. (B) RNAfold reliability information of nucleotide pairing probabilities is labeled by color. (C) The AU-Rich element caused rapid decay of wild-type *pou1f1* mRNA, deletion resulted in the enhanced stability of mutant *pou1f1* mRNA. (D) Deletion of the AU-Rich element resulted in the accumulation of reporter gene protein. (E) MicroRNA *miR-223-3p* suppressed the wild-type and AU-Rich element mutant reporter gene expression. (F-G) *Pou1f1* reporter gene activities were up-regulated in an *miR-223-3p* dependent manner with serum starvation but independent of the AU-Rich element, n=4. All data were represented as the mean $\pm$ SEM, * p <0.05, ** p <0.01 and *** p <0.001.

Figure-1

Figure-2

Figure-3

Figure-4