Biochemical and Biophysical Research Communications 426 (2012) 294–298

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

miR-429 regulation of osmotic stress transcription factor 1 (OSTF1) in tilapia during osmotic stress

Biao Yan [1], Li-Hui Zhao [1], Jin-Tao Guo, Jin-Liang Zhao *

Key Laboratory of Freshwater Aquatic Genetic Resources, Shanghai Ocean University, Ministry of Agriculture, Shanghai Ocean University, Shanghai 201306, China

ARTICLE INFO

Article history:
Received 31 July 2012
Available online 23 August 2012

Keywords:
miR-429
Nile tilapia
Osmotic stress
OSTF1

ABSTRACT

The Nile tilapia represents an excellent model for osmoregulation study. Osmotic stress transcription factor 1 (OSTF1) identified in tilapia gill epithelium is a critical element of osmosensory signal transduction by means of transcriptional regulation. Thus, tight regulation of OSTF1 level is necessary for tilapia osmotic adaptation. microRNAs (miRNAs), have emerged as a crucial regulator of gene expression at post-transcriptional level. We reasoned that OSTF1 expression could be regulated by miRNAs. By bioinformatics analysis, we identified a putative miR-429 binding site in the OSTF1 mRNA. Interestingly, miR-429 is down-regulated in tilapia upon osmotic stress, consistent with OSTF1 protein up-regulation. miR-429 directly regulates OSTF1 expression by targeting its 3′-UTR, and inhibition of miR-429 substantially increases OSTF1 level *in vivo*. Moreover, miR-429 loss of function could influence the regulation of plasma osmolality and ion concentration responding to osmotic stress. Taken together, miR-429 is an endogenous regulator of OSTF1 expression, which participates in a regulatory circuit that allows rapid gene program transitions in response to osmotic stress.

1. Introduction

microRNAs (miRNAs) are a class of 22–25 nucleotide noncoding RNA molecules. They negatively regulate gene expression at the post-transcriptional level either by blocking translation through incomplete binding to the 3′-UTR of target gene, or by directing degradation of target gene [1,2]. miRNAs have been implicated in diverse biologic processes, including developmental timing, cell proliferation, apoptosis, metabolism, and morphogenesis. In particular, multiple evidences indicate that miRNAs play important roles in stress responses. Environmental stress can alter the biogenesis of miRNAs, the expression of mRNA targets, and the activity/mode of miRNA–protein complexes [3,4]. In turn, these changes determine the specificity, timing, and concentration of gene products expressed during stresses.

Fish spend all their life in direct contact with environmental water. They are able to maintain intracellular inorganic ion concentrations within a tightly regulated range in their internal milieu. Tilapiine fishes, despite being a freshwater species, is able to tolerate a wide range of environmental salinities [5,6]. Of them, the Nile tilapia (*Oreochromis niloticus*) has dominated freshwater aquaculture due to its adaptability and fast growth performance. Although *O. niloticus* is not considered to be the most salt-tolerant tilapia species, it still offers a considerable potential for culture in brackish water. Thus, tilapia should have evolved perfect mechanisms of osmotic adaptation [7,8].

Many physiological acclimations to osmotic stress are mediated by alteration of gene expression. Inducible transcription factors would contribute to the change in gene expression. Osmotic stress transcription factor 1 (OSTF1) is identified in tilapia gill epithelium through suppression subtractive hybridization. It could be rapidly and transiently induced upon osmotic stress. Moreover, OSTF1 up-regulation depends on RNA stabilization and the presence of an osmotic gradient between the extracellular and intracellular fluid of tilapia gill cells [9,10]. A role of OSTF1 in osmosensory signal transduction is not limited to fishes, but also is evident in mammalian cells. TSC22D2, an OSTF1 ortholog of mammals, is activated and alternatively spliced in response to osmotic stress in mouse and human kidney cells with similar kinetics as in fish gill cells. However, the regulatory mechanism of OSTF1 expression remains to be thoroughly studied. miRNAs confer a novel layer of posttranscriptional regulation of gene expression [11,12]. This mechanism is found widely in plants and animals. Given miRNA regulation of OSTF1 expression is still unclear, we mainly examined the role of miRNAs in the regulation of OSTF1 expression in tilapia.

* Corresponding author. Address: Shanghai Ocean University, Huchenghuan Road #999, 201306 Shanghai, China. Fax: +86 21 65988326.
E-mail address: jlzhao@shou.edu.cn (J.-L. Zhao).
[1] These authors contribute equally to this paper.

0006-291X/$ - see front matter Crown Copyright © 2012 Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.08.029

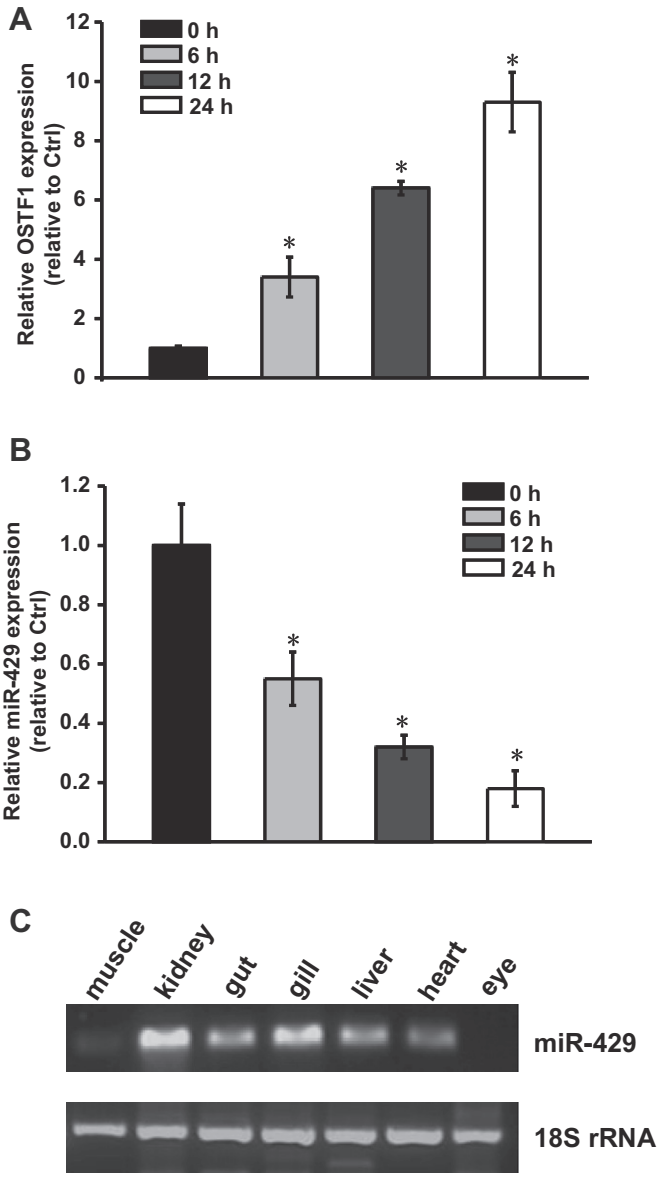

Fig. 1. OSTF1 is a potential target of miR-429 regulation. (A) Tilapia was exposed to 20 g/L salinity for 0 h, 6 h, 12 h, and 24 h. The expression of OSTF1 in gill was detected using a real-time PCR. 18S rRNA expression was detected as the internal control. The group exposed to 20 g/L for 0 h was taken as the control group. The data were expressed as the relative change compared with the control group. (B) Tilapia was treated as shown in Fig. 1A. The expression of miR-429 in gill was detected using a stem-loop PCR technique. The data were expressed as the relative change compared with the control group. Asterisk (*) indicates significant difference compared with the control group ($P < 0.05$). (C) miRNA sample was extracted from different tissues and organs, including skeletal muscle, heart, gut, liver, kidney, gill, and eye. miRNAs expression were detected by RT-PCR. 18S rRNA was detected as the loading control. Shown is the representative image.

2. Material and methods

2.1. Fish

Nile tilapias used in this study were from stocks maintained at the fishery farm of Shanghai Ocean University. The fish were raised in a water circulation system, and water temperature was kept at 25 ± 2 °C under a 12-h light/12-h dark photoperiod. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals of China. This study was also approved by the Committee on the Ethics of Animal Experiments of Shanghai Ocean University.

2.2. RNA isolation and real-time PCR

Total RNA was isolated using Trizol reagent (Invitrogen), and reversely transcribed by PrimeScript RT-PCR Kit (Takara) with Oligo dT primers for cDNA transcript generation. Small RNA (<200 bp) isolation was conducted using an Ambion mirVana miRNA Isolation Kit. Poly (A) tail was added to the small RNAs by Ambion Poly (A) Polymerase (Ambion). Real-time PCR was run by using the SYBR RT-PCR kit in a Bio-rad CFX 96 (Bio-rad) machine and results were analyzed by the CFX Manager software. The PCR reaction for each gene was performed in triplet with the housekeeping gene 18S rRNA as control.

2.3. Target validation by luciferase assay

To generate the 3′-UTR luciferase reporter construct, the full length of the 3′-UTR from OSTF1 was cloned into the downstream of the firefly luciferase gene in pGL3-control vector (Promega). Six base pairs in the UTR region were deleted to generate pGL3-OSTF1 mutant. For luciferase reporter assay, HEK 293T cells were plated at 4×10^5 cells per well in 12-well dishes. Cells were transfected with either wild-type or mutant constructs, with and without miR-429 mimic or negative control mimic. Luciferase assays were performed using the Dual-Glo luciferase Assay System (Promega) according to the manufacturer's instructions. Experiments were repeated at least three times for each reporter, and the *P*-value was calculated by Student's *t* test.

2.4. Blood plasma osmolality, [Na^+], [K^+], and [Cl^-] measurements

Plasma osmolality and ion concentrations were measured in tilapia after specific treatment. The treated fish were anesthetized with 0.1% 2-phenoxyethanol, and blood was collected from the caudal vessels with a heparinized syringe and needle. Blood samples were centrifuged for 15 min at 5000*g* to separate plasma from red blood cells. Plasma osmolality was measured using a freezing point osmometer. Plasma [Na^+], [K^+], and [Cl^-] were measured using the Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Blood plasma analyses were performed in triplicate or duplicate, depending on blood plasma volume available.

2.5. Data analysis

Data are presented as the mean ± S.E.M. unless otherwise stated. Comparisons of plasma osmolality, ion ([Na^+], [K^+], [Cl^-]) concentration and miRNA or mRNA expression level were performed using One-way ANOVA. Statistical significance was defined as $P < 0.05$.

3. Result

3.1. OSTF1 is a potential target of miR-429 regulation

Osmotic stress transcription factor 1 (OSTF1) is thought to be a crucial regulator of osmoregulatory signaling. Because tilapia OSTF1 nucleotide sequence was published as cDNA and the tilapia OSTF1 genomic sequence was unavailable, we carried out a homology analysis between tilapia OSTF1 cDNA and genomic sequences of *Danio rerio*. Based on the structure of the orthologous loci, we were able to determine miRNAs binding sites of OSTF1 3′UTR in tilapia. Target scan analysis suggests that OSTF1 may be regulated by these miRNAs, including miR-30a, miR-200b, miR-200c, miR-429 and miR-429b. OSTF1 is rapidly and specifically induced in response to osmotic stress. OSTF1 up-regulation is mainly mediated

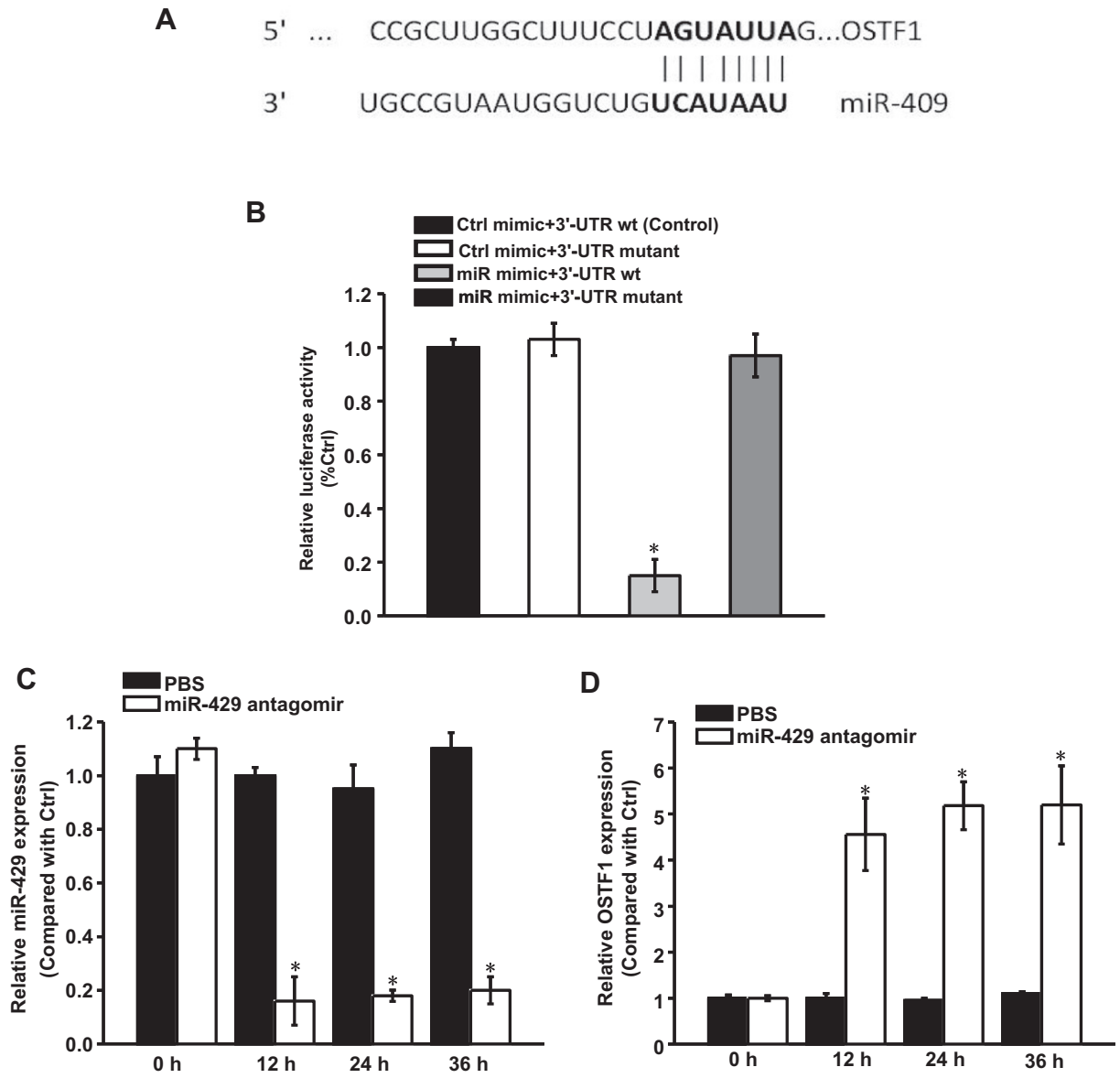

Fig. 2. miR-429 acts directly at the 3′UTR of OSTF1. (A) The alignment between miR-429 and the 3′UTR segment of OSTF1. (B) Luciferase reporters were linked with OSTF1 3′-UTRs containing either putative miR-429-binding sites (3′-UTR wt) or mutated miR-429 binding sites (3′-UTR mutant). miR-429 mimic or control mimic (Ctrl mimic) plasmids were cotransfected with HEK 293T cells with luciferase-UTR constructs, and then luciferase activity was determined. The cells transfected with Ctrl mimic plus 3′-UTR wt were used to serve as the control group. Data represent the mean ± S.E.M. from three independent experiments. $^{*}P < 0.05$. (C and D) Tilapia weighing about 10 g received a tail-vein injection of PBS, or miR-429 antagomir at a dose of 60 mg/kg body weight for indicated time. The untreated group was taken as control. The relative expression of miR-429 (C) and OSTF1 (D) was detected using real-time PCR. The data were expressed as the relative change compared with the untreated group. 18S rRNA expression was detected as the internal control. Asterisk (*) indicates significant difference compared with the control group ($P < 0.05$).

through the mRNA stabilization mechanism decreasing the degradation rate of the mRNA, but not through an increase in the transcription rate [9,10]. Thus, we postulated that the expression of OSTF1-inhibitory miRNAs would downregulate upon osmotic challenge. The result showed that OSTF1 was significantly increased upon osmotic stress (Fig. 1A), which is consistent with the findings from other studies [13–15]. Meanwhile, we also observed a significant decrease in miR-429 expression. However, we did not detect any expression change for other miRNAs (Fig. 1B). The inverse expression correlation between miR-429 and OSTF1 suggests that miR-429 may directly regulate OSTF1 expression in tilapia.

In addition, we performed the RT-PCR experiment to detect miR-429 expression pattern in tilapia. miR-429 was found to be expressed ubiquitously in heart, gut, liver, kidney and gill (Fig. 1C). Importantly, miR-429 expression level in gill is obviously higher than that in other tissues. The gill is regarded as the primary site of net sodium and chloride transport through the activity of chloride cells. It is thought to be a crucial regulator of internal osmotic balance. Given that OSTF1 is also found to be mainly expressed in gill, we therefore investigated whether there is a regulatory relationship between OSTF1 and miR-429 in the following study.

3.2. miR-429 acts directly at the 3′UTR of OSTF1

In animals, miRNA function generally involves uninterrupted base-pairing between nucleotides 2–7 (commonly called the seed sequence) of the miRNA and a complementary sequence in the 3′UTR of the target mRNA. Based on sequence complementarity, OSTF1 has the potential binding site of miR-429 (Fig. 2A). To determine whether miR-429 directly regulates OSTF1 expression, we

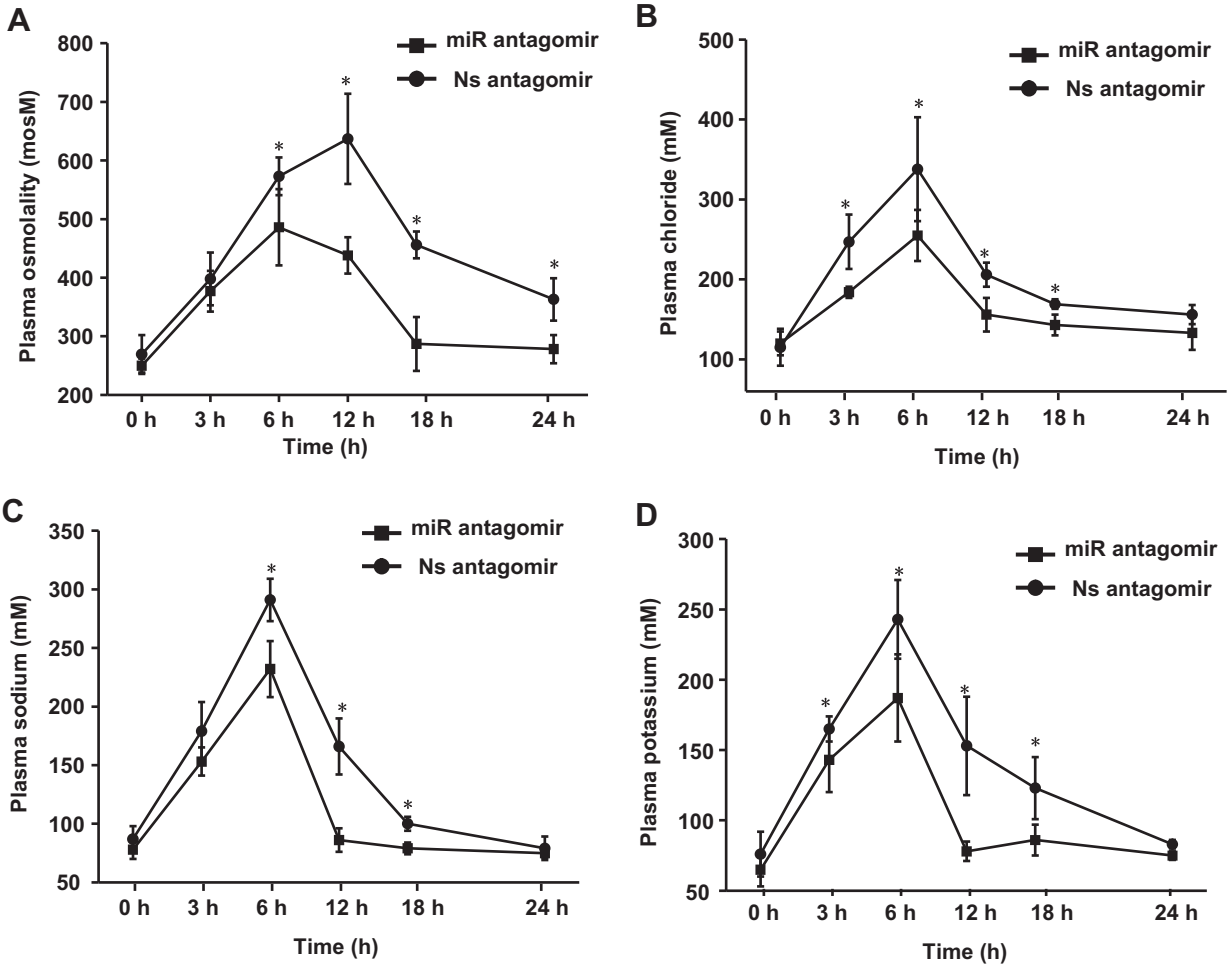

Fig. 3. miR-429 silencing affects salt tolerance performance in tilapia. Tilapia weighing about 10 g received a tail-vein injection of nonspecific antagomir (Ns) or miR-429 antagomir at a dose of 60 mg/kg body weight. After 36 h, these treated fish were exposed to 20 g/L salinity for indicated time. Tilapia blood plasma osmolality (A), [Cl^-] (B), [Na^+] (C), and [K^+] (D) were detected as described in Section 2. Asterisk (*) indicates significant difference compared with the nonspecific antagomir treated group ($P < 0.05$).

engineered luciferase reporters that have either the wild-type 3′-UTR, or the mutant 3′-UTR of OSTF1 gene. These luciferase reporters were cotransfected with miR-429 mimic into HEK 293T cell. A scrambled miRNA mimic with no homology to the tilapia genome was used to control the nonspecific effects of expression. The transfection of scrambled miRNA mimic did not affect the reporter activity. Indeed, miR-429 mimic significantly decreases the luciferase activity of the wild-type OSTF1 reporter compared to the negative control, which strongly suggests that OSTF1 mRNA is a direct target of miR-429. Mutation of the target sequence prevented the down-regulation of luciferase activity by miR-429 mimic (Fig. 2B). These results demonstrate that the negative effect of miR-429 on OSTF1 expression was direct.

In addition, we employed the antagomir method to perform miRNA loss of function experiment. The result showed that antagomir but not PBS treatment leads to a significant decrease in endogenous miR-429 expression. By contrast, OSTF1 expression was found to be significantly increased (Fig. 2C and D). The inverse expression correlation between miR-429 and OSTF1 also suggests that miR-429 can directly regulate OSTF1 expression *in vivo*.

3.3. The role of miR-429 in tilapia osmoregulation

To determine the role of miR-429 in regulating salt tolerance *in vivo*, we knocked down miR-429 by using miRNA antagomir. Endogenous miR-429 expression could be suppressed by miR-429 antagomir but not by mutant antagomir-429 treatment (data not shown). We then subjected tilapia to osmotic stress, and compared salt tolerance performance between miRNA antagomir and mutant antagomir-429 group. When tilapia was transferred from freshwater to brackish water (BW, 20 g/L), all fish behaved normally. In the miR-429 antagomir group, plasma osmolality elevated significantly within 3 h post-transfer but declined to a level similar to the freshwater sample by 24 h post-transfer (Fig. 3A). Meanwhile, plasma [Na^+], [Cl^-], and [K^+] also increased significantly, and reached a peak at 6 h post-transfer, but then declined to the level of freshwater samples by 12 h post-transfer. In the miR-429 antagomir mutant group, plasma osmolality, [Na^+], [Cl^-], and [K^+] also increased significantly upon 20 g/L salinity challenge. However, plasma osmolarity, [Na^+], [Cl^-], and [K^+] at 20 g/L were significantly higher than that in antagomir-429 administrated group before plasma osmolality and ions returned to normal level (Fig. 3B–D). These results indicate that miR-429 silencing could affect tilapia osmoregulation *in vivo*, implying that miR-429/OSTF1 interaction would play roles in modulating transcription levels of various water channels and ion transporters for proper hypertonic response.

4. Discussion

Tilapia lives directly in water environment, and often faces with sudden changes or frequent fluctuations in environmental salinity.

These stresses can damage the existing macromolecules, including proteins, mRNAs, DNA, and lipids. Thus, they should either reestablish cellular homeostasis to the former state or adopt an altered state in the new environment. The stress responses can be mediated via multiple mechanisms, such as induction of molecular chaperones, rapid clearance of damaged macromolecules, growth arrest, and activation of certain gene expression programs [4,16,17]. miRNAs provide multi-cellular organisms with elaborate strategies for posttranscriptional gene regulation [18]. The exact roles of miRNAs in environmental stress and the precise mechanisms by which they exert a regulatory function are currently under intense experimental scrutiny. For example, osa-miR-393 expression is altered during salinity and alkaline stress. Transgenic rice and Arabidopsis thaliana that over-expressed osa-miR-393 are more sensitive to salt and alkali treatment compared to wild-type plants [19]. miR-395 is found to be the most sensitive to salt stress and up-regulated upon salinity stress in tobacco [20]. miR-8 family is expressed in zebrafish ionocytes, and functions as a crucial osmoregulator in zebrafish embryos [21]. In this study, we found that miR-429 can directly regulate the expression of OSTF1, an osmotic stress transcriptional factor identified in tilapia, revealing the role of miR-429 in tilapia osmoregulation.

OSTF1 was first identified in tilapia in 2005. Numerous studies have investigated its regulation and expression profile in fish gill tissues in relation to osmoregulation [9]. During osmotic stress, OSTF1 is found to be significantly up-regulated. In principle, mRNA levels can be upregulated either through an increase in the transcription rate or through a mRNA stabilization mechanism decreasing the degradation rate of the mRNA [7,9]. In cells kept under isosmotic conditions, a decline in OSTF1 mRNA amount was observed, which is due to its degradation over time. However, OSTF1 degradation rate was significantly decreased when cells were exposed to hypertonic stress. These results confirm that mRNA stabilization is involved in the rapid upregulation of OSTF1 mRNA during hyperosmotic stress [10]. miRNAs have been reported to be important regulators of mRNA stabilization in eukaryotes. Experimental verification confirmed the direct interaction between miR-429 and OSTF1, implying that miRNA in tilapia would play a crucial role in the regulation of sustained transcriptional induction of OSTF1 during hyperosmotic stress.

Although tilapias are euryhaline teleosts, they are also required to maintain plasma osmotic homeostasis even in the face of fluctuating environmental salinity. The gill is one of the most important tissues to maintain the right concentration of solutes and the amount of water in their body fluids [22]. In the gill epithelium numerous ion and water transporters are regulated at the mRNA level by salinity. Examples of this regulation include subunits of the Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransporter, urea transporter, taurine transporter and aquaporin 3 water channel. In addition, other mRNAs such as 14-3-3 and C-type lectin are also regulated by osmolality in fish gills [7,23–25]. These responses could potentially be regulated by increases in the synthesis rate of the corresponding mRNAs and/or by mRNA stabilization mechanisms [26]. miR-429 is found to be a gill-enriched miRNA. miR-429 silencing could affect plasma osmolality and ionic balance. Thus, we infer that miR-429/OSTF interaction would provide a new insight into the mechanism of tilapia osmoregulation, indicating feedback regulation and a high degree of complexity of osmosensing and signaling networks in euryhaline fishes.

In summary, we reveal that miR-429 directly regulates OSTF1 by targeting its 3′-UTR, and miR-429 suppression could substantially increase OSTF1 mRNA level *in vivo*. miR-429 participates in a regulatory circuit that allows rapid gene program transitions in response to osmotic stress. In this manner, miRNAs confer robustness to participate in a feed-forward genetic circuit to resist osmotic stress. This study would provide a novel insight into the post-transcriptional regulation mechanism of salt tolerance in tilapia.

Acknowledgments

This work was supported by China Agriculture Research System (CARS-49-04B to J.-L.Z.). This work was supported by the Doctoral Fund (B-8812-11-0197 to B.Y.).

References

[1] E.V. Makeyev, T. Maniatis, Multilevel regulation of gene expression by microRNAs, Science's STKE 319 (2008) 1789.
[2] N. Bushati, S.M. Cohen, MicroRNA functions, Annu. Rev. Cell Dev. Biol. 23 (2007) 175–205.
[3] I. Alvarez-Garcia, E.A. Miska, MicroRNA functions in animal development and human disease, Development 132 (2005) 4653–4662.
[4] A.K.L. Leung, P.A. Sharp, MicroRNA functions in stress responses, Mol. Cell 40 (2010) 205–215.
[5] T. Evans, Co-ordination of osmotic stress responses through osmosensing and signal transduction events in fishes, J. Fish. Biol. 76 (2010) 1903–1925.
[6] A. Velan, G. Hulata, M. Ron, A. Cnaani, Comparative time-course study on pituitary and branchial response to salinity challenge in Mozambique tilapia (*Oreochromis mossambicus*) and Nile tilapia (*O. niloticus*), Annu. Rev. Cell Dev. Biol. (2011) 1–11.
[7] S. Fridman, J. Bron, K. Rana, Ontogenic changes in the osmoregulatory capacity of the Nile tilapia (*Oreochromis niloticus*) and implications for aquaculture, Aquaculture 356–357 (2012) 243–249.
[8] B. Yan, Z. Wang, Growth, salinity tolerance and microsatellite analysis of the F2 reciprocal hybrids of *Oreochromis niloticus* × *Sarotherodon galilaeus* at different salinities, Aquac. Res. 41 (2010) e336–e344.
[9] D.F. Fiol, D. Kültz, Rapid hyperosmotic coinduction of two tilapia (*Oreochromis mossambicus*) transcription factors in gill cells, Proc. Natl. Acad. Sci. U S A 102 (2005) 927.
[10] D.F. Fiol, S.Y. Chan, D. Kültz, Regulation of osmotic stress transcription factor 1 (Ostf1) in tilapia (*Oreochromis mossambicus*) gill epithelium during salinity stress, J. Exp. Biol. 209 (2006) 3257–3265.
[11] D.F. Fiol, D. Kültz, Osmotic stress sensing and signaling in fishes, FEBS J. 274 (2007) 5790–5798.
[12] D.F. Fiol, S.K. Mak, D. Kültz, Specific TSC22 domain transcripts are hypertonically induced and alternatively spliced to protect mouse kidney cells during osmotic stress, FEBS J. 274 (2007) 109–124.
[13] W.K.F. Tse, S.C. Chow, C.K.C. Wong, Eel osmotic stress transcriptional factor 1 (Ostf1) is highly expressed in gill mitochondria-rich cells, where ERK phosphorylated, Front. Zool. 9 (2012) 3.
[14] J.P. Breves, B.K. Fox, A.L. Pierce, T. Hirano, E.G. Grau, Gene expression of growth hormone family and glucocorticoid receptors, osmosensors, and ion transporters in the gill during seawater acclimation of Mozambique tilapia, *Oreochromis mossambicus*, J. Exp. Zool. 313 (2010) 432–441.
[15] W. Tse, S. Chow, C. Wong, The cloning of eel osmotic stress transcription factor and the regulation of its expression in primary gill cell culture, J. Exp. Biol. 211 (2008) 1964–1968.
[16] A. Buchberger, B. Bukau, T. Sommer, Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms, Mol. Cell 40 (2010) 238–252.
[17] K. Richter, M. Haslbeck, J. Buchner, The heat shock response: life on the verge of death, Mol. Cell 40 (2010) 253–266.
[18] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[19] P. Gao, X. Bai, L. Yang, D. Lv, X. Pan, Y. Li, H. Cai, W. Ji, Q. Chen, Y. Zhu, Osa-MIR393: a salinity-and alkaline stress-related microRNA gene, Mol. Biol. Rep. 38 (2011) 237–242.
[20] T.P. Frazier, G. Sun, C.E. Burklew, B. Zhang, Salt and drought stresses induce the aberrant expression of microRNA genes in tobacco, Mol. Biotechnol. 49 (2011) 159–165.
[21] A.S. Flynt, E.J. Thatcher, K. Burkewitz, N. Li, Y. Liu, J.G. Patton, miR-8 microRNAs regulate the response to osmotic stress in zebrafish embryos, J. Cell Biol. 185 (2009) 115–127.
[22] J.M. Whittamore, Osmoregulation and epithelial water transport: lessons from the intestine of marine teleost fish, J. Comp. Physiol. (2012) 1–39.
[23] C.K. Tipsmark, S.S. Madsen, M. Seidelin, A.S. Christensen, C.P. Cutler, G. Cramb, Dynamics of Na^+, K^+, $2Cl^-$ cotransporter and Na^+, K^+-ATPase expression in the branchial epithelium of brown trout (*Salmo trutta*) and atlantic salmon (*Salmo salar*), J. Exp. Zool. 293 (2002) 106–118.
[24] T.G. Evans, G.N. Somero, A microarray-based transcriptomic time-course of hyper-and hypo-osmotic stress signaling events in the euryhaline fish Gillichthys mirabilis: osmosensors to effectors, J. Exp. Biol. 211 (2008) 3636–3649.
[25] C.P. Cutler, G. Cramb, Branchial expression of an aquaporin 3 (AQP-3) homologue is downregulated in the European eel Anguilla anguilla following seawater acclimation, J. Exp. Biol. 205 (2002) 2643–2651.
[26] D.H. Evans, Cell signaling and ion transport across the fish gill epithelium, J. Exp. Zool. 293 (2002) 336–347.