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MiR-30c: A novel regulator of salt tolerance in tilapia

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ABSTRACT

miRNAs comprise a class of ~22 nt noncoding RNAs that modulate the stability and/or translational potential of their mRNA targets. Emerging data suggest that stress conditions can alter the biogenesis of miRNAs, thereby changing the expression of mRNA targets. Here, we reveal that miR-30c, a kidney-enriched miRNA, emerges as a crucial osmoregulator in Nile tilapia. miR-30c loss of function leads to an inability to respond to osmotic stress. We identify HSP70 as one of the direct regulatory targets of miR-30c. miR-30c directly regulates HSP70 by targeting its 3′-UTR, and inhibition of miR-30c substantially increases HSP70 mRNA level *in vivo*. Taken together, our experiments suggest that miRNAs participate in a regulatory circuit that allows rapid gene program transitions in response to osmotic stress. miR-30c may be developed as a molecular marker to assist to breed or genetically engineer salt tolerant species.

1. Introduction

Tilapias are euryhaline fish that can live in a wide range of salinity from freshwater to full seawater. It makes them a good model organism for studies on ionic and osmotic acclimation in euryhaline teleosts [1,2]. The Nile tilapia, *Oreochromis niloticus*, is one of the most important cultured-commercially species. It is mainly cultured in freshwater systems, but still has a good growth performance in salinity up to 20 g/L [3]. Recently, there is growing concern about the genetic improvement of salt tolerance of Nile tilapia. Marker assisted selection is a valuable tool in selecting organisms for traits of interest, such as color, meat quality, or disease resistance [4,5]. It has enormous potential to improve the efficiency and precision of conventional fish breeding. The key for marker-assisted selection is to find the suitable molecular marker associated with economically important characteristics. Thus, to probe gene resources related to salt tolerance is becoming the premise of marker-assisted selection in salt tolerant species breeding.

MicroRNAs (miRNAs) are short (18–22 nucleotides), evolutionary conserved noncoding RNA molecules. They regulate gene expression by binding target messenger RNAs (mRNAs), leading to translational repression or degradation [6,7]. In animal models, miRNAs are predicted to regulate the activity of approximately 50% of all protein-coding genes. Functional studies indicate that miRNAs participate in regulating almost every cellular process. Changes in their expression are associated with many pathophysiological processes [8].

The kidney is an essential organ required for osmoregulation. It is one of the most important organs to maintain the right concentration of solutes and amount of water in their body fluids, which is referred to as osmoregulation [9]. Previous studies have suggested that miRNAs are important in controlling spatial gene expression, which is instrumental in kidney development and disease [10,11]. Among these, the miR-30 family shows the most prominent kidney-restricted expression in many animals. Moreover, knockdown of miR-30a-5p can result in pronephric defect [12]. However, the role of miR-30 family in tilapia is still unknown. In this study, we investigate miR-30 expression pattern, and explore whether miR-30 family plays a role in tilapia osmoregulation.

Here, we show that miR-30c is abundantly expressed in tilapia kidney. By expression analysis and computational predictions, we identify miR-30c as a temporally regulated miRNA in response to osmetic stress, and validate it can target 3′-UTR of HSP70. By miRNA loss-of function approach, we show that the endogenous levels of HSP70 are controlled posttranscriptionally by miR-30c *in vivo*, and miR-30c loss of function can affect the salt tolerance performance of tilapia. We uncover a direct link between miRNA expression and tilapia salt tolerance. miR-30c would be developed as a maker to conduct marker-assisted selection for salt-tolerant tilapia.

2. Materials and methods

2.1. Experimental animal

The Nile tilapias were obtained from the fishery farm of Shanghai Ocean University. They were raised in a water circulation

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system in 100-liter tanks, 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. Quantitative RT-PCR (qRT-PCR)

Total RNA sample was extracted using Trizol reagent (Invitrogen), and miRNA sample was extracted using the miRNeasy kit (Qiagen) in accordance with the manufacturer's instructions. Isolated total RNA was run on an agarose gel (1.5%) for checking RNA quality and integrity. First strand cDNA was synthesized from 2 μg of total RNA using high capacity cDNA reverse transcription kit (Takara). qRT-PCR was performed using the MyiQ5 Real-time PCR Detection System (Bio-Rad). The relative amount of miRNA was detected using stem-loop PCR method. Relative gene or miRNA expression was calculated using comparative C_T method [13].

2.3. 3′-UTR luciferase reporter assay

To generate the 3′-UTR luciferase reporter construct, the full length of the 3′-UTR from HSP70 was cloned into the downstream of the firefly luciferase gene in pGL3-control vector (Promega). Six base pair in the UTR region was deleted to generate pGL3-HSP70 mutant. For luciferase reporter assays, HEK 293T cells were plated at 3×10^5 cells *per* well in 12-well dishes. Cells were transfected with either wild-type or mutant constructs, with and without miR-30c mimic or negative control mimic. Luciferase activity was measured on a scintillation counter using a dual luciferase reporter system [14].

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. The blood plasma was separated by centrifugation and stored at −20 °C. Plasma osmolality was measured using a freezing point osmometer. Plasma [Na^+], [K^+], and [Cl^-] was measured by using the Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing city, 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 ± SD 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. Expression pattern of miR-30 family in tilapia

Our deep sequencing data reveals that miR-30 family in tilapia is consisted of six members, including miR-30a, miR-30b, miR-30c, miR-30d, miR-30e, and miR-30e* (unpublished data). miR-30 family is reported as the most prominent kidney-restricted expression in many animals [10,12]. We performed RT-PCR experiments to detect the expression pattern of these miRNAs in tilapia. Of the 6 miRNAs analyzed in this study, miR-30a, miR-30e, and miR-30e* were expressed ubiquitously in skeletal muscle, heart, gut, liver, kidney and gill. Importantly, they were strongly expressed in the kidney. miR-30b, miR-30c, and miR-30d were found to be kidney-enriched miRNAs (Fig. 1).

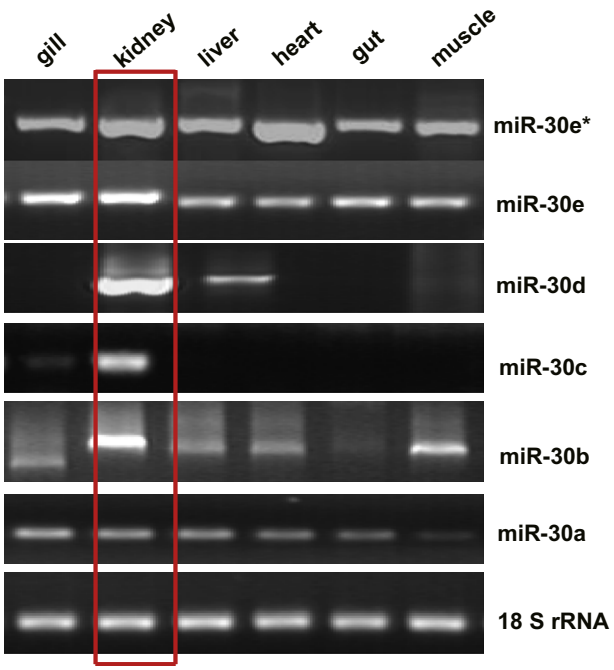

Fig. 1. Expression pattern of miR-30 family in tilapia. miRNA sample was extracted from different tissues and organs, including skeletal muscle, heart, gut, liver, kidney, and gill. miRNAs expression were detected by RT-PCR. 18S rRNA was detected as the loading control. Shown is representative image.

3.2. miR-30 expression in response to osmotic stress

To determine the expression change of miR-30 after salinity challenge, tilapia was exposed to different salinity levels, including 0, 15, 20, and 25 g/L. The expression of miRNAs in kidney was detected 12 h after salinity treatment using stem-loop PCR technique. As shown in Fig. 2A, miR-30c was constantly increased with increasing salinity up to 25 g/L. There was a significant negative correlation between salinity and relative expression of miR-30c. However, the expression levels for other miRNAs were not changed during this test (Data not shown). In addition, miR-30c was found to be down-regulated as early as 6 h after salinity stimulation, and the expression level continued to decrease until 24 h (Fig. 2B). Taken together, these data suggest that miR-30c expression is changed in a salinity- and time-dependant manner.

3.3. miR-30c silencing affects salt tolerance performance in tilapia

To determine the role of miR-30c in regulating salt tolerance *in vivo*, we knocked down miR-30c in tilapia using miRNA antagomir method. Administration of antagomir-30c, but not PBS or mutant antagomir-30c (in which four mismatch mutations were introduced into each miRNA sequence), resulted in a profound decrease in endogenous expression of miR-30c *in vivo*.

We then subjected fish to osmotic stress, and compared the salt tolerance performance between miRNA antagomir and mutant agomir-30c treated group. The plasma osmolality, [Na^+], [Cl^-], and, [K^+] increased significantly upon 20 g/L salinity challenge. In antagomir-30c administrated group, plasma osmolarity, [Na^+], [Cl^-], and, [K^+] at 20 g/L were significantly higher than that in mutant agomir-1 administrated groups at corresponding time point

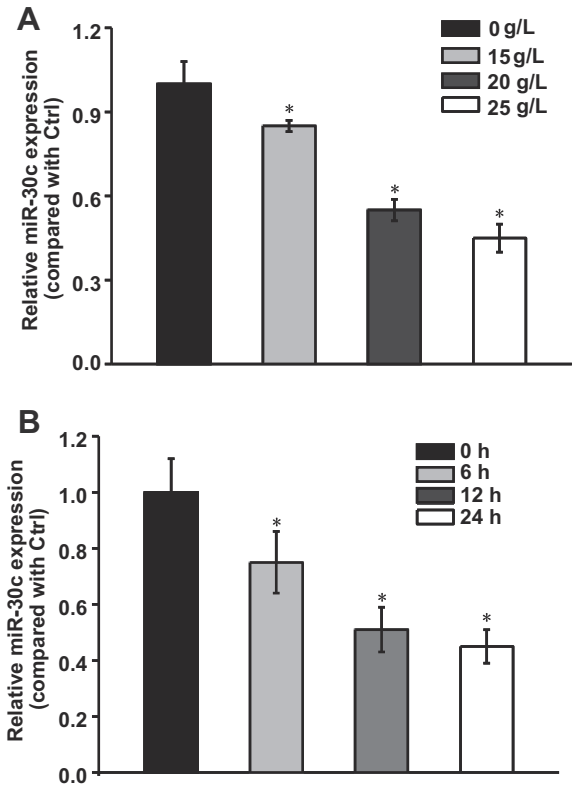

Fig. 2. miR-30 expression in response to osmotic stress. (A) Tilapia was exposed to different salinity levels, including 0, 15, 20, and 25 g/L. The expression of miR-30c in kidney was detected 12 h after salinity treatment using stem-loop PCR technique. The group exposed to 0 g/L was taken as the control group. (B) Tilapia was exposed to 20 g/L salinity for 0, 6, 12, and 24 h. The expression of miR-30c in kidney was detected using stem-loop PCR technique. The group exposed to 20 g/L for 0 h was taken as the control group. 18S rRNA expression was detected as the internal control. The data was expressed as the relative change compared with the control group. Asterisk (*) indicates significant difference compared with the control group (*$P < 0.05$).

(Fig. 3B–E). These results show that miR-30c silencing group would experience more serious plasma ionic imbalance, suggesting that miR-30c is an important regulator of salt tolerance *in vivo*.

3.4. HSP70 emerges as a direct target of miR-30c

To better understand how miR-30c regulates osmotic stress, we sought to identify miR-30c target genes that could be responsible for regulating salt tolerance. 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 3́UTR of the target mRNA. Based on sequence complementarity [15,16], HSP70 was predicted as one of the potential target genes of miR-30c (Fig. 4A).

To investigate whether HSP70 can be directly targeted by miR-30c, we engineered luciferase reporters that have either the wild-type 3′-UTR of HSP70 gene, or the mutant UTR of HSP70 gene. The luciferase reporters were cotransfected with miRNA 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. However, miR-30c mimic significantly reduced luciferase activity of the wild-type HSP70 reporter compared to the negative control. In contrast, mutant reporters were not repressed by miR-30c mimic, which indicates that the target site directly mediates the repression (Fig. 4B).

In addition, we used the antagomir method to conduct miRNA loss of function experiment *in vivo*. Administration of antagomir but not PBS results in a profound decrease in the endogenous expression of miR-30c. Meanwhile, the expression of HSP70 was significantly up-regulated (Fig. 4C and D). The inverse expression correlation between miRNAs and putative target gene also suggests that miR-30c can directly regulate HSP70 expression *in vivo*. Taken together, these results show that miR-30c directly repress HSP70 expression through targeting of the 3′-UTR of HSP70 gene.

4. Discussion

miRNAs have emerged as crucial regulators in many biological processes, including responding to changes in environmental stress. miRNA can restore or reprogram gene expression patterns *in vivo*, thereby changing the amount of mRNA targets. In turn, these changes determine the specificity, timing, and amount of mRNA products expressed upon stresses [17,18]. Thus, understanding miRNA-guided stress regulatory networks would provide a new tool for the genetic improvement of species stress tolerance. In this study, we reveal an osmoregulatory role of miR-30c in tilapia. Change in endogenous miR-30c expression would affect salt tolerant performance in tilapia.

The role of miRNAs in osmoregulation has attracted much attention and research interest. Soil salinity is a major abiotic stress in plant agriculture worldwide. High salinity can cause ion imbalance and hyperosmotic stress. miR-169 family is found to be induced by salt stress. miR-169 members selectively cleaves one of the NF-YA genes, Os03g29760, which participates in transcriptional regulation of large number genes that are associated with salt tolerance [19]. The osa-miR-393 expression level changes under salinity and alkaline stress. Transgenic rice and Arabidopsis thaliana that over-expressed miR-393 are more sensitive to salt and alkali treatment compared to wild-type plants [20]. Salinity stresses can significantly alter miRNA expression in a dose-dependent manner in tobacco. miR-395 is found to be the most sensitive to salt stresses and up-regulated by 2810-folds by 0.171 M NaCl treatment [21]. In addition, the miR-8 family is found to be tightly associated with zebrafish osmoregulation. miR-8 family is expressed in ionocytes and enables precise control of ion transport by modulating the expression of Nherf1, a regulator of apical trafficking of transmembrane ion transporters [22]. Here, we reveal that miR-30c in tilapia plays a key regulatory role in regulating salt tolerance performance by direct targeting of HSP70. Taken together, the above-mentioned evidences suggest that miRNAs appear to be important players in salt stress response.

Tilapia acclimated to higher salinity environments experienced a crisis period in which there was a rapid increase in ion exchange accompanied by elevated plasma ions and osmolality [23,24]. Osmoregulation in tilapia is achieved by integrated water and ion transport in osmoregulation organs such as the gills, kidney and intestine [9,25]. Tilapia would rapidly activate osmoregulatory mechanisms after transfer between environments of different salinities within the range of tissue tolerance. miR-30c is found to be a kidney-enriched miRNA. miR-30c silencing group has higher ion concentration and osmolality compared to the wild-type group. Thus, we infer that miR-30c silencing group would experience more serious plasma ionic imbalance during the crisis period, indicating a key role of miR-30c in salt stress acclimation.

HSP70 proteins are constitutively expressed in cells under normal conditions. They are central components of the cellular network of molecular chaperones and folding catalysts [26]. They assist a large variety of protein folding processes in the cell by transient association of their substrate binding domain with short

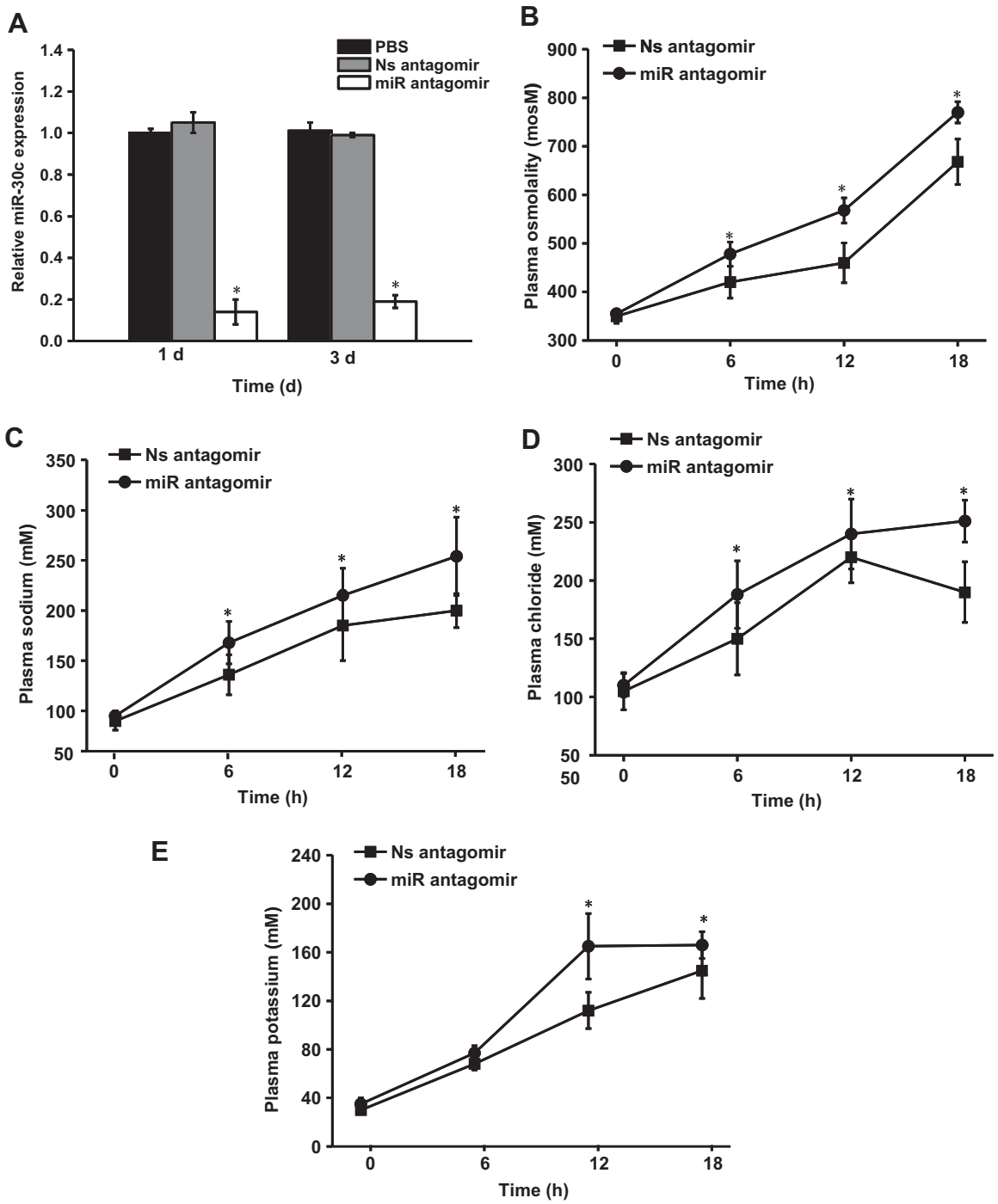

Fig. 3. miR-30c silencing affects salt tolerance performance in tilapia. (A) Tilapia weighing about 10 g received tail-vein injection of PBS, nonspecific antagomir (Ns) or miR-30c antagomir at a dose of 60 mg/kg body weight. The group treated with PBS was taken as control. The relative expression of miR-30c was detected 1 d or 3 d after treatment using Real-time PCR. 18S rRNA expression was detected as the internal control. The data was expressed as the relative change compared with the control group. Asterisk (*) indicates significant difference compared with the control group (*$P < 0.05$). (B–E) Tilapia weighing about 10 g received tail-vein injection of nonspecific antagomir (Ns) or miR-30c antagomir at a dose of 60 mg/kg body weight. After thirty-six hours, these treated fish were exposed to 20 g/L salinity for 6, 12, and 18 h. The fish treated with nonspecific antagomir (Ns) at 0 h was taken as control. Tilapia blood plasma osmolality, [Na^+], [Cl^-], and [K^+] was detected as described in Section 2. Asterisk (*) indicates significant difference compared with the nonspecific antagomir treated group (*$P < 0.05$).

hydrophobic peptide segments within their substrate proteins. Aside from this function, HSP70 are also implicated in the general protection of stressed cells and organisms [27,28]. Exposure of organisms to environmental stressors, such as temperature extremes, pollutants, anoxia, or competition would result in reversible increase in HSP70 expression, which will protect the organism against cellular damage [29–31]. The involvement of HSP70 in the acclimation of fish to salt stress has also been well documented experimentally [32,33]. In the silver sea bream, *Sparus sarba*, branchial expression of HSP70 is increased in response to hypo- or hyper-osmotic shock. In blackchin tilapia, *Sarotherodon melanotheron*, HSP70 is shown to be differentially expressed once they are exposed to different salinities [34]. In light of the above-mentioned studies, HSP70 is shown a critical regulator in response

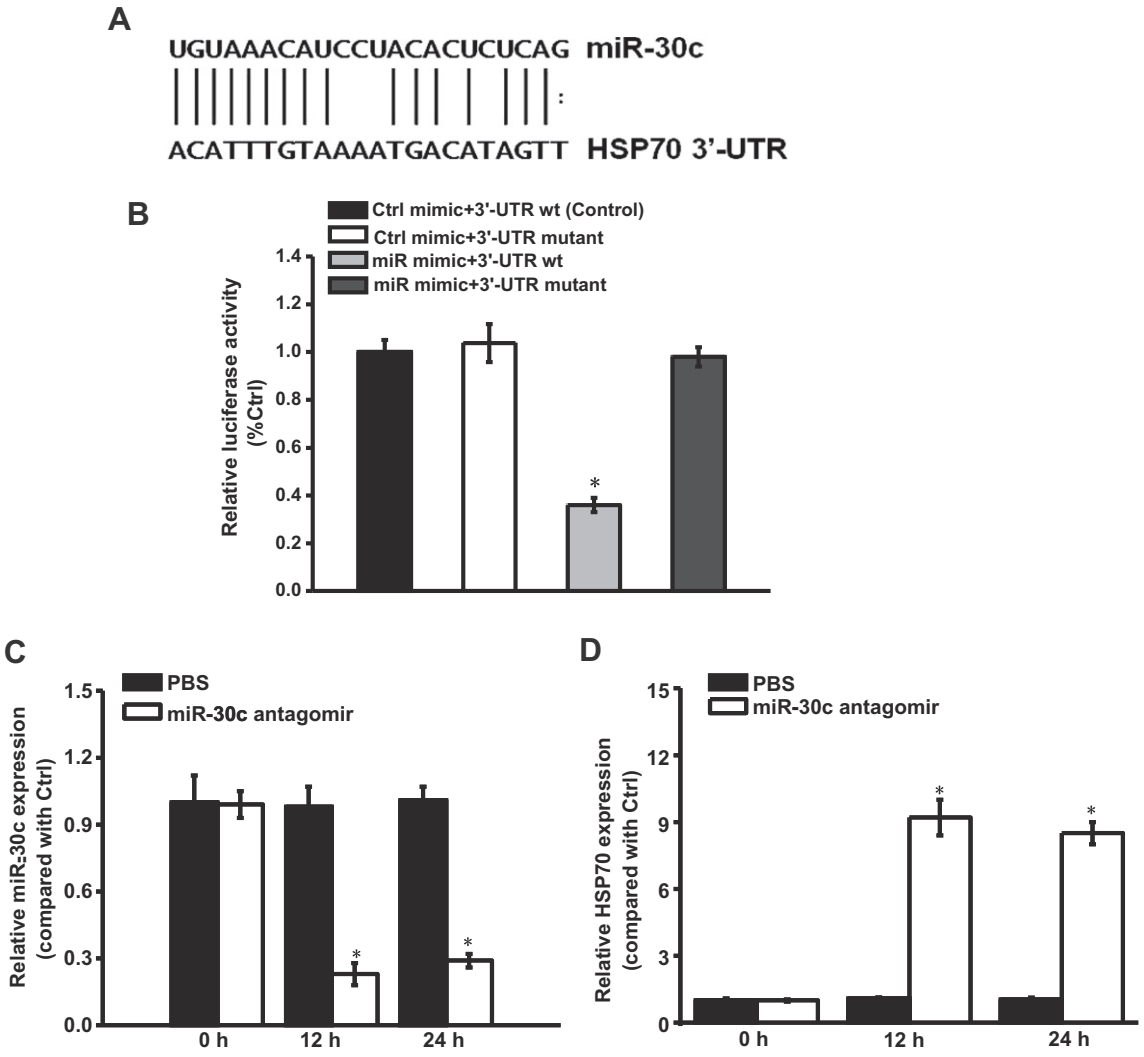

Fig. 4. miR-30c directly represses HSP70 expression through targeting 3′UTR of HSP70 gene. (A) The alignment between miR-30c and the 3′UTR segment of HSP70. (B) Luciferase reporters were linked with HSP70 3′-UTRs containing either putative miR-30c-binding sites (3′-UTR wt) or mutated miR-30c binding sites (3′-UTR mutant). miR-30c mimic or control mimic (Ctrl mimic) plasmids were cotransfected into HEK 293T cells with luciferase-UTR constructs, and then luciferase activity was determined. The cells tranfected with ctrl mimic plus 3′-UTR wt were used to serve as the control group. Data represent the mean ± SD from three independent experiments. $^{*}P < 0.05$. (C and D) Tilapia weighing about 10 g received tail-vein injection of PBS, or miR-30c antagomir at a dose of 60 mg/kg body weight for indicate time. The untreated group was taken as control. The relative expression of miR-30c (C) and HSP70 (D) was detected using real-time PCR. The data was 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 ($^{*}P < 0.05$).

to stress both *in vitro* and *in vivo*. Thus, it is not surprising that HSP70 production is tightly controlled by multiple mechanisms. miRNAs play fine-tuning roles in the control of gene expression. miR-30c targeting of HSP70 in coordination with transcriptional controls is a perfect example of that principle.

In summary, we reveal that miR-30c plays an important role in osmoregulation in tilapia. miRNA do so, at least partially, by limiting and refining the expression of HSP70 gene that are responsible for osmoregulation. In this manner, miRNAs confer robustness to participate in a feed-forward genetic circuit to resist salt stress. This finding provides insight into the post-transcriptional regulation mechanism of salt tolerance in tilapia. miR-30c may be developed as a novel molecular maker to assist selection of salt tolerant species.

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