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miRNA-directed regulation of VEGF in tilapia under hypoxia condition

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ABSTRACT

The Nile tilapia represents an excellent model for hypoxia tolerance. Vascular endothelial growth factor (VEGF) plays a key role in physiological blood vessel formation and pathological angiogenesis under hypoxia conditions. Tight regulation of VEGF level is necessary for hypoxia adaptation in tilapia. MicroRNAs (miRNAs) function as important regulators of gene expression at the post-transcriptional level, which are usually involved in stress responses. We reasoned that VEGF level could be regulated by miRNAs. Through bioinformatics analysis, we identified a putative miR-204 binding site in the VEGF mRNA. We found that hypoxia leads to a marked up-regulation in VEGF level, but a decrease in miR-204 level. miR-204 directly regulates VEGF expression by targeting its 3′-UTR, and inhibition of miR-204 substantially increases VEGF level *in vivo*. Moreover, we found that miR-204 loss of function could affect blood O_2-carrying capacity, anaerobic metabolism, and antioxidant enzyme activity. Taken together, miR-204 is an endogenous regulator of VEGF expression, which participates in a regulatory circuit that allows rapid gene program transitions upon hypoxia stress.

1. Introduction

MicroRNAs (miRNA) are a class of 20–24 nt non-coding RNAs that regulate gene expression primarily through post-transcriptional repression or mRNA degradation in a sequence-specific manner [1]. They play important roles in many fundamental cellular processes, including metabolism, cellular proliferation, differentiation, apoptosis, and developmental timing [2]. Under stress conditions, miRNA expression is altered to cope with the stress response. For example, miR-208 deficient are not capable of coping with cardiac overload [3]. Inactivation of miR-8 renders zebrafish incapable of responding to osmotic stress[4]. miR-7 knockout flies no longer develop their eyes properly when subjected to alternating temperatures [5].

Teleost fish (about 27,000 species) are the largest and most diverse group of vertebrates. They are in direct contact with the aquatic environment. Aquatic environments exhibit much wider temporal and spatial variations in their oxygen. Inadequate dissolved oxygen is a major cause of fish morbidity and mortality in the aquatic environment [6]. Nile tilapia (*Oreochromis niloticus*) has dominated freshwater aquaculture due to its adaptability and fast growth performance. It can naturally deal with abrupt fluctuations in oxygen availability without having to face the danger of excessive oxidative stress [7]. Thus, they may have evolved perfect mechanisms to deal with hypoxia stress.

The regulation of angiogenesis by hypoxia is an important component of homeostatic mechanisms that link vascular oxygen supply to metabolic demand [8,9]. Hypoxia stress usually leads to a significant increase in the level of vascular endothelial growth factor (VEGF), a specific endothelial cell mitogen with potent angiogenic properties. VEGF subsequently activates hypoxia-inducible signaling pathways, promoting the secretion of various angiogenic, inflammatory, and profibrotic cytokines. This highly coordinated process eventually leads to alleviating hypoxia injury and restoring normoxia [10]. VEGF expression has been reported to be regulated by many gene factors, such as HIF and HuR [11,12], but it is still not clear whether miRNA is involved in VEGF regulation under hypoxia in Fish. In this study, we used Nile tilapia as the model to investigate whether VEGF level is regulated by miRNA under hypoxia condition.

2. Materials and methods

2.1. Fish husbandry

Nile tilapia juveniles were obtained from the fishery farm of Shanghai Ocean University. They 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. Dissolved oxygen level was maintained at 80–85% air saturation (6.8 mg L^{-1}). All experiments were

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A

TargetScan	MicroCosm at EMBL
miR-15c	**miR-19a**
miR-1/206	miR-223
miR-15ab/16abc/457ab	**miR-19b**
miR-29	miR-135c
miR-205	miR-135a
miR-430	**miR-204**
miR-17a/20ab/93	miR-135b
miR-204	miR-31
miR-23	
miR-19abcd	
miR-9	
miR-429/200bc	
miR-144	

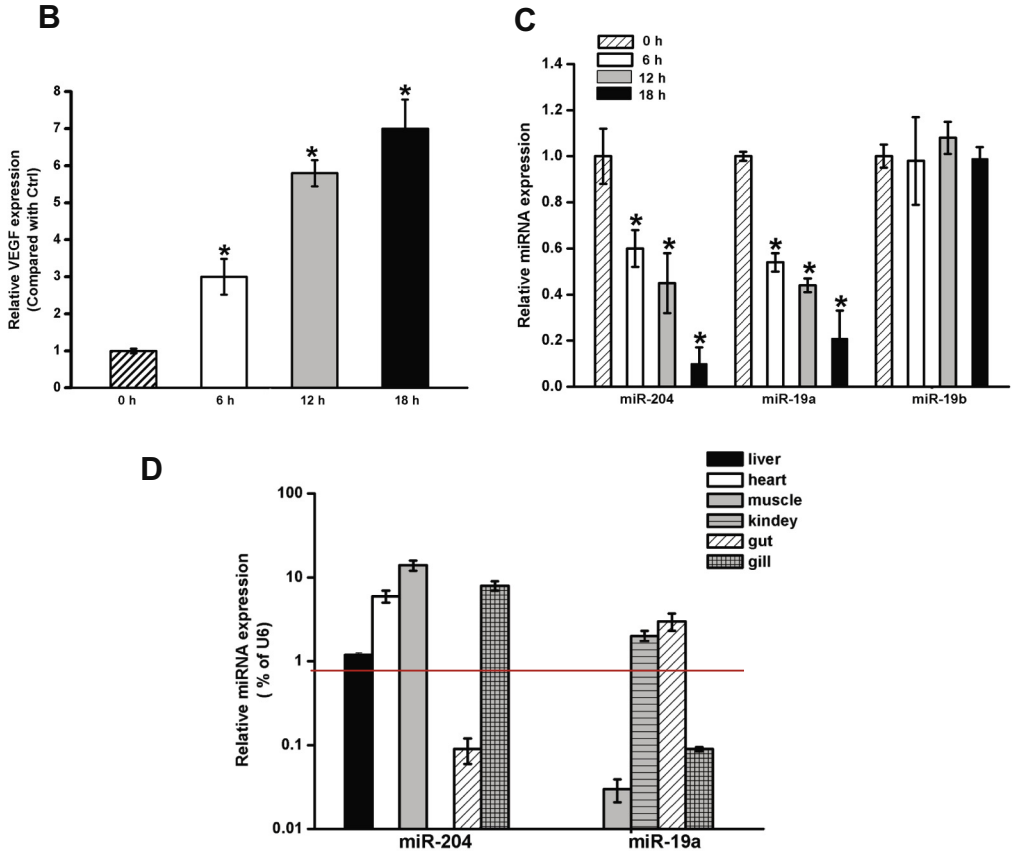

Fig. 1. Prediction of VEGF-binding miRNAs. (A) MicroCosm and TargetScan was used to predict VEGF-binding miRNAs; (B and C) Tilapia weighing about 5 g was exposed to hypoxia condition for 0 h, 6 h, 12 h, and 18 h. The expression of VEGF was detected using qRT-PCRs. 18S rRNA expression was detected as the internal control. The group exposed to 20 g/L O_2 level for 0 h was taken as the control group. The expression of miR-204, miR-19a, and miR-19b was detected using stem-loop PCRs. The data was expressed as the relative change compared with the control group. "$*$" indicates a significant difference compared with the control group ($P < 0.05$). (D) miRNA sample was extracted from different tissues and organs, including skeletal muscle, heart, gut, liver, kidney, and gill. miRNAs expression were detected by qRT-PCRs. 18S rRNA was detected as the loading control.

conducted 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 was isolated with TRIzol Reagent (Invitrogen), and miRNA sample was extracted using the miRNeasy kit (Qiagen) according to the manufacturer's protocol. RT-PCR was performed using TaKaRa one step RNA PCR kit (TaKaRa). The level of VEGF expression was normalized against a housekeeping gene, 18S rRNA. qRT-PCR was performed using the MyiQ5 Real-time PCR Detection System (Bio-Rad). The relative amount of miRNA was detected using the stem-loop PCR method. Relative gene or miRNA expression was calculated using comparative C_T method [13].

2.3. Luciferase activity assay

To perform the luciferase activity assays, VEGF 3′-UTR was cloned into the downstream of the firefly luciferase gene in

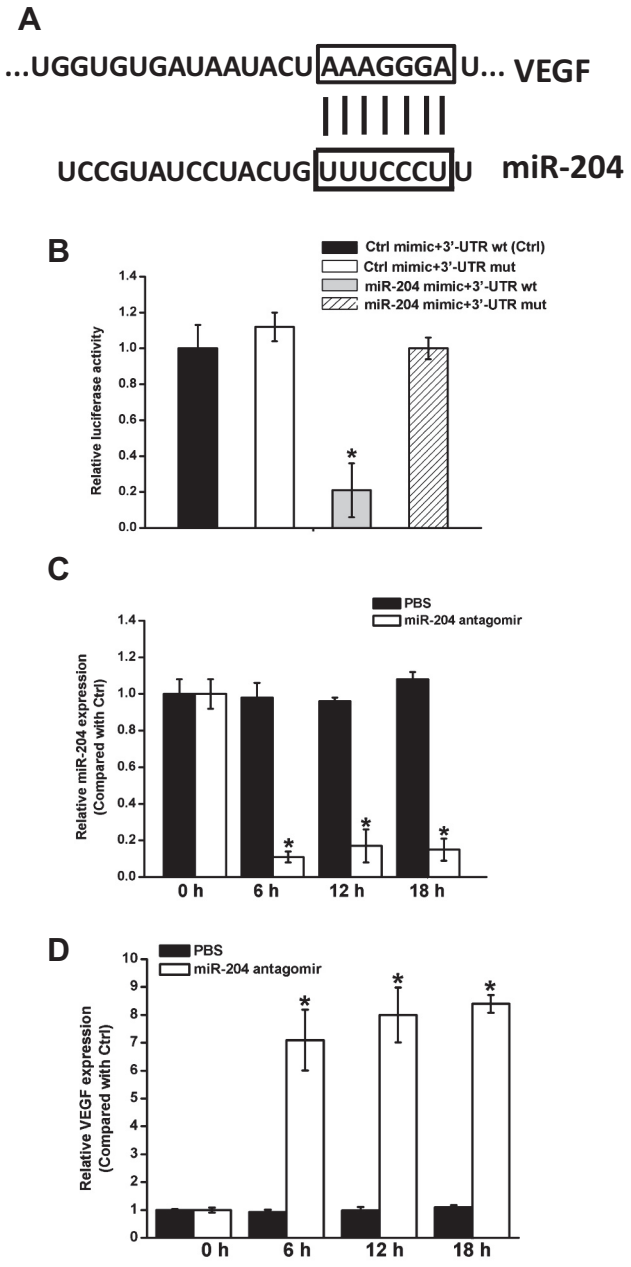

Fig. 2. miR-204 acts directly at the 3′-UTR of VEGF. (A) The alignment between miR-204 and the 3′-UTR segment of VEGF. (B) Luciferase reporters were linked with VEGF 3′-UTRs containing either putative miR-204-binding sites (3′-UTR wt) or mutated miR-204 binding sites (3′-UTR mutant). miR-204 mimic or control mimic (Ctrl mimic) were cotransfected with luciferase-UTR constructs into HEK 293T cells, and then luciferase activity was determined 36 h after transfection. The group transfected with Ctrl mimic plus 3′-UTR wt were used as the control group. (C and D) Tilapias weighing about 5 g were received a tail-vein injection of PBS or miR-204 antagomir at a dose of 50 mg/kg body weight for the indicated time. The untreated group was taken as the control. The relative expression of miR-204 (C) and VEGF (D) was detected using qRT-PCRs. The data was expressed as the relative change compared with the untreated group. 18S rRNA expression was detected as the internal control. "∗" indicates a significant difference compared with the wide-type group ($P < 0.05$).

pGL3-control vector (Promega). Six base pair in the UTR region was deleted to generate pGL3-VEGF mutant. For the luciferase reporter assays, HEK 293T cells were plated in 24-well culture plates 24 h prior to transfection. These cells were transfected with either wild-type or mutant construct with and without miRNA mimic or negative control mimic using lipofectamine 2000. Cell lysate was collected and assayed 36 h after transfection. Firefly and *Renilla* luciferase activities were measured using a Dual Luciferase Reporter Assay System (Promega) and each transfected well was assayed in triplicate [14].

2.4. Hematological parameters assay

Blood samples were kept in an ice bath during all analyses. Hematocrit (Ht) was determined by the microhematocrit centrifugation technique. Red blood cell count (RBC) was determined optically with a Neubauer chamber. Hemoglobin concentration (wHbx) was determined with Drabkin's reagent at 540 nm absorbance [15].

2.5. Enzyme activity assay

Lactate dehydrogenase activity was measured by observing the oxidation of NADH at 340 nm (pH 7.5). Citrate synthase activity was measured by observing the reduction of DTNB [5,5V-dithiobis (2-nitrobenzoic acid)] at 412 nm (pH8.0). SOD activity was measured by observing the inhibition of ferricytochrome *c* reduction by superoxide radicals at 550 nm using the reaction of xanthine and xanthine oxidase as the source of superoxide radicals. Catalase activity was measured by observing the decomposition of hydrogen peroxide into oxygen and water at 240 nm [16].

2.6. Statistical analysis

Data were expressed as means ± S.E.M. unless otherwise indicated. Significant differences across treatments were evaluated using one way ANOVA with the Bonferroni *post hoc* test. The significance level was chosen at $P < 0.05$.

3. Result

3.1. Prediction of miRNAs targeting VEGF

The increase in secreted biologically active VEGF protein could protect against hypoxia-induced cellular injury [17]. To identify miRNAs that potentially interact with VEGF, we employed the MicroCosm and TargetScan programs for bioinformatics prediction. The result shows that miR-204, miR-19a, and miR-19b are the common potential regulators of VEGF expression (Fig. 1A). We used qRT-PCRs to investigate their expression patterns in response to hypoxia stress. We found that hypoxia results in a significant increase in VEGF level in a time-dependant manner (Fig. 1B). The expression of miR-204 and miR-19a is inversely related to VEGF expression. By contrast, the expression of miR-19b is not affected by hypoxia stress (Fig. 1C). We then used qRT-PCR to detect the tissue distribution of miR-204 and miR-19a. The result shows that miR-204 is mainly expressed in heart, muscle, and gill, while miR-19a is mainly expressed kidney and gut. Heart, muscle, and gill are found to be important organs or tissues involved in the regulation of hypoxia stress (Fig. 1D). Thus, we mainly investigated the relationship between VEGF and miR-204 in the following study.

3.2. miR-204 acts directly at the 3′-UTR of VEGF

We employed a luciferase reporter assay to determine whether miR-204 directly regulates VEGF expression. The alignment of miR-204 with VEGF 3′-UTR is illustrated in Fig. 2A. We constructed two different luciferase reporters, including the wild-type VEGF 3′-UTR, and the mutant VEGF 3′-UTR deficient of miR-204 binding site. These luciferase reporters were cotransfected with miR-204

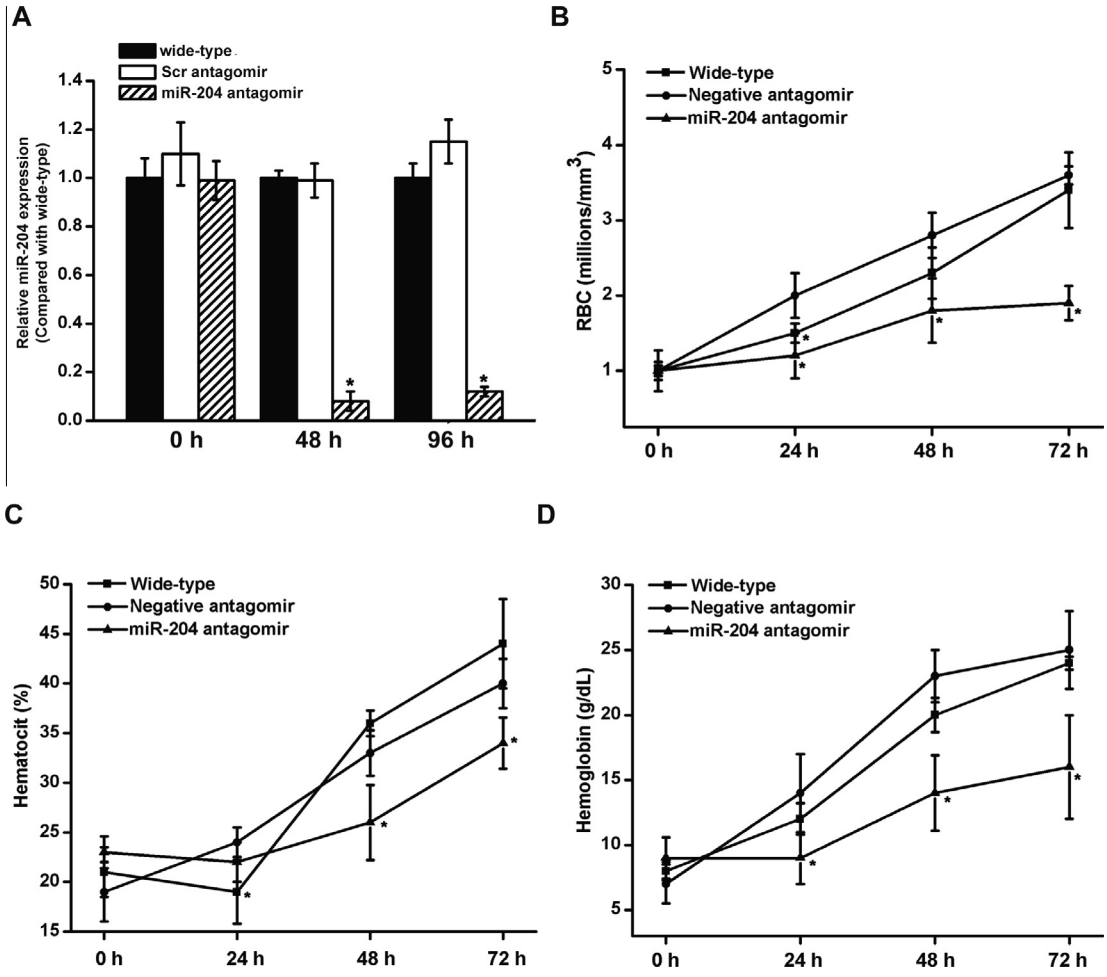

Fig. 3. miR-204 silencing affects hypoxia tolerance performance in tilapia. (A) Tilapias weighing about 5 g were received a tail-vein injection of scramble antagomir (scr) or miR-204 antagomir at a dose of 50 mg/kg body weight or left untreated. The expression of miR-204 was detected using stem-loop PCR. (B–D) Tilapias were received a tail-vein injection of scramble antagomir (scr), miR-204 antagomir, or left untreated, and then exposed to hypoxia condition for the indicated time. Hematological parameters, including Hematocrit (Ht), red blood cell count (RBC), and hemoglobin concentration were detected. "$*$" indicates a significant difference compared with the wide-type group ($P < 0.05$).

mimic into HEK 293T cells. Meanwhile, a scrambled miRNA mimic with no homology to the tilapia genome was used as the negative control. The result demonstrates that miR-204 mimic significantly decreases the luciferase activity of wild-type VEGF 3′-UTR. By contrast, miR-204 mimic does not affect the luciferase activity of mutant VEGF 3′-UTR, suggesting that miR-204 directly suppresses VEGF expression by binding to its 3′-UTR sequence.

To determine whether miR-204 directly targets VEGF *in vivo*, we used the antagomir method to knockdown miR-204 level. The result shows that miR-204 antagomir but not PBS treatment leads to a significant decrease in endogenous miR-204 expression at the different time points. Meanwhile, we detected an increased VEGF expression at the same time points (Fig. 2C and D). The inverse expression correlation between miR-204 and VEGF further verifies that miR-204 directly regulates VEGF expression.

3.3. The role of miR-204 in hypoxia tolerance in tilapia

To determine the role of miR-204 in hypoxia tolerance *in vivo*, we knocked down miR-204 through antagomir injection. Endogenous miR-204 expression could be suppressed by miR-204 antagomir but not by the scramble antagomir injection (Fig. 3A). Tilapias with different miR-204 level were exposed to hypoxia stress, and the control groups were exposed to normoxia condition.

Lactate dehydrogenase (LDH) or citrate synthase is the indicator of anaerobic metabolism or aerobic metabolism. Hypoxia results in a significant increase in LDH activity but a decrease in citrate synthase activity to protect against hypoxia stress. Compared with the wild-type and scramble antagomir group, miR-204 antagomir group has poor ability of anaerobic metabolism, as shown by lower LDH activity and higher citrate synthase activity (Table 1). Catalase and SOD are antioxidant enzymes involved in the elimination of ROS. Hypoxia results in a marked increase in their activity. Compared with the wild-type and scramble antagomir group, miR-204 antagomir group has poor ability of antioxidant ability, as shown by decreased catalase and SOD activity (Table 1).

Further, we collected the blood samples from different experimental groups with differential miR-204 levels. We investigated the effect of miR-204 knockdown on blood O_2-carrying capacity, as shown by Hematocrit (Ht), red blood cell count (RBC), and hemoglobin (Hb) concentration. Hypoxia treatment results in a significant increase in Ht, RBC counts, and Hb concentration, suggesting a significant up-regulation of blood O_2-carrying capacity. Compared with the wide-type group, scramble antagomir injection does not further change these hematological parameters, whereas miR-204 antagomir injection could significantly decrease the change of these hematological parameters (Fig. 3B–D).

Table 1
Antioxidant and metabolic enzyme activity in different tissues after 48 h hypoxia and normoxia exposure.

Enzyme	Group	Oxygen treatment	Tissue		
			Gill	Liver	Muscle
LDH	Wt	Normoxia	0.54 ± 0.04	US	1.95 ± 0.06
	Ng		0.65 ± 0.02	US	1.82 ± 0.04
	miR-204		0.50 ± 0.08	US	1.98 ± 0.02
	Wt	Hypoxia	1.34 ± 0.12	US	23.66 ± 3.21
	Ng		1.55 ± 0.21	US	21.55 ± 5.14
	miR-204		0.89 ± 0.45*	US	14.55 ± 2.98*
Citrate synthase	Wt	Normoxia	0.25 ± 0.04	0.44 ± 0.13	1.09 ± 0.02
	Ng		0.33 ± 0.13	0.38 ± 0.04	0.89 ± 0.15
	miR-204		0.22 ± 0.08	0.42 ± 0.08	0.98 ± 0.07
	Wt	Hypoxia	0.08 ± 0.02	0.24 ± 0.09	0.69 ± 0.03
	Ng		0.09 ± 0.08	0.21 ± 0.06	0.63 ± 0.09
	miR-204		0.16 ± 0.11*	0.35 ± 0.03*	0.88 ± 0.21*
SOD	Wt	Normoxia	4.89 ± 0.16	20.15 ± 2.21	12.32 ± 3.09
	Ng		5.35 ± 1.09	21.00 ± 1.78	13.59 ± 1.93
	miR-204		4.76 ± 0.23	21.56 ± 3.13	11.50 ± 2.70
	Wt	Hypoxia	9.06 ± 1.28	41.82 ± 5.21	20.39 ± 3.23
	Ng		8.87 ± 0.29	40.18 ± 2.62	21.25 ± 2.11
	miR-204		6.18 ± 0.62*	31.04 ± 3.03*	16.82 ± 0.59*
Catalase (CAT)	Wt	Normoxia	US	828 ± 47.08	5.08 ± 0.32
	Ng		US	783 ± 32.82	5.65 ± 0.87
	miR-204		US	802 ± 23.95	4.98 ± 0.42
	Wt	Hypoxia	US	1321 ± 80.42	10.02 ± 1.08
	Ng		US	1459 ± 60.36	10.55 ± 0.76
	miR-204		US	1060 ± 79.21*	6.89 ± 0.67*

US: undetected; LDH: lactate dehydrogenase; SOD: superoxide dismutase; Wt: wild-type; Ng: negative antagomir; miR-204: miR-204 antagomir; normoxia: 6 mg/L; hypoxia: 0.6 mg/L.

4. Discussion

Upon environmental stress, the organisms either choose to restore or reprogram their gene expression patterns. This response is sometimes mediated by miRNA function through regulating the amount of miRNAs, the amount of mRNA targets, or the interaction of miRNA–protein complexes. Thus, clarifying miRNA-mediated regulatory networks would provide a novel insight into the genetic improvement of species stress tolerance [18,19]. Herein, we found that the role of miR-204 in hypoxia tolerance in tilapia. Change in endogenous miR-204 level could change VEGF expression level, which in turn affect hypoxia tolerance performance in tilapia as shown by hematological parameters and enzymatic activities assay.

VEGF plays a key role in physiological blood vessel formation and pathological angiogenesis. The induction of VEGF is a critical step in the angiogenic response to hypoxia. Molecular studies have determined VEGF is regulated primarily at the level of the mRNA. Specifically, hypoxia leads to a marked increase in the transcription of VEGF as well as an increase in its mRNA stability. HIF-1 can bind to VEGF promoter and forms a complex that activates the transcription of VEGF gene [17,20]. The mechanism of HuR stabilization of VEGF mRNA appears to act by displacing RNases that mediates rapid degradation of VEGF mRNA [21]. miRNAs are endogenous regulators of gene expression. They bind to specific mRNA targets, causing their degradation or translational repression [1]. Here, we identified that miRNA is involved in the regulation of VEGF expression. *In vivo* experiment revealed the inverse expression correlation between miR-204 and VEGF. miR-204 directly suppresses VEGF expression by binding to its 3′-UTR sequence. The study provides a novel regulatory layer for VEGF expression in fish.

Hematocrit, hemoglobin concentration and RBC counts are important hematological parameters to reflect blood O_2-carrying capacity [22]. Under hypoxia condition, the tilapia should enhance the blood O_2-carrying capacity to alleviate hypoxia-induced injury. In this study, we observed the gradual increase in hematological parameters with the hypoxia went on. Scramble miRNA antagomir injection has no effect on these parameters. By contrast, miR-204 knockdown partially prevents the increase of hematological parameters-induced by hypoxia stress, implying that miR-204 knockdown could decrease the blood O_2-carrying capacity.

Organisms generally have two different metabolic strategies to cope with the hypoxic stress: the reduction in metabolic rate or a shift in the aerobic and anaerobic contributions to total metabolism. The more severe the hypoxia, the greater the contribution of anaerobic metabolic pathways would occur [16,23]. Lactate dehydrogenase is an indicator of anaerobic metabolism, while citrate synthase is the indicator of aerobic metabolism [24]. Compared with wild-type group, miR-204 knockdown group has lower LDH activity but higher citrate synthase activity. Oxygen limitation should increase usage of glycolytic pathways and decrease use of aerobic pathways [16]. miR-204 knockdown group obviously disobeys this principle.

The antioxidant defense (AD) system of organisms provides a means of dealing with oxidative stress. The key role of the AD system is protecting cellular components from ROS damage [16]. The antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) act to remove oxygen radicals produced within the cells [25]. They are expected to increase under hypoxia to detoxify ROS. Hypoxia results in a significant increase in the activity levels of SOD and CAT, while miR-204 knockdown fish has lower could activity levels, suggesting that miR-204 knockdown could affect the antioxidant activity.

In summary, we found that miR-204 directly regulates VEGF by targeting its 3′-UTR, and miR-204 inhibition is able to significantly up-regulate VEGF mRNA level *in vivo*. miR-204 is involved in the

regulatory circuit that allows rapid gene expression adaption upon hypoxia stress. In this manner, miRNAs confer robustness to participate in a feed-forward genetic circuit to cope with hypoxia stress. This research would provide a novel insight into the potential mechanism of fish hypoxia tolerance.

Conflict of interest

The authors have declared that no competing interests exist.

Acknowledgments

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