microRNA regulation of skin pigmentation in fish

Biao Yan¹², Ban Liu³, Chang-Dong Zhu², Kang-Le Li¹²,
Li-Jia Yue², Jin-Liang Zhao¹², Xiao-Ling Gong², Cheng-Hui Wang¹²

¹Key laboratory of Freshwater Aquatic Genetic Resources, Shanghai Ocean University,
Ministry of Agriculture, Shanghai 201306, China

²College of Fisheries and Life Science, Shanghai Ocean University, Shanghai, 201306, China

³Department of Cardiology, Shanghai Tenth People's Hospital,
Tongji University, Shanghai, 200072, China

Correspondence to: Cheng-Hui Wang
Address: Shanghai Ocean University, Huchenghuan Road 999#, 201306, Shanghai, China
Tel: 086-21-61900439
Fax: 086-21-61900439
E-mail: wangch@shou.edu.cn

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Abstract

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that play crucial roles in numerous biological processes. However, the role of miRNAs in skin color determination in fish has not been completely determined. Here, we identified that 13 miRNAs are differentially expressed between red and white skin. The analysis of miRNA spatial and temporal expression patterns suggests that miR-429 is potential regulator of skin pigmentation. miR-429 silencing results in a obvious change in skin pigmentation. Bioinformatics analysis and luciferase reporter assay shows that miR-429 directly regulates Foxd3 expression by targeting its 3′ untranslated (3′-UTR) region. miR-429 silencing leads to a significant increase in Foxd3 expression in vivo, thereby repressing the transcription of MITF and its downstream genes such as TYR, TYRP1, or TYRP2. These findings would provide a novel insight into the determination of skin color in fish.

Key words: miRNA, skin color, fish, Foxd3

Introduction
Some fish usually display fascinating color patterns in their skin, which play important roles in numerous biological processes, such as mate choice, camouflage, and the perception of threatening behavior (Kelsh, 2004; Protas and Patel, 2008). The determination of skin color is a very complicated process in fish, which is associated with a series of cellular, genetic, environmental and physiological factors (Aspengren et al., 2009). How skin color patterns form, however, is a longstanding question among biologists. The genetic of skin pigmentation in vertebrates has been extensively studied in a range of laboratory animal models. To date, a series of genes have been reported to be involved in the determination of skin color, such as pro-opiomelanocortin (POMC), melanocyte-stimulating hormone (MSH), microphthalmia-associated transcription factor (MITF), kit oncogene (KIT), tyrosinase (TYR), tyrosine related protein-1 (TYRP1), and tyrosine related protein-2 (TYRP2) (Hubbard et al., 2010; Kelsh, 2004). Yet surely the majority of gene resources involved in the determination of fish skin color remain to be discovered.

Skin color pattern is governed by complex and well-balanced programs of gene activation and silencing. MicroRNAs (miRNAs) are a set of single-stranded, non-coding RNA molecules with an average size of about 22 nucleotides. They recognize and bind 3'-untranslated regions (UTRs) of mRNA, blocking translation of the gene or inducing cleavage of the mRNA. The crosstalk between miRNAs and mRNAs is important for the steadiness of signal transduction and the transcriptional activities as well as the maintenance of homeostasis in many organs, including the skin (Mo, 2012; Sand et al., 2009; Yi and Fuchs, 2009). Accordingly, skin-expressed miRNAs may play a crucial role in skin development, body color formation, and skin diseases. In previous study, miR-203 was found to define a molecular boundary between proliferative basal progenitors
and terminally differentiating suprabasal cells, ensuring proper identity of neighboring layers (Yi et al., 2008). miR-137 expression level can affect the body color pattern in mice (Dong et al., 2012). In Drosophila, the loss of miR-8 shows a significant decreased pigmentation of the dorsal abdomen (Kennell et al., 2012). These evidences suggest that miRNAs could be involved in the formation of body color. However, no miRNA has been reported in the process of the determination of fish skin color.

The common carp (Cyprinus carpio) is a widespread freshwater fish of eutrophic waters in lakes and large rivers in Europe and Asia. They have numerous skin colors in the natural environment, such as red, white, orange, or black body color (Wang et al., 2009). In the past ten years, we have built two pure strains of common carp, which have white or red skin color respectively. Moreover, the trait of skin color is inherited in accordance with the law of Mendelian inheritance as shown in Fig. S1. Thus, they offer an appealing model system to investigate the genetic basis of skin pigment patterns. However, the unclear genetic background hinders its application for basic biological study. Tilapia is an important model for the research of fish physiology, endocrinology, and the evolutionary mechanisms in vertebrates. It also has a diversity of color patterns including red and white skin color. Scientists have successfully completed tilapia whole-genome sequencing project in, providing a relative clear genome information (Guyon et al., 2012). Common carp and tilapia would be adopted as research subjects to investigated the role of miRNAs in the determination of skin color.

In this study, we employed RNA-Seq method to identify skin-related miRNAs, and quantitative PCR to screen these differentially expressed miRNAs between white and red skin. We found that the miR-429 is a positive regulator of skin color in fish, and loss of miR-429 affects the
pigment content in the skin. These effects are found to be mainly mediated by the direct interaction between miR-429 and Foxd3, suggesting a role of miRNA in the determination of skin color in fish.

Result

miRNA expression signature in the skin of common carp

To obtain miRNA expression signature in the skin of common carp, we constructed a small RNA library from RNA sample pools isolated from different color skin. Total RNA was size-fractionated via polyacrylamide gel electrophoresis and small RNAs corresponding in molecular weight to the mature miRNA population (18-24 nt fraction) were gel extracted and processed for reverse transcription and PCR amplification to create cDNA libraries. Small RNA sequencing using the Illumina Genome Analyzer II generated 15,505,485 raw reads. After removing low quality sequences and adapter sequences, 15,326,145 clean reads were left for further analysis. Of them, 60.06% of the obtained small RNA sequences were 20-24 nt in size, which is the typical size range for Dicer-derived products (Fig. 1). Currently, the complete genome sequence of common carp is still unavailable. We selected zebrafish genome as the reference genome for subsequent analysis. The above-mentioned clean reads were mapped to zebrafish genome using SOAP software. The result shows that about 76,595 unique sRNAs (7,976,459 total sRNAs) were found to match the zebrafish genome perfectly (Table S1). Next, we performed a database search to find out rRNA, tRNA, snRNA and snoRNA deposited at Rfam and NCBI GenBank database (Fig. 1).

To identify the conserved miRNA homologs in the skin of common carp, BLASTn search with an E-value cutoff of 10 was employed to search for the remnant small RNAs with predicted
hairpin structures against the miRBase (http://microrna.sanger.ac.uk/sequences/) (Kozomara and Griffiths-Jones, 2011). With this similarity search, 73 miRNAs were found to be the same as at least one published miRNAs in the miRBase. All of these conserved miRNA precursors were identified, and had a hairpin structure (Data not shown). Based on sequence similarity, these known miRNAs were divided into 37 families (Table S2).

**miRNAs show differential expression in white and red skin**

To gain insight into the functional role of miRNAs in physiological process, it is essential to have precise information on their temporal and spatial expression patterns. Firstly, we detected the expression levels of skin-related miRNAs in different color skin in common carp. The result indicates that 14 distinct miRNAs are differentially expressed between white and red skin. miR-25, miR-15a-3p, miR-146b, miR-184, miR-429, and miR-141 are abundantly expressed in red skin, while miR-18a, miR-137, miR-17a, miR-203a, miR-9-3p, miR-9-5p, miR-129-5p and miR-204 are highly abundantly expressed in white skin (Table 1). In tilapia, we also detected the expression levels of these differentially expressed miRNAs identified in common carp. The result shows that except miR-141, miR-129-5p or miR-17a, other differentially expressed miRNAs have the similar expression pattern between common carp and tilapia (Table S3).

The expressions of miRNAs are tightly regulated in a time-and space-dependent manner. Tissue-specific miRNAs or high-abundance miRNAs expressed in certain tissues implies that these miRNAs play critical roles in the maintenance of tissue function. The observation that miR-25, miR-137, miR-203a, and miR-429 are strongly expressed in the skin, implies an important role for these miRNAs in the skin constitutive process (Table 2). In addition, we examined miRNAs expression profiling during the development of common carp at five different
developmental stages, including zygote, blastula, gastrula, segmentation, and larvae. Interestingly, we found that miR-429 shows a dynamic expression pattern. miR-429 expression is first detected at the beginning of the gastrula stage, and its expression is significantly up-regulated in segmentation stage and this expression then sustains through to larvae stage (Table 2 and Fig. S2). Given pigment cells are initially derived from the neural crest during gastrula stage (Betancur et al., 2010), we speculated that miR-429 is potential regulator of pigmentation process.

**miR-429 silencing affects the pigmentation process**

In previous studies, we have employed miRNA antagomir method to conduct miRNA loss of function experiment *in vivo* (Yan et al., 2012; Yan et al., 2013). In this study, we also observed that miR-429 expression could be efficiently blocked by its corresponding antagomir (miR-429 antagomir) but not the mismatched miRNA (miR-203 antagomir) *in vivo*. Furthermore, the silencing effect of miRNA antagomir was detected at different time points (Fig. S3). To evaluate the effect of miR-429 silencing on skin pigmentation, we injected red tilapia weighing about 5g with miR-429 antagomir for thirty days. miR-429 expression is efficiently silenced by miR-429 antagomir but not by the mistched antagmor (Fig. 2A). There is no significant difference observed between the wild-type fish and miR-429 silencing fish in survival rate (Data not shown). We compared the melanin production between wild-type and miR-429 silencing fish. We found that the red fish injected with miR-429 antagomir have lower melanin content relative to its matched red group (Fig. 2B). In red common carp, we also observed that the treatment of miR-429 antagomir is able to significantly decrease the melanin content in skin (Fig. S4). These data suggest that miR-429 expression levels could affecte the process of skin pigmentation in fish.

**miR-429 acts directly at the 3'-UTR of Foxd3**
To determine the role of miR-429 in the pigmentation process, we performed an in silico functional annotation analysis of its predicted target genes using the TargetScan and miRanda program based on tilapia genome sequence (Chen et al., 2005). We screened candidate genes of melanogenesis signaling pathway in the KEGG pathway database (http://www.kegg.jp/kegg/) (Kanehisa et al., 2008), and found that Foxd3 is potential target gene of miR-429. The sequence alignment between miR-429 and the 3′-UTR segment of Foxd3 is shown in Fig. 3A. Foxd3 has shown to play a functional role in the specification of various downstream neural crest derivatives. To verify Foxd3/miR-429 interaction, we engineered two luciferase reporters, which are the wild-type 3′-UTR of Foxd3 gene, or the mutant UTR of Foxd3 gene. One luciferase reporter with or without miR-429 mimic or a scrambled miRNA mimic were transfected with into HEK 293T cell. The scrambled miRNA mimic with no homology to the tilapia genome was used to control the nonspecific effects. As shown in Fig. 3B, the transfection of scrambled miRNA mimic does not affect the luciferase activity of Foxd3 3′-UTR wildyte reporter. However, miR-429 mimic transfection results in a significant decrease in the luciferase activity of Foxd3 3′-UTR wildyte reporter. By contrast, the luciferase activity of Foxd3 3′-UTR mutant reporters is not repressed by miR-429 mimic (Fig. 3B). In addition, we found that in vivo administration of miR-429 antagonir but not NaCl results in a profound decrease in the endogenous expression of miR-429 (Fig. 3C). Meanwhile, we detected a significant increase in Foxd3 expression at mRNA level and protein level (Fig. 3D and 3E). The inverse expression correlation between miRNAs and putative target gene also suggests that miR-429 can directly regulate Foxd3 expression in vivo. Taken together, these data indicate that miR-429 regulates Foxd3 expression through targeting of the 3′-UTR of Foxd3 gene.
Altered expression of pigmentation genes in miR-429 silencing tilapia

Skin color is primarily determined by the amount, the type, and the distribution of melanin (Parra, 2007). MITF has been reported as a key gene in the regulation of melanin production. It is involved in melanocyte development and melanogenesis. Foxd3 can bind MITF promoter and repress MITF transcription, thereby affecting the process of melanogenesis. Since miR-429 silencing results in up-regulation of Foxd3 expression, MITF would be up-regulated during this process. Indeed, quantitative PCR analysis reveals that miR-429 silencing results in a significant increase in Foxd3 expression, thereby up-regulating MITF expression (Fig. 4A and 4B). MITF is required for the expression of TYR, TRP-1, and TRP-2 genes that encode enzymes implicated in the production of the pigment melanin (Levy et al., 2006). Quantitative PCR and western blot analysis indicates that the expression of MITF downstream genes, TYR, TYRP1, and TYRP2, are markedly downregulated in miR-429 silencing fish (Fig. 4C and 4D). Taken together, these results indicate miR-429 silencing can increase the level of Foxd3, which represses the transcription of MITF and its downstream genes.

miR-429 mediated pigmentation is distinct from α-MSH-mediated pigmentation upon UV radiation

Ultraviolet (UV) radiation is a major environmental hazard that can lead to skin inflammation, photoaging, and skin cancer. The skin pigment can provide essential protection against UV radiation (Matsumura and Ananthaswamy, 2004). Here, we found that UV radiation significantly up-regulates the level of miR-429 expression in the melanocyte. The change in miR-429 expression could be detected as early as 1 h after UV treatment (Fig. 5A). UV treatment also could significantly increase MC1R and α-MSH expression at mRNA and protein level, which stimulates
the keratinocytes to secrete to enhance the synthesis of skin melanin (Fig. 5A). By comparison, we found that the change of UV-mediated miR-429 expression is significantly earlier than that of UV-mediated gene and protein expression, suggesting that miRNA regulation occurs earlier than most gene transcription response (Fig. 5A). In addition, we also found that miR-429 expression was significantly increased in fish upon UV radiation. Meanwhile, we detected a significant reduction in Foxd3 expression and a marked increase in MITF expression at mRNA level and protein level (Fig. 5B-D). To determine whether miR-429 is involved in MSH-mediated pigmentation, we compared the expression profiling of α-MSH and MC1R between miR-429 silencing group and wild-type group in response to UV radiation. The result shows that miR-429 level does not change the expression of α-MSH and MC1R level under the same condition, suggesting that miR-429-mediated pigmentation is distinct from α-MSH-mediated pigmentation (Fig. 5E, 5F, and 5G).

Discussion

miRNAs post-transcriptionally regulate gene expression by promoting mRNA degradation or inhibiting mRNA translation. They play pivotal roles in a variety of developmental processes, and their dysregulations are linked to numerous skin diseases (Mo, 2012). When miRNAs are globally ablated in skin epithelium by conditionally targeting Dicer 1, a miRNA-processing enzyme, this operation could distort epidermal morphology, implying a key role of miRNAs in skin development (Yi et al., 2006). To gain the insight into the possible significance of skin miRNAs in fish, we first identified miRNAs expression profile in the skin tissue in fish, and then compared miRNAs expression pattern between white and red skin. We found that 11 miRNAs are differentially expressed in different color skin, implying these differential expressed miRNAs may
be involved in skin pigmentation. The knowledge of tissue-specific and cell-specific expression patterns of miRNAs can directly inform functional studies (Aboobaker et al., 2005). Previous studies have revealed that miR-375 is specifically expressed in pancreatic islet cells, where it regulates the expression of insulin secretion (Poy et al., 2004). miR-1 is expressed exclusively in muscle, where it regulates cardiomyocyte proliferation in vertebrates and muscle physiology in flies (Sokol and Ambros, 2005; Zhao et al., 2005). Here, we found that miR-429, miR-25, and miR-137 is highly expressed in fish skin. Specially, miR-429 displays dynamic expression patterns during embryonic development. miR-429 is first detected at the gastrulae stage, and then its expression is gradually up-regulated until the larvae stage. Since pigment cells are initially derived from the neural crest during gastrula stage, we speculated that miR-429 may play a key role in regulating skin pigmentation.

Skin pigmentation in fish is a complex process that involves a series of cellular, genetic and physiological factors (Colihueque, 2010). The role of miRNAs in pigmentation has been reported in some species, including mouse, alpaca, and Drosophila (Dong et al., 2012; Kennell et al., 2012; Zhu et al., 2010). In this study, we revealed that miR-429 is a potential regulator of fish pigmentation. Our previous study found that miR-429 directly regulates the expression of OSTF1, an osmotic stress transcriptional factor, revealing a role of miR-429 in fish osmoregulation (Yan et al., 2012). Here, we found that miR-429 is highly expressed in red skin, and inhibition of miR-429 function causes a significant decrease in skin pigmentation. This study further extends the biological role of miR-429 in fish. miR-429 is a member of miR-8 family, which has been predicted or experimentally confirmed in a wide range of species. Prior studies have identified miR-8 as a regulator of osmoregulation, growth, apoptosis, and neuronal survival by targeting
multiple mRNAs (Hyun et al., 2009; Loya et al., 2009; Vallejo et al., 2011). In Drosophila, miR-8 is required for proper spatial patterning of pigment on adult female abdomens. Loss of miR-8 in the developing cuticle results in cell-autonomous loss of pigmentation (Kennell et al., 2012). Sequence alignment suggests that miR-8 family is highly conserved between invertebrates and vertebrates, which might indicate that its function has been conserved. Our study provides an example which suggests that the role of miR-8 in pigmentation is highly conserved between invertebrates and vertebrates.

miRNAs play the regulatory roles in biological processes through regulating the expression of their target genes. Here, we found that a binding site of miR-429 in the 3’-UTR region of Foxd3, and characterized their effects on Foxd3 using a reporter assay. Foxd3 is one of the earliest molecular markers of the neural crest lineage, it is expressed in many organisms in premigratory and migrating neural crest and its expression is down-regulated as the cells differentiate into most derivatives (Abel and Aplin, 2010; Thomas and Erickson, 2009). Foxd3 can also control the lineage choice between neural/glial and pigment cells by repressing MITF during the early phase of neural crest migration. In this study, we found that miR-429 expression begins in the gastrula stage, which leads to a gradual decrease in Foxd3 expression. The ablation of MITF repression could contribute to the generation of pigment cells.

MITF is a member of the Myc-related family of basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factors and is highly conserved across different vertebrate species. MITF positive cells are also observed in the optic neuroepithelial layer, the presumptive retinal pigment epithelium (RPE), as well as in cells behind the optic cup that were likely neural-crest derived and could develop into choroidal and iris pigment cells (Nakayama et al., 1998).
Mutations of the MITF gene cause a variety of phenotypes, most notably in pigmented cells (Levy et al., 2010). These evidences suggest that MITF expression is tightly associated with the development of pigment cell. MITF is begun to be expressed early in neural crest-derived cells in mouse embryo (Hou et al., 2000). Here, we found a similar result, which shows that miR-429 is activated in gastrula stage, which directly represses Foxd3 expression, thereby releasing MITF inhibition by Foxd3. MITF directly regulates the expression of multiple genes necessary for melanophore development, including tyrosinase, tyrosinase-related protein-1, tyrosinase-related protein-2, and so on (Levy et al., 2006). We also observe the expression change in MITF downstream gene upon the level of miR-429 expression is altered.

Ultraviolet radiation is a major environmental hazard, and brings harmful effect on skin tissue. Protection against UV-mediated damage is afforded by melanin production in melanocyte. Melanin absorbs UV radiation and dissipates the energy as harmless heat, blocking the UV from damaging skin tissue. A key component of this process is the UV-induced α-MSH, pro-opiomelanocortin (POMC) and MC1R gene expression (Miller and Tsao, 2010). α-MSH is the main physiological regulator of skin pigmentation, which is produced by the proteolytic cleavage of the large precursor protein, POMC. α-MSH-bound MC1R can activate adenylyl cyclase, lead to phosphorylation of cAMP responsive-element-binding protein (CREB) transcription factor family members, and in turn transcriptionally activate various genes, such as MITF. Our results show that UV treatment in fish leads the activation of α-MSH and MC1R gene. The result is similar as previously reported in keratinocytes (Dong et al., 2012). By contrast, miR-429 knockdown has no effect on the level of α-MSH and MC1R expression with or without UV radiation. Based on these results, we speculate that miR-429-mediated melanin production is distinct from α-MSH-mediated
melanin production. Based on these results, we proposed a model for the avoidance of UV injury in fish (Fig. 6). Once fish is exposed to UV radiation, miR-429 expression is rapidly up-regulated, and earlier than other pigmentation gene activation. miR-429 up-regulation could release Foxd3-mediated MITF inhibition, thus activating its downstream pathway and pigment synthesis (Segura et al., 2009). If fish are exposed to sustained UV injury, the pituitary and keratinocytes secrete α-MSH and induce the production of MC1R in melanocytes, which activates the α-MSH pathway. miRNA-mediated gene regulation operates earlier than most transcriptional responses. The fast regulation of miRNAs after UV treatment indicates that miRNA-mediated gene silencing acts earlier than most gene transcriptional responses after UV damage. Fish have evolved with numerous strategies for effectively escaping UV injury through distinct signaling pathways. miRNAs are implicated in buffering developmental processes against the effects of environmental fluctuations.

In summary, we revealed a novel regulatory mechanism for skin pigmentation in fish from miRNA viewpoint. We found that miR-429 is differentially expressed between white and red skin, and its silencing using antagonir leads to a significant change in skin melanin content. The post-transcriptional regulation of Foxd3 by miR-429, could affect the expression of MITF and its downstream genes, including TYR, TYRP1, and TYRP2, which in turn affect the pigmentation process in fish skin. miRNA-mediated melanogenesis may provide an additional option for fish to avoid UV injury.

**Materials and methods**

**Experimental fish**

Tilapia and common carp was obtained from the fishery farm of Shanghai Ocean University.
They were kept in a water circulation system in 200-liter tanks, and water temperature was kept at 26 ± 2°C under a 12-h light/12-h dark photoperiod. All experiments were conducted under the Guidance of the Care and Use of Laboratory Animals in China. This research was approved by the Committee on the Ethics of Animal Experiments of Shanghai Ocean University.

**Small RNA library construction and sequencing**

RNA samples were harvested from different color skin of common carp, and immediately frozen in liquid nitrogen. Small RNA libraries were constructed using a Small RNA Cloning Kit (Takara). Approximately 20 μg of small RNA were submitted for sequencing. Briefly, the Solexa sequencing was performed as follows. RNA was purified by polyacrylamide gel electrophoresis (PAGE) to enrich for the molecules in the range of 17-27 nt, then was ligated with 5’ and 3’ adapters. The resulting samples were used as templates for cDNA synthesis followed by PCR amplification. The obtained sequencing libraries were subjected to Solexa sequencing-by-synthesis method. After the run, image analysis, sequencing quality evaluation and data production summarization were performed with Illumina/Solexa pipeline.

**Identification of skin-related miRNAs**

The sequencing data was pretreated to discard low quality reads, no 3’-adaptor reads, 5’-adaptor contaminants and sequences shorter than 18 nucleotides. After trimming the 3’ adaptor sequence, sequence tags were mapped onto the zebrafish genome using SOAP software (http://soap.genomics.org.cn) with a tolerance of one mismatch. The matched sequences were then queried against non-coding RNAs from Rfam database (http://www.sanger.ac.uk/Software/Rfam) and NCBI GenBank database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) by performing BLASTn search. Any small RNAs having exact matches to these sequences were excluded from...
further analysis. The remnant reads were compared to the miRBase (19.0) to annotate conserved miRNAs. The secondary structures of the predicted miRNAs were confirmed by RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

**Real-time RT-PCR analyses of mRNAs and miRNAs**

Total RNA was extracted using Trizol reagent (Invitrogen), and miRNAs were extracted using the miRNeasy kit (Qiagen) according to the manufacturer’s instruction. RNA integrity was assessed by electrophoresis on 1.0 % agarose gel. For mRNA quantification, reverse transcription was performed using a High Fidelity primeScript™ RT-PCR Kit as instructed (Takara, Dalian, China). Real-time RT-PCR was performed by using the SYBR Green Real-time PCR Master Mix (Toyobo, Shanghai) and the StepOne Real-time PCR system (Applied Biosystems Inc., Foster City, CA) according to the manufacturer’s protocol. miRNA abundance was detected using stem-loop PCR method, and 18S rRNA expression was detected as the internal control. The relative gene or miRNA expression was detected using the comparative threshold cycle (C_T) method also referred to as the 2^ΔΔC_T method. All reactions were performed in triplicate on the MyiQ5 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA).

**Luciferase reporter assay**

The 3′-UTR of Foxd3 was amplified from tilapia genomic DNA and individually cloned into the pGL3 vector (Promega) by directional cloning. The mutant Foxd3 3′-UTR reporters were created by mutating the seed region of the predicted miR-429 site. HEK293T cells were co-transfected with 0.4 µg of firefly luciferase reporter vector and 0.02 µg of the control vector containing Renilla luciferase (PRL-CMV, Promega) using lipofectamine 2000 (Invitrogen) in 24-well plates (Costar). Each transfection was performed in four wells. Luciferase assays were
carried out 24 hours after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

Spectrophotometric assay of melanin

Skin sample suspensions were solubilized in 8 M urea/1 M sodium hydroxide and cleared by centrifugation at 10,700 g for 10 min. Chloroform was added to the supernatants to remove fatty impurities. Skin containing pheomelanins were cleared by centrifugation at 10,700g for 10 min and analyzed for absorbance at 400 nm. A400/mg = spectrophotometric pheomelanin (Sp.PM) (Dong et al. 2010).

Western blot

The samples were lysed in the lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) with 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM Na3VO4, and protease inhibitor cocktail. The protein lysates were cleared by centrifugation at 4°C for 15 min at 12,000g, quantified using Bio-Rad protein assay, resolved in 8-12 % SDS-PAGE gels, and transferred onto PVDF membranes (Millipore). After blocking, the PVDF membranes were incubated with the primary antibody, and then the membrane was washed and incubated with goat anti-mouse IgG (H+L)-HRP conjugate (Santa Cruz). Specific complexes were visualized by echo-chemiluminescence (Amersham).

Data analysis

Data was expressed as mean ± S.E.M. unless otherwise stated. Statistical significance was assessed by one-way ANOVA followed by Bonferroni’s multiple comparison tests. Statistical significance was defined as P < 0.05.
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Fig. 1 Overview of small RNA library sequencing

(A) The raw reads were primarily analyzed to obtain clean reads; (B) Length distribution of small RNAs in common carp. (C) The clean reads were blasted against the GenBank and Rfam database to annotate rRNA, tRNA, snRNA, or snoRNA.

Fig. 2 miR-429 silencing affects the pigmentation process

(A) Red tilapia weighing about 5 g was received miR-429 antagomir (or mismatched antagomir) at a dose of 60 mg/kg body weight or left untreated for 30 d. miR-429 expression was detected by using real-time PCR. 18S rRNA expression was detected as the internal control. The data were expressed as the relative change compared with the wildtype group. (B) Tilapia was treated as shown in Fig. 2A. Total melanin content in the skin was detected as described in Materials and methods section. Data represent the mean ± S.E.M. from three independent experiments. Asterisk (﹡) indicates significant difference compared with the wildtype group (P <0.05).

Fig. 3 miR-429 acts directly at the 3’-UTR of Foxd3

(A) The alignment between miR-429 and the 3’-UTR segment of Foxd3. (B) Luciferase reporters were linked with Foxd3 3’-UTRs containing either putative miR-429-binding sites (3’-UTR wt) or mutant miR-429 binding sites (3’-UTR mutant). miR-429 mimic or scrambled mimic (Scr mimic) were cotransfected with luciferase-UTR construct into HEK 293T cells, and then the luciferase activity was determined. The cells transfected with Scr mimic plus 3’-UTR wt were served as the control group. Data represent the mean ± S.E.M. from three independent experiments. P<0.05. (C and D) Tilapia weighing about 5 g received a tail-vein injection of NaCl, or miR-429 antagomir at a dose of 60 mg/kg body weight for the indicated times. The untreated group was taken as the control group. The relative expression of miR-429 (C) and Foxd3 (D) was detected using real-time
PCR. 18S rRNA expression was detected as the internal control. The data were expressed as the relative change compared with the untreated group. Asterisk (﹡) indicates significant difference compared with the control group (P < 0.05). (E) Tilapia was treated as shown in Fig.3D, and the expression of Foxd3 was detected by using Western blots. GAPDH was detected as the internal control. Shown in representive image.

**Fig.4 miR-429 silencing changes the expression of MITF and its downstream genes**

Tilapia weighing about 5 g was received miR-429 antagomir at a dose of 60 mg/kg body weight or left untreated for indicated times. The untreated group was taken as the control group. The relative expression of Foxd3 (A), MITF (B), and MITF downstream genes, including TYR, TRP-1, and TRP-2 was detected using real-time PCR. 18S rRNA expression was detected as the internal control. The data were expressed as the relative change compared with the untreated group. Asterisk (﹡) indicates significant difference compared with the control group (P < 0.05). The expression of TYR, TYRP1, and TYRP2 protein was detected by using Western blot. GAPDH was detected as the internal control. Shown in representive image.

**Fig.5 miR-429 mediated pigmentation is distinct from α-MSH-mediated pigmentation upon UV radiation**

(A) The melanocyte was exposed to UV radiation or left untreated for the indicated times. The expressions of miR-429, MC1R, and α-MSH levels were detected by real-time PCR or western blot. The results were expressed as the change in treated groups relative to the untreated group. (B-D) Tilapia was exposed to UV radiation for the indicated time. The untreated group was taken as the control group. The relative expressions of MC1R and α-MSH levels were detected by
real-time PCR or western blot. 18S rRNA expression (for real-time PCR) or GAPDH (for western blot) was detected as the internal control. The data were expressed as the relative change compared with the untreated group. (E, F, and G) The melanocyte was transfected with miR-429 inhibitor or left untreated, and then they were exposed to UV radiation for the indicated times. The relative expressions of MC1R and α-MSH levels were detected by real-time PCR and Western blot. Each sample was analyzed in triplicate. Asterisk (*) indicates significant difference compared with the control group (P < 0.05).

**Fig. 6 The biological effects of miR-429 in the determination of skin color**

**Table 1:** Differential miRNA expression in white and red skin of common carp

**Table 2:** Overview of the expression pattern of these differential expressed miRNAs in different color skin

**Table S1:** Mapping statistics of sRNAs in carp sample

**Table S2:** miRNAs identified in the skin tissue of common carp

**Table S3:** Differential miRNA expression in white and red skin of tilapia

**Fig S1:** The laws of Mendelian inheritance in skin color in common carp

**Fig S2:** Detection of miR-429 expression in different developmental stages

The level of miR-429 expression in zygote, blastula, gastrula, segmentation, or larvae stage was detected by using stem-loop PCR. 18S rRNA expression was detected as the internal control. The data was expressed as the relative change compared with the expression in larvae stage (Control
Fig S3: Effect of antagonir treatment on miR-429 expression

Tilapia was injected with miR-429 antagonir, miR-203 antagonir or left untreated (untreated), respectively. The expression of miR-429 was detected using stem-loop PCR. The relative amount of miR-429 was normalized to the endogenous control, 18SrRNA expression. Results are expressed as means ± S.E.M. of three independent experiments. Asterisk (*) indicates a significant difference compared with the control group. Each sample was analyzed in triplicate. The results were expressed as the relative change compared with the untreated group.

Fig S4: Effect of expression antagonir treatment on pigmentation in common carp

Red common carp weighing about 5g was received miR-429 antagonir (or mismatched antagonir) at a dose of 60 mg/kg body weight or left untreated for 30 d. Total melanin content in the skin was detected as described in Materials and methods section. Data represent the mean ± S.E.M. from three independent experiments. Asterisk (*) indicates significant difference compared with the wildtype group (P<0.05).
### Table A

<table>
<thead>
<tr>
<th>type</th>
<th>count</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>total_reads</td>
<td>15505485</td>
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</tr>
<tr>
<td>high_quality</td>
<td>15445081</td>
<td>100%</td>
</tr>
<tr>
<td>3'adapter_null</td>
<td>16198</td>
<td>0.10%</td>
</tr>
<tr>
<td>insert_null</td>
<td>3871</td>
<td>0.03%</td>
</tr>
<tr>
<td>5'adapter_contaminants</td>
<td>24375</td>
<td>0.16%</td>
</tr>
<tr>
<td>smaller_than_18nt</td>
<td>777271</td>
<td>0.50%</td>
</tr>
<tr>
<td>polyA</td>
<td>221</td>
<td>0.00%</td>
</tr>
<tr>
<td>clean_reads</td>
<td>15326145</td>
<td>99.21%</td>
</tr>
</tbody>
</table>

### Diagram B

**Length Distribution**

![Length Distribution Chart]

### Diagram C

**Pie chart for ncgb_carp-uniq**

- rRNA (2342)
- other (3518607)

**Pie chart for Rfam_carp-total**

- rRNA (161023)
- other (14997507)
- snRNA (944)
- lncRNA (1648931)
- miRNA (1780)
UGCGUA—AUGGUCUGUCAUAA  miR-429
|   |   |   |   |   |   |   |   |   |   |
ACAGCATAATATCA—ACAGTATT  Foxd3
### Differential miRNA expression in white and red skin of common carp

<table>
<thead>
<tr>
<th>White skin</th>
<th>Highly expressed</th>
<th>Red skin</th>
<th>Highly expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-18a</td>
<td></td>
<td></td>
<td>miR-429</td>
</tr>
<tr>
<td>miR-137</td>
<td></td>
<td></td>
<td>miR-146b</td>
</tr>
<tr>
<td>miR-9-5p</td>
<td></td>
<td></td>
<td>miR-184</td>
</tr>
<tr>
<td>miR-9-3p</td>
<td></td>
<td></td>
<td>miR-25</td>
</tr>
<tr>
<td>miR-204</td>
<td></td>
<td></td>
<td>miR-15a-3p</td>
</tr>
<tr>
<td>miR-129-5p</td>
<td></td>
<td></td>
<td>miR-141</td>
</tr>
<tr>
<td>miR-203a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-17a</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Overview of the expression pattern of these differential expressed miRNAs

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Strong expression in adult tissue</th>
<th>Temporal expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-429</td>
<td>skin, gill, fin</td>
<td>zygote blastula gastrula</td>
</tr>
<tr>
<td>miR-146b</td>
<td>heart, gonad</td>
<td>2 2 2</td>
</tr>
<tr>
<td>miR-184</td>
<td>brain, gonad</td>
<td>1 1 1</td>
</tr>
<tr>
<td>miR-25</td>
<td>liver, muscle, skin</td>
<td>0 0 0</td>
</tr>
<tr>
<td>miR-15a-3p</td>
<td>brain, gonad, heart</td>
<td>1 1 1</td>
</tr>
<tr>
<td>miR-18a</td>
<td>kidney</td>
<td>2 2 2</td>
</tr>
<tr>
<td>miR-137</td>
<td>Intestine, skin, brain</td>
<td>0 0 2</td>
</tr>
<tr>
<td>miR-9-5p</td>
<td>brain, gonad, heart</td>
<td>0 0 1</td>
</tr>
<tr>
<td>miR-9-3p</td>
<td>brain, gonad</td>
<td>0 0 1</td>
</tr>
<tr>
<td>miR-204</td>
<td>gill, heart</td>
<td>0 0 0</td>
</tr>
<tr>
<td>miR-203a</td>
<td>liver, skin, gill</td>
<td>1 1 2</td>
</tr>
</tbody>
</table>

0: no expression;  
1: weak ubiquitous or background expression;  
2: strong ubiquitous expression.
Fig S1. The law of Mendelian inheritance in skin color in common carp.
**Fig S2. Detection of miR-429 expression in different developmental stages.** The level of miR-429 expression in zygote, blastula, gastrula, segmentation, or larvae stage was detected by using stem-loop PCR. 18S rRNA expression was detected as the internal control. The data was expressed as the relative change compared with the expression in larvae stage (Control group). Asterisk (*) indicates significant difference compared with the control group ($P < 0.05$).

**Fig S3. Effect of antagomir treatment on miR-429 expression.** Tilapia was injected with miR-429 antagomir, miR-203 antagomir or left untreated (untreated), respectively. The expression of miR-429 was detected using stem-loop PCR. The relative amount of miR-429 was normalized to the endogenous control, 18S rRNA expression. Results are expressed as means ± S.E.M. of three independent experiments. Asterisk (*) indicates a significant difference compared with the control group. Each sample was analyzed in triplicate. The results were expressed as the relative change compared with the untreated group.
**Fig S4. Effect of expression antagonir treatment on pigmentation in common carp.** Red common carp weighing about 5 g was received miR-429 antagonir (or mismatched antagonir) at a dose of 60 mg/kg body weight or left untreated for 30 d. Total melanin content in the skin was detected as described in Materials and methods section. Data represent the mean ± S.E.M. from three independent experiments. Asterisk (*) indicates significant difference compared with the wild-type group (P <0.05).

### Table S1: Mapping statistics of sRNAs in carp sample

<table>
<thead>
<tr>
<th></th>
<th>Unique sRNAs</th>
<th>Percent(%)</th>
<th>Total sRNAs</th>
<th>Percent(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sRNAs</td>
<td>3520949</td>
<td>100%</td>
<td>15326145</td>
<td>100%</td>
</tr>
<tr>
<td>Mapping to genome</td>
<td>76595</td>
<td>2.18%</td>
<td>7976459</td>
<td>52.04%</td>
</tr>
</tbody>
</table>

### Table S2. miRNAs identified in the skin tissue of common carp.

Download Table

### Differential miRNA expression in white and red skin of tilapia

<table>
<thead>
<tr>
<th>White skin</th>
<th>Highly expressed</th>
<th>Red skin</th>
<th>Highly expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>miR-18a</td>
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<td></td>
<td>miR-204</td>
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<td>miR-15a-3p</td>
</tr>
<tr>
<td></td>
<td>miR-203a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table S3. Differential miRNA expression in white and red skin of tilapia.