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miR-196a regulates the skin pigmentation of koi carp (*Cyprinus carpio* L.) by targeting transcription factor *mitfa*

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Abstract

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that play important roles in many biological processes. The function of miRNAs in skin colour differentiation and pigmentation has been reported in many animals. However, the researches about their molecular mechanisms of regulating the formation of different skin colour of koi carp (*Cyprinus carpio* L.) are deficient. In this study, we detected the temporal and spatial expression levels of ccr-miR-196a (hereinafter referred to as miR-196a) and then predicted its target mRNA of *microphthalmia-associated transcription factor a* (*mitfa*), which is a key regulatory transcript factor in the melanogenesis pathway, with bioinformatics and dual-luciferase reporter methods. Furthermore, we silenced the miR-196a in vivo with an antagomir to detect whether the miR-196a would affect the expression levels of *mitfa* mRNA and its downstream genes including *tyrosinase* (*tyr*), *tyrosinase-related protein* 1 (*tyrp*1) and *dopachrome tautomerase* (*dct*). The results revealed that *mitfa* and its downstream genes' mRNA expressions all significantly increased in the treatment group compared with the control group. We presumed that miR-196a might regulate the pigmentation of koi carp through targeting the *mitfa*. The finding provided the fundamental information regarding miRNA–mRNA interaction on different skin colour pigmentation in koi carp.

KEYWORDS

koi carp, miR-196a, *mitfa*, pigment, skin colour

1 | INTRODUCTION

Body colour of fish is an important phenotypic trait that closely related to its survival and reproduction, which involved in many biological processes such as mimicry, courtship, camouflage and environmental adaptation (Kelsh, 2004; Protas & Patel, 2008). Compared with mammals and birds, fish are ideal models for skin colour differentiation and pigmentation research, which possess various pigment cells including melanophores (black), xanthophores (yellow), erythrophores (red), iridophores (iridescent, blue, silver or gold) and leucophores (dull, whitish) (Braasch, Schartl, & Volff, 2007). Koi carp (*Cyprinus carpio* L.) is an ornamental fish selected from the common carp (*C. carpio*) with several colour variants including Kohaku, Taisho sanke and Showa sanshoku, thus provides good materials for the researches related to fish skin colour (De Kock & Gomelsky, 2015).

To date, a series of genes have been identified in the differentiation and pigmentation in koi carp, such as melanogenesis pathway genes including *tyrosinase* (*tyr*), *tyrosinase-related protein* 1 (*tyrp*1), *dopachrome tautomerase* (*dct*), *melanocortin* 1 *receptor* (*mc1r*), *agouti signalling protein* (*asip*) and *microphthalmia-associated transcription factor a* (*mitfa*). Among them, *mitfa* is an important member of the *mitf* gene family that located in the centre of gene regulatory networks in which act as a 'master regulator', and its product has a basic/

helix-loop-helix/leucine zipper (b-HLH-Zip) domain (Goding, 2000). The b-HLH-Zip domain can form homodimers or heterodimers with its own or similarly structured transcription factors to function and specifically bind to the 5′-CACGTG-3′ or 5′-TCATGTG-3′ (E-box or M-box) structure of the promoter region (Hemesath et al., 1994; Vachtenheim & Borovanský, 2010). In addition, *tyr*, *tyrp*1 and *dct* can directly affect the melanin synthesis and their promoter regions all contain E-box or M-box sequence which are regulated by b-HLH-Zip domain (Bertolotto et al., 1998; Goding, 2000; Levy, Khaled, & Fisher, 2006; Yasumoto, Yokoyama, Shibata, Tomita, & Shibahara, 1995). Therefore, further understanding the relationship between *mitf* and its upstream and downstream targets is becoming more important.

MicroRNAs (miRNAs) are a set of single-stranded ~22 nucleotides non-coding RNAs, which block translation or induce cleavage of the mRNA through recognizing and binding the 3′-untranslated regions (UTRs) of the targeted gene (Ambros, 2004). At present, some studies have illustrated that miRNAs are essential for skin colour pigmentation and differentiation processes. For instance, miRNA-508-3p regulated melanin production of alpaca by targeted to the *Mitf* mRNA (Zhang et al., 2017), miR-21a-5p inhibited *Sox* gene expression level and regulated the melanin deposition in mouse skin (Wang, Zhao, Fan, Chen, & Dong, 2016), miR-429 regulated the skin pigmentation of tilapia and common carp through targeting the *foxd3* gene (Yan et al., 2013), and miR-138-5p and miR-722 were predicted to play important roles in tilapia skin pigmentation (Wang et al., 2018). Therefore, it is necessary to further explore the crosstalk between miRNA and mRNA function on skin colour differentiation and pigmentation in koi carp.

At present, a mass of studies have been conducted on skin colour regulation of koi carp, such as inheritance of body colour (David et al., 2004; Gomelsky, Cherfas, & Hulata, 1998; Gomelsky, Cherfas, Hulata, & Dasgupta, 2003), functional analysis of skin pigment-related genes (Bar, Kaddar, Velan, & David, 2013; Liu et al., 2015), feed additives (Maiti et al., 2017; Sun et al., 2012) and transcriptome analysis (Luo et al., 2018, 2019; Feng et al., 2018). In our previous study, we screened several miRNAs including ccr-miR-196a (hereinafter referred to as miR-196a), which regulated pigmentation on different skins of koi carp by small RNA sequencing (Luo et al., 2018). Herein, we further analysed the regulatory molecules of miR-196a in the skin pigmentation of koi carp in order to provide some useful information for the research of miRNAs in fish skin colour.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All experiments in our study were handled in accordance with the guidelines for the Care and Use of Experimental Animals of China. This study was approved by the Animal Care and Use Committee of the Centre for Applied Aquatic Genomics at the Chinese Academy of Fishery Sciences (CAFS) (BC 2013863, 9/2013).

2.2 | Experimental fish and sample collection

Koi carps were obtained from the Qiting Pilot Research Station (Yixing, Jiangsu, China) affiliated to the Freshwater Fisheries Research Center (FFRC), Chinese Academy of Fishery Sciences. Koi carps were kept in 200-L tanks in a water circulation system. The water temperature was maintained at 24 ± 1°C, pH = 7–8, dissolved oxygen > 6 mg/L and NH_4-N < 0.5 mg/L. Aeration was continuously supplied to each tank. Fish were anaesthetized before sampling by adding clove oil (Zhanyun Chemical Co., Ltd.) into the water; then, different tissues including white skin (Ws), red skin (Rs), black skin (Bs), gonad (Go), liver (Li), eye (Ey), muscle (Mu), intestine (In), brain (Br), head kidney (Hk), kidney (Ki), fin (Fi), spleen (Sp), gill (Gi) and heart (He) were collected and stored at −80°C. 90–100 fertilized eggs (Hi-Utsuri × Hi-Utsuri) were regularly observed with a light microscope (Olympus), and the developmental stages were divided by the developmental characteristics shown by 50% of the observed embryos. Koi carps of eight developmental stages including zygote (Zy), cleavage (Cl), blastula (Bl), gastrula (Ga), neurula (Ne), organogenesis (Or), hatching (Ha) and 20 days post-hatching (20d) were collected and stored at −80°C.

2.3 | Identification of miRNA target genes linked to skin colour

In our previous study, we found the expression level of miR-196a was different in the skin of three colours (black, red and white) in koi carp through Illumina sequencing (Luo et al., 2018). Here, three bioinformatic programs including TargetScan (http://www.targetscan.org), RNAhybrid (https://omictools.com/rnahybrid-tool) and miRanda (http://www.microRNA.org/) were further used to predict the target mRNAs of miR-196a.

2.4 | Plasmid vectors for luciferase reporter assays and mutagenesis

The 3′UTR region of *mitfa* gene was obtained from the NCBI (GenBank accession No. KC565527), and the sequence was synthesized and cloned into the pmirGLO vector (Promega) and downstream of the luciferase minigene. Then, we mutated the putative miRNA target sequence within the 3′UTR with the Site-Directed Mutagenesis Kit (Umibio). Finally, all products were sequenced.

2.5 | Plasmid co-transfection and luciferase reporter detection

The day prior to transfection, HEK293 cells, which did not express miR-196a, were seeded into 48-well plates at 1×10^5 cells per well. All operations were carried out with Lipofectamine 2000 Kit (Invitrogen) according to the manufacturer's instructions. Cells were

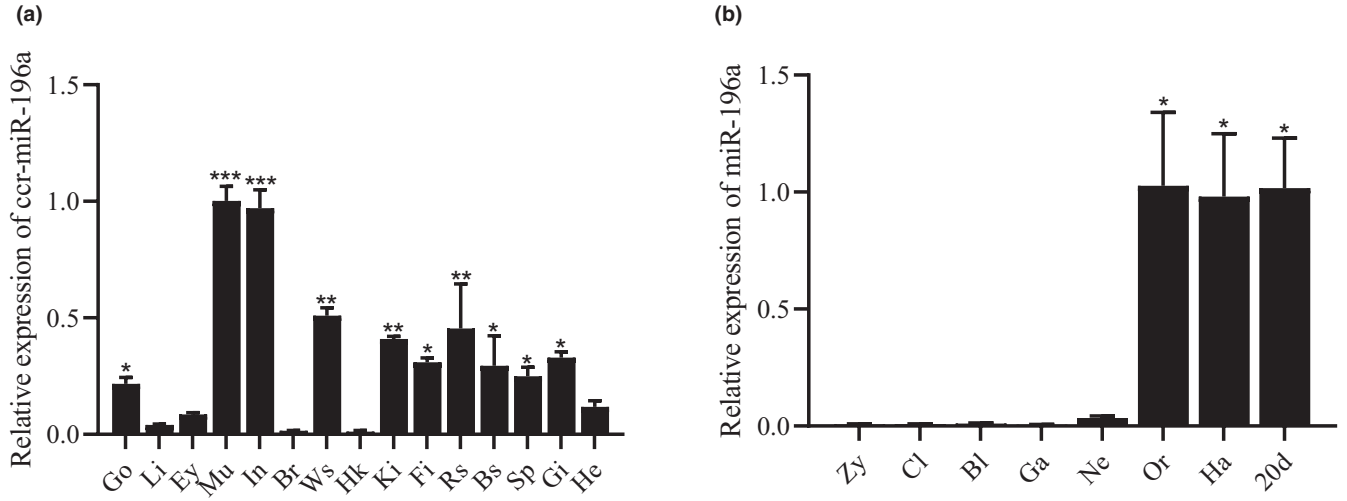

FIGURE 1 Expression pattern of miR-196a in koi carp. (a) Expression level of miR-196a in different tissues of koi carp. Go: gonad; Li: liver; Ey: eye; Mu: muscle; In: intestine; Br: brain; Ws: white skin; Hk: head kidney; Ki: kidney; Fi: fin; Rs: red skin; Bs: black skin; Sp: spleen; Gi: gill; He: heart. (b) Expression level of miR-196a in early development of koi carp. Zy: zygote; Cl: cleavage; Bl: blastula; Ga: gastrula; Ne: neurula; Or: organogenesis; Ha: hatching; 20d: 20 days post-hatching. *means $p < .05$, **means $p < .01$, ***means $p < .001$

transfected with pmirGLO luciferase expression construction containing the 3′UTR of *mitfa* and miRNA precursor or negative control (Ambion). After 48 hr, the luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and normalized to *Renilla* luciferase activity. All experiments were performed in duplicate with data pooled from three independent experiments.

2.6 | Silencing miR-196a in vivo with the antagomir

The antagomir used in this experiment is as follows: miR-196a antagomir, 5′-U_sA_sGGUAGUUUCAUGUUGUU$_sG_sG_sG_s$-Chol-3′, and negative antagomir, 5′-C_sA_sCGGUUCCAGGCACUGU$_sG_sU_sA_s$-Chol-3′. All nucleotides are 2′-OMe-modified. Subscript 's' represents a phosphorothioate linkage and 'Chol' represents cholesterol linked through a hydroxyprolinol linkage. 60 koi carps of Hi-Utsuri family with 5 months old and weighted ~10 g were evenly divided into three groups which subjected to the tail vein injection of miR-196a antagomir, negative antagomir and phosphate-buffered saline (PBS), respectively, and fed in the 200-litre tanks with the circulation water system. Each treatment had three replicates. At 0, 12, 24, 48, 72 and 96 hr after treatment, skin tissues of fish from each group were sampled, immediately snap-frozen in liquid nitrogen and then stored at −80°C.

2.7 | RNA isolation and quantitative PCR

MiRNAs were extracted through a miRNeasy Kit (Takara) and the total RNA was extracted using TRIzol® Reagent (CWBio) according to the manufacturer's protocol. The concentration of RNA was measured with a NanoDrop 2000 UV-spectrophotometer (Thermo Fisher Scientific), and the quality and integrity were checked through OD 260/280 and 1% agarose gel electrophoresis. The total RNAs were reversely transcribed using the Prime-Script RT Master Mix (Takara), and miRNAs were reversely transcribed by the Mir-XTM miRNA First-Strand Synthesis Kit (Takara) respectively. Primers (Table S1) were designed according to the sequence of *mitfa* (KC565527), *tyr* (XM_019097445.1), *tryp1* (XM_019099711), *dct* (XM_019097549.1) and *β-actin* (XM_019114275.1) in NCBI. All quantitative real-time polymerase chain reactions (qRT-PCR) were performed using SYBR Premix Ex Taq II (Takara) on a CFX-96 Real-Time PCR System (Bio-Rad). All samples were run in triplicate, and the relative amount of miRNAs and mRNAs were normalized to the amount of U6 snRNA and *β-actin* mRNA respectively. The relative expression was calculated with the comparative threshold cycle (C_T) method, which referred to as the $2^{-\Delta\Delta C_T}$ algorithm.

2.8 | Statistical analysis

All the results were presented as means ± standard error of mean (*SEM*). Statistical analysis was performed using SPSS 20.0 (SPSS Inc.). All data were analysed by one-way ANOVA after homogeneity of variance test. Significant differences were considered at $p < .05$. When significant differences were found, Duncan's multiple range test was used to identify differences among experimental groups.

3 | RESULTS

3.1 | Expression pattern of miR-196a in koi carp

In order to explore the role of miR-196a in the physiological process of koi carp, its expression level in different tissues and developmental stages was detected by qRT-PCR. We found miR-196a was highly

expressed in muscle and intestine, then in white skin, kidney and red skin. The lowest expression was observed in the brain and head kidney (Figure 1a). The expression level of miR-196a in the black skin was the lowest among the different skin tissues (red, white and black); thus, we speculate whether miR-196a probably acts as a crucial role in the pigmentation process in koi carp. Then, we further explored its temporal expression level during the early development. The results showed that there was almost no expression of miR-196a before the neurula stage, but it highly expressed from the organogenesis stage and then sustained to the larval stage (20 days post-hatching) (Figure 1b).

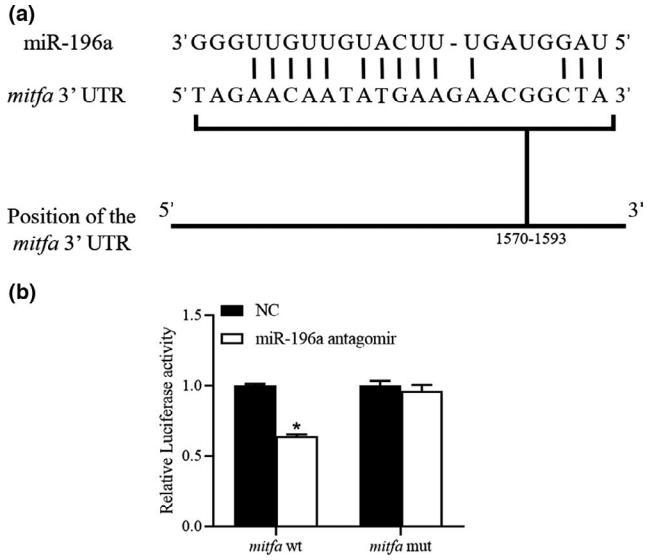

FIGURE 2 miR-196a targets the 3′ untranslated region (ULR) of *mitfa* mRNA. (a) Schematic diagram of predicted miR-196a binding sites in the *mitfa* 3′UTR. (b) Luciferase reporter assays in the HEK293T cells. Luciferase reporter constructs containing a wild-type and mutated *mitfa* 3'UTR were co-transfected with miR-196a mimic into the HEK293T cells. The cells transfected with negative control + *mitfa* wt were used as the control group. Data represent the mean ± *SEM* from three independent experiments. Asterisk (*) indicates a significant difference compared with the control group ($p < .05$)

3.2 | miR-196a target in the 3′ untranslated region (UTR) of *mitfa* mRNA

We further used bioinformatics software to verify the correlation between miR-196a and *mitfa*. Based on the analysis results from three software, we found that 1,570–1,593 nt of the 3′-UTR of *mitfa* mRNA would be the best target of miR-196a (Figure 2a).

To further verify the interaction between miR-196a and *mitfa*, we then performed the dual-luciferase reporter assays using luciferase reporter constructed with either the wild-type *mitfa* (pmirGLO-c.*mitfa*-3′UTR-wt) or the mutant *mitfa* (pmirGLO-c.*mitfa*-3′UTR-mut). And the two vectors were co-transfected with the miR-196a mimic or negative control (NC) into the HEK293T cells respectively. The results of the luciferase reports demonstrated that miR-196a repressed the luciferase activity of wild-type *mitfa* 3′UTR vector by 40% compared with NC ($p < .05$), whereas miR-196a mimic had no effect on the mutant *mitfa* 3′UTR vector ($p > .05$) (Figure 2b). Therefore, we speculated miR-196a could inhibit the expression of *mitfa* by binding the *mitfa* 3′UTR sequence.

3.3 | Interaction between miR196a and *mitfa* mRNA in vivo

Furthermore, miR-196a was silenced in vivo through the tail vein injection to explore the interaction relationships with antagomir method. The injection concentration of 40 mg/kg was chosen to

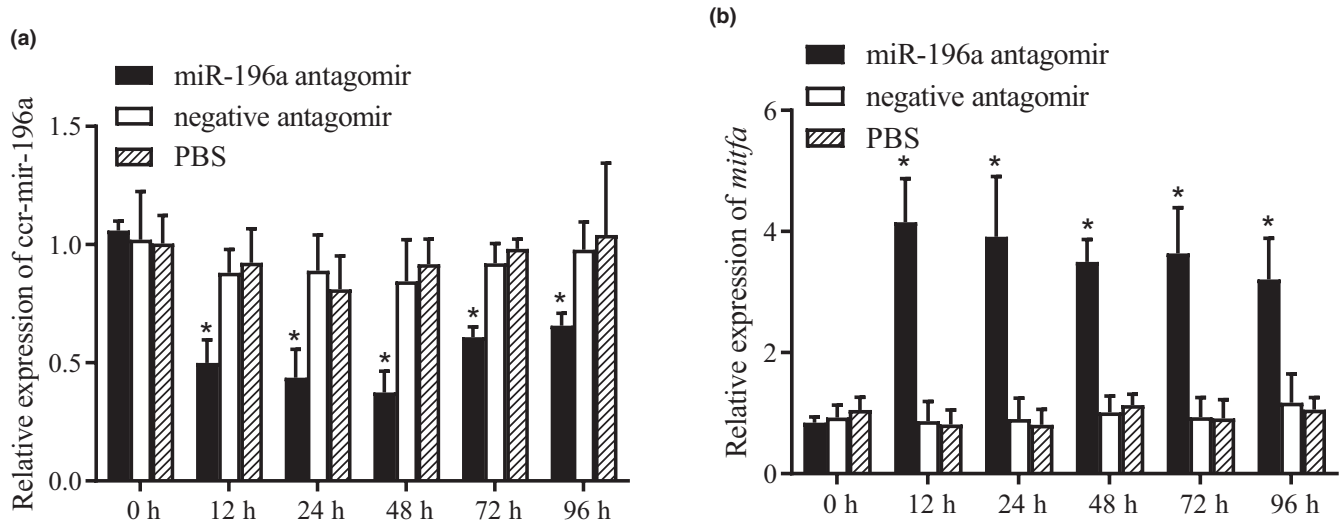

FIGURE 3 Interaction between miR-196a and *mitfa* mRNA in vivo. Koi carps weighing about 20 g received a tail vein injection with miR-196a antagomir, negative antagomir or PBS (dose, 40 mg/kg). The group injected with PBS was used as a control. The relative expression of miR-196a (a) and *mitfa* mRNA (b) was detected using real-time PCR. U6 snRNA or *β-actin* mRNA expression was used as the internal control respectively. Asterisk (*) indicates a significant difference compared with the control group ($p < .05$)

ensure the best interference efficiency after the pre-experiment (Figure S1). The results showed that the miR-196a expression level was significantly decreased ($p < .05$) in the antagomir group within 96 hr of treatment when compared with the negative control and PBS group (Figure 3a). Interestingly, we found the expression level of *mitfa* mRNA was significantly increased in the miR-196a antagomir group compared with the other two groups ($p < .05$) (Figure 3b). The inverse expression correlation between miR-196a and *mitfa* mRNA also suggested that miR-196a could directly target *mitfa* mRNA expression in vivo.

3.4 | Expression changes of the *mitfa* downstream genes

In order to explore whether the silencing of miR-196a would affect other genes expression levels in the melanogenesis pathway, we further detected the expression levels of *mitfa* downstream genes including *tyr*, *tyrp1* and *dct*. The results showed that the expression levels in the miR-196a antagomir injection group were all significantly increased compared with the negative control and PBS group within 96 hr ($p < .05$) (Figure 4). The results indicated that miR-196a silencing can increase the expression level of *mitfa*, then promoting the expression of its downstream genes and regulating the production of melanin.

4 | DISCUSSION

Researches on the molecular mechanisms of physiological process regulated by miRNAs have been mushroomed since the first miRNA was excavated in *C. elegans* in 1993 (Lee, Feinbaum, & Ambros, 2004). Naturally, the different skin colour formation and differentiation are also regulated by some miRNAs. To date, a mass of miRNAs have been reported acting as the crucial roles in the pigmentation process, such as miR-8 (Kennell, Cadigan, Shakhmantsir, & Waldron, 2012),

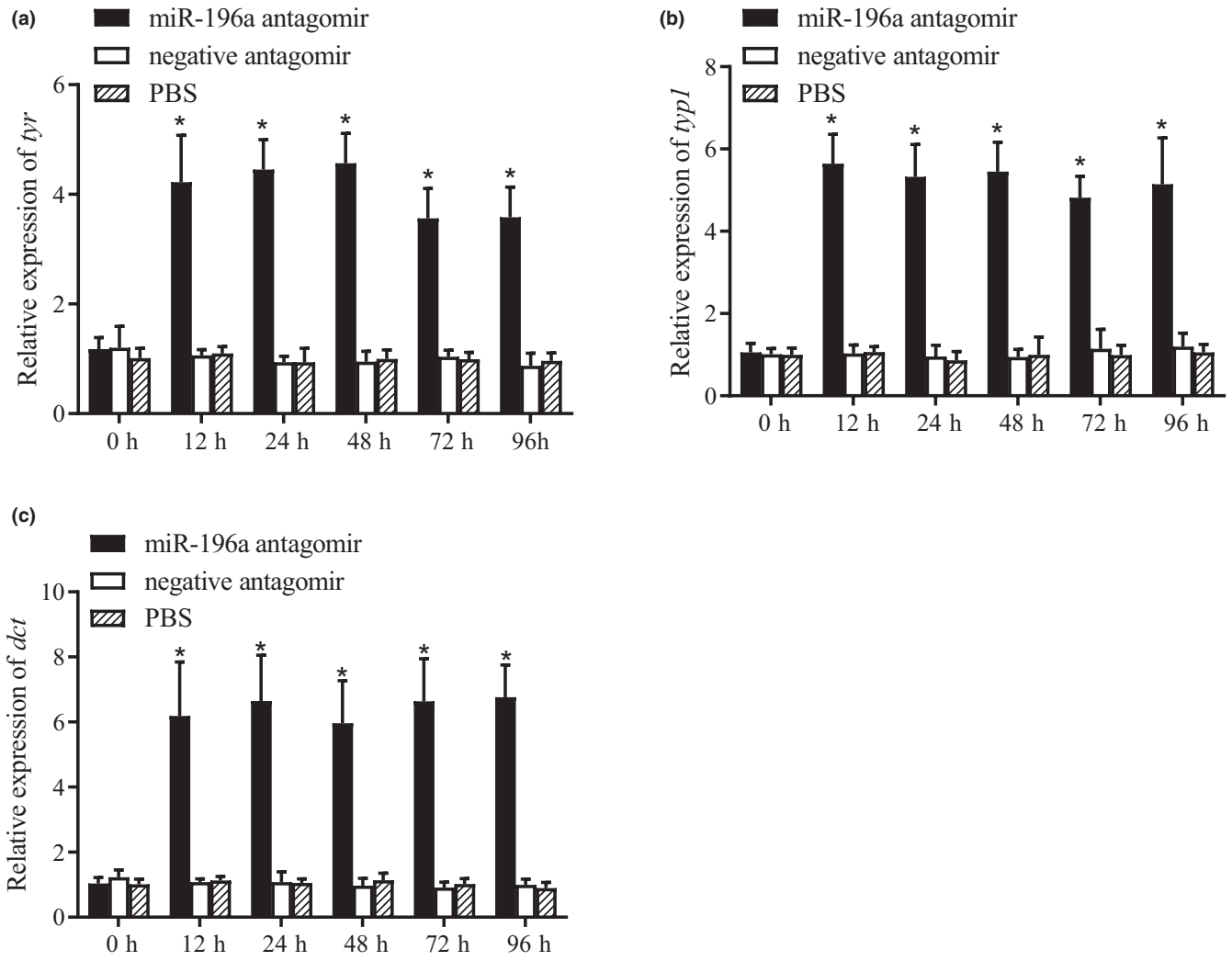

FIGURE 4 Altered expression of *mitfa* downstream genes in miR-196a silencing koi carp. The group injected with PBS was used as a control. The relative expression of *tyr* mRNA (a), *tyrp1* mRNA (b) and *dct* mRNA (c) was detected using real-time PCR. *β-actin* mRNA expression was used as the internal control. Asterisk (*) indicates a significant difference compared with the control group ($p < .05$)

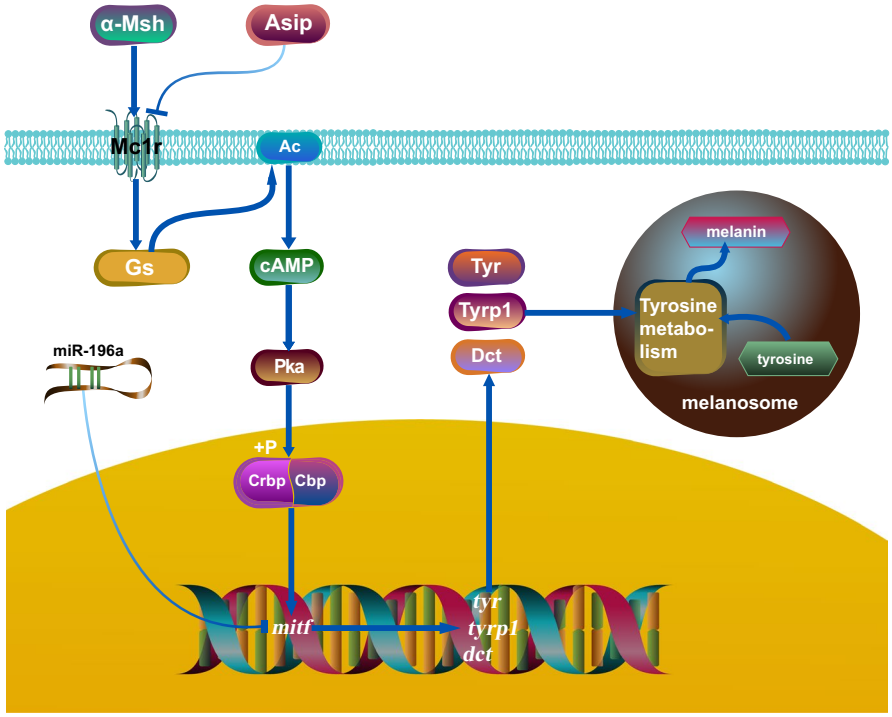

FIGURE 5 The biological effects of miR-196a on the skin colour regulation in melanogenesis pathway

miR-25 (Zhu et al., 2010), miR-145 (Dynoodt et al., 2013), miR-211 (Dai et al., 2015) and miR-508-3p (Zhang et al., 2017). The material of our study, koi carp, has a high market value due to its colourful plaque on the skin. It is an ideal model for studying fish skin colour differentiation. Therefore, this research aims to explore the molecular mechanism of miR-196a in the skin pigmentation of koi carp and may provide some basic information for the molecular selection and breeding.

Herein, we first analysed miR-196a temporal and spatial expression patterns. The different expression level in three colour skins presented the tissue-specificity expression pattern, in which the expression level in black skin was significantly lower than red and white skin ($p < .05$). Therefore, we guess miR-196a may play a role in the process of melanin formation in koi carp. Meanwhile, the expression levels during early development stages showed that miR-196a began to increase after the neurula stage, which was similar to the results of zebrafish (He et al., 2011), *Xenopus laevis* (Qiu et al., 2009) and mouse (Kloosterman, Wienholds, de Bruijn, Kauppinen, & Plasterk, 2006). We suggest that miR-196a is not only involved in the limb differentiation during the early development of vertebrates (Hornstein et al., 2005), but also in pigment cells differentiation in fish (Parichy, 2006). So, we further speculate that miR-196a may play an important role in the pigmentation process of koi carp.

The target genes of miR-196a that have been reported so far include *hoxb8*, *shh*, *ranbp10*, *foxo1* and *bach1* (Go et al., 2016; Her et al., 2017; Hornstein et al., 2005; Yang, Peng, Qin, Zhou, & Wang, 2017), but little researches of miR-196a function related to animal's pigmentation were presented. In this study, the *mitfa* 3'UTR region was predicted as the target mRNA of miR-196a that involved in the melanogenesis pathway through bioinformatics software. In the melanogenesis pathway, the product of the combination of α-Msh and *mc1r* can activate adenylyl cyclase, which belongs to the phosphorylate *cAMP-responsive element-binding protein* (*crbp*) transcription factor family members, and then transcriptionally activate various genes such as *mitf* (Buscà & Ballotti, 2000; Price et al., 1998; Wolf Horrell, Boulanger, & D'Orazio, 2016). While *asip* competitively binds to the *mc1r* and inhibits this response (Lin & Fisher, 2007), meantime, the *mitf* gene can directly regulate many genes for melanocyte/melanophore development and melanocyte/melanophore proliferation, such as *tyr*, *tyrp1* and *dct* (Costin et al., 2005; Goding, 2000; Levy & Fisher, 2011; Zhang et al., 2007). *Tyr* catalyses the conversion of tyrosine to dopaquinone, and then, dopaquinone is catalysed by *tyrp1* and *dct* through a series of reactions to finally produce melanin (Lin & Fisher, 2007; Simon, Hong, & Peles, 2008). In our study, we found *mitfa*, a member of the *mitf* gene family, was very likely to be the target gene of miR-196a. Therefore, the dual-luciferase reporter assay was then performed to verify the results of the software predictions. The luciferase activity of the wild-type 3′UTR vector of *mitfa* gene was suppressed when the miR-196a was over-expressed, while the mutation within the 3′UTR abrogated the repressive ability of the miR-196a. These results further confirmed that the *mitfa* was the target gene of miR-196a.

We also used antagomir method to silence miR-196a in vivo to further study its function. Antagomirs are chemically engineered oligonucleotides which are specific, effective and durable for silencing of endogenous miRNA (Krützfeldt et al., 2005). After injection with miR-196a antagomirs, the expression level of miR-196a was obviously suppressed, but *mitfa* mRNA expression level was significantly up-regulated compared with the negative antagomir and the PBS control group. Meanwhile, the expression levels of *tyr*, *tyrp1* and *dct* mRNA were significantly up-regulated in miR-196a silencing fish.

In previous studies of miRNAs' role in skin pigmentation, the method of silencing the miRNAs in cultured cells in vitro has been widely

adopted, while the in vivo silencing studies are fewer. Dong et al.'s study of miR-206 silencing in vivo observed the expression of its target *mc1r* gene and its downstream genes gives us new inspiration (Dong et al., 2020). This experiment used a similar method of miRNAs silencing in vivo, upregulation of the target gene *mitfa* and its downstream genes was observed in miR-196a silencing koi carp. Therefore, we speculate that miR-196a is involved in regulation of the pigmentation in koi carp by targeting the *mitfa* in the melanogenesis pathway (Figure 5).

5 | CONCLUSIONS

In this study, we found a key miRNA, miR-196a, acting as a crucial role in the melanogenesis pathway of koi carp by targeting the 3′ UTR region of *mitfa* mRNA. The spatial and temporal expression levels showed miR-196a in black skin is significantly lower than that in red and white skins, and significantly up-regulated after the pigment cells began to differentiate. Furthermore, the expression levels of its target gene *mitfa* and downstream genes including *tyr*, *tyrp1* and *dct* were all significantly up-regulated in the miR-196a silencing fish. Overall, our study found a new miRNA closely related to regulation of the body skin colour in koi carp and would provide the useful information for the similar researches in fish.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors have declared that no competing interest exists.

AUTHOR CONTRIBUTIONS

Zaijie Dong designed and supervised the study. Haoran Yin performed experiments and analysed the data. Mingkun Luo performed luciferase reporter assays. Wentao Luo and Jianjun Fu performed qRT-PCR experiments. Zaijie Dong, Lanmei Wang and Wenbin Zhu analysed the data and prepared tables and figures. Haoran Yin wrote the manuscript. Zaijie Dong modified the manuscript. All authors read, reviewed and approved the manuscript for submission.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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