# miR-206 regulates the growth of the teleost tilapia (*Oreochromis niloticus*) through the modulation of IGF-1 gene expression

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**Abstract**

MicroRNAs (miRNAs) are ~22 nt noncoding RNAs that play a crucial role in regulating muscle development. Our previous study shows that miR-206 is specifically expressed in tilapia skeletal muscle, and exhibits dynamic expression pattern at different developmental stages. Here, we reveal that miR-206 emerges as a crucial regulator of tilapia growth. miR-206 loss of function leads to the acceleration of tilapia growth. IGF-1 is identified as the target gene of miR-206. miR-206 directly changes IGF-1 expression by targeting its 3'-UTR, and inhibition of miR-206 substantially increases IGF-1 mRNA level *in vivo*. Thus, miR-206 would be developed as a molecular marker to assist fish breeding.

**Key words:** Nile tilapia, miR-206, growth performance, IGF-1

## Introduction

Nile tilapia, *Oreochromis niloticus*, is one of the most commonly farmed species in freshwater aquaculture. It has become a major source of protein around the world because of its superior growth performance and its outstanding adaptability to a wide range of environment (Davis et al., 2010; Ponzoni et al., 2010). Thus, better understanding of the regulatory mechanism of tilapia growth will provide important information for both developmental biologists and fish breeding experts.

miRNAs are endogenous ~22 nucleotide noncoding RNAs, which act as post-transcriptional regulators in animals and plants (Winter et al., 2009). They result in mRNA cleavage or translational repression by forming imperfect base pairing with the 3'-UTR region of the target gene (Bartel, 2004; Winter et al., 2009). miRNAs have been implicated in regulating numerous physiological processes such as embryogenesis, organ development, cellular differentiation, and cellular apoptosis. miRNA deregulation can influence normal cell growth and development, resulting in numerous disorders (Fiore et al., 2008; Zhao and Srivastava, 2007). Furthermore, miRNAs are usually expressed in a tissue-specific manner (Kim et al., 2006; Makeyev and Maniatis, 2008; Sweetman et al., 2008). Our previous study has revealed that miR-206 is specially expressed in tilapia skeletal muscle, and its expression level is tightly associated with the developmental stage of tilapia (Yan et al., 2012a). Thus, we speculated that miR-206 may be involved in the regulation of growth in tilapia. However, the precise role of miR-206 in tilapia is still unclear.

Antagomirs are cholesterol-conjugated single-stranded RNA molecules, which are 21-23

nucleotides in length and complementary to the mature target miRNA (Krützfeldt et al., 2005). They are perfectly complementary to specific miRNA target with either mispairing at the cleavage site of Ago2 or some sort of base modification to inhibit Ago2 cleavage (Krützfeldt et al., 2005). Antagomirs can block endogenous miRNA expression in mouse, zebrafish, and cell lines (Ma et al., 2010; Morton et al., 2008; Selvamani et al., 2012). Here, we employed this method to inhibit endogenous miR-206 expression, and then examine the regulatory role of miR-206 in tilapia growth. We found that the administration of miR-206 antagomir results in a significant reduction in endogenous miR-206 expression, and a marked growth acceleration in tilapia through up-regulating IGF-1 expression.

## Materials and methods

### Fish and growth experiment

Nile tilapias were obtained from the fishery farm of Shanghai Ocean University. They were temporarily raised in a water circulation system in 100-liter tanks, and water temperature was kept at 28 ± 3°C under a 12-h light/12-h dark photoperiod. Each fish was anesthetized in sodium bicarbonate-buffered MS-222 (200 mg $L^{-1}$, Sigma, St. Louis, MO, USA) prior to collection of required tissue sample. The study was performed according to the Guide for the Care and Use of Laboratory Animals of China.

Tilapia weighing about 1 g was injected with miR-206 antagomir, mismatched antagomir, or left untreated, respectively. Thirty fish for each treatment were cultured in 50 L fiberglass tank, and four replicates were set for this experiment. During the growth experiment, fish were fed with commercial feed, floating pellets containing 30% protein. Daily water was changed about 20% of the total volume. Dissolved oxygen, pH, total ammonia and temperature were detected every day.

Water quality parameters were not analyzed in depth but tests were conducted to determine whether there were significant differences in the key parameters (pH, DO, total ammonia) among different treatments. At the termination of the experiment, all the fish in each treatment was weighed collectively, and the average final weight was recorded. The following indices were calculated to interpret growth performance (El-Sayed and Kawanna, 2008; Yan and Wang, 2010; Chen et al., 2011).

- Initial weight (g) = wet weight of fish at the beginning of culture
- Final weight (g) = wet weight of fish at the final of culture
- Daily weight gain (DWG) = (final weight - initial weight) / culture days
- Coefficient of variation final weight (CV) = mean standard deviation of the final weight/mean final weight
- Feed conversion ratio (FCR) = feed consumption / weight gain
- Feed intake (FI) =100 × feed consumption / [days × (final weight + initial weight) / 2)]

**Quantitative PCR**

Total RNAs were extracted using Trizol reagent (Invitrogen), and miRNAs were extracted using miRNeasy kit (Qiagen). Real-time PCR for IGF-1 was performed using SYBR Green PCR mixture (Takara) in MyiQ5 Real-time PCR Detection System (Bio-Rad). Gene expression was normalized relative to the housekeeping gene (β-actin). The primer sequences are as follows: IGF-1: 5’- GTCTGTGGAGAGCGAGGCTTT-3’ (forward); 5’- CACGTGACCGCCTTGCA-3’ (reverse); β-actin: 5’-GTCCCTGTACGCCTCTGGTCG-3’ (forward); 5’-GCCGGACTCATCGTA CTCCTG -3’ (reverse). The relative amount of miRNA was detected using stem-loop PCR, and U6 RNA was detected as the internal control. Relative gene or miRNA expression was determined

using the comparative Ct method, which is also referred to the $2^{-\Delta\Delta Ct}$ method (Chen et al., 2005; Schmittgen and Livak, 2008).

**Silencing of miR-206 *in vivo* using antagomir method**

The antagomirs used in the study are single-stranded RNAs, which consist of a 21-23-nucleotide length with modification as specified:

antagomir-133a: CsAsGCUGGUUGAAGGGGACCsA s As As-Chol-3';

antagomir-206: CsCsACACACACUUCCUUACAUUs CsC sAs-Chol-3';

mismatch antagomir: CsAsCGGUUCCAGGCACUGUsG sU s As -Chol-3';

All nucleotides are 2'-OMe-modified; subscript 's' represents a phosphorothioate linkage; 'Chol' represents cholesterol linked through a hydroxyprolinol linkage. They were deprotected, desalted and purified by high-performance liquid chromatography. Antagomir was dissolved in PBS buffer before injection. Tilapia weighing about 1 g received tail-vein injection of saline or antagomir at a dose of 60 mg/kg body weight in 0.02 ml/injection twice a week. Tissues were harvested, snap-frozen and stored at -80℃.

**Cell culture and 3'- UTR luciferase reporter assay**

HEK 293T cells were obtained from American Type Culture Collection (ATCC; Rockville, MD). They were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B, and maintained at 37°C in a humidified 5% $CO_2$ incubator.

To generate the 3'-UTR luciferase reporter construct, the full length of the 3'-UTR from IGF-1 was cloned into the downstream of the firefly luciferase gene in pGL3-control vector (Promega). The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce one

or two point mutations into the seed region of pGL3-IGF-1, giving pGL3-IGF-1 m1 and pGL3-IGF-1 m2, respectively. For luciferase reporter assays, HEK 293T cells were plated at $2\times10^5$ cells per well in 12-well dishes. Cells were transfected with either wild-type or mutant IGF-1-3'-UTR constructs, with and without miR-206 mimic or negative control mimic using lipofectamine 2000 (Invitrogen). Luciferase activity was measured on a scintillation counter using a dual luciferase reporter system.

**Statistical analysis**

Values were expressed as mean ± S.E.M. unless otherwise indicated. Significant differences across treatments were evaluated by using one-way analysis of variance (ANOVA) with the Bonferroni post hoc test. The significance level was chosen at $P < 0.05$.

**Results**

**Antagomir treatment represses endogenous miR-206 expression in tilapia**

In our previous study, we have successfully used antagomir method to suppress endogenous miR-30c expression in tilapia (Yan et al., 2012b). Here, we determined whether antagomir method is able to efficiently repress miR-206 expression *in vivo*. As shown in figure 1, miR-206 expression could be efficiently blocked by its corresponding antagomir (miR-206 antagomir) but not the mismatch miRNA (miR-133 antagomir). Furthermore, the silencing effect of antagomir could be detected at different time points (Fig. 1A and B). Thus, antagomir method is suitable for miRNA loss-of-function experiment in tilapia.

**Effect of miR-206 silencing on the growth performance of tilapia**

To determine the role of miR-206 in regulating tilapia growth, we employed antagomir technique to achieve efficient miR-206 loss-of-function *in vivo*, and then compared the growth

performance between different treatments. During the 45-day culture period, water temperature ranged from 22°C to 28°C. Aeration was provided continuously, and DO was maintained greater than 5 mg $L^{-1}$. pH was maintained between 7.4 and 8.4. Total ammonia ($NH_3$ /$NH_4$) ranged from 1.2 to 2.5 mg $L^{-1}$. The survival rate of all fish was above 95%.

As shown in table 1, no significant difference was observed for the initial weight and feed intake value across different groups. The growth rate (DWG) and feed conversion ratio (FCR) were significantly affected by miR-206 intervention ($P$<0.05). The growth rate of miR-206 antagomir injection group was about 30% greater than that in the group injected with mismatch antagomir or the untreated group. Moreover, better FCR was observed in miR-206 antagomir injection group than in the other two groups. These results suggest that miR-206 silencing results in the growth acceleration in tilapia.

**Identification of the target gene of miR-206 in tilapia**

To understand the mechanism of miR-206 regulation of tilapia growth, we sought to identify miR-206 target genes that are responsible for regulating tilapia growth. In generally, miRNA function involves uninterrupted base-pairing between nucleotides 2-7 of the miRNA and a complementary sequence in the 3´ UTR of the target mRNA (Ulitsky et al., 2010). Based on this evidence, IGF-1 was predicted as a potential target gene of miR-206. We first evaluated the expression correlation between miR-206 and IGF-1 in HEK 293T cells. Compared with the control group, transfection with miR-206 inhibitor but not the negative control miRNA inhibitor caused a significant decrease in miR-206 expression. Meanwhile, IGF-1 expression was markedly up-regulated upon miR-206 inhibition (Fig. 2 A and B). Moreover, we found that miR-206 inhibition by antagomir treatment resulted in a significant reduction in miR-206 expression, but a

significant increase in IGF-1expression in tilapia (Fig. 2 C and D).

Next, we tested whether IGF-1 expression is post-transcriptionally regulated by miR-206. The 3'-UTR of IGF-1 was cloned into the downstream of luciferase gene, and luciferase reporter assay was conducted in HEK 293T cells. Cotransfection of miR-206 mimic with the luciferase reporter gene linked to the wild-type 3'-UTR of IGF-1 resulted in a significant decrease in luciferase activity. In contrast, cotransfection of a control miRNA mimic (not complementary to the 3'-UTRs of IGF-1) with the wild-type 3'-UTR constructs did not result in a decrease in luciferase activity. Furthermore, cotransfection of miR-206 mimic with the constructs containing mutated IGF-1 3'-UTR sequences did not result in a marked decrease in luciferase activity (Fig. 2E). Taken together, these results suggest that IGF-1 is a target gene of miR-206.

**Discussion**

Growth in fish involves the recruitment and hypertrophy of muscle fibres. Fish muscle growth is not linear and occurs through a combination of hyperplasia and hypertrophy in post-juvenile stages. miRNAs are a class of non-coding RNAs that have emerged as critical regulators in the expression and function of eukaryotic genomes. They can regulate skeletal muscle development through targeting of the crucial genes controlling myogenesis (Eisenberg et al., 2009; Williams et al., 2009). Given fish growth is tightly associated with skeletal muscle development, the expression of muscle specific-miRNAs may be involved in regulating fish growth (Johnston et al., 2011; Rescan, 2008). Thus, the elucidation of miRNA function in muscle development will enhance our understanding of skeletal muscle biology and may give some hints for marker-assisted fish breeding. Previous studies reveal that cardiac and/or skeletal muscle are highly enriched in many miRNAs, including miR-1, miR-133, miR-206, miR-208, miR-486 and

miR-499 (Güller and Russell, 2010; Ge and Chen, 2011). Here, we show that miR-206 participates in a regulatory circuit that allows rapid gene program transition during the regulation of tilapia growth.

miR-206 is a muscle-specific miRNA, and its role in muscle development has been verified in some animal models, including mouse, rat and zebrafish (Anderson et al., 2006; Kim et al., 2006; Mishima et al., 2009; Shan et al., 2009). Our previous study reveals that miR-206 is specifically expressed in tilapia skeletal muscle, and shows dynamic expression pattern among different developmental stages (Yan et al., 2012a). These evidences promote us to consider whether its expression level can affect the growth performance of tilapia. As expected, miR-206 loss of function *in vivo* significantly improves tilapia growth performance. It is well known that miRNA-guided regulatory network induces mRNA cleavage or translational repression by forming imperfect base pairing with the 3'-UTR region of the target gene. We further reveal that IGF-1 is direct target gene of miR-206.

IGF-1 is one of the important members in IGF signaling, and involved in the regulation of growth and development of skeletal muscle in many vertebrates (Duan et al., 2010). For example, the birth weight of IGF-1 or IGF-2 knockout mice is about 60% of their wild type littermates (Liu et al., 1993). Over-expression of IGF-1 in mice increases the body weight by 30% (Mathews et al., 1988). In many fish species, IGF-1 blood level or tissue IGF-1 mRNA level positively correlate with dietary ration, dietary protein content, and body growth rate (Carnevali et al., 2006; Beckman et al., 2004). Treatment of fish with IGF-1 implants contributes to accelerating growth (McCormick et al., 1992). In this study, miR-206 inhibition results in a significant increase in IGF-1 expression. Thus, we speculate that miR-206 regulation of tilapia growth is mainly

mediated by the up-regulation of IGF-1expression. IGF-1 mRNA is detected in all life stages of fish ranging from the unfertilized egg to the adult. The temporal and spatial expression patterns of fish IGF-1 seem to be similar to those in mammals. In addition, nutrition condition or hormone treatment changes IGF-1 level in fish as they do in mammals. These features suggest that the IGF system is highly conserved between teleost fish and mammals (Duan, 1998; Bower et al., 2008; Johnston et al., 2011). Thus, understanding of tilapia IGF-1 action would contribute to the basic growth physiology of vertebrates and provide important implication in muscle hypertrophy, muscle atrophy, and muscle regeneration of other vertebrates.

In summary, we found that miRNA antagomir is able to repress endogenous miRNA expression in tilapia. miR-206 antagomir results in a significant reduction in endogenous miR-206 level, and an obvious acceleration of tilapia growth through targeting IGF-1 expression. Given the role of miR-206 in tilapia growth, it would be developed as a promising molecular marker to assist the selection of faster-growing fish strains.

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353

354 **Figure legend**

355 **Fig.1 Effect of antagomir treatment on endogenous miRNA expression**

356 (A and B) Tilapia was injected with miR-133 antagomir or miR-206 antagomir, respectively. The
357 expression of miR-133 or miR-206 level was detected using stem-loop PCR. The relative amount
358 of miR-133 or miR-206 was normalized to the endogenous control, U6 snRNA expression. Results
359 are expressed as means ± S.E.M. of three independent experiments. Asterisk indicates significant
360 difference compared with the control group. Each sample was analyzed in triplicate. The results
361 were expressed as the change in treated groups relative to the untreated group.

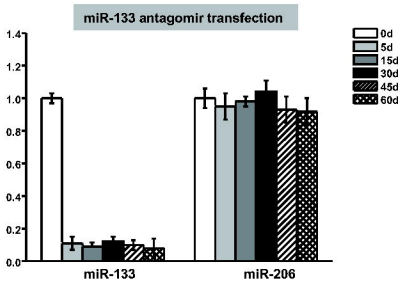
362 **Fig.2 Identification of IGF-1 as the target gene of miR-206 in tilapia**

363 (A) HEK 293T cells were transfected with negative control miRNA inhibitor, miR-206 inhibitor or
364 left untreated for 48 h. The level of miR-206 expression was determined using stem-loop PCR.
365 miR-206 relative amount was normalized to endogenous control U6 snRNA expression. (B) HEK
366 293T cells were treated as Fig. 2A. The level of IGF-1 expression was detected using real-time
367 PCR. IGF-1relative amount was normalized to β-actin expression. (C and D) Tilapia was injected
368 with miR-206 antagomir, mismatched antagomir or left untreated, respectively. The expression of
369 miR-206 or IGF-1 was detected as described in “Material and Method” Section. Results were
370 expressed as means ± S.E.M. of three independent experiments. Asterisk indicated significant
371 difference compared with the control group. (E) Luciferase assays were carried out to address
372 whether IGF-1 was directly targeted by miR-206. Luciferase activities were normalized to the
373 group transfected with PGL3. HEK 293T cells were transfected as shown, and luciferase activity
374 was determined 48 h after transfection. The cells tranfected with PGL3 plus miR-206 were used as

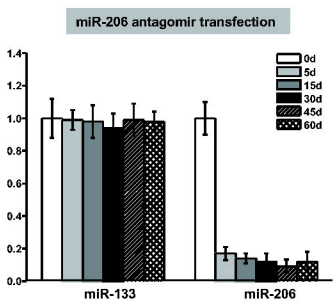
the control group. Data represent the mean ± S.E.M. from three independent experiments. *, $P <$ 0.05. wt: PGL3-IGF-3'-UTR; m1: PGL3-IGF-3'-UTR mutant 1; m2: PGL3-IGF-3'-UTR mutant 2; miR-NG: miRNA which is not complementary to the 3'-UTRs of IGF-1.

**Table 1: Effects of miR-206 intervention on the growth performance of tilapia**

A


miR-133 antagomir transfection
0d
5d
15d
30d
45d
60d
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
miR-133
miR-206

B


miR-206 antagomir transfection
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15d
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45d
60d
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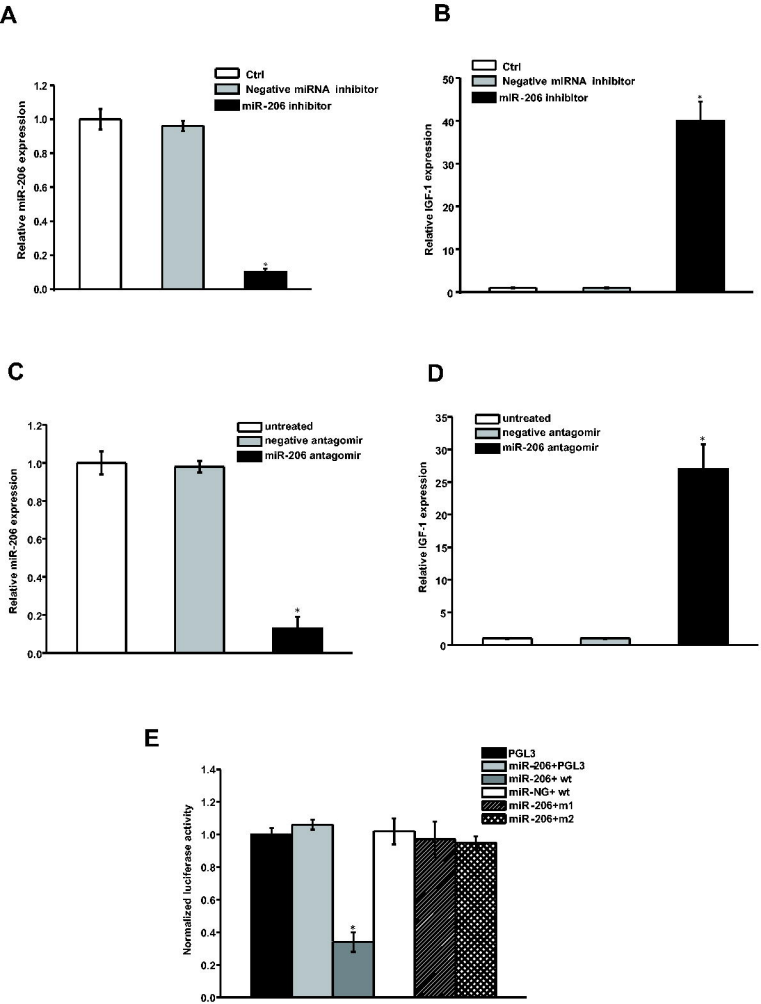
0.8
0.6
0.4
0.2
0.0
miR-133
miR-206


A
Ctrl
Negative miRNA inhibitor
miR-206 inhibitor
Relative miR-206 expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
B
Ctrl
Negative miRNA inhibitor
miR-206 inhibitor
Relative IGF-1 expression
50
40
30
20
10
0
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C
untreated
negative antagomir
miR-206 antagomir
Relative miR-206 expression
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untreated
negative antagomir
miR-206 antagomir
Relative IGF-1 expression
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15
10
5
0
*
E
PGL3
miR-206+PGL3
miR-206+ wt
miR-NG+ wt
miR-206+m1
miR-206+m2
Normalized luciferase activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*

**Table 1: Effects of miR-206 intervention on the growth performance of tilapia**

| | untreated | mismatched antagomir | miR-206 antagomir |
|---|---|---|---|
| Initial weight (g) | 1.0 ± 0.09 a | 0.95 ± 0.04 a | 0.98 ± 0.11 a |
| Final weight (g) | 25 ± 2.4 a | 23 ± 4.0 a | 36 ± 5.2 b |
| Survival rate (%) | 98 a | 96 a | 95 a |
| CV | 0.10 a | 0.17 a | 0.14 a |
| DWG | 0.53 a | 0.49 a | 0.78 b |
| FCR | 3.3±0.26 a | 3.6±0.75 a | 2.4±0.39 b |
| Feed intake (%) | 3.2 ± 0.23 a | 3.0 ± 0.49 a | 2.94 ± 0.31 a |

Values are expressed as means ± SEM of four replicates. Means in the same row with different superscripts are significantly different from each other determined by using one-way analysis of variance (ANOVA) with the Bonferroni post hoc test ( $P<0.05$ ).