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RESEARCH ARTICLE

miR-203b: a novel regulator of MyoD expression in tilapia skeletal muscle

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SUMMARY

MyoD is one of the helix-loop-helix proteins regulating muscle-specific gene expression in tilapia. Tight regulation of the MyoD protein level is necessary for the precise regulation of skeletal muscle development. MicroRNAs (miRNAs) are a class of regulatory RNAs that post-transcriptionally regulate gene expression. An increasing amount of evidence has suggested that miRNAs play an important role in regulating skeletal muscle development. We reasoned that MyoD expression may be regulated by miRNAs. Predictions from bioinformatics have identified a putative miR-203b target site in the 3′-UTR of the MyoD gene. Interestingly, miR-203b expression is negatively correlated with MyoD expression, whereas miR-203b suppression leads to a significant increase in MyoD expression, thereby activating MyoD downstream genes. A 3′-UTR luciferase reporter assay further verifies the direct interaction between miR-203b and MyoD. Taken together, our results reveal a novel molecular mechanism in which miRNA participates in transcriptional circuits that regulate gene expression in tilapia skeletal muscle.

Key words: Nile tilapia, miR-203b, microRNA, skeletal muscle development.

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INTRODUCTION

Nile tilapia [*Oreochromis niloticus* (Linnaeus 1758)] is one of the most widely cultured fish in the world. They are adaptable to a wide range of environmental conditions, and have gradually become a major protein source around the world (Yan and Wang, 2010). Skeletal muscle is the major edible part in fish. The development and growth of skeletal muscle ultimately determines fish growth performance (Johnston et al., 2011; Rescan, 2008). Thus, better understanding of the regulatory mechanism of muscle development will provide important information for both developmental biologists and fish breeding experts.

Skeletal muscle development is well-defined process, and is regulated by the sequential expression of muscle-specific myogenic regulatory transcription factors (MRFs) (Chen and Tsai, 2008). MRFs belong to a group of helix-loop-helix transcription factors, including myogenin, MRF4, MyoD and Myf5. Of these, MyoD is thought to be the key transcription factor that initiates the cascade of regulatory events during muscle development. MyoD can be regulated at the transcriptional level through the recruitment of both chromatin remodeling complexes and p300/CBP and PCAF acetyltransferases onto the MyoD promoter, whereas histone deacetylase (HDAC) recruitment inhibits MyoD expression (Aguiar et al., 2008; Francetic et al., 2012; Lassar, 2012); the PC4 (Tis7/Ifrd1) protein can cooperate with MyoD to induce the transcriptional activity of myocyte enhancer factor 2C (MEF2C), and repress the transcriptional activity of NF-κB to inhibit MyoD mRNA accumulation (Micheli et al., 2011). In addition, β-catenin interacts with MyoD and regulates its transcription activity (Kim et al., 2008). Given its crucial role in the regulation of muscle development, it is highly likely that MyoD expression is under additional modes of regulation yet to be discovered.

MicroRNAs (miRNAs) are short, non-coding RNAs that repress gene expression by binding to target mRNAs. They have emerged as crucial regulators for many developmental processes, including skeletal muscle development (Carthew, 2006; Carthew and Sontheimer, 2009). Previous studies have identified and characterized several muscle-specific miRNAs that control various aspects of myogenesis. These miRNAs include miR-1, miR-133a, miR-133b, miR-206, miR-208, miR-486 and miR-499. Aberrant regulation of some of these muscle-enriched miRNAs can disrupt intracellular signaling networks, which may result in pathological conditions (Williams et al., 2009). In general, one gene can be regulated by several miRNAs, while one miRNA may inhibit the expression of multiple target genes, which results in the formation of complex regulatory feedback networks. We reasoned that MyoD expression may be regulated by miRNAs (Makeyev and Maniatis, 2008). However, miRNAs regulation of MyoD expression has never been reported, despite its notable expression in skeletal muscle. The objective of this study was to examine the role of miRNAs in regulation of MyoD expression in tilapia.

MATERIALS AND METHODS

Experimental fish and tissue sample preparation

Nile tilapia were obtained from the fishery farm of Shanghai Ocean University. They were maintained in a water circulation system in 100 l tanks, and water temperature was kept at 26±2°C under a 12 h:12 h light:dark photoperiod. Skeletal muscle samples were collected from different developmental stages, including juveniles, adults and senile fish. These tissue samples were stored in liquid nitrogen before RNA isolation.

Table 1. Primer sequences used for the quantification of mRNA expression

Gene	GenBank accession no.	Annealing temperature (°C)	Primer
MHC	AF050035	58	ACAACATTAGAAATTTGCTGCGG CGGGCGTACTCGATCTTGTT
Sp1	XM_003451834	58	CCAGGCCGGTACCGTTGCAG GCAGCAGGATGGCCCCTGTG
Cdc6	XM_003454088	55	GGCAACAGACGCCCCCTTCC GGCGCTCAGGGATGGCAGTG
Utrn	XM_003449882	56	CGGAGACGTCGCTGGGGGA TGGCCGTCTTCCCTGTCTTGG
18S rRNA	JF698683.1	54	GGCCGTTCTTAGTTGGTGGA TTGCTCAATCTCGTGTGGCT

Prediction of MyoD-binding miRNAs

To identify miRNAs that potentially bind MyoD, we queried the MicroCosm targets (http://www.ebi.ac.uk/enright-srv/microcosm/) and TargetScan prediction program (http://www.targetscan.org/) based on the zebrafish genome (Alexiou et al., 2011; Rajewsky, 2006; Saito and Sætrom, 2010). We compared the sequence conservation of miRNA target sites between zebrafish and tilapia using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and miRNAs were extracted using the miRNeasy kit (Qiagen, Germantown, MD, USA). For mRNA analysis, total RNA was reverse-transcribed with the SuperScript III First-Strand Synthesis System for RT-PCR (Takara, Dalian, China) and amplified with the SYBR Green PCR Master Mix (Takara). 18S rRNA was detected as the internal normalization control. The primers used for mRNA detection are listed in Table 1. For miRNA expression assays, RNA was reverse-transcribed using specific miRNA stem-loop primers. Mature miRNA expression was detected using Taqman miRNA assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. miRNA expression was normalized against the expression of the house-keeping gene, 18S rRNA, using the comparative Ct method (Schmittgen and Livak, 2008).

Luciferase assay

MyoD 3′-UTR segments predicted to interact specifically with miR-203b were subcloned by standard procedures into the pGL3 vector (Promega, Madison, WI, USA) immediately downstream of the stop codon of the luciferase gene. The mutant MyoD 3′-UTR reporters were created by mutating the seed region of the predicted miR-203b site. These reporters were transfected into HEK 293T cells, and transfection efficiency was corrected by a *Renilla luciferase* vector (PRL-CMV, Promega). Luciferase activity was detected using the Luciferase Assay Systems kit (Promega) according to the manufacturer's protocol.

Regulation of miR-203b expression level *in vivo*

Chemically modified antisense oligonucleotides (antagomir) and agomir were synthesized to regulate miR-203b expression (Ribobio, Guangzhou, China). The 3′ end of the oligonucleotides was conjugated to cholesterol, and all the bases were 2′-OMe modified. The antagomir or agomir oligonucleotides were deprotected, desalted and purified by high-performance liquid chromatography. Tilapia weighing ~5 g received tail-vein injection of saline, agomir or antagomir at a dose of 60 mg kg^{-1} body mass on every other day. They were killed 24 h after the last injection for experimental analysis (Morton et al., 2008; van Solingen et al., 2009).

Statistical analysis

Values are expressed as means ± 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 $P<0.05$.

RESULTS

Prediction of miRNAs targeting MyoD

BLAST search suggests that the sequence of MyoD 3′-UTR is conserved between zebrafish and tilapia. To identify miRNAs that interact with MyoD protein, we employed the MicroCosm and TargetScan prediction programs based on the zebrafish genome. Bioinformatic prediction revealed that miR-375, miR-722, miR-203b, miR-142a-5p, miR-138, miR-190, miR-190b, miR-122 and miR-23b may regulate MyoD expression (Table 2). Furthermore, these miRNAs are highly conserved between tilapia and zebrafish. Among these predicted miRNAs, miR-122, miR-375 and miR-722 have been reported to be abundantly expressed in liver, pancreas and retina (Chang et al., 2008; O'Quin et al., 2011; Poy et al., 2009). Thus, they were ruled out for further analysis. We then used RT-PCR to detect the expression pattern of other miRNAs. As shown in Table 2, miR-23b, miR-190, miR-190 and miR-203b can be detected in skeletal muscle. Therefore, we speculated that these miRNAs would potentially regulate MyoD expression in tilapia skeletal muscle.

Table 2. Predicted MyoD-binding miRNAs and their expression pattern

Predicted miRNA	Expression pattern	Method
miR-23b	Heart, liver, intestine, skeletal muscle	RT-PCR
miR-122	Liver	Reference and RT-PCR
miR-138	Liver, brain, heart	RT-PCR
miR-142a-5p	Liver, intestine	RT-PCR
miR-190	Skeletal muscle, pancreas, intestine	RT-PCR
miR-190b	Skeletal muscle, pancreas, intestine	RT-PCR
miR-203b	Skeletal muscle, heart, intestine	RT-PCR
miR-375	Pancreas	Reference and RT-PCR
miR-722	Eye	Reference and RT-PCR

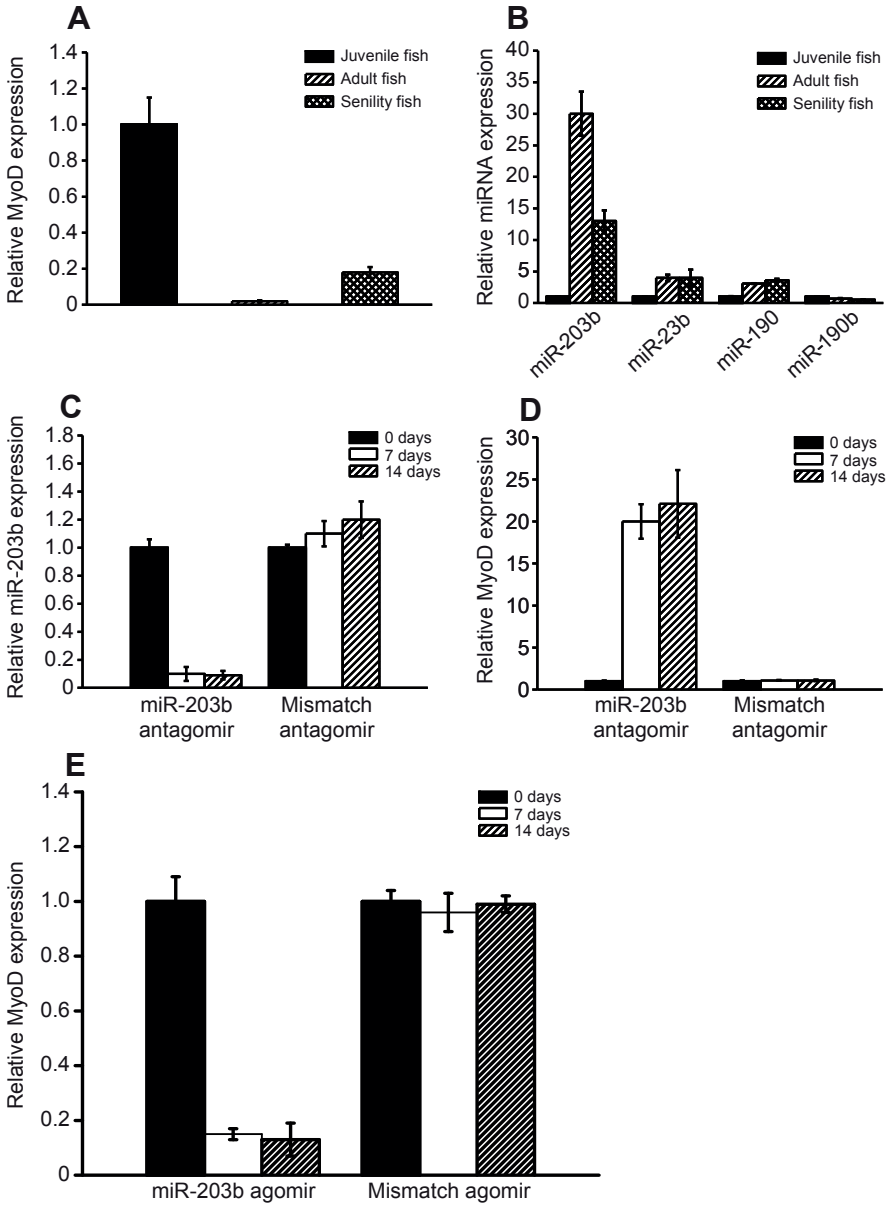

Fig. 1. Identification of miR-203b as a regulator of MyoD expression. (A) Total RNA sample was extracted from the skeletal muscle in different developmental stages, including juvenile fish, adult fish and senility fish. The level of MyoD expression was detected using real-time PCR, and 18S rRNA was detected as the internal control. Data are expressed as the relative change compared with the expression level in juvenile fish. (B) miRNA sample was extracted from the same samples as shown in A. The expression of miR-23b, miR-190, miR-190 or miR-203b was detected using real-time PCR, and 18S rRNA was detected as the internal control. Data are expressed as the relative change compared with the corresponding miRNA expression of juvenile fish. (C,D) Tilapia weighing ~5 g received tail-vein injection of miR-203b antagomir or mismatch antagomir at a dose of 60 mg kg^{-1} body mass on every other day. They were killed after 7 or 14 days antagomir treatment, respectively. The expression of miR-203b or MyoD level was detected using real-time PCR, and 18S rRNA was detected as the internal control. Data are expressed as the relative change compared with the untreated group. Four technical replicates were used for each treatment. (E) Tilapia weighing ~5 g received tail-vein injection of miR-203b agomir or mismatch agomir at a dose of 60 mg kg^{-1} body mass every other day. They were killed after 7 or 14 days of agomir treatment, respectively. The expression of MyoD level was detected using real-time PCR, and 18S rRNA was detected as the internal control. Data are expressed as the relative change compared with the untreated group. Four technical replicates were used for each treatment.

MyoD expression is negatively associated with miR-203b expression

MyoD was found to be highly expressed in juvenile fish and hardly detected in adult fish, but expression gradually increased in senility fish (Fig. 1A). We postulated that MyoD-inhibitory miRNAs would have an opposite expression pattern. Real-time PCR analysis demonstrated that among these potential miRNA regulators of MyoD, one interesting hit is miR-203b (Fig. 1B).

To examine the possibility of miR-203b targeting MyoD, we asked whether alternation in miR-203b expression would change MyoD expression. The result shows that miR-203b antagomir treatment results in a significant reduction in miR-203b expression, while MyoD expression is significantly upregulated. The miR-203b mismatch antagomir treatment had no effect on miR-203b expression, and thereby does not affect MyoD expression (Fig. 1C,D). By contrast, mimicking miR-203b with an miR-203b agomir but not control agomir results in a significant reduction in MyoD expression (Fig. 1E). Taken together, these findings suggest that miR-203b is involved in the regulation of MyoD expression *in vivo*.

miR-203b directly targeting MyoD 3′UTR

To verify that miR-203b directly inhibits MyoD expression, we employed a luciferase reporter assay. The alignment of miR-203b with MyoD 3′UTR insert is illustrated in Fig. 2A. Cotransfection of HEK 293T cells with the parental luciferase construct (PGL3, without MyoD 3′UTR) plus the miR-203b expression vector does not significantly change expression of the reporter (Fig. 2B). However, when the miR-203b target site from the MyoD 3′UTR is inserted into the luciferase construct, luciferase expression is strongly decreased when cotransfected with miR-203b, but the suppression is relieved by a single base mutation in the binding site (Fig. 2B). These results suggest that miR-203b directly suppresses MyoD expression by binding to its 3′UTR target sequence.

miR-203b silencing activates MyoD downstream genes

MyoD is an important myogenic transcription factor that controls the spatial and temporal expression of muscle-specific genes. Alternation in MyoD expression would affect the expression of MyoD downstream genes. In this study, we found that miR-203b silencing results in a significant increase in MyoD expression. MyoD

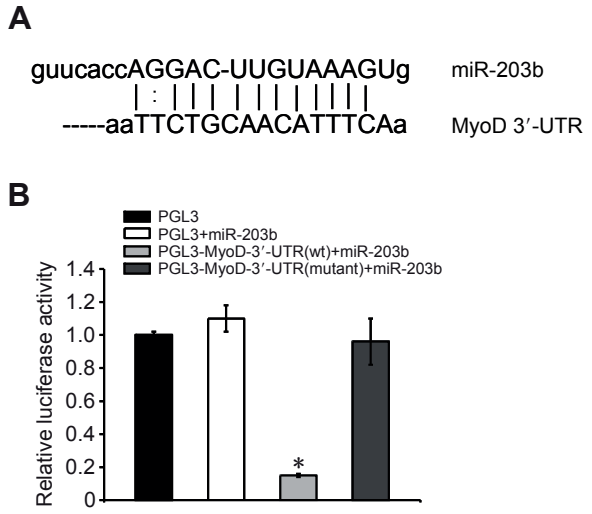

Fig. 2. miR-203b directly targeting MyoD 3′UTR. (A) The alignment between miR-203b and the 3′UTR segment of MyoD. (B) Luciferase assays were carried out to address whether MyoD is directly targeted by miR-203b. HEK 293T cells were transfected with the plasmid as described in Fig. 2B, and transfection efficiency was corrected by a *Renilla luciferase* vector (PRL-CMV, Promega). Luciferase activity was detected using the luciferase Assay Systems kit (Promega) according to the manufacturer's protocol. Results are expressed as means ± s.e.m. of four independent experiments.

downstream genes, including myosin heavy chain (MHC), utrophin (Utrn), cell division control protein 6 (Cdc6) and Sp1 transcription factor (Sp1) genes, were significantly upregulated (Rosenberg et al., 2006; Seward et al., 2001; Viñals et al., 1997; Zhang et al., 2010). In contrast, miR-203b mismatch antagomir treatment had no effect on miR-203b expression, and did not affect the expression of MyoD or its downstream genes (Fig. 3). Taken together, these results suggested that miR-203b can affect MyoD expression, thereby changing the expression of MyoD downstream genes.

DISCUSSION

Nile tilapia is one of the most important commercial fish species, and is widely used in aquaculture. The central goal of tilapia aquaculture is the production of skeletal muscle. It is therefore important to understand the regulatory mechanism of muscle development at the molecular level. Muscle development is a well-defined process, and is regulated by various environmental factors and distinct signaling pathways, resulting in the activation of specific transcription factors and gene expression. Gene expression in skeletal muscle is controlled by a family of basic helix-loop-helix transcription factors known as the myogenic regulatory factors, including Myf5, MyoD, myogenin and MRF4, which are crucial for controlling myogenesis (Chen and Tsai, 2008; Johnston, 1999; Johnston et al., 2011). MyoD has been proposed to be a 'pioneer' transcription factor required to initiate the cascade of regulatory events necessary to initiate expression of muscle-specific genes. MyoD recruits chromatin-modifying activities that alter both the regional histone modifications and the chromatin remodeling at promoter binding sites (Cao et al., 2006; Gerber et al., 1997; Londhe and Davie, 2011). Tight regulation of the MyoD protein level is necessary for the precise regulation of skeletal muscle development. In this study, MyoD expression can be regulated at the miRNA level.

The roles of miRNAs in muscle development have attracted much attention and research interest (Ge and Chen, 2011). miR-133a can promote myoblast proliferation through the repression of serum response factor (SRF) expression (Liu et al., 2008). miR-206 can affect the myoblast differentiation program through the indirect downregulation of the helix-loop-helix protein Id, a repressor of MyoD (Kim et al., 2006). And miR-181 inhibits the expression of Hox-A1, which results in the inhibition of MyoD expression (Yamamoto and Kuroiwa, 2003). Recently, Huang et al. (Huang et al., 2012)

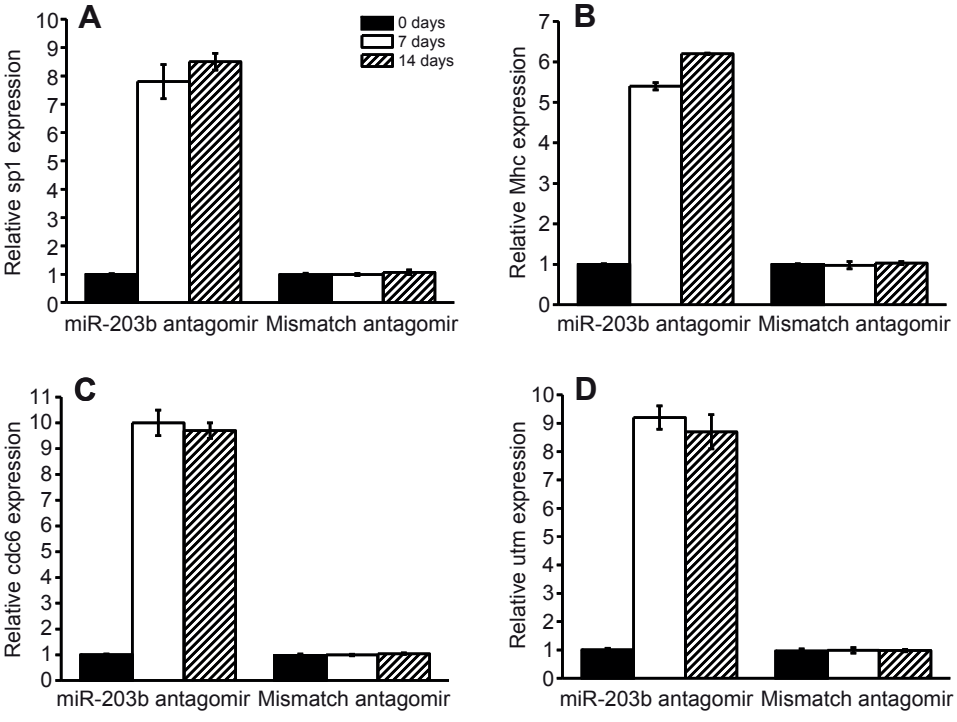

Fig. 3. miR-203b silencing activates MyoD downstream genes. Tilapia were treated as described in Fig. 1C. The expression of (A) Sp1, (B) MHC, (C) cdc6 or (D) utrn was detected using real-time PCR, and 18S rRNA was detected as the internal control. Data are expressed as the relative change compared with the untreated group. Four technical replicates were used for each treatment.

investigated the phenotypic variation in the body growth of Nile tilapia and identified the differential expression of growth-related miRNA in skeletal muscle. They found that eight downregulated miRNAs and eight upregulated miRNAs were associated with body growth in tilapia. Their findings suggest that miRNA may be involved in the regulation of fish growth, and that differential expression of growth-related miRNAs may serve as a molecular marker to guide tilapia breeding programs (Huang et al., 2012). In light of these studies, it is not surprising that the regulation between myogenic transcription factors and miRNAs is very complex. Thus insight into the miR-203b/MyoD interaction would shed new light on the molecular mechanism of muscle development.

As a crucial inducer of skeletal myogenesis both *in vitro* and *in vivo*, it is not surprising that the myogenic production of MyoD is tightly controlled by multiple mechanisms. p300/CBP, PCAF acetyltransferases and histone deacetylases (HDACs) can be recruited onto the MyoD promoter and regulate MyoD gene expression at transcriptional level. In addition, IFRD1 can repress the transcriptional activity of NF-κB, and indirectly inhibit MyoD expression; β-catenin interacts directly with MyoD, and enhances both its binding to E box elements and its transcriptional activity; and miR-203b can regulate muscle development by direct targeting of MyoD (Francetic et al., 2012; Kim et al., 2008; Micheli et al., 2011). Thus, cells have numerous mechanisms to quantitatively regulate the dosage of MyoD expression. Our findings reveal that miR-203b targets the protein MyoD for repression, which highlights an important facet of miRNA-mediated regulation of crucial cellular events. The event suggests that cells bear back-up mechanisms and regulatory pathways that titrate the dose of this crucial regulator of apoptosis in a very controlled manner (Bushati and Cohen, 2007; Kloosterman and Plasterk, 2006).

Conclusions

In summary, we reveal that MyoD is regulated by miR-203b expression in tilapia. miR-203b silencing leads to the upregulation of MyoD expression, and thereby the activation of miR-203b downstream genes. This study extends our knowledge about the regulation of MyoD expression, and sheds new light on the understanding of the molecular mechanism of muscle development and growth.

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