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

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23 **SUMMARY**

24 MyoD is one of the helix-loop-helix proteins regulating muscle-specific gene expression in
25 tilapia. Tight regulation of MyoD protein level is necessary for the precise regulation of skeletal
26 muscle development. MicroRNAs (miRNAs) are a class of regulatory RNAs that
27 post-transcriptionally regulate gene expression. Increasing evidences have suggested that miRNAs
28 play an important role in regulating skeletal muscle development. We reasoned that MyoD
29 expression may be regulated by miRNAs. Bioinformatics prediction identify a putative miR-203b
30 target site in the 3'-UTR of MyoD gene. Interestingly, miR-203b expression is negatively
31 correlated is negatively correlated with MyoD expression. miR-203b suppression leads to a
32 significant increase in MyoD expression, thereby activating MyoD downstream gene. 3'-UTR
33 luciferase reporter assay further verifies the direct interaction between miR-203b and MyoD.
34 Taken together, our studies reveal a novel molecular mechanism in which miRNA participates in
35 transcriptional circuits that regulates gene expression in tilapia skeletal muscle.

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37 Key words: Nile tilapia, MyoD, microRNA, skeletal muscle development

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45 **Introduction**

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

53 Skeletal muscle development is well defined process, and regulated by the sequential
54 expression of muscle-specific myogenic regulatory transcription factors (MRFs) (Chen and Tsai,
55 2008). MRFs belong to a group of helix-loop-helix transcription factors, including myogenin,
56 MRF4, MyoD and Myf5. Of them, MyoD is thought as the key transcription factor that initiates
57 the cascade of regulatory events during muscle development. MyoD can be regulated at
58 transcriptional level through the recruitment of both chromatin remodeling complexes and
59 p300/CBP and PCAF acetyltransferases onto MyoD promoter, whereas histone deacetylases
60 (HDACs) recruitment inhibits MyoD expression(Aguiar et al., 2008; Francetic et al., 2012; Lassar,
61 2012);M. PC4 (Tis7/Ifrd1) can cooperate with MyoD to induce the transcriptional activity of
62 myocyte enhancer factor 2C (MEF2C), and repress the transcriptional activity of NF-κB to inhibit
63 MyoD mRNA accumulation (Micheli et al., 2011). In addition, β-Catenin is found to be interacted
64 with MyoD and regulates its transcription activity (Kim et al., 2008). Given its critical role in the
65 regulation of muscle development, it is highly likely that MyoD expression is under additional
66 modes of regulation yet to be discovered.

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67 MicroRNAs (miRNAs) are short, non-coding RNAs that repress gene expression by binding
68 to target mRNAs. They have emerged as crucial regulators for many developmental processes,
69 including skeletal muscle development (Carthew, 2006; Carthew and Sontheimer, 2009). Previous
70 studies have identified and characterized several muscle-specific miRNAs that control various
71 aspects of myogenesis. These miRNAs include miR-1, miR-133a, miR-133b, miR-206, miR-208,
72 miR-486 and miR-499. Aberrant regulation of some of these muscle-enriched miRNAs can disrupt
73 intracellular signaling networks which may result in pathological conditions (Williams et al.,
74 2009). In general, one gene can be regulated by several miRNAs, while one miRNA may inhibit
75 the expression of multiple target genes, which results in the formation of complex regulatory
76 feedback networks. We reasoned that MyoD expression may be regulated by miRNAs (Makeyev
77 and Maniatis, 2008). However, miRNAs regulation of MyoD expression has never been reported
78 despite its notable expression in skeletal muscle. The objective of this study was to examine the
79 role of miRNAs in regulation of MyoD expression in tilapia.

80 **Materials and methods**

81 **Experimental fish and tissue sample preparation**

82 Nile tilapias were obtained from the fishery farm of Shanghai Ocean University. They were
83 maintained in a water circulation system in 100-liter tanks, and water temperature was kept at 26 ±
84 2°C under a 12-h light/12-h dark photoperiod. Skeletal muscle samples were collected from
85 different developmental stages including juvenile fish, adult fish, and senility fish, respectively.
86 These tissue samples were stored in liquid nitrogen before RNA isolation.

87 **Prediction of MyoD-binding miRNAs**

88 To identify miRNAs that potentially bind MyoD, we queried the MicroCosm targets

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89 (http://www.ebi.ac.uk/enright-srv/microcosm/) and TargetScan prediction program
90 (http://www.targetscan.org/) based on zebrafish genome (Alexiou et al., 2011; Rajewsky, 2006;
91 Saito and Sætrom, 2010). We compared the sequence conservation of miRNA target sites between
92 zebrafish and tilapia using blast search.

93 **Real-time PCR**

94 Total RNA was extracted using Trizol reagent (Invitrogen), and miRNAs were extracted using
95 the miRNeasy kit (Qiagen). For mRNA analysis, total RNA were reversed transcribed with
96 SuperScript III First-Strand Synthesis System for RT-PCR (Takara), and amplified with SYBR
97 Green PCR Master Mix (Takara). 18 SrRNA level was detected as internal normalization control.
98 The primers used for mRNA detection were listed in table 1. For miRNA expression assays, RNA
99 was reverse-transcribed using specific miRNA stem-loop primers. Mature miRNA expression was
100 detected using Taqman miRNA assays (Applied Biosystems) according to the manufacturer's
101 instructions. miRNA expression was normalized against the expression of house-keeping gene,
102 18S rRNA, using comparative Ct method (Schmittgen and Livak, 2008).

103 **Luciferase assay**

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

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

112 Chemically modified antisense oligonucleotides (antagomir) and agomir were synthesized to
113 regulate miR-203b expression (Ribobio, Guangzhou, China). The 3' end of the oligonucleotides
114 was conjugated to cholesterol, and all the bases were 2'-OMe modified. The antagomir or agomir
115 oligonucleotides were deprotected, desalted and purified by high-performance liquid
116 chromatography. Tilapia weighing about 5 g received tail-vein injection of saline, agomir, or
117 antagomir at a dose of 60 mg/kg body weight on every other day. They were sacrificed 24 h after
118 the last injection for experimental analysis (Morton et al., 2008; Van Solingen et al., 2009).

119 **Statistical Analysis**

120 Values were expressed as means ± S.E.M. unless otherwise stated. Statistical significance
121 was assessed by one-way ANOVA followed by Bonferroni's multiple comparison tests. Statistical
122 significance was defined as $P < 0.05$.

123 **Result**

124 **Prediction of miRNAs targeting MyoD**

125 Blast search suggests that the sequence of MyoD 3'-UTR is conserved between zebrafish and
126 tilapia. To identify miRNAs that interact with MyoD protein, we employed MicroCosm and
127 targetscan prediction program based on zebrafish genome. Bioinformatic prediction revealed that
128 miR-375, miR-722, miR-203b, miR-142a-5p, miR-138, miR-190, miR-190b, miR-122, and
129 miR-23b may regulate MyoD expression (Table. 2). Furthermore, these miRNAs are highly
130 conserved between tilapia and zebrafish. Among these predicted miRNAs, miR-122, miR-375 and
131 miR-722 have been reported to be abundantly expressed in liver, pancreas and retina (Chang et al.,
132 2008; O'Quin et al., 2011; Poy et al., 2009). Thus, they were ruled out for further analysis. We then

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133 used RT-PCR method to detect the expression pattern of other miRNAs. As shown in table 2.
134 miR-23b, miR-190, miR-190 and miR-203b can be detected in skeletal muscle. Therefore, we
135 speculated that these miRNAs would potentially regulate MyoD expression in tilapia skeletal
136 muscle.

137 **MyoD expression is negatively associated with miR-203b expression**

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

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

151 **miR-203b directly targeting MyoD 3'UTR**

152 To verify that miR-203b directly inhibits MyoD expression, we employed a luciferase
153 reporter assay. The alignment of miR-203b with MyoD 3'UTR insert is illustrated (Fig. 2A).
154 Cotransfection of HEK 293T cells with the parental luciferase construct (PGL3, without MyoD

155 3'UTR) plus the miR-203b expression vector does not significantly change expression of the
156 reporter (Fig. 2B). However, when the miR-203b target site from the MyoD 3'UTR is inserted into
157 the luciferase construct, luciferase expression is strongly decreased when cotransfected with
158 miR-203b, but the suppression is relieved by a single base mutation in the binding site (Fig.2B).
159 These result suggest that miR-203b directly suppresses MyoD expression by binding to its 3'UTR
160 target sequence.

161 **miR-203b silencing activates MyoD downstream genes**

162 MyoD is an important myogenic transcription factors that control the spatial and temporal
163 expression of muscle-specific genes. Alternation in MyoD expression would affect the expression
164 of MyoD downstream genes. In this study, we found that miR-203b silencing results in a
165 significant increase in MyoD expression. MyoD downstream genes, including Myosin heavy chain
166 (MHC), utrophin (Utrn), cell division control protein 6 (Cdc6), and Sp1 transcription factor (Sp1)
167 genes, were significantly upregulated (Rosenberg et al., 2006; Seward et al., 2001; Viñals et al.,
168 1997; Zhang et al., 2010). In contrast, miR-203b mismatch antagomir treatment has no effect on
169 miR-203b expression, and does not affect the expression of MyoD and its downstream genes
170 (Fig.3 A-D). Taken together, these results suggested that miR-203b can affect MyoD expression,
171 thereby changing the expression of MyoD downstream genes.

172 **Discussion**

173 Nile tilapia is one of the most important aquaculture species widely used in aquaculture for
174 commercial use. The central goal of tilapia aquaculture is the production of skeletal muscle. It is
175 therefore important to understand the regulatory mechanism of muscle development at the
176 molecular level. Muscle development is a well-defined process, which is regulated by various

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177 environmental factors and distinct signaling pathways, resulting in the activation of specific
178 transcription factors and gene expression. Gene expression in skeletal muscle is controlled by a
179 family of basic helix-loop-helix transcription factors known as the myogenic regulatory factors,
180 including Myf5, MyoD, myogenin and MRF4 are found to be crucial for controlling myogenesis
181 (Chen and Tsai, 2008; Johnston, 1999; Johnston et al., 2011). MyoD has been proposed to be
182 a" pioneer" transcription factor required to initiate the cascade of regulatory events required to
183 initiate expression of muscle-specific genes. MyoD recruits chromatin-modifying activities that
184 alter both the regional histone modifications and the chromatin remodeling at promoter binding
185 sites(Cao et al., 2006; Gerber et al., 1997; Londhe and Davie, 2011). Tight regulation of MyoD
186 protein level is necessary for the precise regulation of skeletal muscle development. In this study,
187 MyoD expression can be regulated at miRNA level.

188 The roles of miRNAs in muscle development have attracted much attention and research
189 interest (Ge and Chen, 2011). miR-133a can promote myoblast proliferation through the
190 repression of SRF expression (Liu et al., 2008). miR-206 can affect myoblast differentiation
191 program through the indirect down-regulation of the helix-loop-helix protein Id, a repressor of
192 MyoD (Kim et al., 2006). miR-181 inhibits the expression of Hox-A1, which results in the
193 inhibition of MyoD expression (Yamamoto and Kuroiwa, 2003). Recently, Huang et al. (2012)
194 investigated the phenotypic variation in the body growth of Nile tilapia and identified the
195 differential expression of growth-related miRNA in skeletal muscle. They found that 8
196 down-regulated miRNAs and 8 up-regulated miRNA associated with body growth in tilapia. The
197 finding suggests that miRNA may be involved in the regulation of fish growth, and differential
198 expression of growth-related miRNA may serve as molecular markers to guide tilapia breeding

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199 programs(Huang et al., 2012). In light of these studies, it is not surprise that the regulation
200 between myogenic transcription factors and miRNAs is very complex. The miR-203b/MyoD
201 interaction would shed a novel insight into the understanding the molecular mechanism of muscle
202 development.

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

216 **Conclusion**

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

221 and growth.

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331 **Figure Legend**

332 **Fig. 1 Identification of miR-203b as a regulator of MyoD expression**

333 (A) Total RNA sample was extracted from the skeletal muscle in different developmental stages
334 including juvenile fish, adult fish and senility fish, respectively. The level of MyoD expression
335 was detected using real-time PCR, and 18S rRNA was detected as the internal control. The data
336 was expressed as the relative change compared with the expression level in juvenile fish. (B)
337 miRNA sample was extracted from the same samples as shown in Fig. 1A. The expression of
338 miR-23b, miR-190, miR-190 or miR-203b was detected using real-time PCR, and 18S rRNA was
339 detected as the internal control. The data was expressed as the relative change compared with the
340 corresponding miRNA expression of juvenile fish. (C, D) Tilapia weighing about 5 g received
341 tail-vein injection of miR-203b antagomir or mismatch antagomir at a dose of 60 mg/kg body
342 weight on every other day. They were sacrificed after 7 d or 14 d antagomir treatment, respectively.
343 The expression of miR-203b or MyoD level was detected using Real-time PCR, and 18S rRNA
344 was detected as the internal control. The data was expressed as the relative change compared with
345 the untreated group. Four technical replicates were used for each treatment. (E) Tilapia weighing
346 about 5 g received tail-vein injection of miR-203b agomir or mismatch agomir at a dose of 60
347 mg/kg body weight on every other day. They were sacrificed after 7 d or 14 d agomir treatment,
348 respectively. The expression of MyoD level was detected using Real-time PCR, and 18S rRNA
349 was detected as the internal control. The data was expressed as the relative change compared with
350 the untreated group. Four technical replicates were used for each treatment.

351 **Fig. 2 miR-203b directly targeting MyoD 3’UTR**

352 (A) The alignment between miR-203b and the 3’UTR segment of MyoD. (B) Luciferase assays

353 were carried out to address whether MyoD is directly targeted by miR-203b. HEK 293T cells were
354 transfected with the plasmid as shown in Fig.2B, and transfection efficiency corrected by a *Renilla*
355 *luciferase* vector (PRL-CMV, Promega). Luciferase activity was detected using the luciferase
356 Assay Systems kit (Promega) according to the manufacturer's protocol. Results were expressed as
357 means ± S.E.M. of four independent experiments.

358 **Fig.3 miR-203b silencing activates MyoD downstream genes**

359 Tilapia was treated as shown in Fig. 1C. The expression of Sp1, MHC, cdc6 or utrn was
360 detected using Real-time PCR, and 18S rRNA was detected as the internal control. The data was
361 expressed as the relative change compared with the untreated group. Four technical replicates
362 were used for each treatment.

363 Table 1: Primer sequence used for the quantification of mRNA expression

364 Table 2: Predicted MyoD-binding miRNAs and their expression pattern

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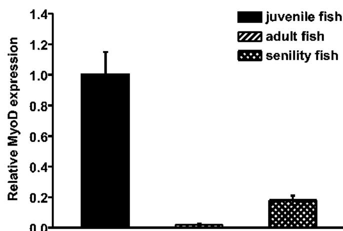
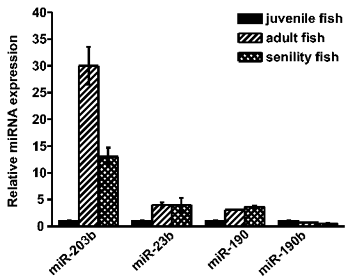
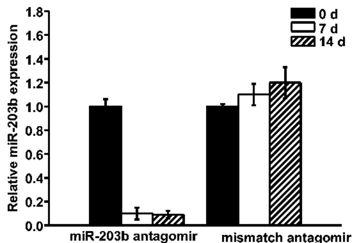
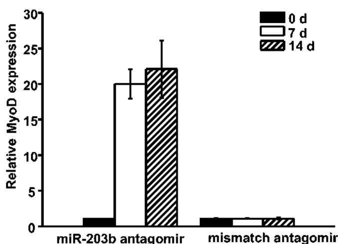
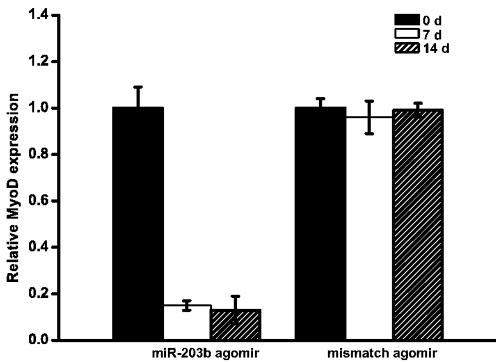

A
Relative MyoD expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
juvenile fish
adult fish
senility fish

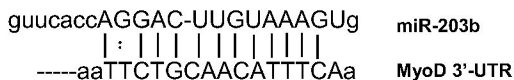

B
Relative miRNA expression
40
35
30
25
20
15
10
5
0
juvenile fish
adult fish
senility fish
miR-203b
miR-23b
miR-190
miR-190b


C
Relative miR-203b expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 d
7 d
14 d
miR-203b antagomir
mismatch antagomir

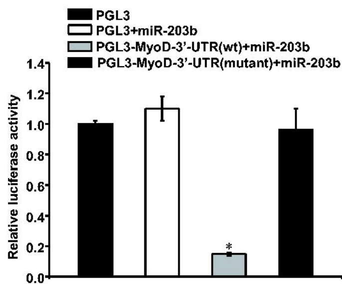

D
Relative MyoD expression
30
25
20
15
10
5
0
0 d
7 d
14 d
miR-203b antagomir
mismatch antagomir

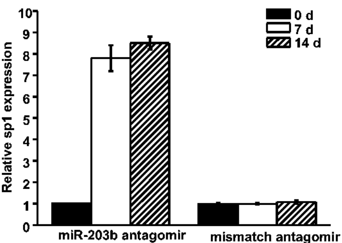

E
Relative MyoD expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 d
7 d
14 d
miR-203b agomir
mismatch agomir

A

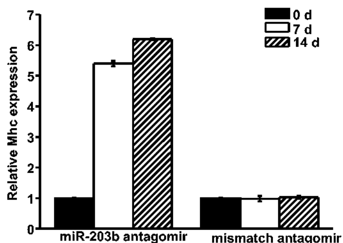


B

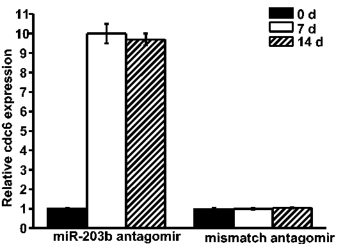




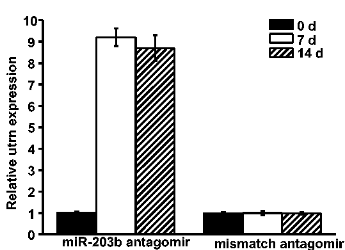
Relative sp1 expression
0
1
2
3
4
5
6
7
8
9
10
0 d
7 d
14 d
miR-203b antagomir
mismatch antagomir



Relative Mhc expression
0
1
2
3
4
5
6
7
0 d
7 d
14 d
miR-203b antagomir
mismatch antagomir



Relative cdc6 expression
0
1
2
3
4
5
6
7
8
9
10
11
0 d
7 d
14 d
miR-203b antagomir
mismatch antagomir



Relative utrn expression
0
1
2
3
4
5
6
7
8
9
10
0 d
7 d
14 d
miR-203b antagomir
mismatch antagomir

Table 1: Primer sequence used for the detection of mRNA expression

Gene	GeneBank accession	Annealing temperature	Primer
MHC	AF050035	58°C	ACAACATTAGAAATTTGCTGCGG CGGGCGTACTCGATCTTGTT
Sp1	XM_003451834	58°C	CCAGGCCGGTACCGTTGCAG GCAGCAGGATGGCCCCTGTG
Cdc6	XM_003454088	55°C	GGCAACAGACGCCCCCTTCC GGCGCTCAGGGATGGCAGTG
Utrn	XM_003449882	56°C	CGGAGACGTCGCTGGGGGA TGGCCGTCTTCCCTGTCTTGG
18S rRNA	JF698683.1	54°C	GGCCGTTCTTAGTTGGTGGA TTGCTCAATCTCGTGTGGCT

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

Predicted miRNAs	Expression pattern	Method
miR-23b	heart, liver, intestine, skeletal muscle	RT-PCR
miR-122	liver	Referance 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	Referance and RT-PCR
miR-722	eye	Referance and RT-PCR