MicroRNA signature in response to nutrient restriction and re-feeding in fast skeletal muscle of grass carp (*Ctenopharyngodon idella*)

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Abstract: The grass carp (*Ctenopharyngodon idella*) is one of the most important cultivated fish species in China. Mounting evidences suggests that microRNAs (miRNAs) may be key regulators of skeletal muscle among the grass carp, but the knowledge of the identity of myogenic miRNAs and role of miRNAs during skeletal muscle anabolic state remains limited. In the present study, we choose 8 miRNAs previously reported to act as muscle growth-related miRNAs for fasting-refeeding research. We investigated postprandial changes in the expression of 8 miRNAs following a single satiating meal in grass carp juveniles who had been fasting for one week and found that 7 miRNAs were sharply up-regulated within 1 or 3 h after refeeding, suggesting that they may be promising candidate miRNAs involved in a fast-response signaling system that regulates fish skeletal muscle growth.

Keywords: MicroRNA**;** Grass carp**;** Fasting; Refeeding**;** Skeletal muscle

The grass carp (*Ctenopharyngodon idella*) is one of the most important cultivated species in Chinese freshwater aquaculture. Generally, fast skeletal muscle comprises the largest tissue of the fish body (Zhang et al, 2009), and forms the main edible part of this species. Given the complicated development process as well as the importance of these tissues, gaining a clearer understanding of the mechanism underlying muscle development may provide key data for both developmental biologists and researchers attempting to improve grass carp musculature for aquaculture.

Several lines of research have implicated growth factors, regulatory proteins, and transcription factors as key actors involved in the regulation and maintenance of skeletal muscle mass among fish (Nihei et al, 2006; Steinbacher et al, 2006; Chu et al, 2010). Recent studies found that alongside transcriptional factors involved in muscle proliferation and differentiation, a set of microRNAs (miRNAs) may also play important roles in skeletal muscle development among vertebrate animals (Ge & Chen, 2011; Güller & Russell, 2010; Chu et al, 2013). MicroRNAs (miRNAs) are approximately 22-nt noncoding RNAs that act as negative regulators of gene expression, either via inhibiting mRNA translation or promoting mRNA degradation through base pairing to the 3' untranslated region (UTR) of target mRNAs (Xie et al, 2005; Liu, 2008; Zhang & Wen, 2010). Furthermore, miRNAs regulate the expression of transcription factors and signaling mediators critical to both cardiac and skeletal muscle development and function (Callis & Wang, 2008; van Rooij et al, 2008). Studies using the mouse myogenic C2C12 cell line demonstrated miR-1 and miR-133 are involved in myoblast proliferation and differentiation via regulation of the expression of HDAC4 and SRF, respectively (Chen et al, 2006). Meanwhile, miR-1, miR-133a and miR-206 (muscle-specific miRNAs), were also noted to be differentially expressed in Japanese flounder (*Paralichthys olivaceus*) during metamorphosis, as well as in Nile tilapia (*Oreochromis niloticus*) among several developmental stages.

Received: 25 March 2014; Accepted: 17 July 2014
Foundation items: This study was supported by the National Natural Science Foundation of China (31230076; 31340054), the Natural Science Foundation of Hunan province (14JJ2135) and the State Key Laboratory of Freshwater Ecology and Biotechnology (2012FB01)
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Taken together, these findings imply that miRNAs likely play an important role in regulating muscle development (Fu et al, 2011; Yan et al, 2012a). Further evidences suggest that miRNAs act as key regulators of myogenesis, but unfortunately characterizing the identity of myogenic miRNAs and delineating the role of miRNAs during skeletal muscle anabolic state remains unclear.

The maintenance of skeletal muscle mass is a complex and controlled process, largely influenced by both nutritional and physiological states of different animals (Fuentes et al, 2012). Fasting-refeeding protocols have been commonly used as models to investigate the regulation of muscle growth in fish species that experience the transition from catabolic to anabolic states. In the present study, we analyzed the expression of 8 select miRNAs during skeletal muscle anabolic state using a fasting-refeeding experiment. The goal of our study was to better parse out the potential role of these miRNAs in skeletal muscle proliferation and differentiation. The 8 miRNAs (miR-1a, miR-133a-3p, miR-133b-3p, miR-146, miR-181a-5p, miR-206, miR-214 and miR-26a) were previously reported to act as muscle growth- related miRNAs (McCarthy & Esser, 2007; Flynt et al, 2007; Kuang et al, 2009; Yan et al, 2012b). In theory, these miRNAs and their target genes may comprise a coordinated regulating net to favor resumption of myogenesis as an early response to refeeding.

MATERIALS AND METHODS

Fasting-refeeding experiments and sampling

All grass carp individuals were reared under standard conditions at the Che Tian Jiang Reservoir in Loudi, Hunan, China. Two homogeneous groups of grass carp juveniles (average body weight 150 g, 90 days post-hatching (dph)) were reared respectively in two net cages (5 m×5 m×2 m) with fifty fish per tank. All juveniles were fed under standard conditions for 3 weeks, after which the juveniles underwent fasting for 1 week, and were then fed a single meal, which was distributed to all individuals until they appeared to be visually satiated. At each time point from 0 h (before the recovery meal), and at 1, 3, 6, 12, 24, 48 and 96 h (hours after the single meal), six fish were sampled, wherein fast muscles were dissected from the dorsal myotome of individuals. The resulting samples were then snap-frozen in liquid nitrogen and stored at -80 °C until further processing.

Quantitative real-time PCR for the miRNAs

Tissue samples were ground in liquid nitrogen, and total RNAs were extracted using TRIzol (Invitrogen, USA), and then treated with RNAse-free DNAse I (Promega, USA) in the presence of RNAse inhibitor (Sigma, China Branch) followed by ethanol precipitation. The obtained RNAs were polyadenylated by poly (A) polymerase, and then reverse transcribed with one step PrimeScript miRNA cDNA synthesis Kit (TaKaRa, Dalian, China) and a Universal Adaptor Primer (a poly (T) primer ligated with an adapter) for miRNA quantitative assays.

The miRNA expression levels were quantified using real-time PCR with grass carp β-actin gene (GenBank No. DQ211096.1) as an internal control. The cDNA samples were used as templates for quantitative RT-PCR assays with SYBR Premix Ex Taq II (TaKaRa, Japan) and its amplification reaction was carried out on a Bio-Rad CFX96 system (USA). Each 2 μL cDNA template was added to a total volume of 25 μL reaction mix containing 12.5 μL SYBR Green mix, 1 μL of each miRNA or gene specific forward primer (as shown in Table 1, 10 μmol/L) and 1μL of universal downstream primer (Uni-miR qPCR Primer, 10 μmol/L, TaKaRa) or gene specific reverse primer, 8.5 μL nuclease-free water. The protocols used are as follows: (i) pre-denaturation at 95 °C for 60 s; (ii) amplification and quantification, repeated 40 cycles of at 95 °C for 5 s and at 60 °C for 25 s; (iii) melting curve program (65-95 °C with heating rate of 0.1 °C/S and fluorescence measurement) (Zhou et al, 2010). The relative expression ratio (R) of target miRNA was calculated by $R=2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001; Bustin et al, 2009), where Ct is the cycle threshold. The basic equation employed was:

$$\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{housekeepi ng gene}})_{\text{experiment}} - (Ct_{\text{target gene}} - Ct_{\text{housekeepi ng gene}})_{\text{cont rol}}$$

The miRNA expression levels were then analyzed by one-way ANOVA procedures and regression analysis of SPSS 17.0 (SPSS inc., Chicago, USA). Duncan's multiple range tests were used to compare the control (0 h before the recovery meal) and experimental (# hours after refeeding) groups. The differences were considered statistically significant when $P<0.05$. Data are shown as means±SE (n=6). Correlation of gene expression was analyzed by the Spearman rank order correlation test. Hierarchical clustering was performed using Cluster3.

Table 1 Primers used for miRNA detection

Name	Primer Sequence (5′−3′)	Name	Primer Sequence (5′−3′)
miR-1a-F	TGGAATGTAAAGAAGTATGTAT	miR-146-F	CGTGAGAACTGAATTCCATAGATGG
miR-133a-3p-F	CGCGTTTGGTCCCCTTCA	miR-133b-3p-F	TTGGTCCCCTTCAACCAGCTA
miR-206-F	CGTGGAATGTAAGGAAGTGTGTGG	miR-214-F	ACAGCAGGCACAGACAGGCAG
miR-181a-5p-F	CGAACATTCAACGCTGTCGGT	miR-26a-F	CGTTCAAGTAATCCAGGATAGGCT
β-actin-F	GCCGTGACCTGACTGACTACCT	β-actin-R	CGCAAGACTCCATACCCAAGAAG

Forward primers used for detection were detailed in the Methods; the reverse primer used for detection was universal downstream primer (Uni-miR qPCR Primer, 10 μmol/L, Takara).

RESULTS

Effect of fasting and refeeding on the expression of the miRNAs

A significant up-regulation of each of the 8 miRNAs was observed between 1-6 hours after the single meal (P<0.05) (Figure 1). MiR-1a was sharply up-regulated within 1 h after refeeding and peaked at 6 h (Figure 1A). The expression of miR-133a-3p, miR-133b-3p, miR-146, miR-181a-5p, miR-206 and miR-214 were significantly increased at 3h (P<0.05), reached the maximal levels at 3, 6 or 48 h postprandial (Figure 1B-G). While miR-26a responded slowly to refeeding, it significantly increased at 6 h (P<0.05) and peaked at 48 h (Figure 1F). However, miR-1a, miR-133b-3p and miR-181a-5p significantly decreased at 12 h. All 8 miRNAs returned to the initial baseline values at 96 h.

Heat map summary of hierarchical clustering of miRNAs in skeletal muscle during fasting-refeeding periods

Hierarchical clustering analysis of miRNAs in muscle was done according to the similarity in their expression across different postprandial times (0-96 h). Hierarchical clustering of the miRNAs throughout the trial showed three clades (Figure 2). The first clade including 2 pairs of closely linked miRNAs (miR-133b-3p and miR-133a-3p, miR-206 and miR-181a-5p) that clustered together. The second clade clustered miR-26a with miR-146 expression.

DISCUSSION

MicroRNAs (miRNAs) are noncoding RNA molecules that regulate the stability and/or the translational efficiency of target mRNAs, and several miRNAs have been found to be specifically expressed or highly enriched in skeletal muscle. The expression of muscle-specific miR-1, miR-133, miR-206, while miR-208 is regulated by muscle transcriptional networks involving SRF, MyoD and MEF2. (Flynt & Lai, 2008; Latronico et al, 2007; Thum et al, 2008; Van Rooij et al, 2008; Callis et al, 2008). Interestingly, non-muscle-specific miRNAs, e.g., miR-26a and miR-181, also regulate skeletal muscle differentiation (Wong & Tellam, 2008; Naguibneva et al, 2006). While a great deal of attention has been paid to miRNAs involved in control of muscle development, a recent study suggests that several other miRNAs, including miR-499, miR-208b and miR-23a, also play an important role in human muscle growth (Drummond et al, 2009). Among fish species, Huang et al (2012) previously detected differentially expressed miRNA between 2 strains of Nile tilapia and identified miR-140, miR-192, miR-204, miR-218a, miR-218b, miR-301c, miR-460, miR-133, miR-152, miR-15a, miR-193a, miR-30b and miR-34 as being associated with body growth in tilapia.

Our present study focused on exploring the potential role(s) of miRNAs as a new layer of control in the postprandial regulation of the muscle development among grass carp. Typically, nutrient availability is among the most important environmental variable altering muscle growth (Valente et al, 2012). As such, starvation and refeeding experiments have served as an effective model for studying the regulation of muscle growth in fish, including the Atlantic salmon (*Salmo salar*) (Bower et al, 2009), rainbow trout (*Oncorhynchus mykiss*) (Montserrat et al, 2007), and Atlantic halibut (*Hippoglossus hippoglossus*) (Hagen et al, 2009). Similarly, in humans MiRNAs turned over quite rapidly (i.e. hours) in skeletal muscle following amino acid ingestion. However, little information is available regarding the early transcriptional changes of miRNA during the postprandial period, especially among fish. We therefore focused on exploring the postprandial regulation of growth-related miRNAs shortly after

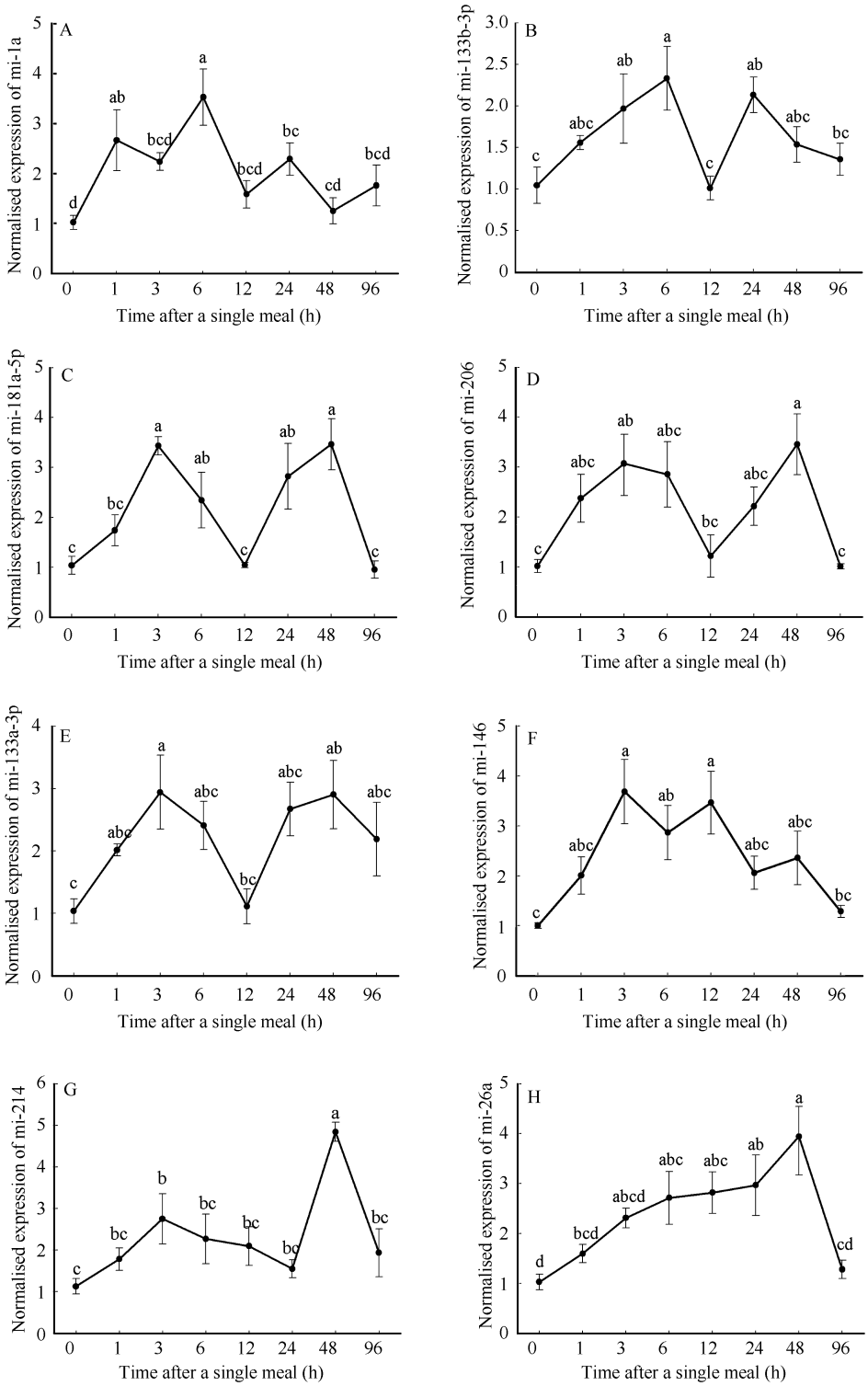

Figure 1 Relative expression of miRNAs with significant up-regulation following refeeding after 1 week fasting β-actin expression was detected as the internal control. All values are presented as mean±*SE*, *n*=6. Different letters indicate significant differences between columns ($P<0.05$).

feeding a single meal in grass carp. Our results showed that miR-1a, miR-133a-3p, miR-133b-3p, miR-146, miR-181a-5p, miR-206 and miR-214 were significantly elevated at 1 or 3 h after refeeding in the fast muscle of grass carp. Drummond et al (2009) previously found a rapid up-regulation of the miR-1, miR-208b, miR-23a

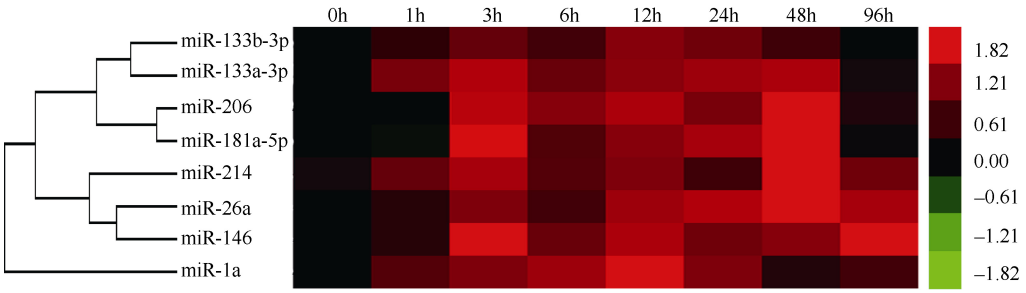

Figure 2 Heat map summary of hierarchical clustering of miRNAs in skeletal muscle during fasting-refeeding periods

Blue and yellow respectively denote a decrease or increase. The absolute signal intensity ranged from -1.82 to +1.82, with corresponding color changes from green to red.

and miR-499 following the amino acid ingestion in humans. These findings suggest that the identified miRNAs may be promising candidate miRNAs involved in a fast-response signaling system that regulates fish skeletal muscle growth. The other finding of significant decreased of miR-1a, miR-133b-3p and miR-181a-5p at 12 h after single meal suggests there may be other signaling pathways regulated by the miRNAs that limit excessive regulation of muscle growth.

A further finding of our study was that miR-206 and miR-181a-5p showed a dramatic and simultaneous up-regulation following feeding by a single meal. MiR-206 is known to be a muscle-specific miRNA, with its role in muscle development having been verified in some animal models, including mice, rats and zebrafish (Anderson et al, 2006; Kim et al, 2006; Mishima et al, 2009; Shan et al, 2009). MyoD acts as a transcriptional activator of the miR-206 pre-miRNA transcript, and subsequently the induced high levels of the mature miR-206 result in the down-regulation of specific target muscle growth-related genes (Rosenberg et al, 2006). MiR-181 is also thought to function partly through inhibition of Hox-A11 expression, a known repressor of MyoD, which is required for new muscle growth (Naguibneva et al, 2006). Taken together, these different lines of evidence suggest that miR-181 may indirectly promote miR-206 expression, though some further study is needed. Moreover, our finding that two miRNAs (miR-181a-5p and miR-206) clustered together suggest a close relation and coordination regulation of these miRNAs towards resumption of myogenesis following refeeding.

In conclusion, the present results show that several miRNAs likely involved in fast skeletal muscle in grass carp respond quickly to refeeding of a single meal following fasting. Results of our analysis indicate that refeeding induced a coordinated regulation of several miRNAs involved in a strong resumption of myogenesis, wherein the 8 tested miRNAs transcripts were sharply up-regulated in muscle tissues in response to refeeding. This finding suggests that these miRNAs may be promising candidate miRNAs involved in regulating fish fast muscle growth. Further study is needed to experimentally assess the targets of these miRNAs and elucidate how they contribute to the regulation of skeletal muscle growth during anabolic state.

Acknowledgements: We would like to thank Yu-Long LI, Dun-Xue CHEN, Kai-Zhuo WANG and Jun-Zhi ZHANG for their help and support in sample collection.

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