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Short communication

Pm-miR-133 hosting in one potential lncRNA regulates *RhoA* expression in pearl oyster *Pinctada martensii*

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ABSTRACT

Long non-coding RNAs (LncRNAs) are abundant in the genome of higher forms of eukaryotes and implicated in regulating the diversity of biological processes partly because they host microRNAs (miRNAs), which are repressors of target gene expression. In vertebrates, *miR-133* regulates the differentiation and proliferation of cardiac and skeletal muscles. *Pinctada martensii miR-133* (*pm-miR-133*) was identified in our previous research through Solexa deep sequencing. In the present study, the precise sequence of mature *pm-miR-133* was validated through miR-RACE. Stem loop qRT-PCR analysis demonstrated that mature *pm-miR-133* was constitutively expressed in the adductor muscle, gonad, hepatopancreas, mantle, foot, and gill of *P. martensii*. Among these tissues, the adductor muscle exhibited the highest *pm-miR-133* expression. Target analysis indicated that *pm-RhoA* was the potential regulatory target of *pm-miR-133*. Bioinformatics analyses revealed that a potential LncRNA (designated as *Lnc133*) with a mature *pm-miR-133* could generate a hairpin structure that was highly homologous to that of *Lottia gigantea*. *Lnc133* was also highly expressed in the adductor muscle, gill, hepatopancreas, and gonad. Phylogenetic analysis further showed that the *miR-133s* derived from chordate and achordate were separated into two classes. Therefore, *Lnc133* hosting *pm-miR-133* could be involved in regulating the cell proliferation of adductor muscles by targeting *pm-RhoA*.

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1. Introduction

Skeletal muscles are connected to the skeleton, and they work together to accomplish movements, to maintain body gestures, and to provide skeletal nutrition and protection in vertebrates. Similar to the skeletal muscles of mammals, the adductor muscles of shellfish are strong and firm. These muscles are connected to their shells and thus control "turn on" and "turn off" actions. Functional molecules of skeletal matrix, such as collagen and metalloproteinase tissue inhibitor, are involved in biomineralization in vertebrates and invertebrates; this phenomenon suggests that the formation of skeletons share partially common substance composition (Kadler et al., 2007; Ke et al., 2013; Robert and Hideaki, 2003; Fang et al., 2014). However, the general regulatory mechanism of skeletal muscle functions remains unclear.

MicroRNAs (miRNAs) are a class of non-coding RNA molecules with 18–24 nucleotides; they regulate target gene expression at post-transcriptional levels in various eukaryotic organisms (Kusakabe et al., 2013; Bartel, 2004; Graves and Zeng, 2012). miRNAs also contribute to the modulation of numerous cellular processes (Bartel, 2004). In animals, some miRNAs recruit long non-coding RNAs (LncRNAs) with polyA as a primary RNA, and then processed by Drosha to produce precursor miRNAs characterized by a hairpin structure. Afterward, precursor miRNAs are exported into the cytoplasm, where they are further cleaved by a dicer to form functional mature miRNAs (Graves and Zeng, 2012; Hinske et al., 2010).

MiR-133, also known as *miR-133a* or *miR-133a-3p*, is a well-studied muscle-specific miRNA expressed in the cardiac and skeletal muscles of mammals (Ivey et al., 2008). In cardiomyocytes, *miR-133* suppresses proliferation by repressing *cyclin D2* expression (Yu et al., 2014). During myogenesis, *miR-133* regulates multiple key regulatory genes, such as small GTPase *RhoA* [11 − 13] and serum response factor (Williams et al., 2009).

Pinctada martensii, an important molluscan mariculture species, is primarily cultured for pearl production in China and Japan. Mature *miR-133* was identified in *P. martensii* through Solexa deep sequencing technology in our previous report (Jiao et al., 2013). In the present study, the existence and potential functions of *miR-133* in adductor muscles were investigated to reveal the common regulatory mechanism in skeletal muscles between vertebrates and mollusks.

Abbreviations: LncRNA, long non-coding RNA; *RhoA*, Ras homolog gene family, member A.

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2. Material and methods

2.1. Sample collection and preparation

Two-year-old *P. martensii* specimens were collected from Liushagang, Zhanjiang, Guangdong Province, China. Adductor muscle, gill, mantle, hepatopancreas, gonad, and foot tissues were collected and stored in liquid nitrogen until use. Total RNAs were extracted using Trizol reagent (Invitrogen). RNA integrity was determined through fractionation on 1% formaldehyde-denatured agarose gels. RNA quantity was measured by obtaining OD260/OD280 with a NanoDrop ND1000 spectrophotometer.

2.2. Verification of mature pm-miR-133 by miR-RACE

To validate the obtained mature *pm-miR-133*, we conducted 5′ and 3′ miR-RACE in accordance with previously reported procedures (Zhang et al., 2012) with slight modifications. Small RNA was extracted by using a *mir*Vana™ miRNA isolation kit (Ambion). The extracted small RNAs were polyadenylated by poly(A) polymerase (Takara). A 5′ adapter (CGACUGGAGCACGAGGACACUGAAA) was ligated to the poly (A)-tailed RNA by using T4 RNA ligase (Takara). The ligated products were recovered through phenol/chloroform extraction followed by ethanol precipitation. Reverse transcription was performed using an RT primer (Supplementary Table 1). miR-RACE universal primers are complementary to 5′ and 3′ adaptors. Gene-specific primers were designed similar to the mature sequence of *miR-133* obtained in our previous research (Jiao et al., 2013). The primers used for miR-RACE are listed in Supplementary Table 1.

2.3. Prediction of precursor pm-miR-133

The potential sequence of *pm-miR-133* precursor was determined in accordance with a similar procedure used to identify new miRNAs, with slight modifications (Weaver et al., 2007). Local blastn was performed between the sequence of mature *pm-miR-133* (TTGGTCCCCTTCAACC AGCTGT) and the sequence of *P. martensii* in transcriptome databases (Zhao et al., 2012). The secondary structure of the obtained unigene sequence was predicted by using M-fold (http://mfold.rna.albany.edu/). Afterward, local blastn was used, and the fragment hosting *pm-miR-133* was mapped back to *P. martensii* genome, which is published on http://marinegenomics.oist.jp/pearl/viewer/info?project_id=20.

2.4. Sequence analysis and tissue expression patterns of pm-miR-133

Multiple alignments of animal precursor *miR-133s* were performed on miRBase (http://www.mirbase.org/search.shtml). A phylogenetic tree was constructed via Le Gascuel (LG) substitution model of PHYML in Geneious 7 software, and the bootstrap value is 500. The tissue-specific expression of mature *pm-miR-133* with 2^ΔΔCt was determined through stem loop quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis by U6 as a reference gene and mantle tissue as a control. The bar represented standard deviation. Stem-loop RT and real-time PCR primers were designed as described in a previous study (Chen et al., 2005). The primers for the qRT-PCR analysis of *Lnc133*, which is the primary *miR-133*, were designed on the basis of unigene75113 sequence, which was predicted as the partial sequence of primary *pm-miR-133*. Beta-actin and mantle tissue were respectively used as an internal reference and a control to quantify primary *miR-133* with 2^ΔΔCt. The primers used in this analysis are listed in Supplementary Table 1.

2.5. Target prediction between miR-133 and RhoA

MiR-133 target gene was analyzed via RNAhybrid (Kruger and Rehmsmeier, 2006). Similar to the amount of energy used in our previous research (Jiao et al., 2013), the free energy of less than −20 kcal/mol likely indicated a potential interaction.

2.6. Statistical analysis

ANOVA in SPSS 19.0 (IBM, USA) was conducted to detect the differences in the relative expression levels of mature *pm-miR-133* and primary *pm-miR-133* among different tissues. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Sequence verification of mature pm-miR-133

We conducted 5′ and 3′ miR-RACE to validate the sequence of the mature *pm-miR-133* obtained by Solexa deep sequencing. The 5′ and 3′ sequences of *pm-miR-133* were consistent with the Solexa deep sequencing (Supplementary Fig. 1). The validated sequence of mature *pm-miR-133* is UUGGUCCCCUUCAACCAGCUGU.

3.2. Tissue-specific expression of pm-miR-133

qRT-PCR analysis was performed to determine the tissue-specific expression of mature *pm-miR-133*. Mature *pm-miR-133* was expressed in the adductor muscle, mantle, gill, foot, gonad, and hepatopancreas. The expression of mature *pm-miR-133* was significantly higher in the adductor muscle than in the other tissues ($p < 0.05$) (Fig. 1a).

3.3. Target analysis of pm-miR-133

We obtained the unigenes encoding *pm-RhoA* in our previous research by analyzing the transcriptome database of the pearl sac from *P. martensii* (Fig. 2). The obtained partial sequence of *pm-RhoA* cDNA was 579 bp, and it contained a 156 bp 3′ UTR and 423 bp open reading frame encoding 141 amino acid residues (Fig. 2a). The deduced protein sequence of *pm-RhoA* comprised a conserved RHO motif (Fig. 2b). Multiple sequence alignments indicated that *RhoA* was highly conserved among species (Fig. 2c). Target analysis between *miR-133* and these genes revealed that the potential interaction was mainly observed

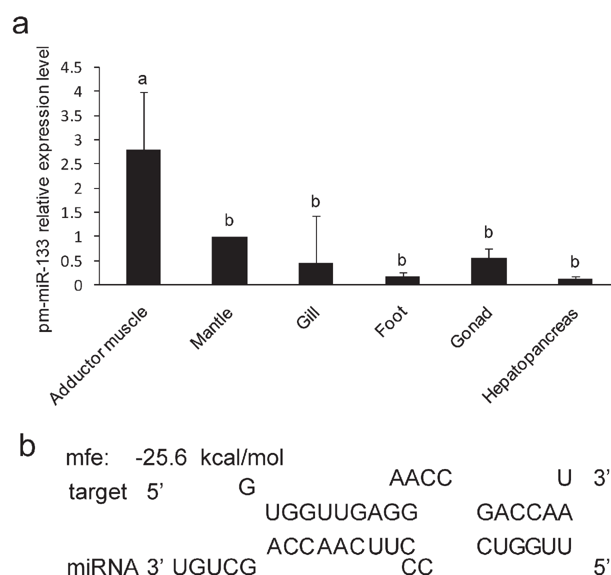

Fig. 1. Expression pattern of (a) mature *pm-miR-133* in different tissues and its target prediction and (b) mature *pm-miR-133* in *P. martensii*. Different letters indicate significant differences ($p < 0.05$) determined through one-way ANOVA and Duncan's multiple comparisons. The bar represents standard deviation.

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