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EXPRESSION ANALYSIS OF MIR-1 AND MIR-133 IN THREE DEVELOPMENTAL STAGES OF GOAT SKELETAL MUSCLE

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ABSTRACT

MiR-1 and miR-133 are evolutionary conserved in most animal species, from drosophila to human, and have been confirmed to take an important role for skeletal muscle development in mice and pigs. In this study, the function of miR-1 and miR-133 was detected through analysis the expression pattern of the two miRNAs and their predicted target genes in three developmental stages of goat skeletal muscle. HDAC4 and SRF, which were the target genes of miR-1 and miR-133 family, respectively, were predicted using MiRanda and Target Scan 6.2 software. Then the expression profiles of miR-1, miR-133, HDAC4 and SRF in 10 normal goat tissues (heart, liver, spleen, lung, kidney, small intestine, fat, longissimusdorsi muscles, pectoral muscles and crural muscles) from three stages (newborn, three months, eighteen months) of Anhui White goat were characterized by real-time RT-PCR. The expression of miR-1 and miR-133 were varied among different tissues, with most abundant in heart and skeletal muscles (longissimusdori muscles, pectoral muscles and crural muscles) in Anhui white goats, while there were the least expression in other tissues. However, the expression levels of HDAC4 and SRF were on the contrary. The results suggested that miR-1 and miR-133 may enhance myoblast proliferation and differentiation by repressing their predicated target genes, thus affecting skeletal muscle growth and development. This study will be a useful reference for further studies about miR-1 and miR-133 for their role in the regulation in animal growth and development processes.

Key words: miR-1 and miR-133, goat, target gene, growth and development, expression profiles.

INTRODUCTION

MicroRNAs (miRNAs) are a class of short (20~25 nucleotides), Single-stranded, non-coding RNA that regulate mRNA expression at the post-transcriptional level (Huang *et al.*, 2013). miRNAs negatively regulate gene expression through complementary base-pair binding of the miRNA" seed sequence" (nucleotides 2-7) to the 3'untranslated region (UTR) of target mRNA, degrading or destabilizing the RNA message, or inhibiting protein translation, depending on the quality of complimentary base-pair matches or the number of miRNA targeting sites with the 3'UTR (Townley-Tilson *et al.*, 2010). Emerging evidence has demonstrated that miRNAs are essential for normal mammalian development, cell proliferation, differentiation, growth, development, remodeling in response to stress, and many other functions (Güller and Russell, 2010; Lu *et al.*, 2012). Studies from recent years have shown that many miRNAs involved in the regulation of skeletal muscle proliferation and differentiation processes (Chen *et al.*, 2011; Yan *et al.*, 2012). Subsets of these miRNAs, miR-1 and miR-133 are muscle specific and have been the focus of intensive investigation (Lu *et al.*, 2012). Both miR-1 and miR-133 have been found in most animal species, from drosophila to human, suggesting that they are evolutionary conserved (Chen *et al.*, 2006). In addition, Studies have confirmed that miRNAs have an important role for skeletal muscle development in mice and pigs (Mcdaneld *et al.*, 2009; Parra *et al.*, 2010).

According to the foxO1 expression profile of human and mouse, indicating that miR-133b may be involved in the regulation of foxO1 in B cell development (Liang *et al.*, 2011). MiR-1 promotes myogenesis by targeting histone deacetylase 4 (HDAC4), a transcriptional repressor of muscle gene expression, while miR-133 enhances myoblast proliferation by repressing serum response factor (SRF) (Chen *et al.*, 2006; Rao *et al.*, 2006).Although miR-1 and miR-133 have been extensively studied, there is no information about their expression and expression of their predicted target genes (HDAC4 and SRF) of goat skeletal muscle.

In this study, real-time quantitative reverse transcription PCR (qRT-PCR) method (Mishina *et al.*, 2007) was used to quantify the expression of miR-1, miR-133, HDAC4 and SRF in the tissues (heart, liver, spleen, lung, kidney, small intestine, fat, longissimusdorsi muscles, pectoral muscles and crural muscles) from neonatal, three-month and eighteen-month Anhui white goats. Studying the regulatory mechanisms of miR-1 and miR-133 and their predicated target genes in muscle proliferation and differentiation will extend our

knowledge of miRNAs in muscle biology and will improve our understanding of the myogenesis regulation.

MATERIALS AND METHODS

Animals, tissue sampling, and RNA extraction: Anhui white goat was bred in the experimental farm of Hefei Bo Da Livestock Technology Development CO, LTD. All goats were allowed free access to water and treated in accordance with the 'Regulation on Animal Experimentation at Anhui Agricultural University'. Goats from the herd of the farm will be referred to as Anhui white goat in this paper. Generally, the goats grow very fast from 0 to three months, and grow slowly after eighteen months. Hence, three healthy, neonatal, three-month-old and eighteen-month-old goats were selected from the herd for this study. Heart, liver, spleen, lung, kidney, small intestine, fat and skeletal muscles (longissimusdorsi muscles, pectoral muscles and crural muscles) tissues were surgically collected from three different goats, and immediately frozen in liquid nitrogen.

Total RNA was extracted from heart, liver, spleen, lung, kidney, small intestine, fat and skeletal muscles (longissimusdorsi muscles, pectoral muscles and crural muscles) tissues using TRIzol (TaKaRa) according to the manufacturer's instructions. The quantity and quality of the RNA were measured using SMA 1000 spectrophotometer (Merinton). The integrity of the RNA was assessed by 0.8% electrophoretic analysis of the 28S, 18S and 5S rRNA subunits. Total RNAs were stored at -80 for analyses.

Primer design and synthesize: The mature sequences of miR-1 and miR-133 were from the preliminary Solexa sequencing for Anhui white goats (Ling *et al.*, 2013). The stem-loop primers and specific PCR primers for miR-1, miR-133 and U6 RNA were obtained from RiboBio (RiboBio, Guangzhou, China). As the goat HDAC4 and SRF gene sequences are not available, according to the high homology between bovine and sheep HDAC4 and SRF gene (GenBank accession number XM_002686597 and XM_004003449; NM_001206016 and XM_004019222), two pairs of primers were designed to amplify goat HDAC4 and SRF gene (Table 1). The primers were synthesized by the BGI (China).

cDNA synthesis: For miR-1, miR-133 and U6, first strand cDNA were synthesized using specific stem-loop RT primers and a cDNA First Strand Synthesis Kit (TaKaRa Code : DRR037S). Briefly, the 20μL reverse-transcriptase reaction volume contained 1μg total RNA, 5×PrimeScript Buffer, Prime Script RT Enzyme Mix I, 5μM specific stem-loop RT primers, and RNase free water. The mixture was incubated for 15 min at 42 and 5 sec at 85 . Moreover, the 20μL reverse-transcriptase reaction volume of HDAC4 and SRF contained 1μg total RNA, 5×PrimeScript Buffer, Prime Script RT Enzyme Mix I, 50μM Oligod TPrimer, 100μM Random 6 mers, and RNase free water. The mixture was incubated for 15 min at 37 and 5 sec at 85 . The first-strand cDNAs were then diluted 1:10 with RNase free water and stored at -20 until further use.

Table 1. Primer sequence of predicated target genes (HDAC4 and SRF)

Gene	Forward primer	Reverse primer	Size (bp)
HDAC4	5'ATGTGGTGCTGGTGTCCTCG 3'	5' GCCCATTAGCTGCTTCGTCA 3'	113
SRF	5' CCCTCCTTTCCCATCACC 3'	5' GCCGCTGCCTGTACTCTT 3'	105

Cloning and sequencing of HDAC4 and SRF gene: Of the 2 pairs of primers for sequencing goat HDAC4 and SRF cDNAs were confirmed by subsequent sequencing. The 25μL volume contained: 2μLcDNA, 10μM each primer, 2.5mMdNTP Mixture, 10×EX Taq Buffer, 5UTaKaRaEX Taq HS, and ddH_2O. The cycling protocol was 30s at 94 , 35 cycles of 94 for 30s, 58 for 30s, 72 for 1min, with a final extention at 72 for 5min. The expected sizes of the application can be found in Table 1. They were confirmed by 1% agarose gel electrophoresis stained with Nucleic Acid Dye (TIAN GEN). The applications were gel-purified (AXYGEN, USA) and ligated into the pMD-18T vector (Takara) for sequencing.

Real-Time Quantitative Reverse Transcription PCR (qRT-PCR): Relative quantification of skeletal muscle growth and development-related gene were performed using real-time qRT-PCR assays based on the gene sequences of miR-1, miR-133, HDAC4 and SRF. These qRT-PCR assays were used to assess gene expression in ten normal tissues (heart, liver, spleen, lung, kidney, small intestine, fat, longissimusdorsi muscles, pectoral muscles and crural muscles), and U6 small RNA was used as an internal reference gene. The specific forward and reverse primers employed for real-time qRT-PCR were designed with Primer Premier 5.0 software and are shown in Table 1. For real-time qPCR, a total of 15μL mixture composed of 2μL of diluted template, 10μM of each primer, and Fast Start Universal SYBR Green Master Mix (Rox) (Roche), was run in a PCR reaction plate in triplicate using Step-One real-time PCR system (Applied Biosystems), and the thermal cycling conditions

were 95 for10 min, followed by 40 cycles of a 95 denaturing step for 15 s, and a 60 annealing/extending step for1min. The conditions were similar for all of the primer pairs, and the dissociation curves were run to ensure single product detection. The reaction conditions of dissociation curves were95 for 15 s ; 60 for 1 min ; 95 for 15 s, and 60 for 1 min. All of the samples were tested for cDNAs contamination by running no-reverse transcriptase controls.

Statistical analysis: The relative expression levels of the miR-1, miR-133, HDAC4 and SRF were normalized with internal control (U6 snRNA) expression level, which was calculated using the 2^{-Ct} method (Livak and Schmittgen *et al.*, 2001), among $Ct = Ct_{Target} - Ct_{U6}$; $\triangle\triangle Ct = Ct_{Target} - \triangle Ct_{Calibrator}$. Data were presented as Means ±SE from at least three separate experiments. Statistical significance was determined using one-way ANOVA analyses, in which a P-value of < 0.05 was considered to be significant. All statistical analyses were conducted by using SPSS 17.0 software.

RESULTS

Target sequences: MiR-1 and miR-133 were amplified with specific stem-loop RT products as a template. The size of miR-1 and miR-133 products was about 80bp. In addition, HDAC4 and SRF were amplified with universal cDNA as a template. The applications were gel-purified (AXYGEN, USA) and ligated into the pMD-18T vector (Takara) for sequencing. The fragment length of HDAC4 and SRF products were about 100bp with 2% agarose gel electrophoresis.

Expression profiling of miR-1 and miR-133: To further understand the biological function of miRNAs in goat, we first detected the expression profiles of miR-1 and miR-133 identified in ten goat tissues (heart, liver, spleen, lung, kidney, small intestine, fat, longissimusdorsi muscles, pectoral muscles and crural muscles) by the real-time qRT-PCR. In the gene expression data, the expression of miR-1 and miR-133 in skeletal muscles, heart tissues and other tissues was found to be significantly different ($P < 0.05$) based on comparison with miRNA expression patterns from 0, 3, and 18-month-old Anhui white goats. The results showed that both of the two miRNAs appeared to be tissue specific (**Figure**1). miR-1 and miR-133 were specifically expressed in heart and skeletal muscle (longissimusdorsi muscles, pectoral muscles and crural muscles), which was reported to be strongly expressed in eighteen-month-old cardiac and three-month-old skeletal muscle tissues and play the important role in regulating muscle development. The results also showed that miR-1 and miR-133 had a similar relative expression pattern in some tissues, respectively. In addition, miR-1 and miR-133 had anupregulated trend from newborn to three months old, and their expression levels were reduced with the individual maturing.

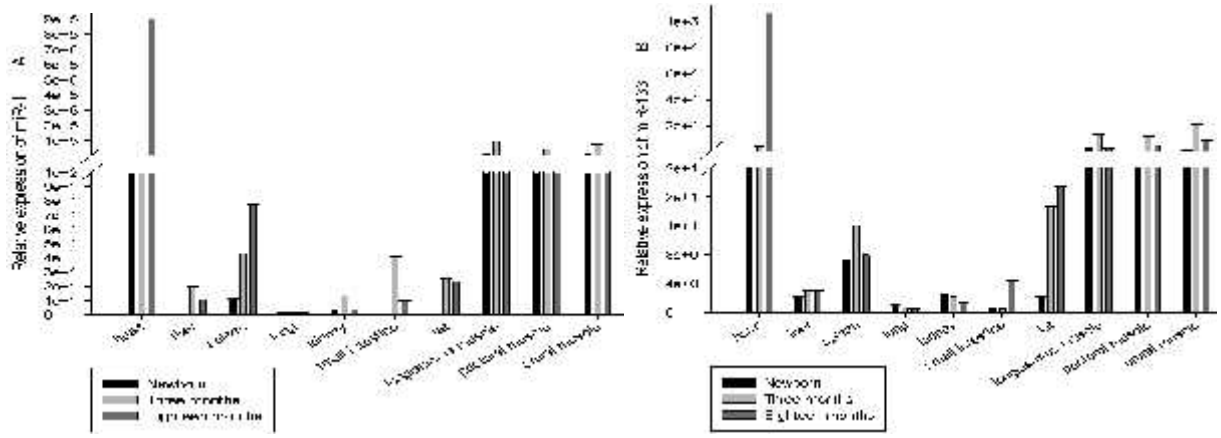

Figure 1 Real-time quantitative reverse transcription-PCR analysis of miR-1 in ten normal tissues (A) and of miR-133 in ten normal tissues (B) during the growth stage (0, 3, and 18 months of age) in Anhui white goats, which exhibit different growth performance. The fold changes were analyzed using the 2^{-Ct} method, with corrections for PCR efficiency. The bars indicate the SE of values from three separate experiments. The reported miR-1 and miR-133 change values indicate significant differences between heart, skeletal muscle (longissimusdorsi muscles, pectoral muscles and crural muscles) and other tissues (0ne-way ANOVA, $P < 0.05$). The data are presented as the mean ± SE (n=3). All of the values were normalized using U6 small RNA as an internal control.

Expression profiling of predicated target genes (HDAC4 and SRF): To validate the differential expression of HDAC4 and SRF in ten normal tissues (heart, liver, spleen, lung, kidney, small intestine, fat,

longissimusdorsi muscles, pectoral muscles and crural muscles), U6 small RNA were selected, and their expression were quantified using real-time qRT-PCR (**Figure**.2). The mean normalized HDAC4 and SRF expression values from each tissue were calculated and expressed as a relative fold change. Both of HDAC4 and SRF could be detected in total tissues. In ten tissues, the expression level of HDAC4 and SRF in lung and small intestine tissues were significantly higher than heart and skeletal muscles tissues ($P<0.05$). In comparison to the high abundance of miR-1 and miR-133 inskeletal muscle tissues, goat HDAC4 and SRF had the least expression. The results also showed that HDAC4 and SRF had anupregulated trend from newborn to three months old, and their expression levels were reduced with the individual maturing, which was similar with miR-1 and miR-133.

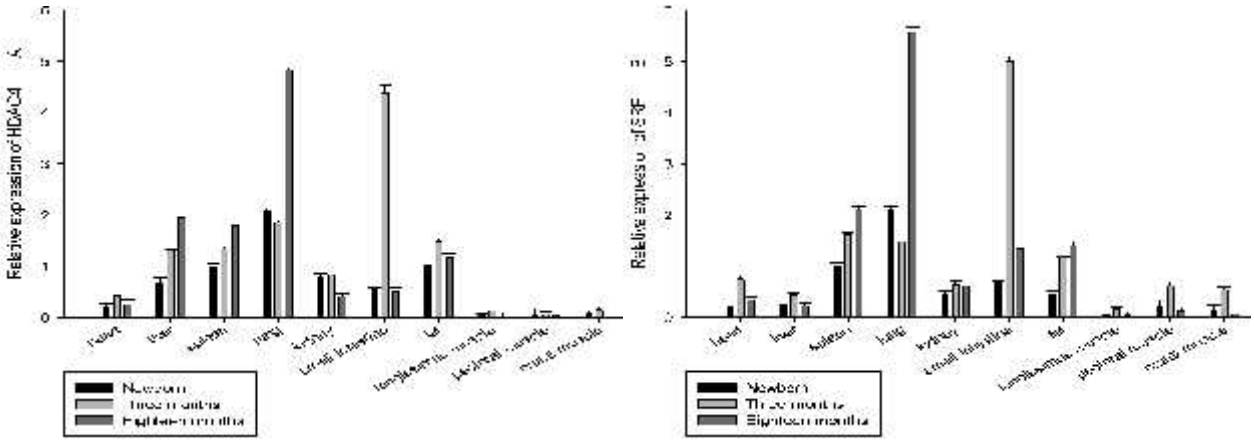

Figure 2 Real-time quantitative reverse transcription-PCR analysis of HDAC4 in ten normal tissues (A) and of SRF in ten normal tissues (B) during the growth stage (0, 3, and 18 months of age) in Anhui white goats. The fold changes were analyzed using the 2^{-Ct} method, with corrections for PCR efficiency. The bars indicate the SE of values from three separate experiments. The data are presented as the mean ± SE (n=3). All of the values were normalized using U6 small RNA as an internal control.

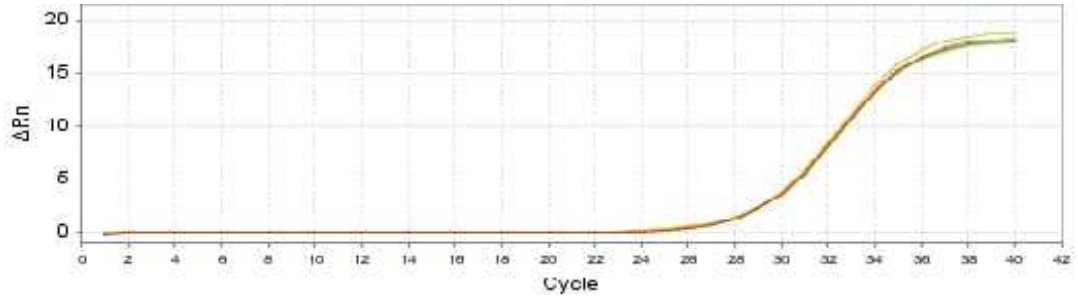

Figure S1.Stype dynamics curve of fluoresecence real-time quantitative amplification

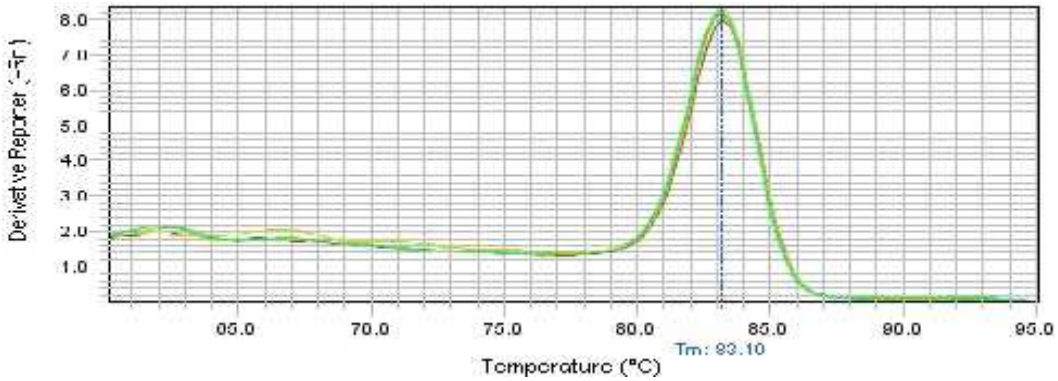

Figure S2. Melt curve peak chart

DISCUSSION

Meat production is a vital characteristic of goat, thus the exploitation of miRNAs involved in regulation the process of muscle development in goat is very important (Sheng *et al.*, 2010). Numerous cellular and developmental processes in various organisms have been reported to be regarded by miRNAs, such as embryonic development, cell proliferation and differentiation, cell apoptosis, and fat metabolism (Kulkarnis *et al.*, 2011; Baley and Li *et al.*, 2012; Nohata *et al.*, 2012; Luo *et al.*, 2013). It is more prevalent for miRNAs to negatively regulate gene expression by promoting degradation of target mRNAs or inhibiting their translation (Podolska *et al.*, 2011). It is a key step to understand miRNAs function by analyzing the expression of tissue-specific miRNAs in different tissues and developmental stages, while the quantitative detection of miRNAs is a premise to study miRNAs function. To date, there are two ways to detect miRNAs expression, such as 3' end plus connectors and stem-loop primers. Due to the stem-loop RT-PCR method is simple, fast, high accuracy, and good specificity that is widely used in miRNAs expression studies.

MiR-1 and miR-133 were initially cloned and identified from human and mouse muscle tissues by Lagos-Quintana *et al* (2001), and were the muscle-specific miRNAs (Sweetman *et al.*, 2008). MiR-1 and miR-133 derived from the same miRNA polycistron and transcribed together can carry out distinct biological functions (Chen *et al.*, 2006). MiR-1 and miR-133 are expressed in muscle tissues and induced during muscle cell differentiation, a process that directs myoblasts to differentiate into mature myotubes, which are organized into myofibers(Koutsoulidoub *et al.*, 2011). Although miR-1 and miR-133 genes have similar expression patterns, they have different biological functions. For example, miR-1 was found to promote muscle cell differentiation, while miR-133 could promote muscle cell proliferation. MiR-1 and miR-133 are highly expressed in skeletal muscles, implying a critical role in the regulation of muscle developmental process.

At present, the functions of most miRNAs remain unclear. Because each miRNA may have many target genes and multiple miRNAs regulate a gene expression at the same time, it is urgent to rapidly and accurately predict and identify the target genes of miRNAs, which is very important to study miRNAs functions. Studies demonstrated that miRNAs regulated cell proliferation and differentiation by regulating some transcription factors and signal transduction factors (Ge *et al.*, 2011; Huang *et al.*, 2012). It has been proved that miR-1 and miR-133 were involved in cell proliferation and differentiation by regulating the expression of target genes (Chen *et al.*, 2006). Hence, to identify target genes that might mediate the observed effects of miR-1 and miR-133 on skeletal muscle proliferation and differentiation, we next examined potential targets of these two miRNAs. Many computational and bioinformatics-based approaches have been used to predict potential target genes of miR-1 and miR-133, such as MiRanda and TargetScan6.2 software. Strikingly, many transcription factors and signal transduction factors have been indicated to be target genes of miRNAs, raising the possibility that miRNAs might be involved in transcriptional regulation of gene expression. Among the predicated target genes of miR-1, miR-1 was shown to bind to the 3'UTR of HDAC4, an inhibitor of muscle differentiation, and suppresses its expression during growth and differentiation conditions (Koutsoulidou *et al.*, 2011). Similarly, miR-133-binding sites are found in the 3'UTR of the mammalian gene encoding SRF, which has been shown to be important in muscle proliferation and differentiation in vitro and vivo (Chen *et al.*, 2006).

qRT-PCR was used to confirm the expression of miR-1, miR-133, and their predicated target genes (HDAC4 and SRF). Using this method, we were able to validate the existence of miR-1, miR-133, HDAC4 and SRF in ten normal tissues (heart, liver, spleen, lung, kidney, small intestine, fat, longissimusdorsi muscles, pectoral muscles and crural muscles) of Anhui white goats. In this study, miR-1 and miR-133 are induced during goat muscle cell differentiation and their expression levels are increased from newborn to three months old, and decreased with the individual maturing. That indicated miR-1 and miR-133 was necessary during the muscle rapid growth, which could promote bulk copy of fibroblasts. The expression levels of miR-1 and miR-133 were relatively reduced when the process of myoblasts proliferation and differentiation was weakened. While the expression changes of miR-1 and miR-133 suggested that miR-1 and miR-133 may be related to the development of goat skeletal muscles. However, their predicated target genes were found to be highly expressed in fat and small intestine tissues, while there was the least expression in skeletal muscles.

In our study, miR-1 and miR-133 and their target genes (HDAC4 and SRF) represented negative correlation of expression patterns by qRT-PCR. MiR-1 and miR-133 was the most abundance in goat skeletal muscles. And the target genes of miR-1 and miR-133, HDAC4 and SRF predicated to be muscle transcription factors which involved in muscle proliferation and differentiation were the least abundant transcript in skeletal muscles. Therefore, our study suggested that the increase of miR-1 and miR-133 expression may lead to decrease of HDAC4 and SRF expression. And more studies will need to be done to determine the regulation patterns of miR-1 and miR-133 and their predicated target genes.

Although miR-1, miR-133, HDAC4 and SRF have been extensively studied, there is no information about their expression of goat skeletal muscle. Results

presented in this study show that miR-1 and miR-133 are induced during goat muscle cell differentiation and their expression levels are increased proportionally to the stage of muscle neonate development. And miR-1 and miR-133 and their predicated target genes (HDAC4 and SRF) represented negative correlation. Since miRNAs regulate important processes during skeletal muscle development, such information would be very important for understanding the mechanism of muscle formation during neonate development.

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