Research Article

Identification and comparison of microRNAs in pituitary gland during prenatal and postnatal stages in sheep by deep sequencing

Xiaoyue Li[1], Cunyuan Li[1], Wei Ni[1,*], Dawei Wang[1], Xiaoxu Hou[1], Zhijin Liu[1], Yang Cao[1], Yang Yao[1], Xiangyu Zhang[1], Shengwei Hu[1,*]

[1]College of Life Sciences, Shihezi University, Shihezi, Xinjiang, 832003, China

Running head: MicroRNA in pituitary gland of sheep

*Corresponding author: Email: niweiwonderful@sina.com; E-mail: hushengwei@163.com

Abstract: MicroRNAs are a class of short-chain RNA molecules of ~22 nucleotides in length and regulate gene expression at post-transcriptional levels by interacting with mRNAs. Although many microRNAs have been identified, the expression and function of microRNAs in the pituitary gland of sheep are unclear. In this study, the identity and abundance of microRNAs were determined in sheep pituitary gland of prenatal and postnatal stages. We showed that 107 microRNAs are significantly ($p<0.05$) differentially expressed in pituitary glands between prenatal and postnatal stages, and 44 new microRNAs candidates were found according to a series of filtration criteria. Reverse transcription PCR (RT-PCR) and DNA sequencing analysis confirmed the presence of several microRNAs. Real-time RT-

PCR analysis showed that sheep microRNAs were expressed in prenatal and postnatal pituitary glands. We found that microRNAs were involved in hormone synthesis, secretion and signaling pathway regulation by gene ontology (GO) and KEGG concentration analysis. Our study provides valuable resources for comprehensive investigation of microRNAs in pituitary gland and microRNAs biology of sheep.

Keywords: Sheep, MicroRNAs, Pituitary gland, Expression profiles, Deep sequencing

Introduction

Pituitary is an oval body and located in the ventral part of the hypothalamus, including the anterior and posterior leaves. Both sides of the pituitary are surrounded by the cavernous sinus and optic nerve bundle passes through the channel (Landgraf *et al*. 2007). The anterior pituitary is the most important endocrine glands in mammals (Lupu *et al*. 2001). It produces many hormones which are not only related to the growth of bones and soft tissue of the body, but also affects the development and hormone secretion activities of endocrine glands in lactation and adolescent. These important hormones includes growth hormone (GH), thyroid stimulating hormone (TSH) and follicle stimulation hormones (FSH) and so on (Müller *et al*. 1999; Lupu *et al*. 2001). The posterior pituitary is a neurohypophysis, which doesn't contain glandular cells and can't synthesize hormones. It secretes neurohypogenic hormones that are produced by neurons. Obviously, pituitary hormone synthesis and secretion are a complex regulatory process. Some studies have shown that 157 genes are associated with key pathways in the pituitary of developmental stages (Brinkmeier *et al*. 2009), and 222 microRNAs (miRNAs) have been identified from pituitary glands and may affect postnatal growth of pigs (Ye *et al*. 2015).

MiRNAs are a class of single-stranded endogenous non-coding RNA molecules that are highly conserved present in most eukaryotes. MiRNAs are capable of modulating gene expression at post-

transcriptional levels (Bartel 2004; Kim and Nam 2006; Kloosterman and Plasterk 2006). Maturation of miRNAs relies on two types of ribonucleases (RNase) III called Drosha and Dicer (Le *et al*. 2003). Matured miRNAs are incorporated into a ribonucleoprotein complex, called RNA-induced silencing complex (RISC), and achieves post-transcriptional gene regulation by specifically targeting 3' untranslated regions of messenger RNA (mRNA) (Novina and Sharp 2004; Bushati and Cohen 2007). The interaction between miRNAs and mRNAs results in degradation of mRNA or incomplete binding of targeted mRNA in animals (Ambros 2004; Valencia-Sanchez *et al*. 2006; Wu *et al*. 2006). Current research has found hundreds of genes encoding miRNAs in animals. These studies have shown that miRNAs are involved in many biological processes, such as cell growth, differentiation, proliferation, and immune response (Landgraf *et al*. 2007; Rodriguez *et al*. 2007).

Sheep is an important economic animals in agricultural production and model animals of medical research (Porada *et al*. 2004; Jäger *et al*. 2011). However, the molecular genetic experiments of sheep are limited due to the lack of in-depth understanding of the sheep gene sequence. So far, we still do not know the expression and function of miRNAs in the pituitary gland of sheep. In this study, we first used Illumina HiSeq 2500 technology to investigate the gene expression profiles of miRNAs in prenatal and postnatal pituitary glands of sheep. These data will contribute to further function analysis of miRNAs in the development and growth of the pituitary gland.

Materials and methods

Ethics Statement

All procedures involving animals were approved by the Animal Care Committee of Shihezi University. The study was performed in accordance with the ethical standards laid down the declarations of Helsinki in 1964 and its later amendments.

Animals

We obtained three adult Kazakh sheep (female) which have been slaughtered from a commercial slaughterhouse in Shihezi City. We obtained three Kazak sheep fetuses (130 days after mating of ewes) from the experimental animal center of Shihezi University by surgery. Under sterile conditions, pituitary glands were separated and immediately frozen in the liquid nitrogen until the RNA isolation.

Library construction and miRNAs sequencing analysis

Briefly, the total RNAs were isolated from frozen pituitary tissues by using TRIzol reagent (Invitrogen, CA, USA) and following the manufacturer's protocol. The RNA quality and the concentration of all samples were tested by using the Bioanalyzer 2100 and RNA6000 Nano Kit (Agilent, CA, USA). The Agilent 2100 was used to accurately detect RNA integrity (Santa Clara, CA, USA). The equivalent concentration of RNAs from three sheep were pooled for constructing the library by using the Small RNA Sample Pre Kit (Invitrogen, CA, USA). MiRNA analysis were performed following flow chart (Figure 1a). Prior to analysis, adaptor reads and low quality reads were removed, and other reads were aligned to sheep reference genome (http:/genome.ucsc.edu/) by using Tophat package.

RT-PCR analysis and DNA sequencing

Total RNAs were extracted from pituitary glands of sheep by using TRIzol (Invitrogen, CA, USA). From purified RNAs, RT-PCR of miRNAs was performed by using stem-loop RT primer (Chen *et al*. 2005). First-strand cDNA synthesis from total RNA was performed with 1µg total RNA, 10 µM each stem-loop RT primer and oligo (dT) primer by using RT-PCR Kit (Takara, Dalian, China) according to manufacturer's protocol. RT-PCR of co-expression mRNAs was performed by oligo (dT) primer by using RT-PCR Kit (Takara, Dalian, China) according to manufacturer's protocol. PCR was carried out using specific primers for miR-143, miR-27, miR-361, miR-129, miR-370, *Kras, PHB, FSHB, CDK6* and *Dnmt3a*. All the Primer sequence were listed in Additional file 1. PCR products were analyzed by gel electrophoresis. Sequence data from PCR products were compared with sheep reference genome and

RNA-seq data by DNAMAN software. PCR was carried out by using the following reaction system: 10 µl Premix (Takara, Dalian, China), 2 µl cDNA, 0.6 µl upstream and downstream primers, respectively, RNase-free ddH$_2$O water 6.8 µl. PCR was performed with the following thermocycling conditions: An initial 3min at 95˚C, followed by 45 cycles of 95˚C for 10s, 58˚C for 15s and 72˚C for 5s.

Real-time RT-PCR analysis

Co-expression analysis of 5 miRNAs (miR-143, miR-27, miR-361, miR-129 and miR-370) and their target mRNAs (*KRAS, FSHB, PHB, CDK6* and *Dnmt3a*), which were related to the development and function of the pituitary, were performed by Real-time RT--PCR. The housekeeping gene β-actin was used as internal reference genes. Total RNAs were directly subjected to cDNA synthesis with a RT-PCR kit (Takara, Dalian, China). Real-time RT-PCR was performed by using SYBR Green (TaKaRa Biotech, Dalian) according to the manufacturer's protocol. Real-time RT-PCR reaction mix consisted of 2 µL cDNA, 10 µL 2×SYBR Premix DimerEraser (TaKaRa Biotech, Dalian), 0.6 µL the upstream and downstream primers, respectively, and 6.8 µL RNase-free ddH$_2$O. The reaction conditions was as follows: 30s at 95˚C, 55 cycles of 10s at 95˚C, 15s at 54˚C and 72˚C for 5s, then extend 10s at 72˚C to generate a melt curve to control the specificity of the amplified product.

Target gene prediction and enrichment analysis

The predictions of animal miRNA targets are computationally difficult relative to plants because animal miRNAs are relatively short and are only complementary to their mRNA target moiety, it possibly due to additional interactions involving RNA binding proteins. Enright *et al*. developed the MiRanda program to rigorously screen target genes for animal miRNAs, and the program selects the target gene for each miRNA based on three properties: sequence complementarity using a position-weighted local alignment algorithm, free energies of RNA-RNA duplexes, and conservation of target sites in related genomes (Enright *et al*. 2003). The method is validated by using known examples. MiRanda was used

for analyzing the interaction between miRNAs and mRNAs (Betel *et al*. 2008; Enright *et al*. 2003; Li *et al*. 2013). The main stringency criteria for the MiRanda (miRanda-3.3a) program are as follows: -sc S Set score threshold to S [DEFAULT: 140.0]; -en -E Set energy threshold to -E kcal/mol [DEFAULT: 1.0]; -scale Z Set scaling parameter to Z [DEFAULT: 4.0]; -strict Demand strict 5' seed pairing [DEFAULT: off]; -loose Remove strict duplex heuristics [DEFAULT: out] (Other unlisted parameters use default parameters). We used the DAVID software to carry out gene ontology (GO) analysis of the target genes of miRNAs (Ashburner *et al*. 2000). The KEGG enrichment analysis of the target genes of the miRNAs were carried out by using KOBAS software (Kanehisa *et al*. 2007). Score (*P < 0.05*) was considered to be significant for enrichment analysis.

Results

Identification of miRNAs expression in sheep pituitary gland by deep sequencing

To confirm miRNAs expression patterns in sheep pituitary gland, we examined the expression, abundance and difference of miRNAs in sheep pituitary glands according to the flow chart (Figure 1a). By means of RNA-seq of sheep embryo pituitary gland (PG_E) and adult pituitary gland (PG_A), 14436357 and 14680329 reads are obtained from the sequencing results respectively. In order to ensure the quality of bioinformatics analysis, a total of 14256079 (PG_E) and 14505228 (PG_A) clean reads were obtained by removing low quality and adapter sequence reads. The distribution peak of the total sRNAs fragments of clean reads was 22 nt in all samples (PG_E and PG_A) (Figure 1b), which was consistent with the common size of the miRNAs in 21 to 22 nt. Then, we put these screened sRNAs positioning to the reference sequence with bowtie (Langmead *et al*. 2009), and a total of 12920436 (PG_E) and 12927117 (PG_A) reads were mapped to the reference sequence. A total of 1075 miRNA matures were perfectly mapped in miRBase (Griffiths-Jones *et al*. 2006). These miRNAs are evenly distributed over 26 autosomes and X chromosomes (Figure 1c). The correlation analysis showed that the expression of miRNAs was quite similar in the pituitary glands of these two stages (Pearson correlation

coefficient, PG_E and PG_A r = 0.947) (Figure 1d).

Analysis of known miRNAs

We obtained the secondary structure of miRNAs that could match the known miRNAs by sequence alignment, which were consistent with previously reported. When the miRNA precursor was developed into a mature body, it had been digested by Dicer. Due to the specificity of the enzyme cleavage site, the first base of the miRNAs maturation sequence has a strong bias. Thus, we analyzed the nucleotide frequency distribution at each position of 22 nt miRNAs (Figure 2a) and the nucleotide frequency distribution at the first base position of different length of miRNAs (Figure 2b), respectively. These miRNAs are focused on this length in sheep embryo pituitary gland (PG_E) and adult pituitary gland (PG_A).

Forecast and analyze novel miRNAs

The hairpin structure of miRNA precursor could be used to predict new miRNAs. We used miREvo (Wen *et al*. 2012) and mirdeep2 (Friedländer *et al*. 2011) software for the prediction and analysis of novel miRNAs. We found a total of 44 novel miRNAs, including 40 novel miRNAs in embryo pituitary (PG_E) and 38 novel miRNAs in adult pituitary (PG_A). The secondary structure of novel miRNAs was explored by intercepting certain length of sRNAs that were aligned with the reference sequence, such as novel_100, novel_104 and so on (Figure 3a). We analyzed the nucleotide base distribution of novel 22 nt miRNAs (Figure 3b) and the first nucleotide base bias of different length of miRNAs (Figure 3c).

Differentially expressed miRNAs between embryo pituitary gland (PG_E) and adult pituitary gland (PG_A) of sheep

To further understand the miRNAs in the pituitary gland, statistical analysis of the expression levels of known and novel miRNAs in each sample was performed and normalized with TPM (Zhou *et al*.

2010) (Figure 4a), and the gene expression pattern of these samples was examined by TPM density distribution (Figure 4b). We found 107 differentially expressed miRNAs in embryo pituitary gland (PG_E) and adult pituitary gland (PG_A), including 61 up-regulated and 46 down-regulated miRNAs (Figure 4c). In order to determine the clustering pattern of differentially expressed miRNAs in both groups, the TPM values of miRNAs obtained from each comparison combination were used for cluster analysis of differential miRNAs.

Enrichment of differentially expressed miRNAs

These known and novel miRNAs target genes were predicted by miRanda software, and we got the corresponding relationship between miRNAs and their target genes (Additional file 2). Some important miRNAs target genes and their functions were shown in Table 1. In order to understand the functions of differentially expressed miRNAs, we used GO and KEGG pathways to enrich the differentially expressed miRNAs target genes. 419 terms were significant (*P <0.05*) enrichment in biological processes, molecular functions and cell composition categories by GO analysis (Figure 5a), which suggested that some miRNAs might be involved in important biological regulation of the pituitary. We analyzed the possible biological pathways which were affected by differentially expressed genes by analyzing the potential KEGG pathway (Figure 5b) between two groups for enrichment of differentially expressed miRNAs target genes A total of 275 enriched terms (Additional file 3) were involved in metabolism, disease, cell transmission and endocrine regulation. It is noteworthy that all of these, including Gap junction, Amoebiasis, Inflammatory bowel disease (IBD), Lysosome Proteasome and ABC transporters, were related to the growth, development, disease and function of pituitary glands. These results suggested that miRNAs might play an important role in the pituitary gland.

Validation of miRNA and their target genes by Real-time RT-PCR

In order to validate the sequencing data in the pituitary gland of sheep, we randomly selected 5 differentially expressed miRNAs (including miR-143, miR-27, miR-361, miR-129 and miR-370) by using RT-PCR and DNA sequencing. The results of RT-PCR amplification showed a single size of the expected band (Figure 6a). At the same time, we used the same method to validate the target genes (including *KRAS, FSHB, PHB, CDK6* and *Dnmt3a*) (Figure 6b) of these miRNAs. DNA sequencing results confirmed that these products were our amplified miRNA and their target genes (The data is not listed). The co-expression analysis of these miRNAs and their target mRNAs in the pituitary gland of sheep were performed by Real-time RT-PCR. As shown in Figure 6d, expression levels of miR-143 and miR-370 were higher in the embryonic group (PG_E) than in adult group (PG_A). In contrast, miR-27, miR-361, miR-129 were higher expressed in the adult group (PG_A) compared with that of the embryonic group (PG_E). The results showed that there was a strong agreement between Real-time RT-PCR and RNA-seq data (Figure 6c and Figure 6d). The Real-time RT-PCR analysis for their target gene showed that there was a strong and consistent correspondence between miRNAs and their predicted target genes, except miR-129 (*CDK6*). The results showed that the identified miRNAs largely reflected the true differential expression in vivo and target genes of identified miRNA were accurately predicted.

Bioinformatics analysis of miRNA-mRNA networks

A large number of studies have shown that miRNAs have the function of regulating pituitary growth and development by interfering with mRNA. To further expound the function of miRNAs in the pituitary gland growth and development, the basic miRNA-mRNA connectivity was established by TargetScan and miRanda. All the differentially expressed miRNAs were predicted and used for further study. A network of miRNA-mRNA interaction between the miRNAs and their target genes was constructed by using Cytoscape (Figure 7). These information might help us to explore the underlying mechanisms of miRNAs involved in the growth and development of pituitary gland in sheep.

Discussion

At present, some breeds of the sheep genome have completed annotation and assembly, but transcriptome research still needs more work. Thousands of unique miRNAs have been identified in different tissues and cell types (Clop *et al*. 2006; Sheng *et al*. 2011; Barozai *et al*. 2012; McBride *et al*. 2012). Although many miRNAs have been shown to be closely associated with animal growth, development, disease and immune regulation, the role of miRNAs in the growth and development of pituitary glands and pituitary regulation is still unclear. As the most complex endocrine gland in mammals, the pituitary gland has strong and important endocrine effect. Pituitary glands produce a number of regulatory factors that are involved in important basic biological processes such as animal development, growth, and so on. In this study, the miRNAs expression profiles of the pituitary gland were analyzed by high-throughput sequencing and bioinformatics in embryos and postnatal sheep, a total of 1075 miRNAs were identified. Rui-Song Ye *et al*. (Ye *et al*. 2015) reported 222 miRNAs in the anterior pituitary of different strains of pigs, and these miRNAs may regulate the different growth conditions of different strains of pigs.

As non-coding RNA with extensive regulation at the post-transcriptional level, miRNAs are involved in many physiological and pathological processes such as development, growth, and disease occurrence. Through this study, we identified 107 differentially expressed miRNAs between the embryonic and adult pituitary glands. Compared with the adult pituitary gland, there are 61 upturned miRNAs and 46 down miRNAs in the embryo pituitary gland. We further confirmed these differentially expressed miRNAs and their target genes (related to pituitary development and function) by Real-time RT-PCR . These miRNAs may play an important biological role in pituitary growth and development and hypothalamic-pituitary secretion regulation axis of adult pituitary. Some studies have reported that changes in the expression of many genes and non-coding RNAs influence the regulation of adult pituitary hormone secretion (Brinkmeier *et al*. 2009; Shan *et al*. 2013; Ye *et al*. 2013). Zhang *et al*. reported that some miRNAs may involve in the development of mouse pituitary gland (Zhang *et al*.

2010). Bottoni *et al*. have shown that miRNAs expression may be involved in pituitary tumorigenesis (Bottoni *et al*. 2007). Ye *et al*. explained that the expression of miRNAs in pituitary cells changed (Ye *et al*. 2013) when the pituitary cells of the pig were treated with hypothalamic gonadotropin-releasing hormone (GnRH). Therefore, we speculated that miRNAs might play an important role in development and endocrine of the pituitary gland in prenatal and postnatal sheep.

Many studies have shown that miRNA plays an important post-transcriptional regulation by targeting or cleavaging targeted mRNA (Zanette *et al*. 2007). We used miRanda software to obtain the correspondence between some miRNAs and their target genes. A lot of miRNAs associated with pituitary function are predicted, such as miR-181, miR-133, etc (Lee *et al*. 2006; Chu *et al*. 2015). Since these miRNAs can interact with many target genes, they may play a broad regulatory role in the pituitary biology process. The function of these miRNAs will be further analyzed in the future.

We know that BMP, FGF, WNT, SHH and NOTCH pathways are related to pituitary development and endocrine function. However, the role of miRNAs in the pituitary of different periods is limited. In our study, we found that miRNAs play an important role in pituitary disease, growth and development by the enriched KEGG pathway and GO pathway. Especially Amoebiasis, Gap junction, Inflammatory bowel disease (IBD), Lysosome, ABC Transporter (Smith and Farquhar 1966; Becker Jr *et al*. 1980; Cardozo 1993; Morand *et al*. 1996; Straub *et al*. 2002; Mitsuishi *et al*. 2013), etc. The results suggested that miRNAs might be associated with regulation of adult pituitary hormone secretion (Treier *et al*. 2001). However, function of miRNAs in the pituitary gland and its target prediction and analysis will be carefully verified through further experiments.

In summary, we understood the expression profile of miRNAs in the pituitary through this study, and screened out some differentially expressed miRNAs in the prenatal and postnatal pituitary gland of sheep. At the same time, some of the miRNAs involved in pituitary development, endocrine regulation and potential pituitary disease were found by GO and KEGG pathway analysis. Our study provides valuable resources for in-depth understanding of miRNA functions and categories in pituitary gland.

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Additional Information

Additional file 1. All the Primersequence for RT-PCR and Real-time RT-PCR.

Additional file 2. Predict the target gene of miRNAs.

Additional file 3. KEGG pathway analysis demonstrated 275 terms were enriched.

Competing Interests: The authors have declared that no competing interests exist.

Authors' contributions

XYL, CYL, WN and SWH conceived and designed the experiment, analyzed and interpreted the data. XYL, CYL, DWW, WN and SWH wrote the manuscript. XXH, ZJL, YC, XYZ, YY collected sheep skeletal muscle sample. All authors read and approved the final manuscript.
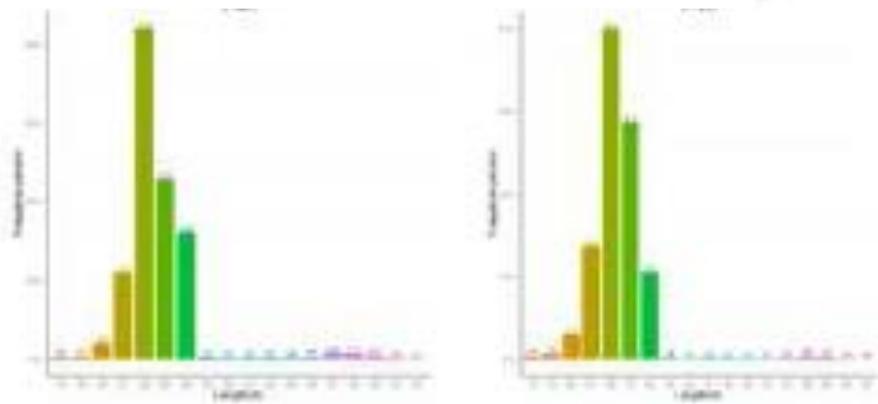
Data Availability

The datasets generated during analyzed and/or during the current study are available from the corresponding author on reasonable request.
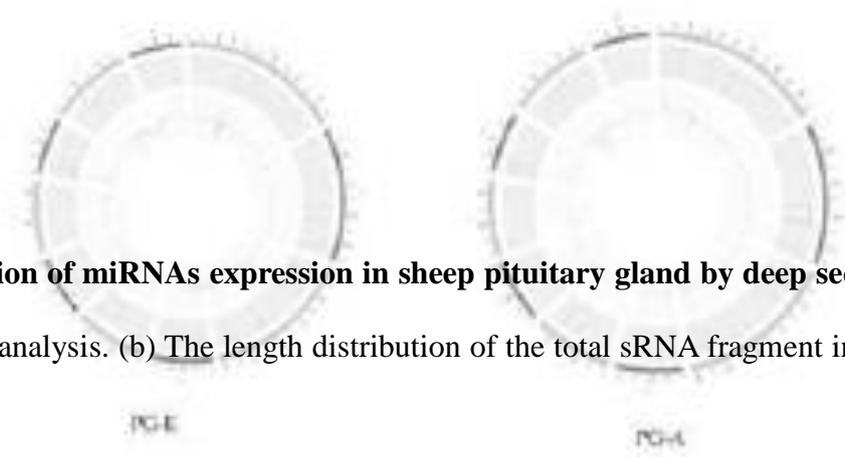
Fig. 1

a

b

c

Fig. 1 Identification of miRNAs expression in sheep pituitary gland by deep sequencing. (a) Flow chart of miRNAs analysis. (b) The length distribution of the total sRNA fragment in the clean reading.

PG-E   PG-A

d

Pearson correlation between samples

(c) The distribution of miRNAs on chromosomes. The outer circle is the chromosome selected for the display; the gray background area in the middle is the distribution of the 10000 reads, the red mapping to the positive chain, the blue mapping to the negative chain; the inside of the circle area is mapped to all the chromosomes Reads, the orange-red is the distribution of the chain, the green is the negative chain coverage, and the singular points above the standard deviation of all coverage sets +3 times the standard deviation will be discarded. (d) Correlative Analysis of Gene Expression Level in Samples. (The abscissa and ordinate are the square of log10 (TPM + 1); R^2 is the square of the pearson correlation coefficient).

Fig. 2 The information of known miRNAs. (a) The nucleotide bases distribution of 22 nt long known miRNAs. The abscissa is the base position of the miRNA, and the ordinate is the percentage of base A / U / C / G in the position where the miRNA appears. (b) The nucleotide bases distribution of the first position of different length known miRNAs. The abscissa is the length of the miRNA, and the ordinate is the percentage of A / U / C / G in the first base of the length of the miRNA (the value above the column is the total number of miRNAs).

Fig. 3

a

Fig. 3 Forecast and analyze novel miRNAs. (a) The secondary structure of novel miRNAs in sheep pituitary gland. The entire sequence is a miRNA hairpin, and the miRNA mature sequence is located in red. (b) The nucleotide bases distribution of novel 22 nt miRNAs. The abscissa is the base position of the miRNA, and the ordinate is the percentage of base A / U / C / G in the position where the miRNA appears. (c) The nucleotide bases distribution of the first position of different length novel miRNAs. The abscissa is the length of the miRNA, and the ordinate is the percentage of A / U / C / G in the first base of the length of the miRNA (the value above the column is the total number of miRNAs)

Fig. 4 Analysis and validation of differentially expressed miRNAs in sheep PG_E and PG_A. (a)

TPM box chart of different groups in sheep PG_E and PG_A. The abscissa is the name of the group, the

ordinate is log10 (TPM), and the box graph for each region is the five statistic (top to bottom, respectively, the maximum, the upper quartile, the median, the under quartile and the minimum). (b) The TPM density profile of different groups. The abscissa is the log10 (TPM) value of the gene, the ordinate is the density corresponding to log10 (TPM), and the different colors represent different groups. (c) The Volcano Plot of analysis volcano plot of all differentially expressed miRNAs in sheep PG_E and PG_A. The logarithm of the significant difference between the two samples was analyzed by log2 (fold change) as the abscissa, and the negative logarithm-log10 (P-value) of the P value was calculated as the ordinate (P<0.05). Red dots indicate genes that were up regulated in PG_A; green dots indicate down regulated genes; the blue dots indicate no significant difference.

Fig 5

a

Enriched GO Terms
(P3EvsPGA)

Fig. 5 Differentially expressed miRNAs annotations and enrichment in sheep PG_E and PG_A.

(a) The GO analysis showed 419 significantly (PG_A VS PG_E) enriched terms (P < 0.05). The abscissa

is the enriched GO Term, and the ordinate is the number of candidate target genes annotated to the term.

Different colors are used to distinguish three major categories of GO Term, red for biological process

(BP), blue for cell composition (CC) and green for molecular function (CC). (b) 275 terms were enriched

of differentially expressed miRNAs in sheep PG_E and PG_A by KEGG pathway analysis. The Gap

junction, Amoebiasis, Inflammatory bowel disease (IBD), Lysosome, Proteasome and ABC transporters

are labeled with red lines. Q value is the statistical testing of p values, and the value of q is indicated by

red to blue (From small to large). The number of genes is displayed in circle size.
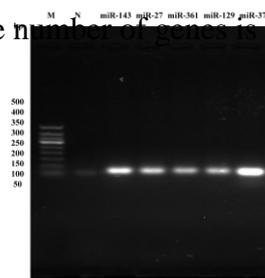
Fig. 6

Fig. 6 Validation of miRNA and their target genes. (a) RT-PCR amplification of miRNAs with stem-

loop RT primer and specific primers. PCR products of miR-143，miR-27，miR-361, miR-129 and miR-

370 were analysed by gel electrophoresis. (b) RT-PCR amplification of target genes with specific primers.

PCR products of *KRAS*，*FSHB*，*PHB*，*CDK6* and *Dnmt3a* were analysed by gel electrophoresis. M is

Marker (Tiangen50bp DNA Ladder: 500 bp, 400 bp, 350bp, 300 bp, 250bp, 200 bp, 150 bp, 100 bp, and

50 bp), and N is the negative control. (c) Change in miRNA levels between the PG_E and PG_A groups. PG_E/PG_A ratios for 5 different miRNAs based on the Illumina next-generation sequencing (RNA-seq) data. (d) Expression of differentially expressed genes and their target genes as determined by Real-time RT-PCR. Error bars indicate ± SD.
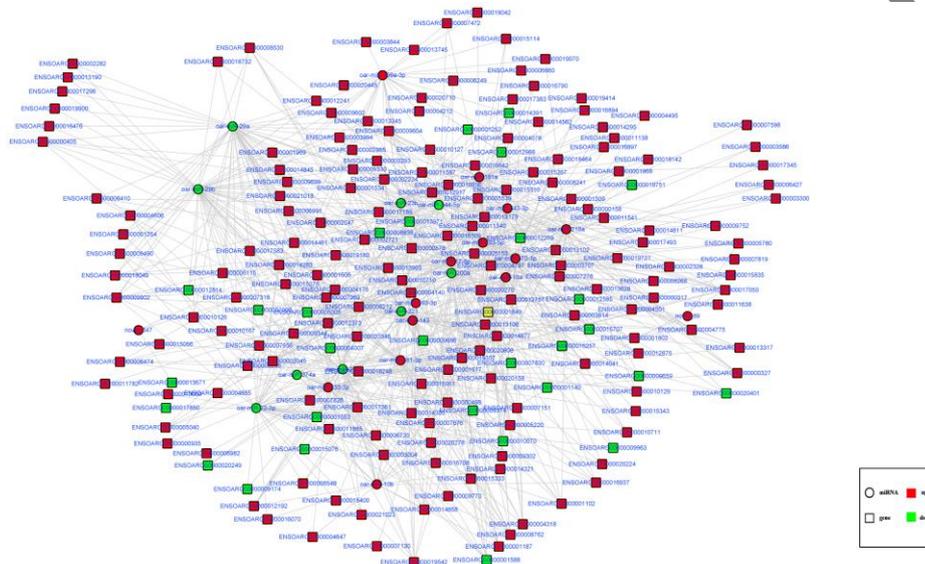
Fig 7

Fig. 7 The miRNA-mRNA networks. In the panorama network, circle nodes represent miRNAs and rectangle nodes represent genes. Red color and green color represents up and down regulation respectively.

Table 1.**Potential target forthe partial important miRNAfamiliesand their function.**

miRNA	Target gene	Target gene function

miR-20	*CYP2E1*	CYP2E1 is a member of the cytochrome P450 mixed-function oxidase system
novel_122	*DDX17*	Probable ATP-dependent RNA helicase DDX17 (p72) is an enzyme
miR-375	*HuD*	Predominantly neuronal RNA-binding protein
miR-361	*FSHβ*	Follicle-stimulating hormone (FSH) is a gonadotropin, a glycoprotein polypeptide hormone. It regulates the development, growth, pubertal maturation, and reproductive processes of the body reproductive system.
miR-27b	*PHB*	Prohibitin(PHB)
miR-132	*SIRT1*	SIRT1, a NAD-dependent deacetylase, has diverse roles in a variety of organs such as regulation of endocrine function and metabolism.
miR-143	*Kras*	Control the regulation of cell growth pathways, and involve in intracellular signaling
miR-181	*SKI*	SKI proto-oncogene is a membrane-bound proteinase，and it is a Ca2+-dependent serine proteinase exhibiting a wide pH optimum for cleavage of pro-brain-derived neurotrophic factor.
miR-493	*LGALS3*	LGALS3 is a member of the lectin family and the beta-galactoside-binding protein family that plays an important role in cell-cell adhesion, cell-matrix interactions, macrophage activation, angiogenesis, metastasis, apoptosis.
miR-370	*Dnmt3a*	DNA (cytosine-5)-methyltransferase 3A is an enzyme that catalyzes the transfer of methyl groups to specific CpG structures in DNA,
miR-19	*SLC13A1*	The solute carrier (SLC) group of membrane transport proteins