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Cloning, Characterization and Bioinformatics Analysis of the Sequences of miR-10a and miR-10b in Sheep (*Hu sheep*)

Zhibo Wu[1], Zhenhan Zhai[1], Mengyao Wang[1], Hongxiang Ding[1], Huibin Shi[1], Zhilong Tian[1], Xiaohui Zhang[1], Yuqin Wang[1], Caihong Wei[2], Fadi Li[3]

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ABSTRACT

Background: MicroRNAs (miRNAs) are active regulators of numerous biological and physiological processes and play an important role in the regulation of animal ovaries and other reproductive related organs. To understand the molecular mechanisms of miR-10 family, we investigated the molecular characteristics and the relative expression of sheep miR-10a and miR-10b (miR-10a/b) and conducted bioinformatics analysis.

Methods: During the period 2018-2019 total of 20 samples including blood and tissues such as hypothalamus, pituitary and ovary were collected from the Hu sheep raised in the National Meat Sheep Experimental Station of China (Luoyang City, Henan Province, China). Blood was collected from jugular vein by vacuum and anticoagulation blood collection tube and stored in refrigerator at -20°C. The tissues were placed into the cryopreservation tube treated with Diethy pyrocarbonate (DEPC) water and stored in liquid nitrogen. All the samples were processed for isolation and confirmed with biochemical analysis and Polymerase chain reaction (PCR) and Real-time fluorescence quantitative PCR.

Result: The target genes were predicted by three kinds of target gene predicting software. The function of target genes and their involved pathways were obtained by gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The relative expression of miR-10a/b in sheep ovary was extremely significantly higher than that in hypothalamus and pituitary gland ($P<0.01$). The relative expression of miR-10b in ovary or pituitary was extremely significantly higher than that of miR-10a ($P<0.01$) and the relative expression of miR-10b in hypothalamus was significantly higher than that of miR-10a ($P<0.05$). These results serve as a foundation for further study on the Sheep miR-10 family.

Key words: Bioinformatics analysis, Expression level, Hu sheep, miR-10a, miR-10b.

INTRODUCTION

MicroRNA (miRNA) was first found in *C. elegans* in 1993 (Lee *et al.,* 1993). It is a kind of small molecular non-coding RNA, with a length of about 22 nt, which causes the degradation or inhibition of target mRNA translation by binding to the 3'UTR of the target mRNA (Bartel, 2004). miRNA is involved in cell proliferation and differentiation, biological development and metabolism in the process of biological growth, in addition, miRNA also plays some roles in the occurrence of cancer (Kloosterman, 2004; Liu *et al.* 2020). Recent studies have revealed that miRNA plays an important role in the regulation of animal ovaries and other reproductive related organs (Yeruva *et al.,* 2016; Toms, *et al.,* 2018). Some studies have also shown that miR-10a can regulate reproductive function by regulating the secretion of steroid hormone in human ovarian granulosa cells (Sirotkin *et al.,* 2009). miR-10b can inhibit brain-derived neurotrophic factor (BDNF), which can regulate follicular development (Varendi, *et al.,* 2014; Dissen, *et al.,* 2002). miR-10b also regulated the function of an inhibitor of follicular atresia, the cytochrome P450 family 19 subfamily A member 1 (CYP19A1) gene, by inhibiting its expression (Li *et al.,* 2018).

In this study, the relative expression of miR-10a and miR-10b (miR-10a/b) in hypothalamus, pituitary and ovary of Hu sheep was determined by fluorescence quantitative PCR. Moreover, the conservation and evolution of sequences were analyzed and the target genes were predicted. Through gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, we explored the possible function of miR-10a/b target genes and the involved signaling pathways. This study will create a foundation for further revealing the regulation of sheep miRNA on reproductive function.

[1]School of Animal Science and Technology, Henan University of Science and Technology, Luoyang-471 003, China.
[2]Key Laboratory of Animal Genetics and Breeding and Reproduction of Ministry of Agriculture, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing-100 193, China.
[3]College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou-730 020, China.

Corresponding Author: Yuqin Wang, School of Animal Science and Technology, Henan University of Science and Technology, Luoyang-471 003, China. Email: wangyq6836@163.com

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MATERIALS AND METHODS

All experimental procedures involving sheep followed the policies and guidelines on the Henan University of Science and Technology Animal Care and use Committee.

Experimental animals

Five sheep (Hu sheep) were from the flock raised in the National Meat Sheep Experimental Station of China (Luoyang City, Henan Province, China). Blood was collected from jugular vein by vacuum and anticoagulation blood collection tube and stored in refrigerator at -20°C. Immediately after slaughtering, the tissues such as hypothalamus, pituitary and ovary were taken out and placed into the cryopreservation tube treated with Diethy pyrocarbonate (DEPC) water and stored in liquid nitrogen.

Experimental materials

Following instruments and materials were used in the current study: NanoDorp 2000 (Thermo Fisher Scientific Co., Ltd, Shanghai), Gentier 48E Polymerase Chain Reaction (PCR) instrument (Tianlong, Xian), CFX ConnectTM Optics Module fluorescence quantitative PCR instrument (Thermo Fisher Scientific Co., Ltd, Shanghai), DNA extraction kit (Tiangen, Beijing), miRNA extraction kit (Tiangen, Beijing), reverse transcription kit (Tiangen, Beijing).

Cloning of the sequences of miR-10a and miR-10b precursor in sheep

The sequence of sheep miR-10a/b precursor was downloaded from Ensemble database. Primers of precursor sequences were designed by Primer 5.0 (Table 1). The primers were synthesized by Beijing TSINGKE Biotechnology Co., Ltd. DNA extraction kit was used to extract blood DNA and 1% agarose gel electrophoresis was used to verify the quality of DNA extraction. The sequences of miR-10a/b precursors were amplified by PCR. The final volume of the reaction system was 50 µl, including 45 µl T3 Super PCR Mix (1.1×T3 Super PCR Mix 10×1.125ml), 1 µl template DNA, 2 µl upstream primers and 2 µl downstream primers. The reaction conditions were as follows: pre-denaturation at 98° for 2 min; 35 cycles of denaturation at 98° for 10 s, annealing at 60°C for 30 s and extension at 72°C for 10 s; extension at 72°C for 2 min; cooling at 16°C for 1 min. The PCR products were detected by 1% agarose gel electrophoresis and the products were sent to Beijing TSINGKE Biotechnology Co., Ltd for sequencing.

Determination of the expression levels of miR-10a and miR-10b in sheep by Real-time fluorescence quantitative PCR

The miRNA of hypothalamus, pituitary and ovary was extracted by miRcute miRNA separation kit, the concentration was detected by Nanodorp 2000 and the integrity was detected by 1.2% agarose gel electrophoresis. The first strand of cDNA was obtained by poly A tailing method through RT-PCR. The volume of the reaction system was 20 µl, including 5 µl template miRNA, 10 µl 2 × miRNA RT Reaction Buttfer, 2 µl miRNA RT Enzyme Mix and 3 µl RNase Free dd H^2O. The reaction conditions were as follows: 42°C for 60 min and 95°C for 3 min. The cDNA products were stored in the refrigerator at -20°C .

According to the known sheep miR-10a/b gene sequences, real-time fluorescent quantitative PCR primers were designed with Primer 5.0. The internal reference gene was 18s (Table 2). The primers were synthesized by Beijing TSINGKE Biotechnology Co., Ltd. CFX Connect TM Optics Module was used for reaction, with 3 repeats in each group. The volume of the reaction system was 20 µl, including 10 µl 2 × miRcute Plus miRNA, 0.4 µl upstream primers, 0.4 µl downstream primers, 2 µl cDNA and 7.2 µl dd H^2O. The reaction conditions were as follows: 95°C for 5 min; 40 cycles of 95°C for 20 s and 60°C for 34 s.

Bioinformatics analysis

The sequencing results of the sequences of miR-10a and miR-10b precursor in sheep were compared with the corresponding sequences, which downloaded from Ensemble database (http://www.ensembl.org/index.html?redirect=no) by using Blast tool in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). TargetScan 7.2 (http://www.targetscan.org/vert_72/), miRWalk 3.0 (http://mirwalk.umm.uni-heidelberg.de/) and miRDB (http://

Table 1: Primers for sheep miR-10S precursor sequences.

Primer name	Primer sequence (5′→3′)	Product size/bp	TM/°C
Oar-miR-10a	F: CATACCCCAAACGAAGGATGC		
	R: AAAAGGAATCTTCGTGTCACC	283	60
Oar-miR-10b	F: CCCCGGCTCTGTTACAAGGA		
	R: ACATTATTGGGCTCCGACGA	164	60

Table 2: Primers of sheep miR-10S.

Primer name	Primer sequence (5′→3′)	TM/°C
Oar-miR-10a	F: CCGCGTGCCCTGTAGATCCGAATTTG	65
Oar-miR-10b	F: CGCGACCCTGTAGAACCGAATTTGTG	65
18S	F: AACATCGATGGGCGGCGGAA	
	R: CGCGTTCCACCTCGTCCTCAGT	65

Note: The downstream primers were provided by the kit.

www.mirdb.org/) were used to predict the target genes of miR-10a and miR-10b, respectively. These results were made into Venn graph to obtain the intersection. miRTarbase database (http://mirtarbase.mbc.nctu.edu.tw/) was used to search the target genes of miR-10a and miR-10b in sheep, g: profiler (http://biit.cs.ut.ee/gprofiler/) was used for GO enrichment analysis and KEGG (https://www.kegg.jp/) was used for pathway analysis. The mature sequences of miR-10a and miR-10b from 10 different species including human, bovine, goat, sheep, pig, horse, dog, rabbit, mouse and zebrafish were downloaded from miRBase database (http://www.mirbase.org/index.shtml). The mature sequences of miR-10a and miR-10b genes of the above 10 species were analyzed by MEGA 7.0, the phylogenetic tree was constructed and the conserved regions of mature sequences among different species were obtained.

Statistical methods

The relative expression of genes was calculated by 2-ΔΔct method and the data were compared by single factor ANOVA analysis and multiple comparisons of Duncan's test using SPSS 21.0 statistical software. The obtained data were plotted using Graphpad Prism 6 in the way of "mean ± standard deviation". P<0.05 represented significant difference and P<0.01 represented extremely significant difference.

RESULTS AND DISCUSSION

Cloning and analysis of the sequences of miR-10a and miR-10b precursor in sheep

miRNA is a kind of endogenously regulated small RNA with length about 22nt after treatment of pre-miRNA with Dicer enzyme (Piletič and Kunej, 2016; Zou *et al*., 2016). miRNA is related to cell growth, differentiation, apoptosis and the process of some cancers such as induction of apoptosis in ovarian cancer cells by miRNA (Liu *et al*., 2019; Vidigal and Ventura, 2015; Kleemann *et al*., 2018). miRNAs are potent regulators of testicular, ovarian, hypothalamic and pituitary hormones (Messina and Prevot, 2017) and also regulate the expression and functions of numerous steroidogenic genes (Reza *et al*., 2019). Micro RNA-mediated gene regulatory mechanisms in mammalian female reproductive health have been showed that microRNAs as a class of non-coding RNAs are also known to be involved in various pathophysiological conditions (Gebremedhn *et al*. 2021). In this experiment, the sequences of miR-10a and miR-10b precursor (pre-miR-10a and pre-miR-10b) in Hu sheep were successfully cloned using blood DNA as template and the length of the amplified fragments was 283bp and 164bp, respectively (Fig 1). After comparison, it was found that the sequence of pre-miR-10a was completely consistent with the sheep data and the length was 109bp. The pre-miR-10b length was 99bp.

Expression of miR-10a and miR-10b in different tissues of sheep

The results showed that the relative expression of miR-10a in ovary was extremely significantly higher than that in hypothalamus and pituitary (P<0.01, Fig 2A). The relative expression of miR-10b in ovary was extremely significantly higher than that in hypothalamus and pituitary (P<0.01, Fig 2B). In addition, the relative expression of miR-10b in ovary or pituitary was extremely significantly higher than that of miR-10a (P<0.01, Fig 2C) and the relative expression of miR-10b in hypothalamus was significantly higher than that of miR-10a (P<0.05). Previous study has shown that miR-10a and miR-10b can inhibit BDNF and TGF-β pathway as well as the growth of granulosa cells in ovary and indicated that BDNF was a direct target of miR-10a and miR-10b in GCs (Tu *et al*., 2017). It is worth noting that although miR-10a is highly expressed in the ovaries, there is not any significant difference before and after sexual maturation in chicken (Li *et al*., 2013).

Conservative and evolutionary analysis of mature sequences of miR-10a and miR-10b in sheep

The seed region of miR-10a mature sequence was completely consistent in 11 species, of which 21 nucleotides were found in 10 species (Fig 3A). Evolutionary analysis indicated that the genetic distance between sheep and goat was the closest (Fig 3B).

Except for horse, the seed region of miR-10b mature sequence was completely consistent with 10 other species (Fig 4A). Evolutionary analysis indicated that the genetic distance between sheep and goat was the closest (Fig 4B). miRNA has been proved to have sequence conservation, tissue specificity and timing specificity (Sempere *et al*., 2004; Kloosterman *et al*., 2006; Silverman *et al*., 2016). We found that the precursor sequences and the mature sequence of Hu sheep miR-10a/b were highly conserved. Similar results were found in a study of smooth muscle cell differentiation (Huang *et al*., 2010).

Prediction of miR-10a and miR-10b target genes in sheep

More scientific and accurate data can be obtained by using different software to predict target genes (Yousef and Allmer,

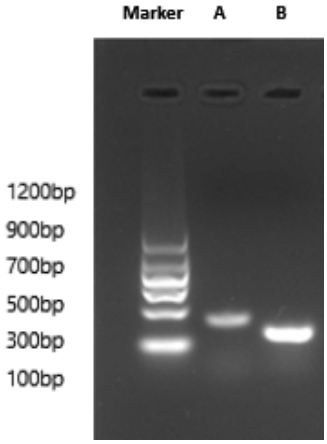

Fig 1: The cloning result of the precursor sequences of miR-10a and miR- 10bin sheep.

A. PCR products of miR-10a. B. PCR products of miR-10b.

2016). According to the predicted results of Target Scan, mi R Walk and mi RDB online prediction software, the number of miR-10a target genes was 243, 1866 and 352, respectively. Among them, 17 genes were intersecting target genes in three software, such as TMEM132B, ARSJ, CRK, SMAP1, IGDCC4, GATA6, RPRD1A, NR5A2, ACTG1, PRRT3, LRRC8B, WDR26, TMOD1, ATCAY, PEX5L, RBMS3 and SLC38A2 (Fig 5). The number of miR-10b target genes in Target Scan, mi R Walk and mi RDB software was 59, 2338 and 463, respectively and among them, 3 genes were intersecting target genes, such as RBM27, POLR3D and MBNL3 (Fig 6). In addition, 200 and 308 target genes of miR-10a and miR-10b were obtained by mi R Tarbase database, respectively. Based on this information, we will further analyze the correlation between the expression of mir-10a /b and the number and expression of target genes and further reveal the growth, reproduction, growth traits related mi RNAs and the differences of their expression levels in different tissues of sheep, which is conducive to the study of genes regulating sheep reproduction. In addition, these target genes were further combined with the predicted target genes for GO and KEGG analysis.

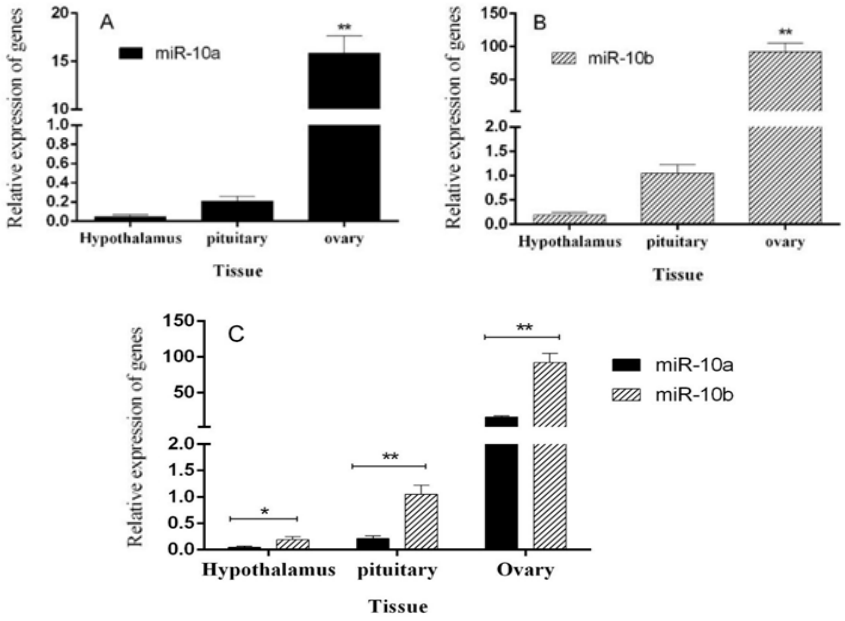

Fig 2: Expression of miR-10a and miR-10b genes in different Hu sheep tissues.

A. Expression of miR-10a in different Hu sheep tissues. B. Expression of miR-10b in different Hu sheep tissues. C. Comparison of expression of miR-10a and miR-10b in the same tissue. Data are expressed as mean ± SD. P<0.05 represented significant difference and shown with *, P<0.01 represented extremely significant difference and shown with **.

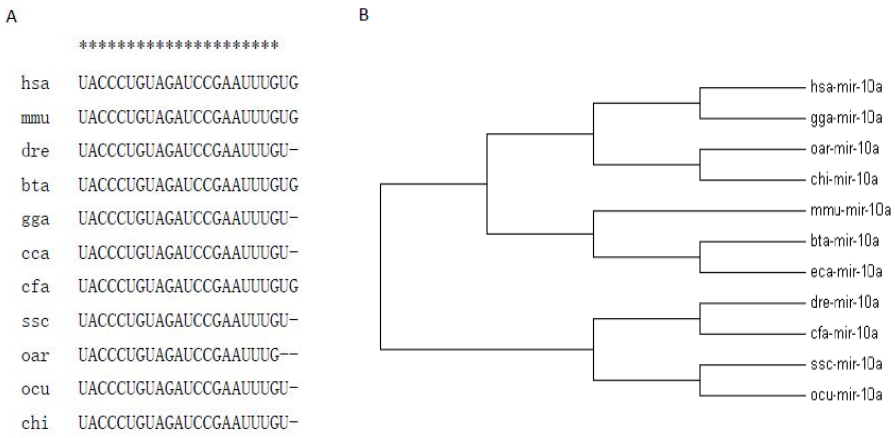

Fig 3: Analysis of miR-10a sequence.

A.conservative analysis of miR-10a mature sequence. The seed region of miR-10a mature sequence was completely consistent in 11 species, of which 21 nucleotides were found in 10 species. B.Evolutionary relationship of miR-10a. The genetic distance between sheep and goat was the closest.

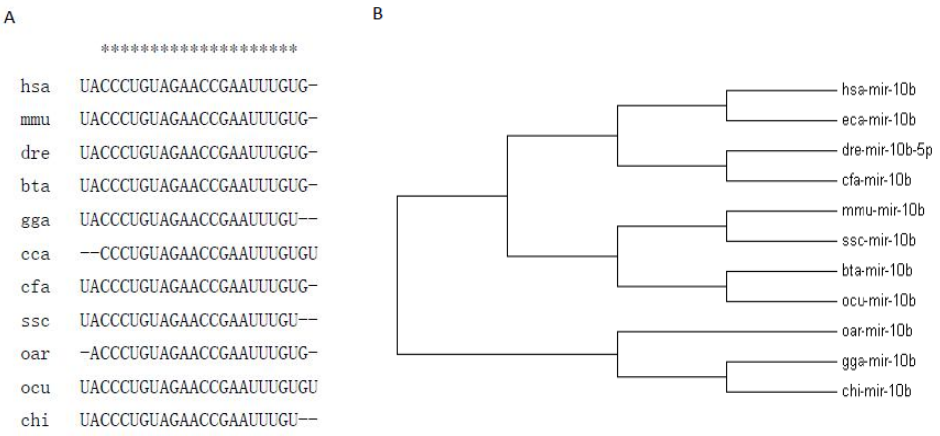

Fig 4: Analysis of miR-10b sequence.

A. conservative analysis of miR-10b mature sequence. The seed region of miR-10b mature sequence was completely consistent with 10 other species. B. Evolutionary relationship of miR-10b. The genetic distance between sheep and goat was the closest.

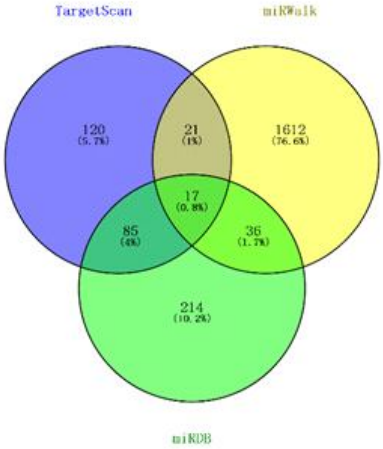

Fig 5: Venn Diagram of miR-10a target genes.

According to the predicted results of TargetScan, miRWalk and miRDB online prediction software, the number of miR-10a target genes was 243, 1866 and 352, respectively. Among them, 17 genes were intersecting target genes, they are TMEM132B, ARSJ, CRK, SMAP1, IGDCC4, GATA6, RPRD1A, NR5A2, ACTG1, PRRT3, LRRC8B, WDR26, TMOD1, ATCAY, PEX5L, RBMS3 and SLC38A2.

GO enrichment analysis of miR-10a and miR-10b target genes in sheep

The significant threshold was P<0.05. Higher Log P value indicated greater significance. The results indicated that miR-10a target genes were enriched to 843 biological processes, 53 molecular functions and 59 cell components (Fig 7); whereas miR-10b target genes were enriched to 355 biological processes, 23 molecular functions and 7 cell components (Fig 8). Recent studies have shown that miR-10a/b are closely related to cell proliferation and differentiation (Eun *et al*., 2018; Jin *et al*., 2019; Li *et al*., 2018). These data provide a new idea for the study of mir-10a/b regulating the proliferation and differentiation of ovine follicular granulosa cells.

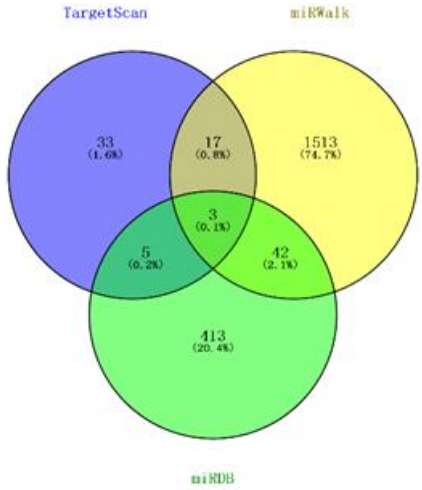

Fig 6: Venn Diagram of miR-10b target genes.

The number of miR-10b target genes in was 59, 2338 and 463, respectively and 3 genes were intersecting target genes such as RBM27, POLR3D and MBNL3.

KEGG analysis of miR-10a and miR-10b target genes in sheep

The top 20 KEGG enrichment results were used to draw bubble map for analysis. The transverse coordinate was the enrichment index of the enrichment factor, the longitudinal coordinate was the name of the signal pathway.

Target genes of miR-10a were enriched to TNF signaling pathway, Ubiguitin mediated proteolysis, Rap 1 signaling pathway, Toll-like receptor signaling pathway, proteoglycans in cancer, platelet activation, neurotrophin signaling pathway, oocyte meiosis, mRNA surveillance pathway, leukocyte transendothelial migration, hippo signaling pathway, insulin resistance, Influenza a HTLV-1 infection, Hepatitis B, clycosaminoglycan biosynthesis-keraten sulfate, circadian rhythm, chemokine signaling pathway, cell cycle signal pathway, *etc*. (Fig 9).

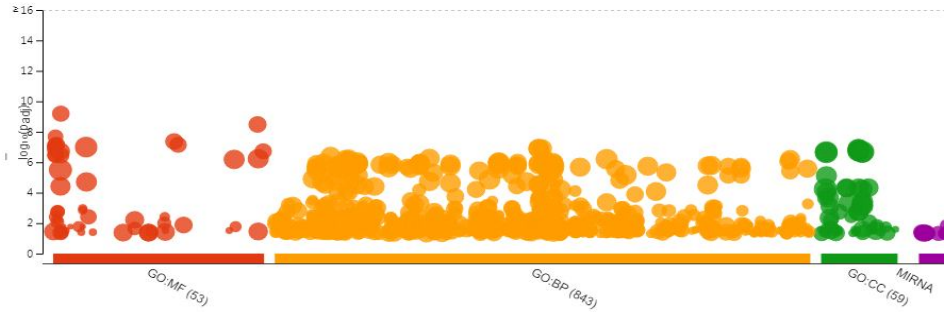

Fig 7: GO enrichment of miR-10a.

The significant threshold was P < 0.05. Red indicates molecular function. Yellow indicates biological processes. Green indicates cell component. The results showed that miR-10a target genes were enriched to 843 biological processes, 53 molecular functions and 59 cell components.

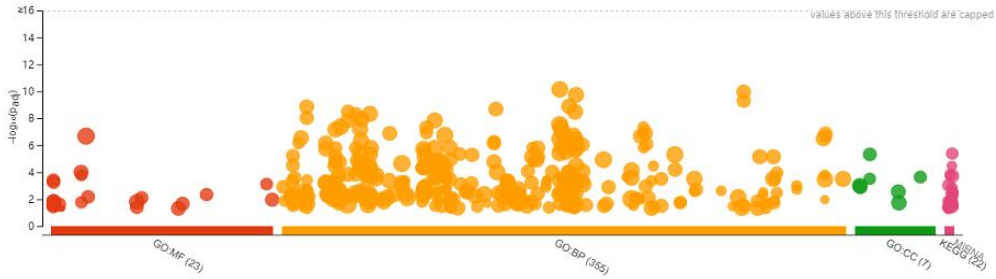

Fig 8: GO enrichment of miR-10b.

The significant threshold was P<0.05 Red indicates molecular function. Yellow indicates biological processes. Green indicates cell component. The miR-10b target genes were enriched to 355 biological processes, 23 molecular functions and 7 cell components.

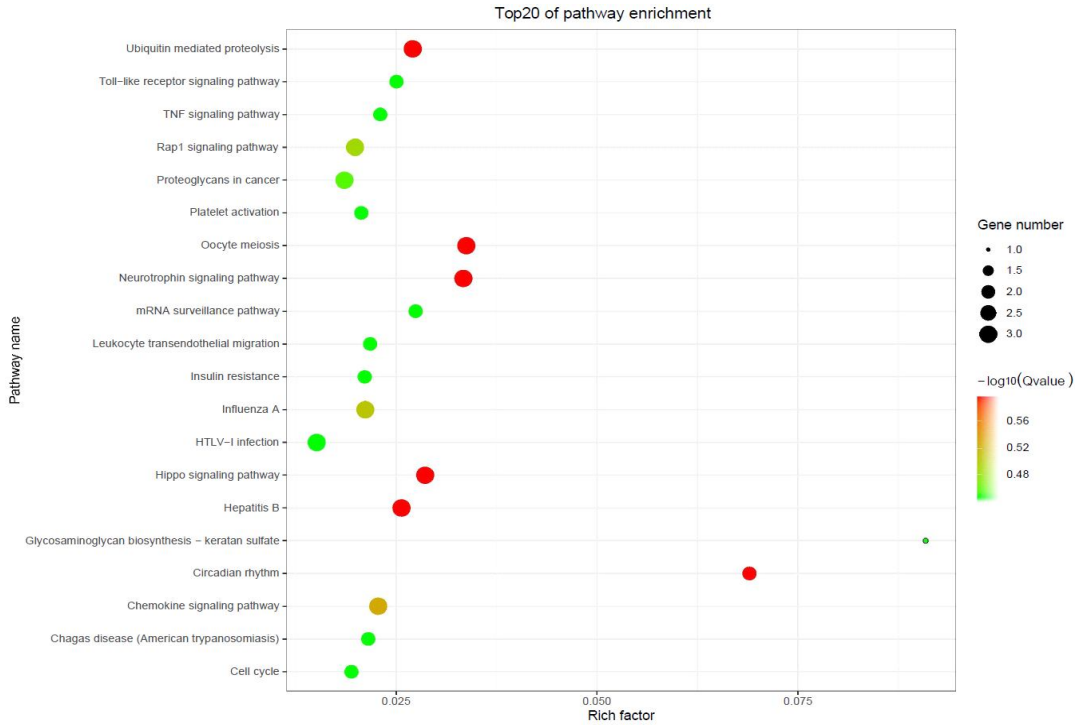

Fig 9: KEGG enrichment of miR-10a.

The bubble size represented the number of genes and the bubble color indicated that the enrichment difference was significant, among them, red represents the higher significance of difference.

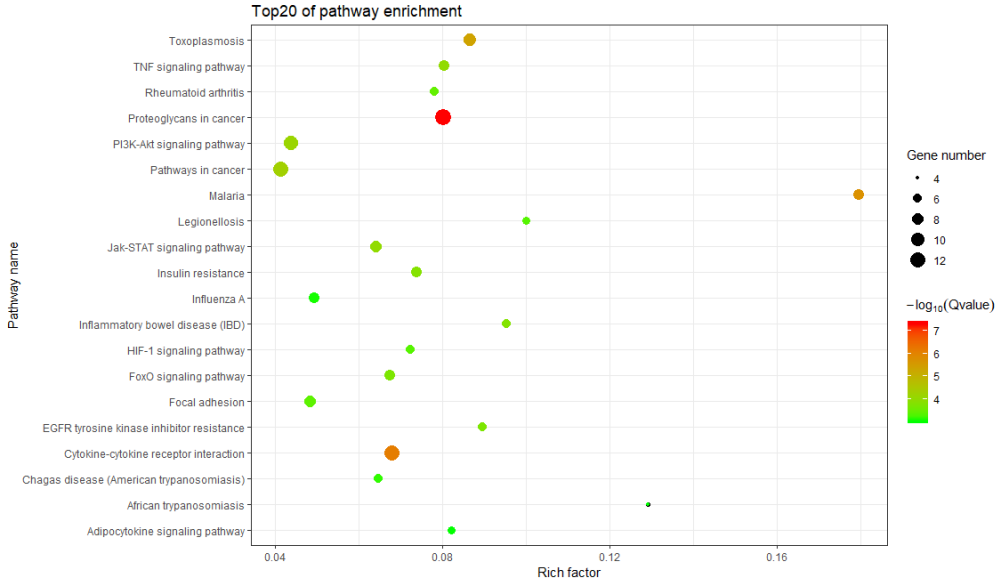

Fig 10: KEGG enrichment of miR-10b.

The bubble size represented the number of genes and the bubble color indicated that the enrichment difference was significant, among them, red represents the higher significance of difference.

Target genes of miR-10b were enriched to toxoplasmosis, TNF signaling pathway, rheumatoid arthritis, P13K-Akt signaling pathway, proteoglycans in cancer, pathways in cancer, Malaria, Legionellosis, Jak-STAT signaling pathway, insulin resistance, Influenza A, inflammatory bowel disease, H hypoxia inducible factor (HIF-1) signaling pathway, Foxo signaling pathway, focal adhesion, EGFR tyrosine kinase inhibitor resistance, cytokine-cytokine receptor interaction, American trypanosomiasis, African trypanosomiasis, adipocytokine signaling pathway, *etc*. (Fig 10).

Oocyte meiotic pathway is an important signal pathway to regulate reproductive physiology, which is closely related to oocyte meiotic division and oocyte maturation (Matsuda-Minehata *et al*. 2006). Adipocytokines, including leptin and adiponectin, can widely affect and regulate energy metabolism and various functions of the body (Kershaw and Flier, 2004; Ogunyemi *et al*., 2013). Insulin resistance is associated with fat metabolism (Shimomura *et al*. 1999), metabolic syndrome (Llaneza *et al*. 2009), polycystic ovary syndrome (Lord and Wilkin, 2004). Previous study discovered that tumor necrosis factor (TNF) may regulate germ cell nests breakdown in rats and TNFα may promote ovulations in the rat ovary (Greenfeld *et al*., 2006; Brannstrom, 1995). It is obvious that mir10 /b is involved fat metabolism, oocyte maturation and ovulation through some important signaling pathways. These results suggested that miR-10a/b might be used in molecular breeding.

CONCLUSION

Our results demonstrate that the high conservation of miR-10a/b between sheep and other species and determined the high expression of miR-10a/b in sheep ovaries. In addition, the function and related pathway of sheep miR-10a/b target gene was specified by target gene prediction, GO enrichment analysis and KEGG pathway analysis. The results provide important information for the potential regulation of the development of sheep ovaries and follicles and contribute to elucidate the processes of miRNA regulation on sheep reproduction.

ACKNOWLEDGEMENT

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Statement of conflict of interest

All authors declare no conflicts of interest.

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