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Bio-characteristics, tissue expression of miR-375 in hypothalamic-pituitary-ovarian axis and its regulation in reproduction-related diseases

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Our study concentrated on the expression of miRNA-375 in the hypothalamic-pituitary-gonadal axis of female Hu sheep. The investigation involved cloning the precursor sequence of miR-NA-375, followed by comparison with database entries and subsequent Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. In our approach, we obtained ovaries, thalamus, cerebellum, brain, uterus, pituitary gland, hypothalamus, and pineal gland from fertile but nonpregnant Hu ewes. MiRNA extraction kit was used to extract miRNA from the above eight tissues. Real-time fluorescent quantitative polymerase chain reaction was used to evaluate the role of miR-375 in the hy-pothalamic-pituitary-gonadal axis. The results of miR-375 precursor sequence cloning were compared with those of *Anopheles gambiae*, *Apis mellifera*, *Bos taurus*, *Drosophila melanogaster*, *Danio rerio*, *Fugu rubripes*, *Gallus gallus*, *Homo sapiens*, *Monodelphis domestica*, *Macaca mulatta*, *Mus musculus*, *Pan troglodytes*, *Rattus norvegicus*, *Tetraodon nigroviridis*, *Xenopus tropicalis* miR-375 in miRBase database. It was found that oar-miR-375 was highly conserved. Notably, miR-375 expression in the pineal gland was significantly higher ($p < 0.01$) than that in the ovaries, thalamus, cerebellum, brain, uterus, pituitary gland, hypothalamus. The study also involved predicting miR-375 target genes. GO and KEGG enrichment analyses of these predicted target genes revealed that miR-375 is involved in 182 biological processes, affects 186 cellular components, and participates in 184 molecular functions. In terms of pathway enrichment, miR-375 was linked to nine pathways, including the Hippo, Wnt, and mTOR signaling pathways. This study has validated the interaction between miR-375 and its target gene FZD4, which can be recognized and bound to produce effects. These findings lead to the inference that miR-375 may play a crucial regulatory role in sheep reproduction through the Hippo pathway and Wnt pathway, laying a foundation for further exploration of miR-375's role in this domain.

Keywords miRNA-375, Precursor sequence, Real-time quantitative PCR, Hypothalamic-pituitary-gonadal axis, Bioinformatics analysis

MicroRNAs (miRNAs) are concise, noncoding RNA molecules, typically comprising 18–25 nucleotides in length[1]. They perform the critical function of governing gene expression[2]. Following enzymatic cleavage by the Dicer enzyme, the pre-miRNA undergoes processing, resulting in double-stranded molecules. Among these, a functional miRNA is chosen to modulate gene expression within cellular environments[3]. This regulatory mechanism is observable across viruses, plants, and higher mammals[4,5].

In the animal kingdom, three pivotal endocrine systems hold sway: the hypothalamic-pituitary-thyroid axis, the hypothalamic–pituitary–adrenal axis, and the hypothalamic-pituitary-gonadal (HPG) axis. Notably, the hypothalamus and pituitary gland assume central roles in endocrine regulation. The HPG axis, overseeing the secretion of sex hormones, assumes primary significance. This study centers its focus on the HPG axis in female animals, given its pivotal role in the domain of reproduction. Within the HPG axis, an array of hormones, including gonadotropin releasing hormone (GnRH), follicle-stimulating hormone (FSH), and luteinizing

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hormone (LH), orchestrate the foundation for reproductive activities in animals. Recent investigations have underscored the indispensable regulatory influence exerted by miRNAs within the hypothalamic-pituitary-ovary (HPO) axis[6].

The year 1993 marked a seminal moment in development research when a 22-nucleotide nonprotein-coding lin-4 gene and let-7 gene were identified, representing the earliest known miRNAs[2]. Since then, the scientific community has harnessed tools such as bioinformatics and molecular cloning to unveil a multitude of miRNAs in animal and plant tissues and organs[5,7].

MiRNAs have surfaced as pivotal regulators of cell proliferation and differentiation, wielding influence over cell fate by modulating the expression of target genes. Notably, miRNAs have been shown to govern the proliferation and differentiation of adipose-derived stem cells[8]. Furthermore, Chen et al. (2006)[9] provided evidence demonstrating that miR-1 and miR-133 exert control over the proliferation and differentiation of skeletal muscle cells, unraveling the involvement of miRNAs in the molecular mechanisms underpinning skeletal muscle gene expression and embryonic development. Recent investigations by Zhao et al. (2022)[10] revealed that mesenchymal stem cell-derived exosomes inhibit fibroblast proliferation, migration, and protein expression through the action of miR-138-5p. Overall, miRNAs have a substantial influence on the precise regulation of fundamental biological processes, including cell proliferation, differentiation, survival, and apoptosis. Comprehensive analyses, as elucidated in another study[11], have elucidated the role of miRNAs in governing the cell cycle in embryos, somatic cells, and cancer stem cells. In summary, miRNAs assume significant roles in an array of biological processes, carrying extensive implications for diverse biological phenomena. The continuous advancement of miRNA research undoubtedly promises to deepen our comprehension of their biological functions and regulatory mechanisms.

The HPG axis consists of the hypothalamus, pituitary and gonads, which are responsible for controlling the production and release of reproductive hormones. The hypothalamus secretes GnRH, which stimulates the pituitary to secrete FSH and LH, which further regulate gonadal function. miRNAs influence the function of the HPG axis by regulating the expression of genes related to hormone synthesis and secretion during this process. It has been shown that changes in the expression of HPG axis-related genes affect reproduction in sheep[12]. miRNAs are able to influence the activity of signaling pathways, such as the PI3K-Akt pathway and the MAPK pathway, which are closely related to the synthesis of reproductive hormones and their biological activities[13]. Studies have shown that chronic stress can alter the expression level of miRNAs, which in turn affects signaling in the HPG axis; under stress, miRNAs may affect reproductive function by regulating the expression of genes associated with the stress response[12,14]. In some endocrine-related tumors, changes in miRNA expression may affect signaling pathways associated with cell proliferation and apoptosis, thereby influencing tumorigenesis and progression[15,16]. The above literature collectively shows that the effect of differential expression of miRNAs in the HPG axis on multiple signaling pathways is a complex and important area of research. The HPG axis plays a key role in regulating reproductive function, and miRNAs, as important factors in regulating gene expression, can affect this process through multiple mechanisms. It is also shown that it is meaningful to study miR-375 and HPO axis.

The pineal gland is a crucial organ involved in transducing light signals to the reproductive axis, thus playing an essential role in seasonal reproduction[17,18]. Its primary function lies in the secretion of melatonin, which inhibits pituitary FSH and LH secretion while also releasing various potent anti-gonadotropin peptide substances, effectively suppressing gonadal activity and sexual characteristic development. Destruction of the pineal gland can lead to premature puberty and excessive reproductive organ growth, significantly impacting animal reproduction. Furthermore, the pineal gland exhibits distinct periodicity with melatonin secretion decreasing during daylight hours and increasing at nightfall. Studies have demonstrated significant variations in miRNA expression within the pineal gland across different reproductive stages[19], as well as notable differences between day and night periods[20]. In pigs' pineal glands, inhibition of miR-7 has been found to enhance AANAT expression levels and subsequently elevate melatonin production[21].

In recent years, Studies have demonstrated that miR-375 exerts a significant influence on reproduction, exemplified by its regulatory role in GnRH production in mice[22]. Moreover, miR-375 has emerged as a key player in reproduction across various animal species, as indicated by other studies[23–25]. miR-375 can affect the proliferation and apoptosis of bovine cumulus cells through BMPR2[23]. In pigs, miR-375 can mediate the corticotropin releasing hormone (CRH) pathway to regulate estradiol synthesis[24]. Some scholars have found that miR-375 can target ADAMTS1 and PGR in bovine cumulus cells to regulate oocyte maturation in vitro[25]. Pathways have an impact on animal reproduction. In particular, Wnt and Hippo pathways are particularly important. Activation of the WNT pathway is an important condition for the culture of embryos and embryonic stem cells[26]. Studies have shown that the Hippo signaling pathway plays an important role in the physiology and pathology of the ovary[27]. However, investigations on the role of miR-375 in sheep reproduction remain limited. Consequently, this study endeavors to bridge this gap by examining the expression of miR-375 in different tissues, predicting its target genes and associated pathways, trying to explain the effect of miR-375 on sheep reproduction from the perspective of pathways, and constructing a gene interaction network specific to miR-375.

Materials and methods

The experimental procedures adhered to the relevant policies and guidelines of the Animal Care and Use Committee of Henan University of Science and Technology, as well as the ARRIVE guidelines. In accordance with both the Chinese guidelines for the Euthanasia of Laboratory Animals (GB/T 39760 – 2021) and ARRIVE guidelines, euthanasia was performed on sheep through intravenous injection of sodium pentobarbital at a dosage of 100 mg/kg.

Experimental animals

In this investigation, we selected six nulliparous Hu ewes, which were accommodated at the National Meat Sheep Luoyang Experimental Station, as the subjects of our research. All Hu ewes were fertile but not pregnant, with an average age of 3.5 ± 0.5 and a weight of 50.3 ± 2.0 kg. According to the Laboratory Animal Guidelines for Euthanasia (GB/T 39760 − 2021) in reference to China, intravenous injection of Sodium pentobarbital at a dosage of 100 mg/kg is recommended for euthanizing sheep. Prior to their euthanization, blood specimens were obtained via the neck vein of the sheep. These blood samples were then preserved at a temperature of -20 °C. Following the euthanasia process, a rigorous collection of various reproductive and neurological tissues, including the ovaries, thalamus, cerebellum, brain, uterus, pituitary gland, hypothalamus, and pineal gland, was undertaken. After isolating the organs (ovary, thalamus, cerebellum, brain, uterus, pituitary gland, hypothalamus, and pineal gland) from the body, the organ tissue surfaces were rinsed with 37 °C saline solution. Subsequently, the tissues were placed in a tray covered with tin foil for sampling purposes. Sterilized forceps and scalpels were used to collect three tissue samples measuring 1 cubic centimeter each from different locations on the same organ tissue. Following three washes with normal saline and diethyl pyrocarbonate water, the samples were transferred into cryopreservation tubes for long-term storage in liquid nitrogen. In cases where tissue samples such as the pineal gland and pituitary gland did not yield sufficient volume for collecting three 1 cm^3 samples individually, efforts were made to obtain as much sample material as possible which was then evenly divided into three parts before being washed and stored in liquid nitrogen for future use. The scalpel and forceps underwent rinsing procedures using normal saline followed by 75% alcohol after each collection of tissue samples.

Cloning of the sequences of the miR-375 precursor in sheep

In this experimental procedure, the Genomic DNA Column Extraction Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing China) was employed for the extraction of genomic DNA from the blood samples of Hu sheep. Subsequently, the concentration of the DNA samples was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific Co., Ltd., Shanghai China), while the size and purity of the extracted DNA fragments were evaluated via agarose gel electrophoresis. The genomic DNA obtained was carefully preserved at a temperature of -20 °C for future utilization.

For the investigation of miR-375, the precursor sequence of miR-375 was retrieved from both the Ensembl website (http://asia.ensembl.org/index.html) and the miRBase database website (http://www.mirbase.org/). Utilizing Oligo 7[28] (Version 7.56) software, primers were rigorously designed for the miR-375 precursor sequence, as outlined in Table 1. These primers were synthesized by Beijing Tsingke Biotechnology Co., Ltd (Beijing, China). Subsequently, PCR was conducted to amplify the miR-375 precursor sequence. The reaction system included a total volume of 20 μL, comprising 18 μL of T3 Super PCR Mix, 0.8 μL of forward and reverse primers and 0.4 μL of the DNA template. The PCR conditions involved an initial denaturation at 98 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 10 s. Subsequently, a final extension step at 72 °C for 2 min was executed, followed by cooling at 16 °C for 1 min. The PCR products underwent evaluation through 1% agarose gel electrophoresis and were subsequently submitted to the primer synthesis company for Sanger sequencing.

One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 8.0.1 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. A significance level of $P < 0.05$ was employed to establish statistical significance.

Determination of the expression levels of miR-375 in sheep by real-time fluorescence quantitative PCR

Initially, miRNA extraction was executed utilizing the Vazyme miRNA extraction kit. The quality and integrity of the obtained miRNA were rigorously evaluated through agarose gel electrophoresis, with a focus on assessing the brightness and width of the resulting band. The concentration of the miRNA was subsequently quantified using a NanoDrop Spectrophotometer. Next, reverse transcription was carried out through the reverse transcription kit (Nanjing Vazyme Biotech Co., Ltd., Nanjing China), utilizing the extracted miRNA as a template and employing the neck loop method to synthesize cDNA with reverse transcriptase.

Subsequently, real-time fluorescent quantitative PCR was undertaken, employing cDNA from different tissues as the template to determine the its expression level. This technique relies on the emission of light by fluorescent dyes that specifically bind to the minor groove of the DNA double-stranded helical structure. Variations in the concentration of cDNA serve as a direct indicator of differential RNA expression across various tissues, facilitating the assessment of tissue-specific expression patterns. It is of utmost importance to perform these procedures on ice and to add samples vertically, thereby ensuring precision throughout the process.

The qRT-PCR system featured a total volume of 20 μL. The details were 10.0 μL of 2×miRNA Universal SYBR qPCR Master Mix, 0.4 μL of Specific Primer (10 μM), 0.4 μL of mQ Primer R (10 μM), 1.0 μL of Template DNA/cDNA, 8.2 μL of ddH2O. Each group underwent triplicate replicates to ensure the robustness and reliability of the results. The reaction conditions for real-time fluorescent quantitative PCR were as follows: Predenatured was repeated at 95℃ for 5 min; The Cyclic reaction was repeated 40 times at 95℃ for 10 s and 60℃ for 30 s. The

Primer name	Primer sequence (5'→3')	TM/℃
oar-miR-375	F: TCCCCGACACCCCCTCCA R: CACAGCCTCTCCGACCCG	60

Table 1. Primers for sheep miR-375 precursor sequences.

Melting curve sessions were repeated once at 95℃ for 15 s, 60℃ for 60 s, and 95℃ for 15 s. In this study, the internal reference gene employed was 18 S rRNA.

Bioinformatics analysis

Following the alignment of the sheep miR-375 precursor sequence using the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the miRBase database (http://www.mirbase.org), mature miR-375 sequences from various species, including *Homo sapiens*, *Mus musculus*, *medaka*, *chimpanzee*, *macaque*, *domestic dog*, *sea lamprey*, sheep, horse, and rabbit, were acquired. To scrutinize the evolutionary relationships and discern distinctions among these diverse species, one can employ the online tool T-Coffee (https://www.ebi.ac.uk/Tools/msa/tcoffee/) to construct an evolutionary tree. Additionally, it is possible to identify a conserved fragment within the mature sequence for subsequent analysis.

For the prediction of miR-375 target genes, online resources such as TargetScan 8.0 (https://www.targetscan.org/vert_80/), miRWalk (http://mirwalk.umm.uni-heidelberg.de/), and miRPathDB (https://mirdb.org/mirdb/index.html) are valuable. By collating the prediction results from these three databases and employing an online Venn diagram tool (https://bioinformatics.psb.ugent.be/webtools/Venn/), one can discern the intersecting target genes. These intersecting target genes can then undergo Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes[29–31] (KEGG) pathway analysis through the DAVID database website (https://david-d.ncifcrf.gov/tools.jsp). To visualize the associated functional signal transduction pathways effectively, the Weishengxin[32] online tool (http://www.bioinformatics.com.cn/plot_basic_gopathway_enrichment_bubbleplot_081) can be harnessed to generate informative bubble plots.

Finally, to construct an interaction network including miR-375, its target genes, and the KEGG pathway, one may utilize Cytoscape[33] (Version 3.8.0) software. This network visualization serves as a valuable resource for gaining insights into the complex interplay between miR-375 and its target genes within the context of the KEGG pathway.

Cell culture

293T cells were derived from Procell (Procell, Wuhan, China). Cells were cultured in DMEM medium (Servicebio, Wuhan, China) with 10% fetal bovine serum (BD Biosciences, Zhejiang, China) and 1% penicillin-streptomycin (Solarbio, Beijing, China) at 37 ℃ and 5% carbon dioxide in a humidified atmosphere.

Cell passage was performed when the cells covered 80–90% of the bottom of the T25 bottle. Cells were rinsed three times with 1x PBS(Servicebio, Wuhan, China). 1 ml of 0.25% trypsin (Thermo Fisher Scientific, Shanghai, China) was added and digested for 3 min. Digestion was terminated by the addition of 3 ml of complete medium after 3 min. The cell suspension was withdrawn from the T25 bottle and added to a new 15 ml centrifuge tube and centrifuged at 1000 RPM for 3 min. The supernatant was discarded and the cells were resuspended by adding 1 ml of complete medium.

The number of cells was counted using a blood cell counting plate, and according to the number of cells, complete medium was added diluted to the desired cell concentration and added to a new T25 bottle or to a 96-well plate.

Luciferase reporter assay

The potential binding site between miR-375 and FZD4 was predicted using TargetScan 8.0 (https://www.targetscan.org/vert_80/), miRanda (WeiShengxin) was used to predict the binding sites and binding free energy values between miR-374 and FZD4. FZD4 wild-type (WT) and mutant type (MUT) recombinant plasmids were constructed using the pmiRGLO plasmid. 293T cells were seeded into 96-well plates and then co-transfected with either the mimic or miR-NC, along with either the pmirGLO-WT or pmirGLO-MUT vectors. All transfections were conducted using the Liposomal Transfection Reagent (Yeasen Biotechnology (Shanghai) Co., Ltd., Shanghai China) following the manufacturer's instructions. Forty-eight hours after transfection, luciferase reporter assays were performed using a dual luciferase reporter assay system (Nanjing Vazyme Biotech Co., Ltd., Nanjing China) to compare firefly luciferase activity with renilla luciferase activity in each well. All assays were performed in triplicate, and the relative luciferase activity was calculated as the ratio of firefly to Renilla luciferase activity.

Results

Cloning and analysis of the sequences of the miR-375 precursor in sheep

In our experimental procedures, we conducted amplification and electrophoresis tests on DNA extracted from the whole blood of Hu sheep. The results yielded the anticipated results, featuring clear and well-defined bands, as depicted in Fig. 1. Within Fig. 1, the band designated "M" signifies the DL2000 DNA marker, while Band 1 corresponds to sample No. 96, with a fragment length spanning from 500 to 750. Similarly, Band 2 corresponds to sample No. 102, also exhibiting a length within the range of 500 to 750.

To determine the precise sequence of the obtained DNA fragments, we used the services of Sangon Co., Ltd (Shanghai, China). During the subsequent comparison of these sequences with the miRBase database, an incongruity between the precursor sequence of miRNA-375 and the cloned sequence emerged. This comparative analysis afforded valuable insights into the genetic variations inherent in the two sheep samples, thereby enhancing our comprehension of miRNA-375 and its role within the genetic framework of Hu sheep.

Notably, in sample No. 96, the length of miRNA-375 measured 644 base pairs. Upon scrutiny and comparison with the oar-miR-375 precursor sequence stored in the database, we identified four base pairs that exhibited disparities and had undergone mutations. Similarly, in sample No. 102, miRNA-375 exhibited a length of 641 base pairs. Upon comparative analysis with the precursor sequence within the database, we detected seven base pairs that displayed distinctions and had experienced mutations.

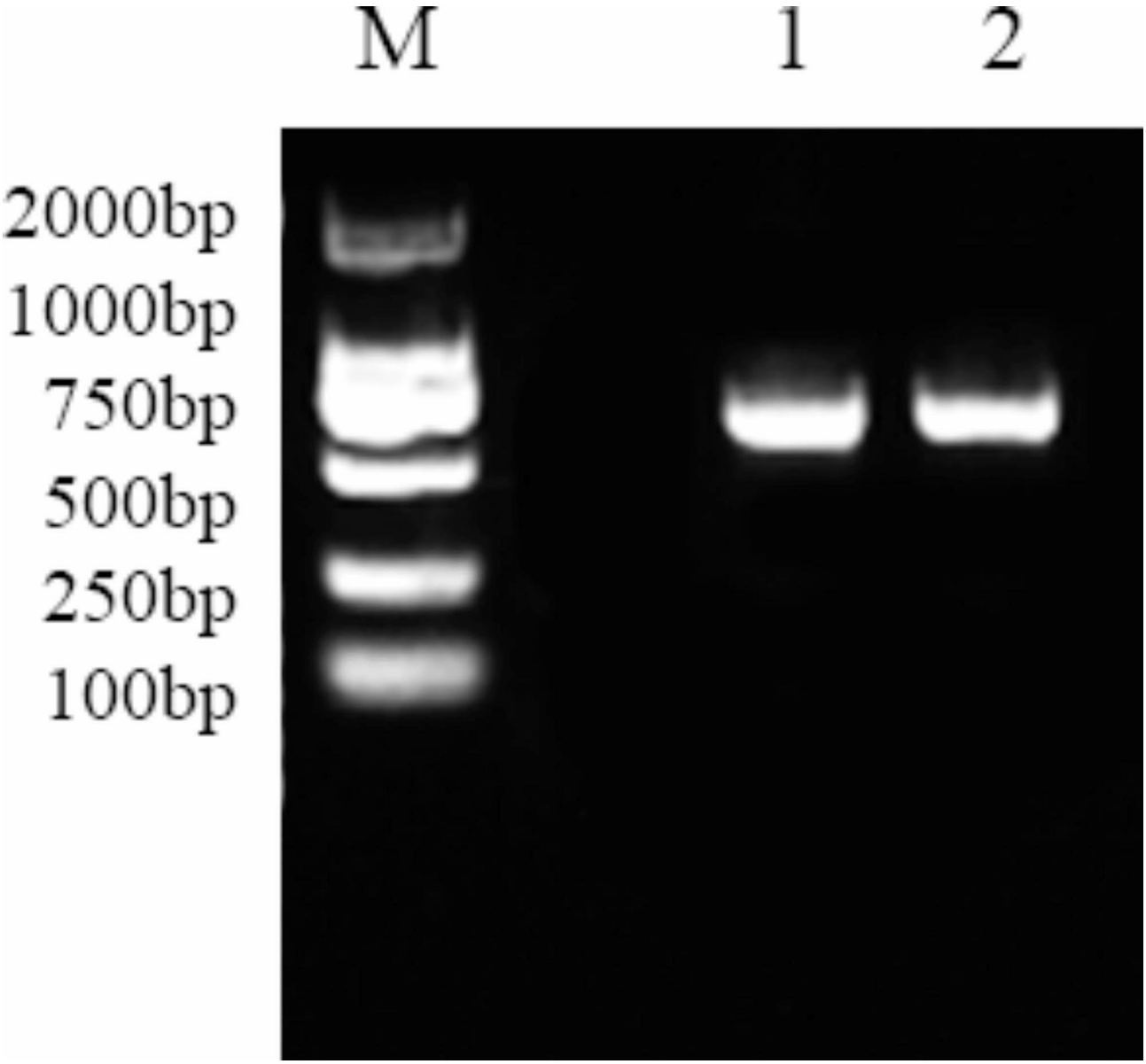

Fig. 1. The cloning result of the precursor sequences of miR-3375 in sheep. (1) PCR products of miR-375 (sample No. 96). (2) PCR products of miR-375 (sample No. 102). M. DL 2000 DNA Marker. The image has been selectively cropped to enhance readability by removing unnecessary areas. The original, uncropped images are depicted in Fig. 10 and supplementary material.

Expression of miR-375 in different tissues of sheep

Upon obtaining the cDNA, we subjected it to 1.0% gel electrophoresis to assess its quality. The results revealed uniformly vivid and well-defined bands, affirming the suitability of proceeding with subsequent steps, such as real-time fluorescent quantitative PCR, for the determination of miR-375 tissue expression levels.

As depicted in Fig. 2, an evaluation of miR-375 expression levels across various tissues was conducted. The findings underscored remarkably elevated expression levels in the thalamus, cerebellum, brain, uterus, pituitary, and hypothalamus, with these disparities holding statistical significance ($P < 0.001$). Notably, the expression of miR-375 within the brain exhibited a notably higher magnitude than that in the ovary, thalamus, uterus, pituitary, and hypothalamus. Furthermore, the relative expression level of miR-375 within the brain significantly surpassed that in the ovary, thalamus, cerebellum, uterus, pituitary, and hypothalamus.

Conservative and evolutionary analysis of mature sequences of miR-375 in sheep

Figure 3 presents the analysis of mature sequence conservation for miR-375. Notably, the seed regions of miRNAs within sixteen species, as depicted in Fig. 3, manifest a high degree of similarity. However, it is worth noting exceptions in the case of bovine miR-375 (bta-miR-375), short-tailed miR-375 (mdo-miR-375), and sheep miR-375 (oar-miR-375). Diverging from other species, these three exhibit a 22-nucleotide mature sequence for miR-

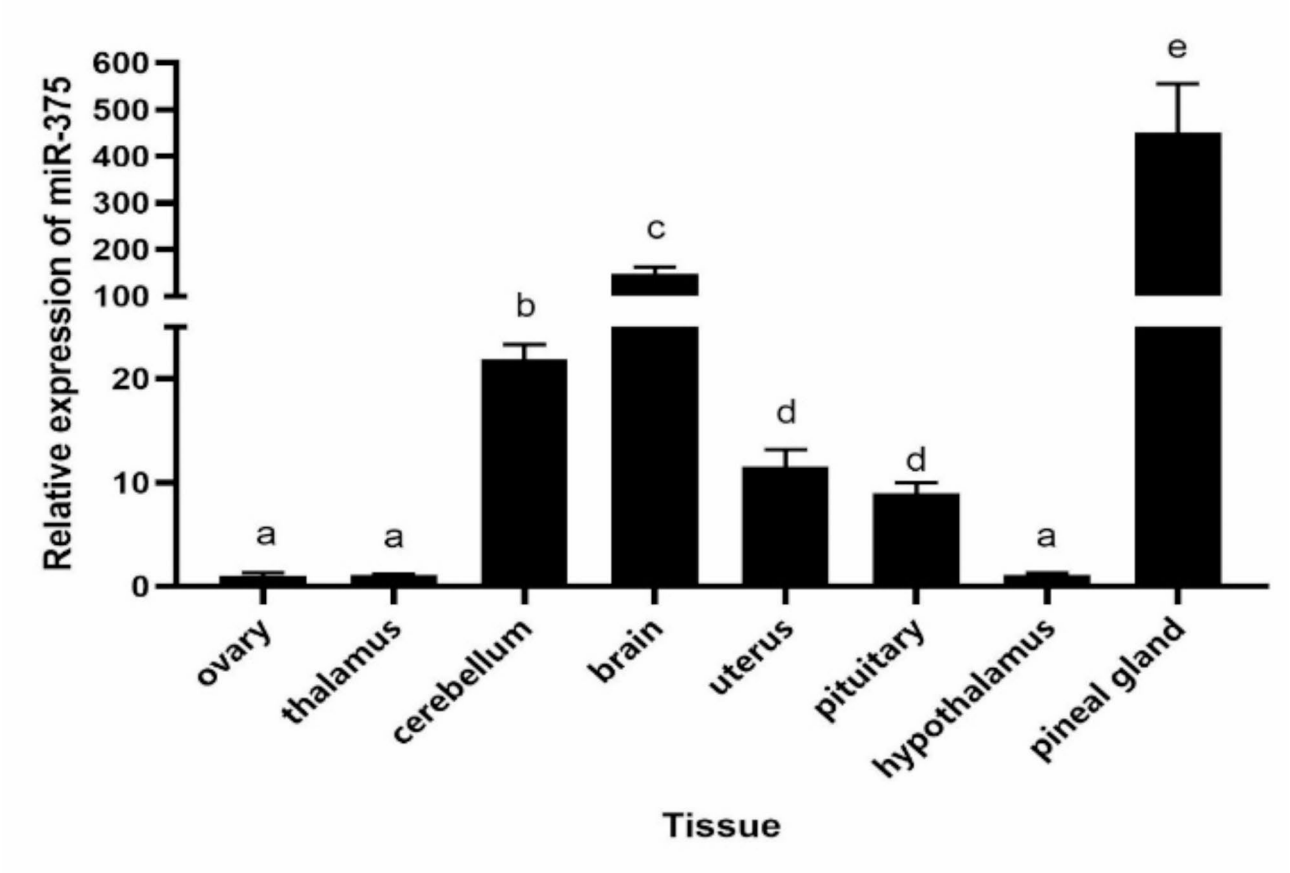

Fig. 2. Relative tissue expression of miRNA-375.

375. Of particular interest, the left image within Fig. 3 underscores the congruence of the mature sequence of miR-375 in sheep (oar-miR-375) with those observed in bovine (mdo) and bovine (bta). This intriguing observation suggests the potential existence of evolutionary conservation pertaining to the miR-375 sequence across these species, thereby accentuating its significance in biological processes under the regulation of this specific miRNA. Further exploration in this domain has the potential to shed illuminating insights into the functional implications and roles of miR-375 within these species.

Prediction of miR-375 target genes in sheep

The Venn diagram, depicted in Fig. 4, was generated by leveraging three online prediction software tools: TargetScan, miRWalk, and miRPathDB. This diagram serves as a valuable resource for gaining insights into the predicted target genes of miR-375. The comprehensive analysis of miR-375 target genes yielded 304 targets according to TargetScan, 5,681 targets according to miRPathDB, and an extensive set of 12,224 targets according to miRWalk.

Subsequently, a more refined analysis was conducted, focusing on the intersection of these predicted target genes, culminating in a subset of 194 genes. This subset then underwent thorough scrutiny via GO enrichment analysis and KEGG pathway analysis, allowing for a deeper exploration of their biological significance and functional roles.

GO enrichment analysis of miR-375 target genes in sheep

The target gene analysis of miR-375, illustrated in Fig. 5 as a bubble diagram, encapsulates a wealth of information. Various elements within the diagram convey distinct meanings, including the abscissa, ordinate, bubble size, and color variations. The abscissa is representative of the fold enrichment of these factors, while the ordinate denotes the enriched GO terms. Bubble size correlates with the number of genes associated, and the color of the bubble reflects the significance of the enrichment difference. Notably, a red bubble signifies a noteworthy variation in the results, with the significance of a pathway amplifying as the -Log P value increases. The analysis revealed that miR-375 exhibited enrichment in 182 biological processes, 186 cellular components, and 184 molecular functions.

KEGG analysis of miR-375 target genes in sheep

We conducted KEGG analysis on the intersecting set of predicted target genes utilizing data from the three databases through the utilization of the DAVID database. This comprehensive analysis revealed that miR-375

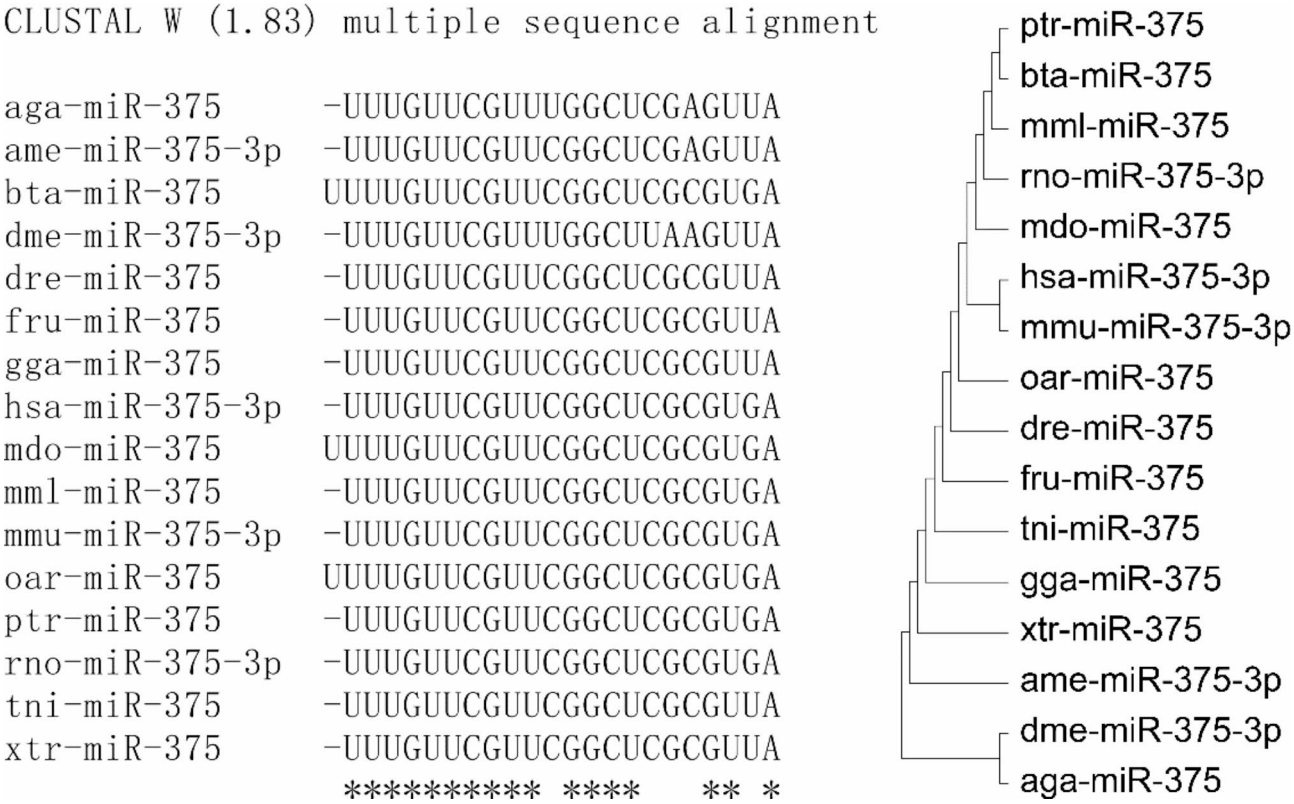

Fig. 3. Conservation analysis of miRNA-375.

exhibited enrichment in a total of 82 pathways. Inclusion criteria were established, necessitating a count greater than 2 and an EASE value exceeding 0.1. As a result, nine pathways met these criteria, namely, ubiquitin-mediated proteolysis, parathyroid hormone synthesis, secretion, and action, Hippo signaling pathway, Wnt signaling pathway, spinocerebellar ataxia, signaling pathways regulating pluripotency of stem cells, mTOR signaling pathway, Alzheimer's disease, and pathways of neurodegeneration - multiple diseases. Figure 6 prominently features the miR-375 KEGG pathway enrichment analysis diagram, showcasing these nine pathways of notable biological significance.

Network of interaction

Figure 7 depicts a complex interaction network graph including miRNA-375, transcription factors, and target genes. This network exhibits enrichment, including 67 nodes and 110 edges, which consist of 4 transcription factors and 53 mRNAs. Notably, oar-miR-375 exerts regulatory control over target genes, including YWHAB, YWHAZ, and FZD4, consequently influencing diverse signal transduction pathways, such as the Hippo signaling pathway and mTOR signaling pathway. This observation underscores the pivotal involvement of miR-375 in these crucial signaling pathways of notable biological significance.

Directly targeting FZD4 by miR-375

Target genes of intersection were identified using Venn diagram (Fig. 4), and KEGG analysis showed that FZD4 was associated with Hippo, Wnt, and mTOR pathways. Notably, our analysis suggests that miR-375 potentially targets FZD4 (Fig. 8). Based on the results of miRanda and target gene prediction, FZD4 was selected as a potential target gene of miRNA-375 for verification.

The dual-luciferase reporter assay (Fig. 9) demonstrated a significant reduction in firefly luciferase activity upon co-transfection of the WT construct with the miR-375 mimic, compared to the negative control ($p < 0.01$). Conversely, no notable difference was observed when utilizing the MUT construct, indicating specific binding of miR-375 to the predicted site within the FZD4 3'UTR.

Discussion

The mature miRNA-375 sequence displays a high degree of conservation across multiple species, establishing a robust foundation for subsequent experiments. To investigate the regulatory function of miR-375 in sheep reproduction, real-time fluorescence quantitative PCR was employed to assess its expression within the HPG axis. Our results revealed significantly elevated expression of miR-375 in the pineal gland ($P < 0.001$) compared to the hypothalamus, pituitary, and ovary. This observation implies a pivotal role for miR-375 in pineal gland regulation, with potential implications for the secretion of pituitary FSH, LH, melatonin, and various anti-gonadotropin hormones, thereby influencing reproductive organ development. To gain deeper insights into

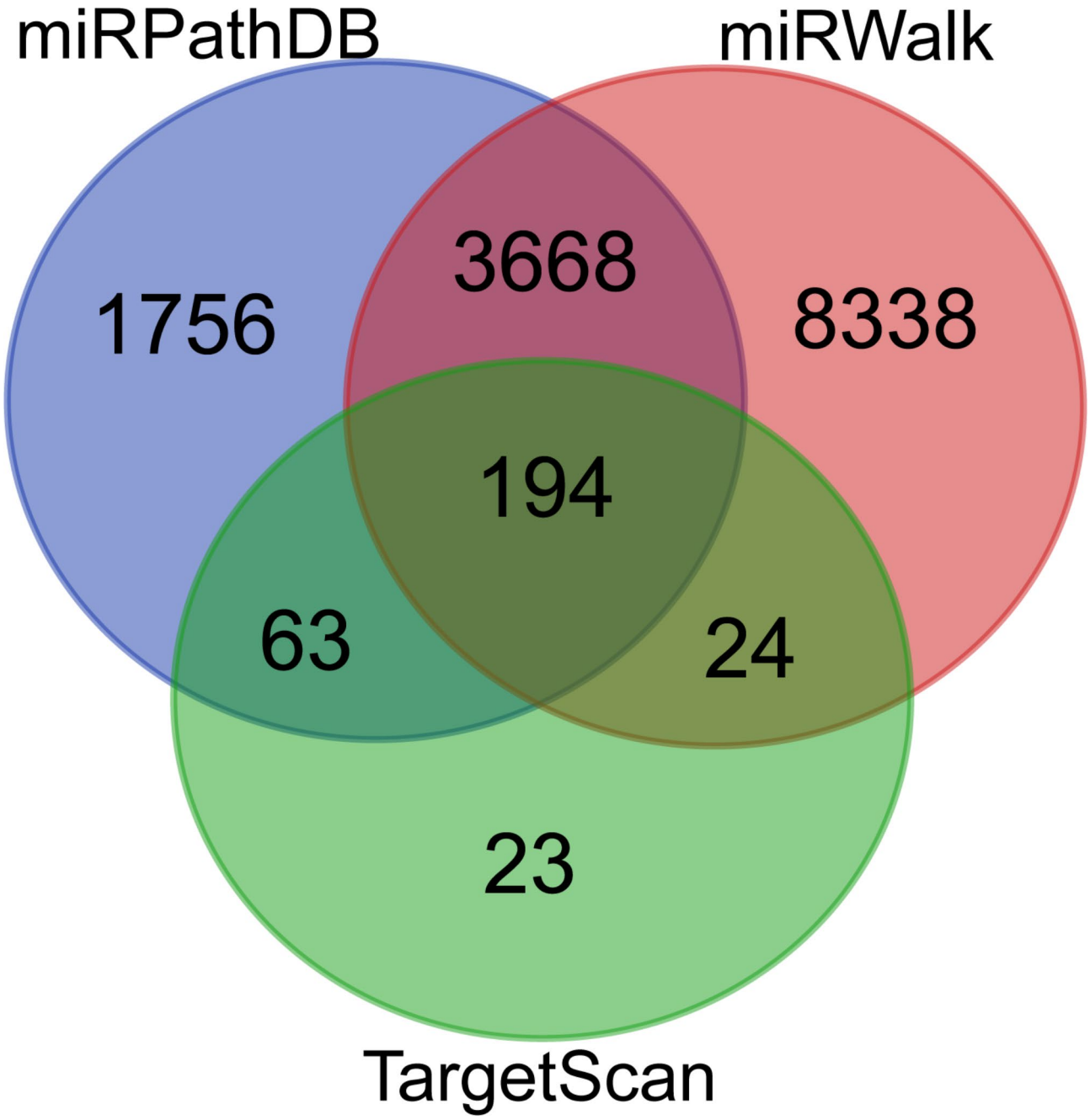

Fig. 4. Venn diagram of miRNA-375 target genes.

the function and mechanism of miRNAs, multiple online databases, such as TargetScan, were utilized for the prediction of their target genes. Subsequently, GO functional enrichment analysis and KEGG pathway enrichment analysis were conducted for the target genes derived from the intersection of these three databases. Dual luciferase assay was designed to verify the interaction between mirNA-375 and its screened target gene FZD4. The luciferase activity of wild type and mutant reporter gene vectors after transfection was compared to determine whether miRNA could specifically regulate the target gene.

Melatonin, a hormone secreted by the pineal gland, assumes a critical role in coordinating cellular responses to external light stimuli. Recent studies underscore the importance of melatonin in the reproductive process, impacting oocyte maturation and enhancing fertilization rates[34]. Given the heightened expression of miR-375 in the pineal gland, it is plausible that miR-375 exerts its influence over the synthesis and secretion of melatonin and other hormones, potentially affecting the reproductive performance of sheep. The role of miR-375 in a variety of diseases has also been investigated. Some scholars[35] found that miR-375 in hypoxic ischemic brain influences the damage degree of the pineal gland function. One group[36]found that the amount of miR-375 exosomes in plasma was associated with castration-resistant prostate cancer (CRPC) in men. The growth of

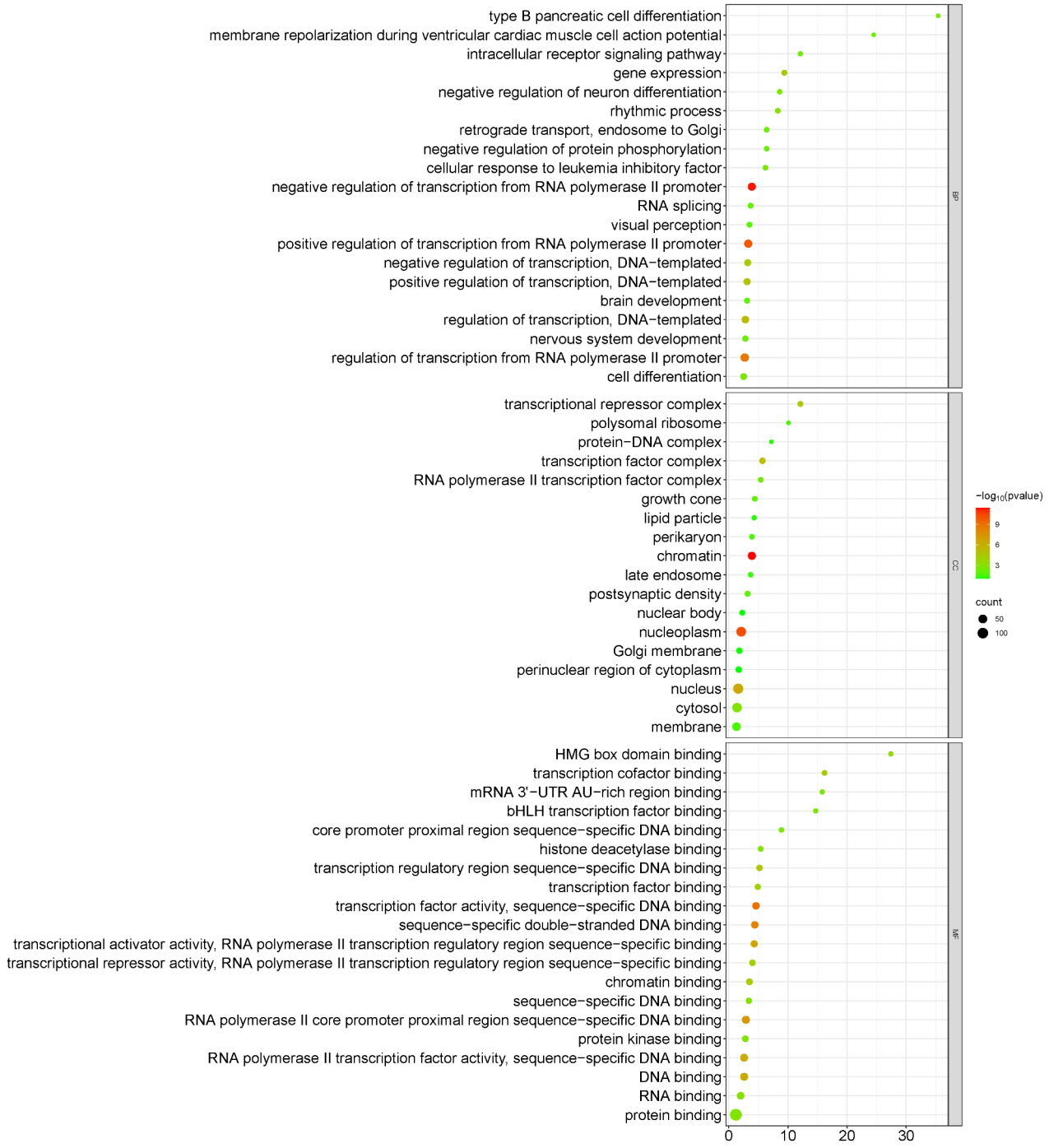

Fig. 5. GO enrichment analysis of miRNA-375. The significance threshold was $P < 0.05$. Red indicates molecular function.

castration-resistant prostate cancer (CRPC) is inhibited by the regulation of ptpn4 by miR-375 in further studies, which provides a new treatment method for prostate cancer. Knockout of miR-375 in the pituitary in mice resulted in a large reduction in the amount of pituitary prolactin (PRL)[37]. Prolactin level is closely related to the reproductive system of mammals, which can stimulate the production of follicle LH receptor. It can be inferred that miR-375 in sheep may affect the reproductive performance of sheep by changing the secretion level of PRL in the pituitary gland, which provides certain data support for studying the effect of miR-375 on PRL and reproductive system in sheep.

This investigation centers on elucidating the role of miR-375 in sheep reproduction regulation and its associated mechanisms, with specific attention to its tissue-specific characteristics, to explain the effect of miR-

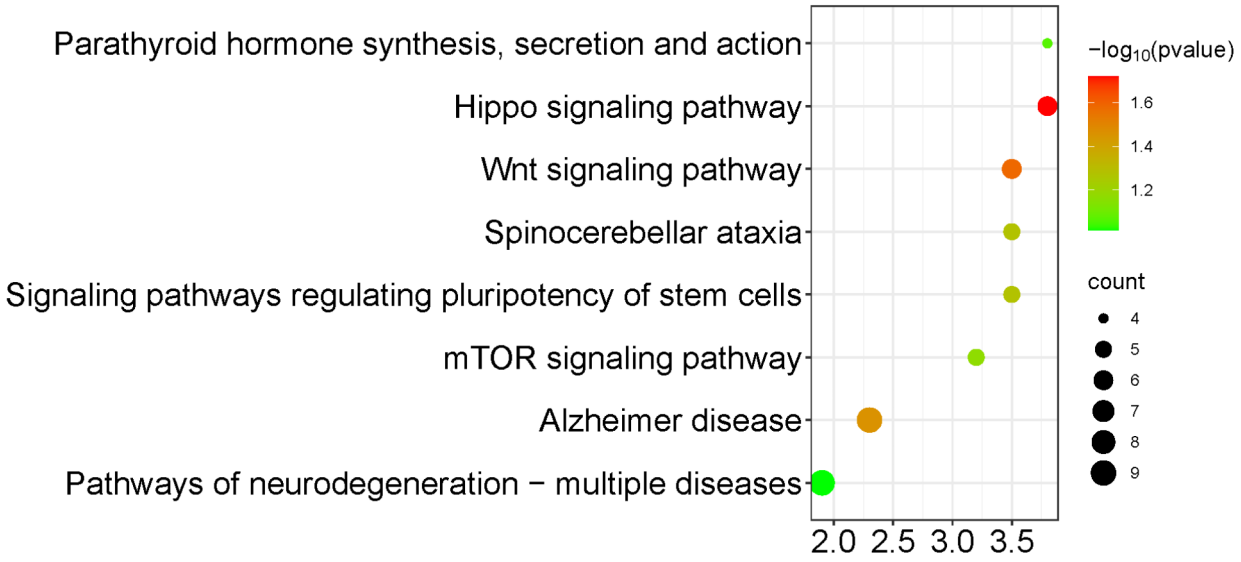

Fig. 6. KEGG pathway enrichment analysis of miRNA-375. The bubble size represents the number of genes, and the bubble color indicates that the enrichment difference was significant; among them, red represents a higher significance of the difference.

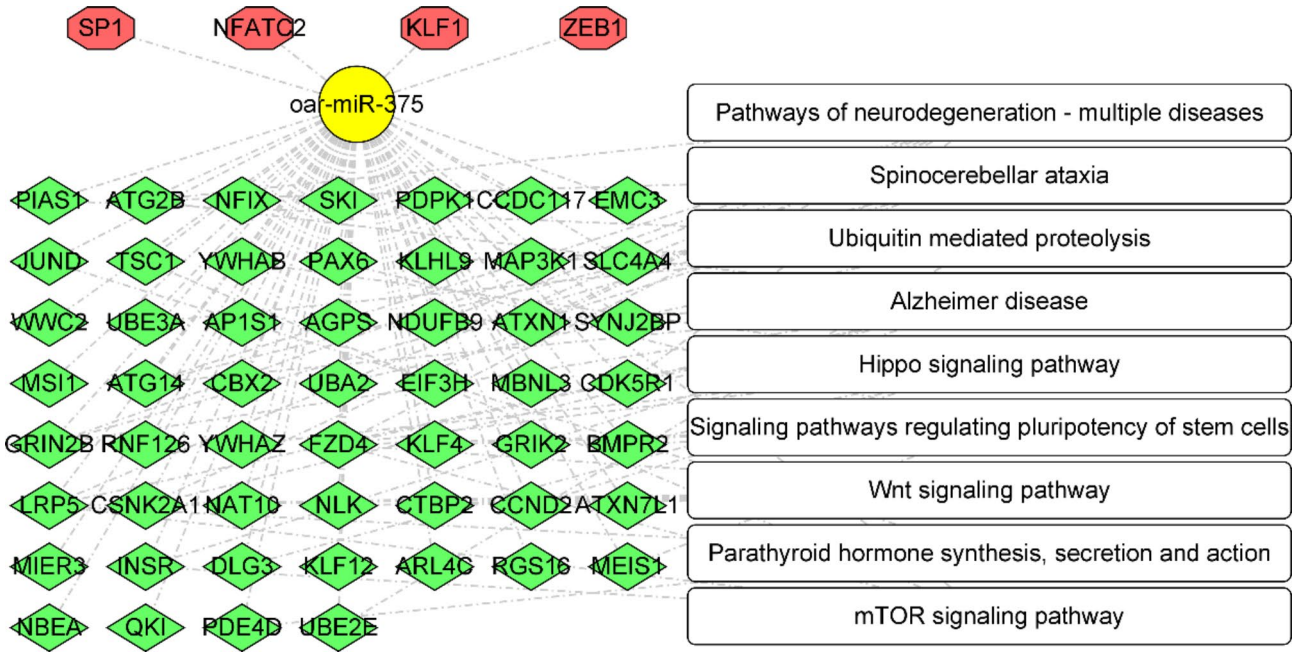

Fig. 7. oar-miR-375 target gene-transcription factor-signaling pathway interaction network diagram. Red hexagons represent transcription factors, yellow circles represent oar-miR-375, green diamonds represent target genes, and white rectangles represent signaling pathways.

375 on sheep reproduction through the perspective of pathways. KEGG analysis revealed that the miR-375 gene primarily participates in processes such as ubiquitin-mediated proteolysis, parathyroid hormone synthesis, secretion, and action, as well as the Hippo and Wnt signaling pathways. The Hippo signaling pathway was first identified in *Drosophila melanogaster*[38]. Later studies showed that Hippo pathway affects ovarian development in *Drosophila*[39–43]. Prior literature consistently underscores the relevance of the Hippo signaling pathway in reproduction, with implications for ovarian development, follicle development, and oocyte maturation[27,44],

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   Query:     3' agugcgCUCGGCUUGCUUGUUUu 5' (Target FZD4)
                     || ::|  :|||||||
   Ref:       5' aaaacaGAATTGTTTGAACAAAc 3' (miRNA miR-375-3p)

   Energy:  -14.400000 kCal/Mol
```

Fig. 8. After predicting the binding site of FZD4 to miR-375 using TargetScan, the binding energy was predicted using miRanda to evaluate the possibility of binding.

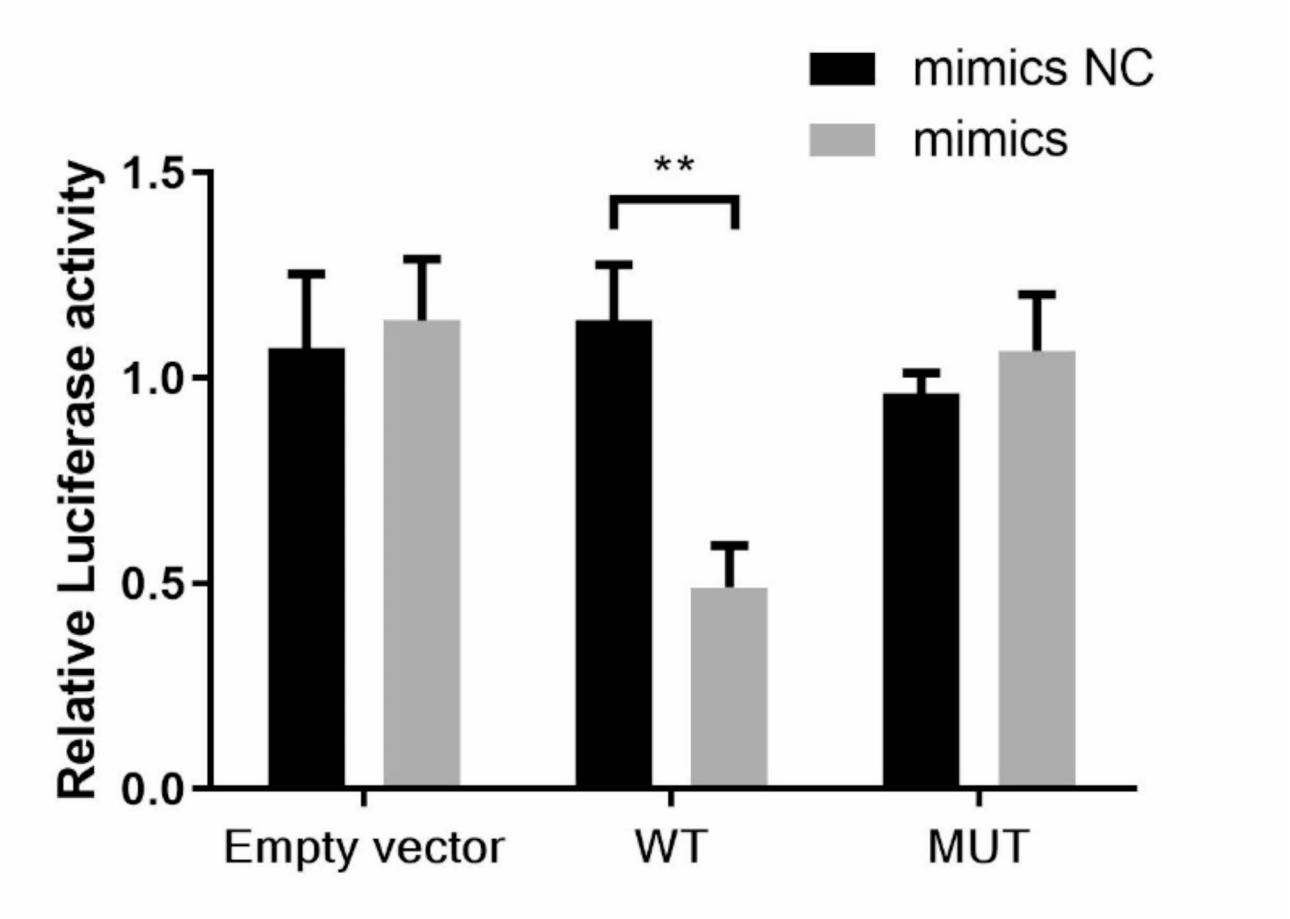

Fig. 9. Figure 9 illustrates the effect of miR-375 mimics on the relative luciferase activity of FZD4 wild-type (WT) and mutant (MUT) reporters in different plasmid vectors. The results showed that the relative luciferase activity of the empty vector and the WT plasmid in the experimental group was significantly lower than that in the control group ($P < 0.01$).

emphasizing its central role in ovarian physiology. The Hippo pathway is a highly conserved regulation pathway, which not only regulates the ovary and fertility, but also is related to certain diseases. Dysregulation of Hippo signaling in the ovary leads to YAP1 overexpression in granulosa cells, resulting in imbalances in tissue homeostasis and reduced fertility[27]. miR-375 may affect human polycystic ovary syndrome (PCOS), ovarian insufficiency and ovarian cancer by regulating the Hippo signaling pathway, which provides new ideas for the treatment of these diseases. The ovary is a dynamic structure that undergoes extensive tissue remodeling during

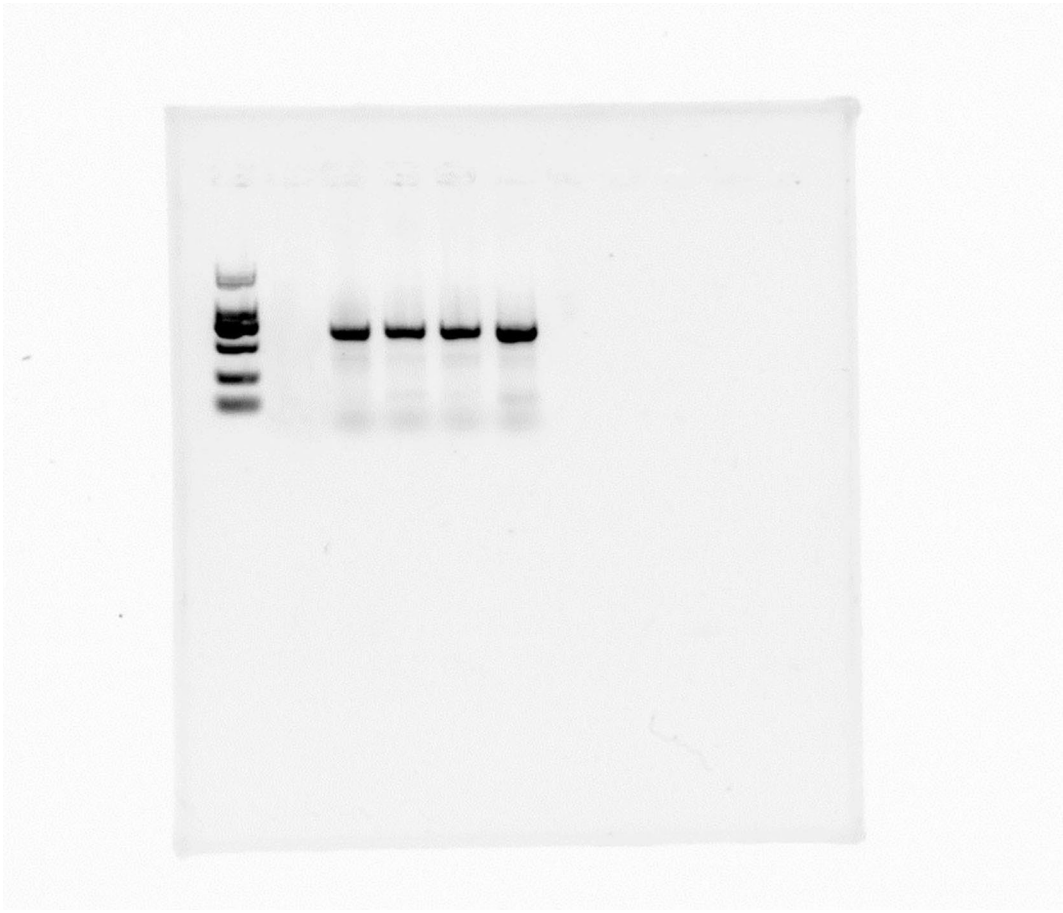

Fig. 10. Cloning results of untrimmed sheep miR-375 precursor sequences.

each reproductive cycle. The periodic remodeling of the ovary includes folliculogenesis and atresia, ovulation, luteinization and regression, and is accompanied by changes in the vasculature and microenvironment. Each step is related to the success of reproduction[45–47]. The in-depth study of miR-375 and Hippo pathway may solve the problem of ovarian dysplasia and infertility, and alleviate the error phenomenon in the process of cell proliferation and differentiation regulated by paracrine, autocrine and endocrine factors. Hippo pathway activates follicles and improves reproductive performance[27], which is similar to the expected results discussed in this study. miR-375 may improve the reproductive performance and economic benefits of sheep through Hippo pathway, but these need to be verified by further experiments. miR-375 may enhance reproductive performance and economic benefits in sheep via the Hippo pathway. This study has demonstrated a direct targeting relationship between miRNA-375 and the Hippo pathway gene FZD4 by validating the associated target genes; however, further experiments are required for confirmation.

In recent studies, it has been found that the oncoprotein YAP1 is targeted by miR-375 and can be inactivated via the Hippo pathway[48,49]. CircSH3BGRL3 isolates miR-375 and inhibits its activity, increases the expression of its target YAP1, and ultimately activates the Hippo signaling pathway[50]. The results of this experiment show that miR-375 can specifically inhibit the expression of FZD4 wild type, but not its mutant type. The direct target relationship between miR-375 and FZD4, the target gene of Hippo signaling pathway, was directly demonstrated. Thus, the target relationship between miR-375 and the Hippo pathway was confirmed, which is consistent with the predicted results in this study.

MiR-375 is a validated regulator of the Wnt pathway. miR-375 targets beta-catenin and FZD8 [51], and FZD8 activates the Wnt signalling pathway[52]. Consistent with the predicted results in this study, the results are plausible. The Wnt signaling pathway, highly conserved across animal species[53], includes various subpathways, including the Wnt/β-catenin pathway, planar cell polarization pathway, Wnt/Ca_2^+ pathway, asymmetric cell division pathway, and spindle orientation pathway. Recent investigations have shed light on the regulatory influence of the Wnt signaling pathway on ovarian granulosa cell development, particularly in cell cycle modulation[54–56]. Notably, the Wnt signaling pathway orchestrates its functions through the interplay of multiple signaling molecules, receptors, and ligands, ultimately governing the growth and development of granulosa cells[54].

A study conducted in 2019 confirmed that XIST can activate the mTOR signaling pathway via miR-375, which further influences the inhibition of cell proliferation[57]. Additionally, miR-375 impacts cell migration, potentially affecting the migration of fertilised eggs to the uterus in sheep, thereby influencing the implantation of sheep blastocysts and impacting reproduction. Some studies have also demonstrated the relationship between

miR-375 and the mTOR signaling pathway in mammals[58]. This finding aligns with the predicted results of our study and holds value for further research.

The aforementioned references clearly illustrate a connection between miR-375 and the Hippo, Wnt, and mTOR signaling pathways, with several key target genes identified, such as YAP1, FZD8, etc., which correspond with our predicted results. Notably, there are common and significant genes: the Hippo signaling pathway, WNT signaling pathway, and mTOR signaling pathway are all enriched for the target gene FZD4; both Hippo and WNT pathways are enriched for the target gene CCND2; and both WNT and mTOR pathways are enriched for the target gene LRP5.

FZD4 is a transmembrane protein belonging to the Wnt signalling pathway receptor family, which plays a key role in a variety of biological processes, including cell proliferation, migration, differentiation, and apoptosis, and FZD4 is found throughout the human body, mainly with the ovaries of adults[59], which suggests that possibly miR-375 can have an effect on the reproductive function of animals through FZD4. FZD4 was found to be associated with Hippo, Wnt and mTOR signaling pathways. miR-375 targets FZD4's 3'utr and potentially impacting sheep breeding. This is important for studying the relationship between miR-375, FZD4 and signaling pathways. But necessitating further research on its regulatory effects.

CCND2 belongs to the family of cell cycle proteins and affects the G1/S phase of cell mitosis[60], which suggests that it is possible that miR-375 can affect cell proliferation through CCND2 by influencing cell mitosis.

LRP5 is an important transmembrane protein mainly involved in the regulation of cellular signal transduction and bone metabolism, which participates in the Wnt signalling pathway by binding to the Frizzled receptor, thus regulating the balance between osteogenesis and bone resorption, which is a key cellular signaling pathway that has an important impact on cell proliferation and differentiation[61,62]. This suggests that it is possible that miR-375 can have an effect on the Wnt signalling pathway via LRP5 and have some effect on neonatal bone development in the ovary.

The results of the present study showed that miR-375 was able to specifically inhibit the expression of FZD4 wild-type without affecting its mutant form, directly confirming a direct targeting relationship between miR-375 and the target gene FZD4. To date, no study has detailed how miR-375 affects hypothalamic, pituitary and ovarian functions by regulating Hippo and Wnt signaling pathways. Therefore, the present study sought to uncover these associations as a direction for future research. The hypothalamus is responsible for regulating the activities of several endocrine glands, such as the pituitary gland.The Hippo signalling pathway affects reproductive and other endocrine functions by regulating the hypothalamic-pituitary-gonadal axis (HPG axis).The expression of YAP and TAZ in the hypothalamus may affect the secretion of gonadotropins, which in turn affects the levels of reproductive hormones[63,64]. The Hippo signalling pathway is also involved in the hypothalamic response to stress. Under stress, the activity of the Hippo signalling pathway may be altered, which in turn affects the function of the hypothalamus. By regulating the neuroendocrine activity of the hypothalamus, the Hippo signalling pathway may play an important role in the body's response to stress[65,66]. Given the relationship between miR-375 and the above signaling pathways, it is speculated that Mir-375 may affect the reproductive ability of sheep through these pathways, but the specific mechanism remains to be verified by further experiments.

The WNT signalling pathway is thought to play an important role in the development of the hypothalamus. It has been found that WNT signalling influences the structural formation of the hypothalamus by regulating the morphogenesis and cellular differentiation of the neuroepithelium of the forebrain[67,68]. WNT signalling also plays a key role in hypothalamic neurogenesis. The activity of WNT signalling was found to be closely related to the proliferation and differentiation of hypothalamic neural precursor cells[69,70]. During embryonic developmental stages, WNT signalling promotes the generation of neurons in the hypothalamus and affects their morphology and function, with implications for ovarian function[71] .

Embryonic stem cells (ESCs), also referred to as pluripotent stem cells, possess remarkable capabilities for differentiation into various cell types. Derived from the inner cell mass of the blastocyst stage, ESCs can be cultured in vitro and exhibit unlimited proliferation potential, self-renewal capacity, and differentiation potential. They find extensive applications in diverse fields, such as cell transplantation, modeling human genetic diseases, and animal breeding. The exploration of the association between miR-375 and target genes such as BMPR2, CCND2, DLG3, FZD4, and YWHAB/Z hints at a potential regulatory role of miR-375 in the Wnt signaling pathway. The present study has validated the interaction between miR-375 and its target gene FZD4, providing further insight into this relationship.

In conclusion, miR-375 may affect the hypothalamus, pituitary and ovary by regulating the Hippo and Wnt signalling pathways and influencing key functions such as cell proliferation, hormone secretion and signalling.

Conclusions

In this study, a precursor sequence cloning experiment was conducted to confirm the conservation of sheep miRNA-375 throughout evolution. The results from real-time fluorescence quantification indicated that miRNA-375 exhibited high expression levels in the pineal gland. Further analysis through GO and KEGG enrichment demonstrated miRNA-375 was most likely involved in pathways related to sheep reproduction through the Hippo pathway and Wnt pathway, suggesting its potential role in regulating the reproductive process. These findings provide evidence that miR-375 directly targets the 3'UTR of FZD4, which may have implications for the breeding process in sheep. Further investigation is warranted to explore the downstream effects of this regulation on cellular functions and disease progression. Overall, the findings of this study provide essential foundational data for investigating the role of miRNA-375 in sheep reproduction and lay the groundwork for future studies aiming to elucidate its underlying mechanisms.

Data availability

Experimental data have been uploaded to the SRA database and can be obtained by searching PRJNA1117672 at NCBI.

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Declarations

Competing interests

The authors declare no competing interests.

Institutional review board statement

The animal study protocol was approved by the Henan University of Science and Technology Animal Welfare and Use Committee (protocol code 2019HkDDk1201).

Conflict of interest

The authors declare no conflicts of interest.

Additional information

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