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LncRNAs 60248.51 and 9826.1 are identified as potential regulators of endometrial receptivity in Tibetan Northwest Cashmere goats

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Abstract

Endometrial receptivity is a key determinant of embryo implantation, which is the initial step of a successful pregnancy. In this study, high-throughput RNA sequencing was conducted in pre-receptive endometrium (PE) and receptive endometrium (RE) derived from Tibetan Northwest Cashmere goats. There were only a handful of miRNAs and circRNAs differentially expressed between PE and RE tissues, while there were 250 upregulated coding genes and 193 upregulated lncRNAs and 135 downregulated coding genes and 123 downregulated lncRNAs in RE tissues, suggesting the predominant role of coding genes and lncRNAs in the regulation of endometrial receptivity. Moreover, gene ontology and Kyoto Encyclopedia of Genes and Genomes analyses revealed that differentially expressed lncRNAs are significantly enriched in Wnt, Hedgehog, and Hippo signaling pathways. Furthermore, the two most upregulated lncRNAs, MSTRG.60248.51 (1264 bp) and MSTRG.9826.1 (1362 bp), were screened as candidate lncRNAs, and both upregulation of 60248.51 and 9826.1 could activate Wnt and Hippo signaling and promote cell viability, migration, and secretion of endothelin-1, epidermal growth factor, and colony stimulating factor in primary goat endometrial epithelial cells. In summary, lncRNAs 60248.51 and 9826.1, identified as potential regulators of endometrial receptivity in Tibetan Northwest Cashmere goats, improve cell viability, migration, and secretion functions in endometrial epithelial cells, which may be associated with the Wnt and Hippo signaling pathways.

Keywords: Tibetan Northwest cashmere goats; endometrial receptivity; lncRNA 60248.51 and lncRNA 9826.1; primary goat endometrial epithelial cells; the Wnt and Hippo signaling pathways.

Practical Application: Transcription factor family clustering for differentially expressed coding genes.

1 INTRODUCTION

Cashmere is a thin layer of fine hair growing on the skin of goats and covering the root of coarse goat hair, which naturally adapts the goat to the climate. Cashmere is very precious not only because of its scarce output (only accounting for 0.2% of the total output of animal fibers in the world), but also because of its excellent quality and characteristics, being considered "fiber jewel," "fiber Queen," and "soft gold" (Duan et al., 2022). Cashmere is incomparable to all textile raw materials that can be used by humans. About 70% of the world's cashmere is produced in China. The cashmere goat, a goat breed with the highest cashmere yield and the best cashmere fiber quality in the world, is an important biological resource in China (Jin et al., 2020). With the increasing demand for quantity and quality of cashmere, the survival and development of cashmere goats are facing great challenges.

The cashmere goat is mainly distributed in cold regions, such as North China, Northeast China, Northwest China, and Southwest China, among which Inner Mongolia and Tibet are the highest-quality cashmere-producing areas. The Tibetan Northwest Cashmere goat is a specific goat breed in the Tibet Autonomous Region that is located in the vast and sparsely populated Qinghai Tibet Plateau. Tibetan Northwest Cashmere goats are mainly raised by semi-grazing, which combines natural grassland and house feeding. This breed has excellent cashmere production characteristics and is a livestock variety under key protection in China. It has outstanding characteristics such as rough feeding resistance, cold tolerance, anoxia tolerance, good cashmere production performance, fine cashmere, and excellent breed quality. Compared with cashmere goat sub-breeds in most other regions, Tibetan Northwest Cashmere goats have great advantages in cashmere color (pure white individuals account for more than 95%), cashmere yield (up to 700 g), cashmere fiber fineness (the main body fineness of pile fiber below 14 μm occupies more than 70%), and cashmere layer height (average tomentum height is larger than 4 cm) (Li et al., 2022). However, affected by various natural and human factors, including the gradual deterioration of the ecological environment in Northwest Tibet and the lack of scientific selection and breeding

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during the traditional and extensive management of farmers and herdsmen for a long time, this breed has a tendency of degradation. Moreover, due to the poor natural environment and the extensive feeding methods of farmers and herdsmen in Tibet, the conception rate of Tibetan Northwest Cashmere goats has been greatly affected. Therefore, it is urgent to take steps to improve the conception rate of Tibetan Northwest Cashmere goats based on a deep understanding of reproductive regulation mechanisms.

There are many factors affecting the conception rate, including estrous cycle, ovulation time after estrus, mating season, the number, time, and site of insemination, as well as the status of the female goat uterus. Embryo implantation is the initial step of a successful pregnancy, consisting of apposition, adhesion, and invasion. Endometrial receptivity is essential for the implantation of embryos, which is a spatially and temporally restricted stage (Ashary et al., 2018). During the development of endometrial receptivity, the ovary produces a series of hormones, including steroids, estrogen, and progesterone, to adjust the structure and function of the endometrium to facilitate embryo implantation (Lessey & Young, 2019). It has been acknowledged that dysfunction of receptive endometrium (RE) can lead to infertility and is a major obstacle for embryo transplantation in assisted reproduction.

High-throughput sequencing has been widely applied in studying human and animal disease pathogenesis, development mechanisms, genetics, and breeding. A couple of studies have given us a certain amount of increased knowledge of the genetic information on the regulation of endometrial receptivity in goats. However, it is still far from a clear understanding of its regulatory mechanism. Here, RNA sequencing was used to comprehensively understand the transcriptional profiles during endometrial receptivity, and lncRNAs MSTRG.60248.51 and 9826.1 were found to be two of the most significantly upregulated lncRNAs, the regulatory role of which was preliminarily investigated in primary goat endometrial epithelial cells.

2 MATERIALS AND METHODS

2.1 Ethics statement

Animal protocols were approved by the Ethics Committee of College of Agriculture and Animal Husbandry of Tibet Autonomous Region.

2.2 Study design and sample collection

A total of 10 healthy, 24-month-old multiparous Tibetan Northwest White Cashmere goats were induced to oestrous synchronization for this study, and they were observed three times daily to ascertain oestrous signs and mated naturally twice during oestrous. The first day of mating was set as day 0 of pregnancy. As very important time points for embryo implantation, gestational days 5 (a presentative time point of pre-receptive endometrium [PE]) and 15 (a presentative time point of RE) are selected as the slaughter days of the goats (three goats in each group were randomly selected) (Zhang et al., 2015), and the endometrial tissue was obtained from the anterior wall of their uterine cavity. The samples were washed with PBS and frozen in liquid nitrogen immediately.

2.3 RNA sequencing and analysis

Total RNA was extracted from the endometrial samples by using the Trizol reagent (Invitrogen, Carlsbad, CA). RNA quantity and purity were analyzed using Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) and RNA 6000 Nano LabChip Kit (Agilent). Library construction, sequencing (paired-end, 2*150 bp, Illumina Hiseq2500), and identification of potential novel transcripts were conducted by the PersonalBio Co., Ltd. (Shanghai, China). Clean reads were aligned to the human genome by HISAT, allowing four mismatches. Expression levels of all of the transcripts, including putative mRNAs, lncRNAs, circRNAs, and small RNAs, were quantified as fragments per kilobase of exon per million fragments mapped using the Cuffdiff program (Cufflinks package). Differential gene expression was determined using Cuffdiff with a $p<0.05$.

2.4 Functional annotation

Unigene annotations provide functional annotations for their expression levels, which were analyzed using protein sequence similarity by PersonalBio Co., Ltd. Protein function information could be predicted from annotations in the following published databases: SWISS-PROT, NR, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Pfam. All searches were conducted with BLASTx using a minimum E-value$<1^{e-10}$ as the threshold.

2.5 RNA extraction and quantitative polymerase chain reaction

Total RNA from tissue samples or cultured cells was extracted by using the TRIzol Reagent (Invitrogen, Carlsbad, CA) and reversely transcribed with the Prime Script TMRT Master Mix kit (Takara, Dalian, China). Then, qPCR reactions were conducted using a SYBR Green PCR kit (Takara) and run in the QuantStudio 6 Flex Real-Time PCR systems (Thermo Scientific). 18S RNA was used as the loading control. The abundance of the transcripts was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.6 Isolation, culture, and treatment of primary goat endometrial epithelial cells

The segment of uterine horn was taken from young Tibetan Northwest White Cashmere goats, placed in D-Hank's balanced salt solution containing double antibodies, and repeatedly rinsed until the uterus was white. The longitudinal layer and the endometrial layer were removed and repeatedly rinsed with D-Hank's balanced salt solution (#QYR6080, QualitYard, Beijing, China) for 3–4 times until the liquid was clear. The obtained tissue was washed with penicillin, cut into small pieces of about 1 mm^3, washed with D-Hank's balanced salt solution for 3–4 times, transferred into a triangle bottle with glass beads, and digested with 2 g/L collagenase I in a water bath at 37°C for 4 h. After digestion, the triangular flask was vigorously shaken to obtain a suspension, and then the suspension was passed through a 200-mesh filter screen. The filtered liquid was transferred into a centrifuge tube and centrifuged at 500 r/min for 5 min. The precipitate was resuspended in D-Hank's balanced salt solution and re-centrifuged, which was repeated three times.

Finally, the precipitate was suspended in DMEM/F12 medium plus 10% fetal bovine serum (FBS), seeded in the dish, and then set in an atmosphere of 37°C, 5% CO_2, and saturated humidity. The cultured cells were identified as cytokeratin and vimentin dual-positive cells so as to verify successful separation.

Adenoviral overexpression vectors of lncRNA MSTRG.60248.51 (Ad-60248.51, $>10^{10}$ plaque forming unit (PFU)/mL) and Ad-9826.1 ($>10^{10}$ PFU/mL) were constructed and packaged by the GenScript ProBio Co., Ltd. (Nanjing, China). For cell infection, 1 mL of 10^{10} PFU/mL Ad-MSTRG.60248.51 or Ad-9826.1 was added in each 30 mm dish together with 1 mL of DMEM/F12 medium plus 10% FBS. After incubation for 48 h, cells were harvested for the following tests.

2.7 Cell Counting-Kit-8 (CCK-8) assay

The 2×10^4 cells (per well) were seeded into 96-well plates and cultured for 24 h. A volume of 10 μL of CCK-8 resolution was added into each well, incubated with the cells for another 2 h, and detected under a Multifunctional Microwell Plate Tester at 450 nm (Thermo Scientific).

2.8 Transwell assay

The Transwell system (Millipore) was used to detect the migration capacity of the endometrial epithelial cells. Briefly, cells in serum-free medium were loaded in the top chamber, and fresh medium with 10% FBS was added to the lower chamber. After incubation for 12 h at 37°C, migrated cells were fixed and stained. Migrated cell numbers were counted under a CX43 optical microscope (OLYMPUS).

2.9 Western blotting

Total protein was extracted from cultured cells with Radio Immunoprecipitation Assay (RIPA) lysis buffer (Sangon Biotech, Shanghai, China) containing cocktails of protease and phosphatase inhibitors. Then, 25 μg of protein was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking, the membranes were respectively mixed with primary antibodies at 4°C overnight and then incubated with horseradish peroxidase-conjugated secondary antibodies. The blots were visualized by using the enhanced chemiluminescence (ECL)-Plus Analysis Detection Kit in a gel imaging system (Thermo Fisher Scientific). Antibodies used in this study are as follows: anti-Wnt1 (1:800; ab63934; Abcam), anti-β-catenin (1:600; ab224803; Abcam), anti-MOB1A (MOB kinase activator 1A; 1:800; ab236969; Abcam), anti-YAP1 (Yes-associated protein 1; 1:500; ab52771; Abcam), anti-SHh (Sonic Hedgehog; 1:500; ab240438; Abcam), anti-GLI1 (1:600; ab134906; Abcam), and anti-β-actin (1:1000; ab8227; Abcam).

2.10 Enzyme linked immunosorbent assay

The secretion levels of endothelin-1 (ET-1), epidermal growth factor (EGF), and colony stimulating factor (CSF) in the supernatant of endometrial epithelial cells were detected with corresponding ELISA kits (Sigma) according to the manufacturer's instructions.

2.11 Statistical analysis

Principal component analysis was performed by the R package factoextra to show the clustering of samples. The heatmap package in R was used to perform the clustering based on Euclidean distance. The Student's t-test was used for the comparison between the two groups.

3 RESULTS

3.1 Differentially expressed miRNAs between PE and RE are significantly associated with tissue, organism and system development

RNA sequencing was conducted in endometrial tissues derived from gestational days 5 (representing the PE period) and 15 (representing the RE period) of Tibetan Northwest Cashmere goats. Expression levels of all of the transcripts, including putative mRNAs, lncRNAs, circRNAs, and small RNAs, were quantified and annotated. Up to 95% of the small RNAs were miRNAs (Figure 1A), and most miRNAs started with the nucleotide A or U (Figure 1B). Unfortunately, there were only four significantly upregulated and four downregulated miRNAs between PE and RE tissues (Supplementary Material 1). Functional annotations showed that these miRNAs were significantly associated with tissue, organism, and system development (Figure 1C) and enriched in cancer-related pathways and kinase pathways, including phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT), mitogen-activated protein kinase (MAPK), and the Hippo signaling pathway (Figure 1D).

3.2 Differentially expressed circRNAs are significantly associated with antigen presentation and enriched in immunological rejection-related pathways

Next, circRNAs were analyzed and annotated. The length distribution of circRNAs basically conformed to the normal distribution; >95% of circRNAs were at lengths of about 10^2 bp to $10^{3.5}$ bp, and the peak is located at about $10^{2.6}$ bp (Figure 2A). Type distribution analysis revealed that nearly 80% of identified circRNAs were exon-derived circRNAs, and nearly 10% of them were intron-exon circRNAs (Figure 2B). Chromosome distribution analysis revealed that these circRNAs were equally distributed on all chromosomes, and circRNAs detected in RE tissues occupied about 60% of most chromosomes (Figure 2C). Volcano and heatmap results indicated that there were 11 significantly upregulated and 8 downregulated circRNAs in RE tissues (Figure 2D). Functional annotations showed that these circRNAs were significantly associated with antigen presentation complexes and antigen processing (Figure 2F) and enriched in immunological rejection-related pathways, such as IgA production, allograft rejection, and graft-versus-host disease (Figure 2G).

3.3 Differentially expressed coding genes are significantly associated with cell motility and shaping and enriched in glycerolipid and pyrimidine metabolism-related pathways

Subsequently, we found that there were 17604 identified coding genes in PE and RE tissues, including 250 significantly upregulated and 135 downregulated (RE/PE, Figures 3A and 3B). Gene ontology (GO) analysis showed they were significantly

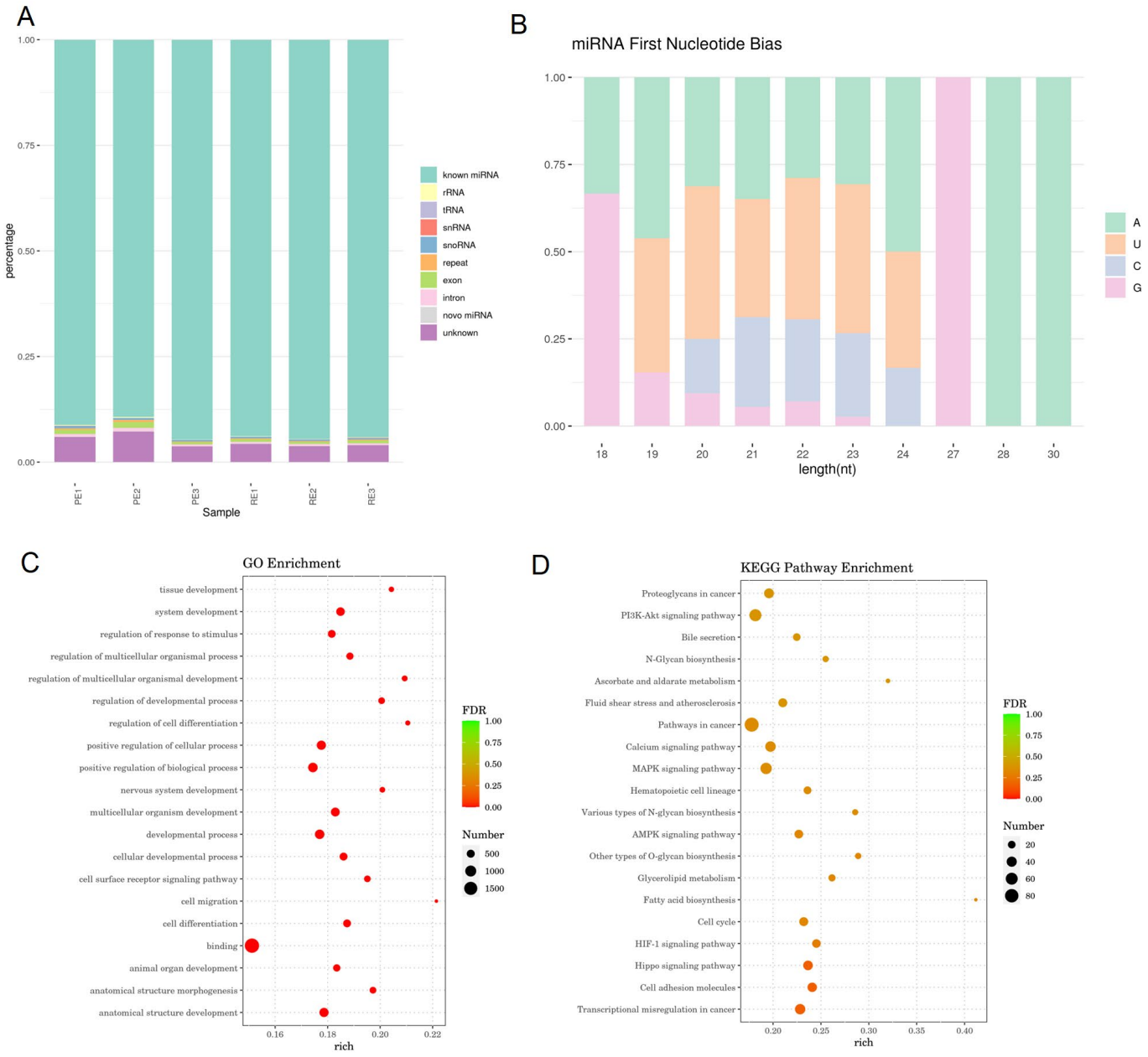


Figure 1. Classification and function prediction of differentially expressed miRNAs between PE and RE. RNA sequencing was conducted in endometrial tissues derived from gestational days 5 (representing the PE period) and 15 (representing the RE period) of Tibetan Northwest Cashmere goats. The expression levels of small RNAs were quantified and annotated. (A) Percentages of various classes of sequenced small RNAs, including miRNAs, other known small RNAs, and unknown small RNAs. (B) First nucleotide bias of miRNAs at different lengths. (C and D) GO and KEGG functional annotations for differentially expressed miRNAs.

associated with cell projection, microtubule-based process, and cilium, which are important in cell motility and shaping (Figure 3C). KEGG analysis showed that they were enriched in glycerolipid and pyrimidine metabolism-related pathways (Figure 3D). Moreover, transcription factor (TF) family clustering showed that nearly 500 of the 17604 coding genes belonged to the zf-C2H2 TF family and nearly 200 genes belonged to the Homeobox TF family. There were respectively 20 to 100 genes that belonged to the TF families: basic helix-loop-helix (bHLH), high-mobility group (HMG), basic (region-leucine) zipper (bZIP), Zinc Finger and BTB Domain-Containing Protein (ZBTB), Fork_head, E-twenty-six (ETS), and V-myb avian myeloblastosis viral oncogene homolog (MYB).

3.4 Differentially expressed lncRNAs are significantly associated with response to multiple signals and regulation of cell cycle and enriched in Wnt, Hedgehog, and Hippo signaling pathways

Furthermore, lncRNAs were analyzed and annotated. The coding potentials of lncRNAs were evaluated (Supplementary Material 2). About 7500 lncRNAs had 2 exons, about

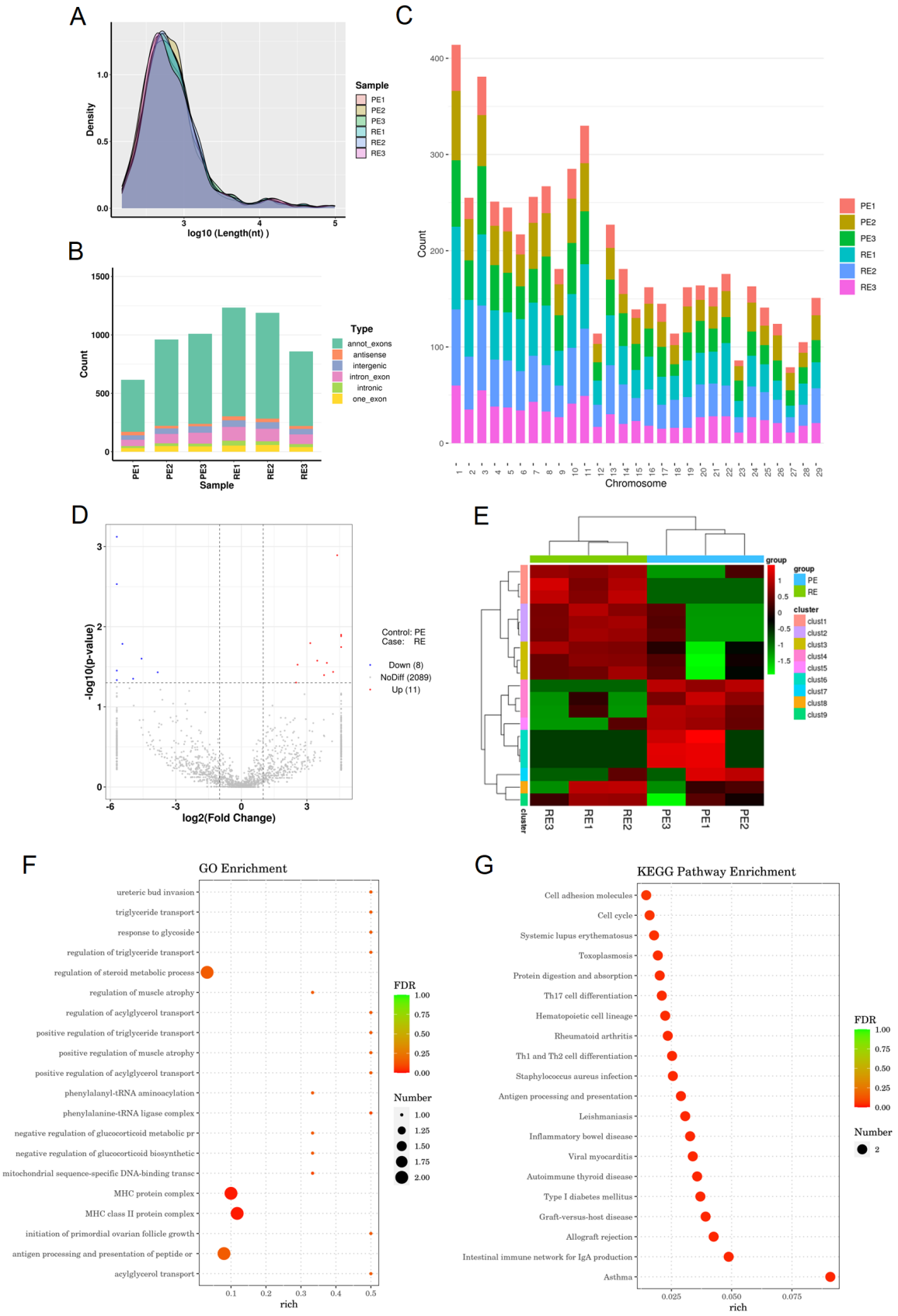


Figure 2. Sequence characteristics and function prediction of differentially expressed circRNAs between PE and RE. (A) The length distribution of sequenced circRNAs in each sample. (B) Proportions of various classes of sequenced circRNAs in each sample. (C) Chromosome distribution of sequenced circRNAs in each sample. (D and E) Volcano plots and heatmaps of differentially expressed circRNAs between PE and RE. (F and G) GO and KEGG functional annotations for differentially expressed circRNAs.

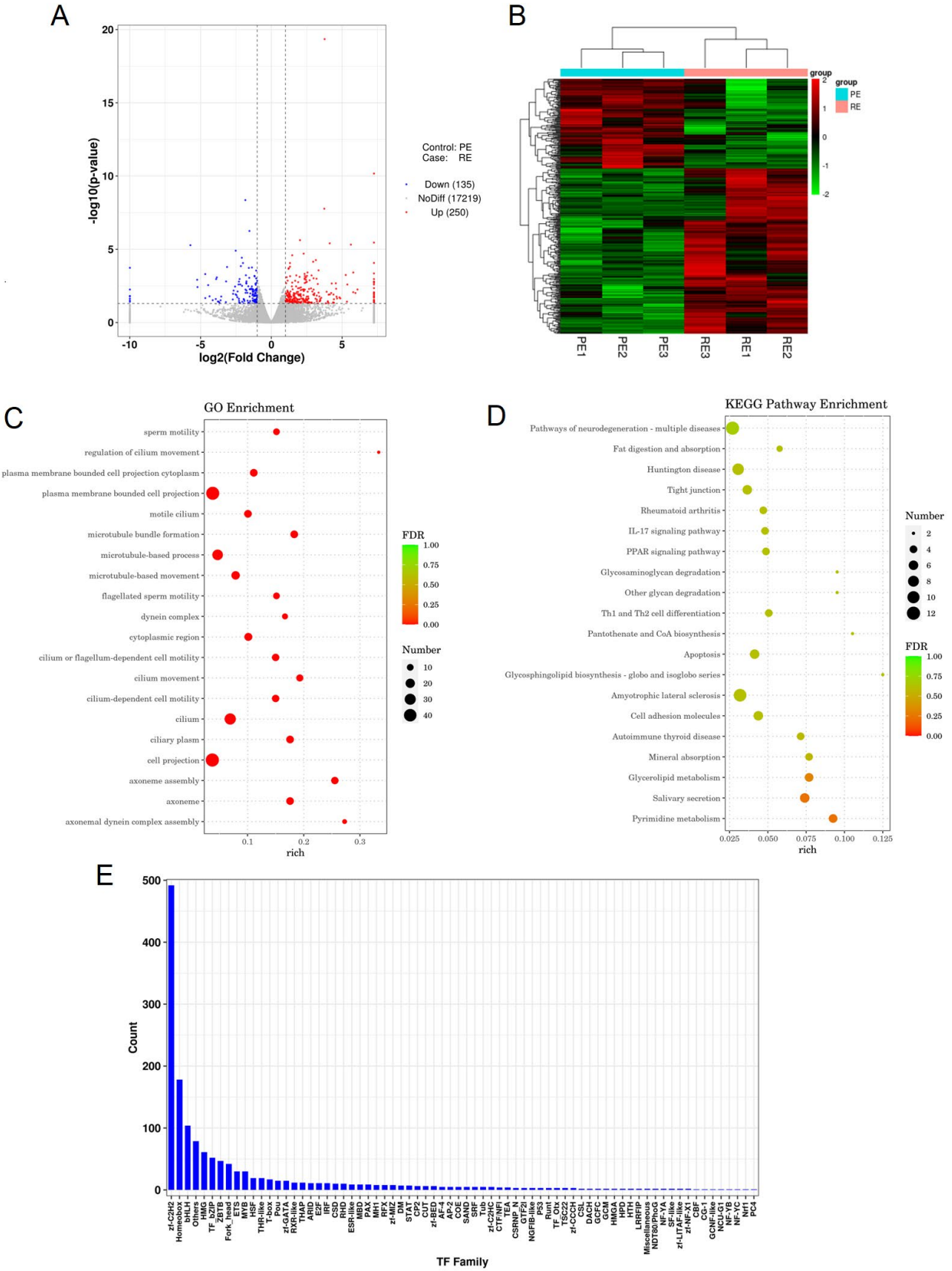


Figure 3. Functional annotations of differentially expressed coding genes. (A and B) Volcano plots and heatmaps of differentially expressed coding genes between PE and RE. (C and D) GO and KEGG functional annotations for differentially expressed coding genes. € Family affiliations of transcription factors in all detected coding genes.

2,500 had 3 exons, about 800 had 4 exons, and only a small part of the lncRNAs had 5 or more exons (Figure 4A). In contrast, more than 13,000 coding transcripts had 11 or more exons, and 1,500–2,000 coding transcripts had 10 or less exons (Figure 4A). Length distribution analysis revealed that the length of most lncRNAs was less than 2,000 bp, especially from 200 to 1,000 bp (Figure 4B). In contrast, the length of most mRNAs was less than 4,000 bp, but that of a considerable number was no less than 5,000 bp (Figure 4B). Density analysis revealed that the number of upregulated lncRNAs is a little larger than the number of downregulated lncRNAs (Figure 4C). There were 193 significantly upregulated and 123 downregulated lncRNAs (Figures 4D and 4E). GO analysis indicated that these lncRNAs are significantly associated with response to multiple signals and regulation of cell cycle and intracellular biological processes (Figure 4F). KEGG analysis showed that they were enriched in Wnt, Hedgehog, and Hippo signaling pathways (Figure 4G).

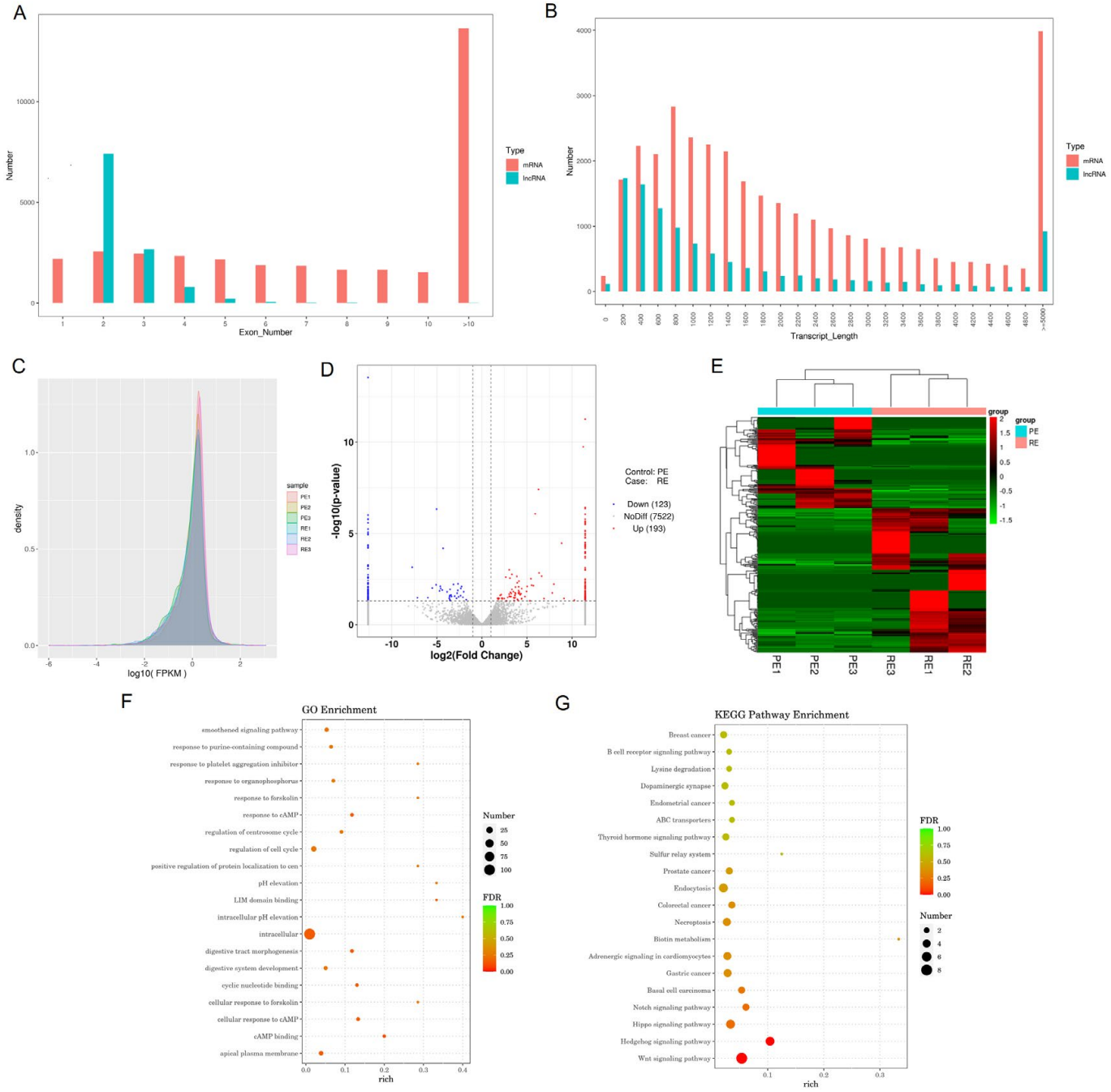


Figure 4. Sequence characteristics and function prediction of differentially expressed lncRNAs between PE and RE. (A) Distribution of number of exons of sequenced lncRNAs. (B) Length distribution of sequenced lncRNAs. (C) Density analysis of sequenced lncRNAs in each sample. (D and E) Volcano plots and heatmaps of differentially expressed circRNAs between PE and RE. (F and G) GO and KEGG functional annotations for differentially expressed lncRNAs.

3.5 LncRNAs 60248.51 and 9826.1 might be key regulators of endometrial receptivity

Supplementary Materials 3 and 4 showed the top 10 upregulated lncRNAs (RE/PE) and their sequences, including MSTRG.26755.8, MSTRG.37411.29, MSTRG.60248.51, MSTRG.60107.10, MSTRG.10519.3, MSTRG.2773.1, MSTRG.9826.1, MSTRG.60169.6, MSTRG.60248.50, and MSTRG.54294.13, and qPCR was used to verify the sequencing results. Our results showed that MSTRG.60248.51 (1,264 bp) and MSTRG.9826.1 (1,362 bp) were the two most upregulated lncRNAs in the RE tissue compared with the PE tissue (Figure 5). Then, adenovirus-mediated overexpression experiments were performed in primary goat endometrial epithelial cells to explore the potential role of 60248.51 and 9826.1 in endometrial receptivity in vitro. CCK-8 and Transwell cell migration assays indicated that both upregulation of 60,248.51 and 9,826.1 could moderately promote cell viability and markedly increase cell migration capacity (Figures 6A–6C). ET-1, EGF, and CSF are important secreted cytokines in endometrial epithelial cells. ELISA results showed that both upregulation of 60,248.51

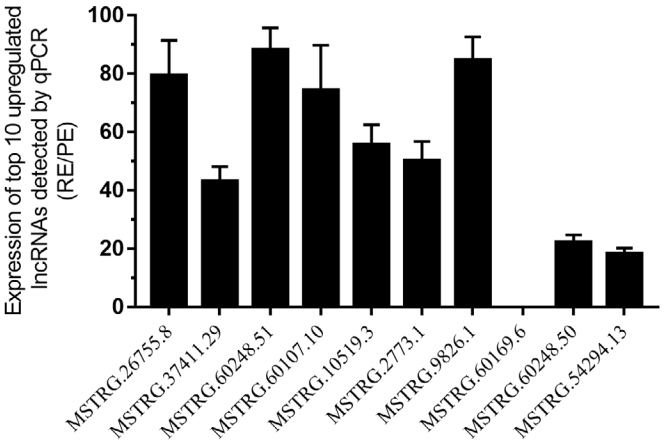
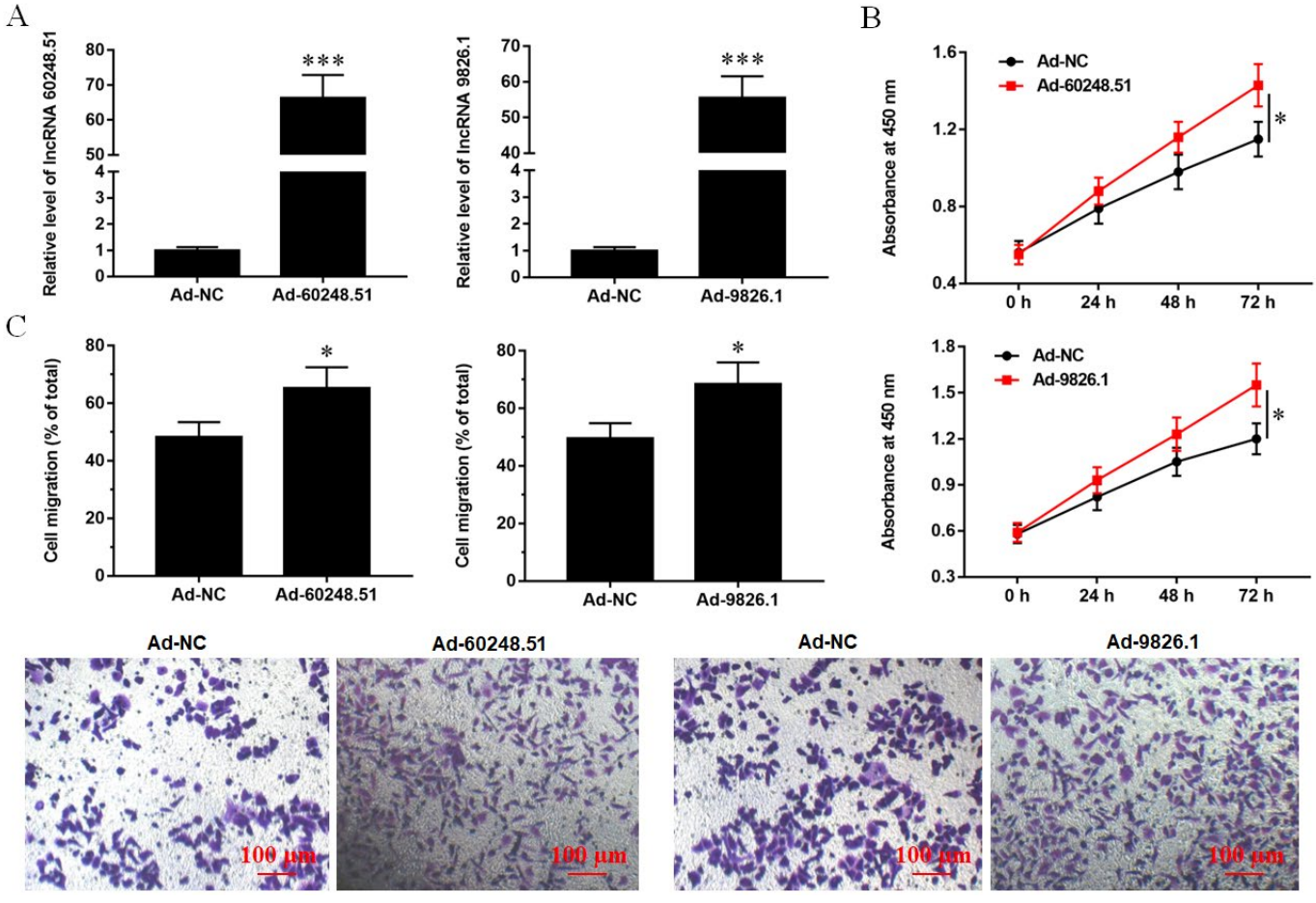


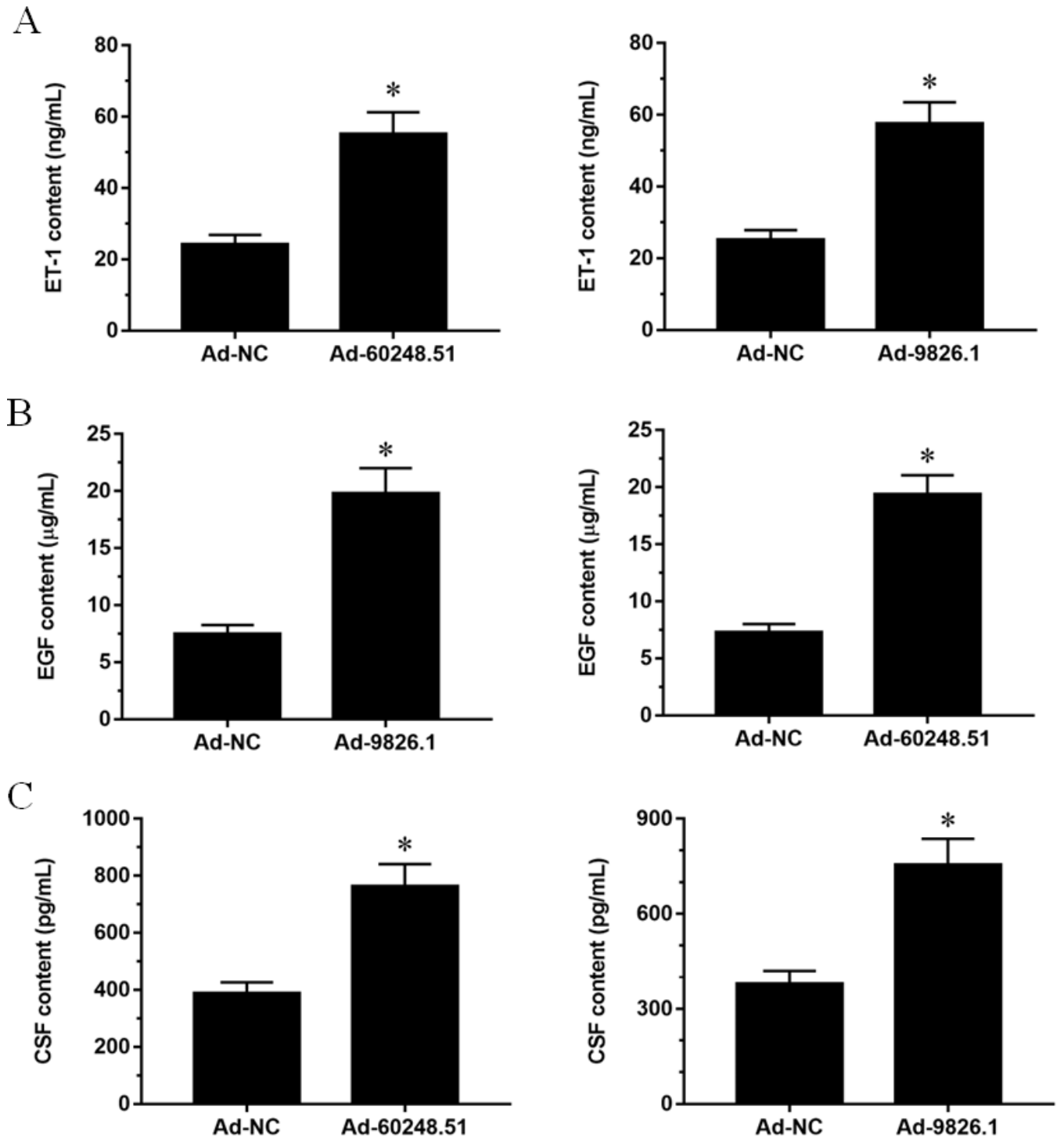
Figure 5. Expression levels of the top 10 upregulated lncRNAs in RE were verified by qPCR. Total RNA was extracted from PE and RE, and qPCR was used to detecte the expression levels of the top 10 upregulated lncRNAs, including MSTRG.26755.8, MSTRG.37411.29, MSTRG.60248.51, MSTRG.60107.10, MSTRG.10519.3, MSTRG.2773.1, MSTRG.9826.1, MSTRG.60169.6, MSTRG.60248.50, and MSTRG.54294.13.



*p<0.05; ***p<0.01.

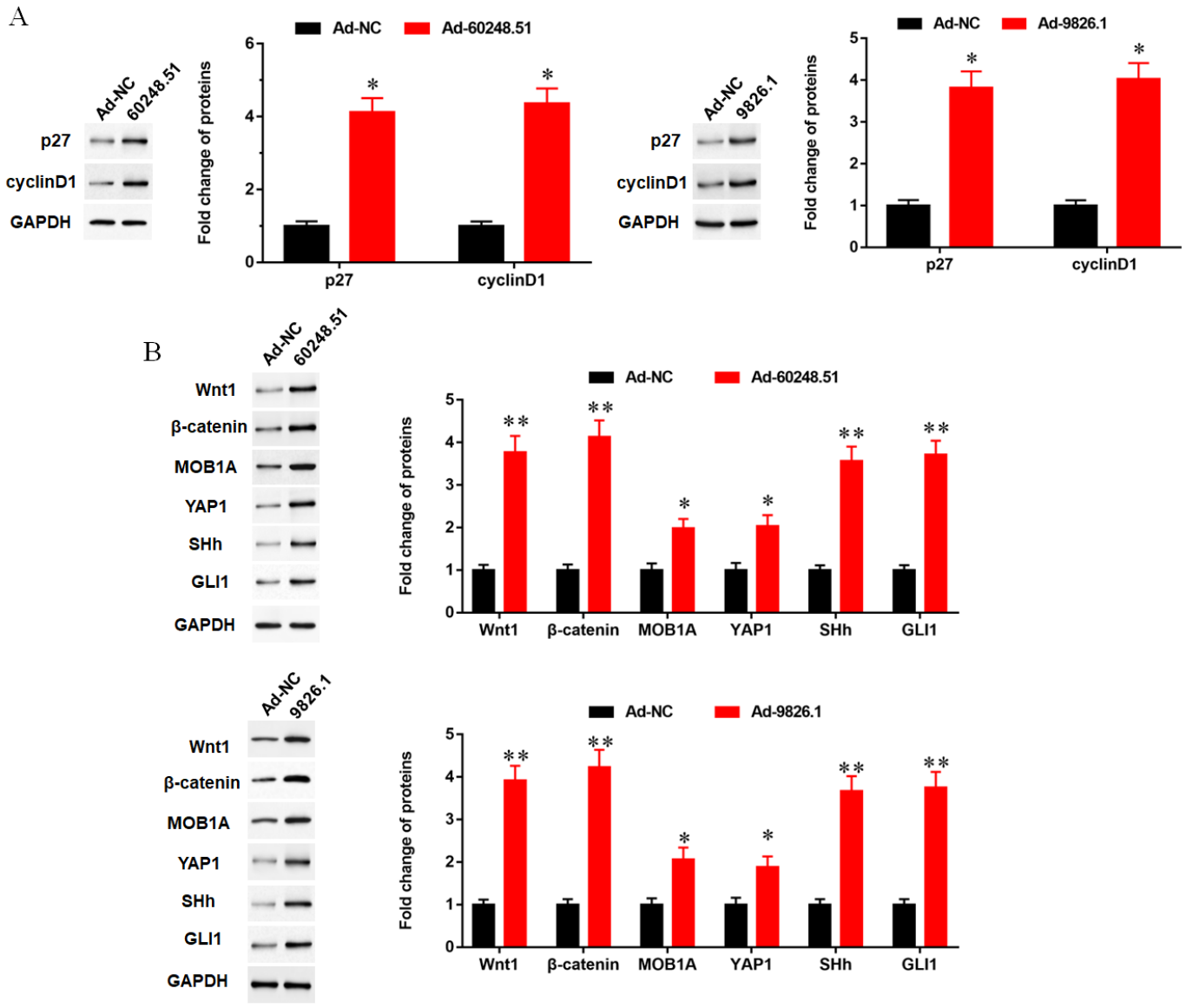
Figure 6. Overexpression of lncRNAs 60248.51 and 9826.1 increased cell proliferation and migration capacities in primary goat endometrial epithelial cells. Primary goat endometrial epithelial cells were isolated from Tibetan Northwest Cashmere goats. Adenoviral overexpression of lncRNAs 60,248.51 and 9,826.1 were respectively used to infect the endometrial epithelial cells. (A) Detection of overexpression efficiencies with qPCR. (B and C) CCK-8 and Transwell cell migration assays were used to evaluate the cell viability and migration capacities.

and 9,826.1 increased the secretion of ET-1, EGF, and CSF in endometrial epithelial cells (Figures 7A–7C), suggesting that 60,248.51 and 9,826.1 may contribute to endometrial receptivity and implantation. Finally, Western blotting showed that upregulation of 60,248.51 and 9,826.1 promoted the expression of cell cycle maker proteins p27 and cyclinD1 (Figure 8A) and activated the Wnt and Hippo signaling pathways (Figure 8B), indicating that Wnt and Hippo might mediate the contributions of 60,248.51 and 9,826.1 to endometrial receptivity and implantation.



*p<0.05.

Figure 7. Overexpression of lncRNAs 60,248.51 and 9,826.1 increased secretion of ET-1, EGF, and CSF in primary goat endometrial epithelial cells. Primary goat endometrial epithelial cells were isolated from Tibetan Northwest Cashmere goats. Adenoviral overexpression of lncRNAs 60,248.51 and 9,826.1 were respectively used to infect the endometrial epithelial cells. (A, B, and C) Contents of key secreted cytokines including ET-1, EGF, and CSF in the supernatant of endometrial epithelial cells were detected with ELISA.



*p<0.05; **p<0.01.

Figure 8. Overexpression of lncRNAs 60,248.51 and 9,826.1 activated the Wnt and Hippo signaling pathways in primary goat endometrial epithelial cells. Primary goat endometrial epithelial cells were isolated from Tibetan Northwest Cashmere goats. Adenoviral overexpression of lncRNAs 60,248.51 and 9,826.1 were respectively used to infect the endometrial epithelial cells. (A) The levels of cell cycle maker proteins p27 and cyclinD1 were detected with Western blotting. (B) The levels of maker proteins in the Wnt, Hedgehog, and Hippo pathways were detected with Western blotting, including Wnt1, β-catenin, MOB1A, YAP1, SHh and GLI1.

4 DISCUSSION

In this study, we performed a high-throughput RNA sequencing analysis and figured out a comprehensive transcriptome profiling in PE and RE tissues from Tibetan Northwest Cashmere goats. Our results revealed that only a handful of miRNAs and circRNAs are differentially expressed between PE and RE tissues, while there were 250 upregulated coding genes and 193 upregulated lncRNAs in PE tissues and 135 downregulated coding genes and 123 downregulated lncRNAs in RE tissues, suggesting that coding genes and lncRNAs are the main forces in the regulation of endometrial receptivity in Tibetan Northwest Cashmere goats. Moreover, differentially expressed transcripts were significantly associated with the PI3K/AKT, MAPK, Wnt, Hedgehog, and Hippo signaling pathways. Kinase pathways, especially MAPK and PI3K/AKT pathways, are vital signal transductors in many developmental and physiological processes. A couple of epigenetic and transcriptomic analyses had revealed that kinase pathways may have important functions in regulating endometrial receptivity in the goat embryo (Liu et al., 2021; Song et al., 2019). Several in vitro studies indicated that MAPK and PI3K/AKT pathways play important roles in apoptosis and proliferation in goat stromal cells and endometrial epithelium cells, suggesting their involvement in goat endometrial receptivity (Cui et al., 2020; Liu et al., 2020; Zhang et al., 2018). Moreover, human clinical studies demonstrated that

traditional Chinese medicines for assisted pregnancy, such as Xiaoyao powder and Bu Shen Zhu Yun Decoction, could improve human endometrial receptivity via growth cytokine-mediated activation of MAPK and PI3K/AKT pathways (He et al., 2022; Li et al., 2019).

The endometrium is necessary for implantation, formation of placenta, and a successful pregnancy and is composed of stromal, epithelial, vascular endothelial, and immune cells (Critchley et al., 2020). Endometrial epithelial cells are the frontline of the endometrium during embryo implantation. Their behaviors and functions, including proliferation, apoptosis, migration, and secretion, are important for the receptivity of fertilized eggs and the intrauterine environment for embryo implantation. A sufficient number of endometrial epithelial cells, balanced by proliferation and apoptosis, guarantees the integrity of the endometrium during the entire process of embryo implantation; the epithelial to mesenchymal transition and capacity of migration endow the endometrium with plasticity and are essential for the progression of embryo implantation (Nishino et al., 2021; Oghbaei et al., 2022); moreover, endometrial epithelial cells secrete some hormones and cytokines to help the uterus receive embryos, including ET, EGF, and CSF (Shen et al., 2021). ET, especially ET-1, is a vasoconstrictor synthesized and secreted by endometrial epithelial cells and stromal cells, which can assist the reproduction of trophozoites and ectodermal cells in the implantation of pregnant eggs (Zhou et al., 2021). EGF stimulates the growth of glandular cells in the proliferative and secretory endometrium and facilitates the epithelial-mesenchymal transition (Okada et al., 2022). CSF stimulates cell proliferation, which has no change in the menstrual cycle, but its concentration increases significantly in early pregnancy (Lin et al., 2022). In our study, based on our sequencing analysis, we screened two of the most upregulated lncRNAs in the RE tissue and explored their roles in primary goat endometrial epithelial cells through adenovirus-mediated overexpression experiments. Our results showed that overexpression of these two lncRNAs caused increased cell viability, migration, and increased secretion of ET-1, EGF, and CSF, suggesting their promotion of the functions of epithelial cells and positive effects on endometrial receptivity.

LncRNAs, as the most heterogeneous class of non-coding RNAs, have been involved in the fine regulation of all stages of gene expression, thus emerging as important regulatory factors in nearly all physiological and pathological processes. In humans, some lncRNAs were identified as signatures in pre-implantation embryos and endometriosis and reported to regulate proliferation, apoptosis, metastasis, cell cycle, and secretion of endometrial epithelial cells in normal endometrial physiology and pathophysiology of endometrial infertility (Aljubran & Nothnick, 2021; Bouckenheimer et al., 2016). However, in domestic animals, especially ruminant livestock, there are very limited reports on the exact roles of lncRNAs in regulating endometrial receptivity. Zhang et al. (2019) first reported that lncRNA882, a screened lncRNA regulated by estrogen (E2) and progestin (P4), sponged miR-15b to promote the transforming growth factor-b-activated kinase 1 binding protein 3 (TAB3) and leukemia inhibitory factor (LIF) to be helpful for the formation of endometrial receptivity in dairy goats. Here, through high-throughput RNA sequencing analysis, 193 upregulated lncRNAs and 123 downregulated lncRNAs were found in RE tissues, and GO and KEGG analyses indicated that these lncRNAs are significantly associated with response to multiple signals and regulation of cell cycle and intracellular biological processes and enriched in Wnt, Hedgehog, and Hippo signaling pathways. Furthermore, the two most upregulated lncRNAs, MSTRG.60248.51 (1,264 bp) and MSTRG.9826.1 (1,362 bp), were screened as candidate lncRNAs, and our functional experiments indicated that both upregulation of 60,248.51 and 9,826.1 could promote cell viability, cell migration capacity, and secretion of ET-1, EGF, and CSF, and mechanism exploration revealed that their functions might be associated with the Wnt and Hippo signaling pathways.

The Wnt/β-catenin signaling pathway is one of the most well-characterized regulatory pathways under physiological and pathological conditions. As appraised by Clevers and Nusse, the Wnt signal controls myriad biological phenomena throughout the development and adult life of all animals. Wnt controls cell fate, differentiation, proliferation, and apoptosis during embryo development and plays a huge potential role in embryo implantation (Tepekoy et al., 2015). Biological or chemical medicines, such as miRNAs, podocalyxin, and benzo(a)pyrene, can regulate endometrial receptivity via Wnt-mediated endometrial epithelial cell proliferation, apoptosis, and migration (Altmäe et al., 2013; Heng et al., 2021; Huang et al., 2020; Yi et al., 2019). Hippo signaling is famous for its key roles in regulating embryogenesis and development, immunity, and carcinogenesis. However, evidence on the regulation of endometrial receptivity is relatively limited. A recent study revealed that the Hippo signaling pathway was associated with differentiation of human embryonic stem cell-derived trophoblastic spheroids and might contribute to early implantation and trophoblast development (Yue et al., 2020). In our study, functional annotation of our sequencing results revealed that Wnt and Hippo signaling pathways might be key pathways in the regulation of endometrial receptivity in Tibetan Northwest Cashmere goats and potential targets of the differentially expressed lncRNAs. Moreover, the two candidate lncRNAs 60,248.51 and 9,826.1 that we screened played a positive role in cell proliferation, cell migration capacity, and secretion of ET-1, EGF, and CSF in endometrial epithelial cells, which might be associated with the Wnt and Hippo signaling pathways.

5 CONCLUSION

In conclusion, we performed comprehensive transcriptome profiling in PE and RE tissues from Tibetan Northwest Cashmere goats and found 193 upregulated and 123 downregulated lncRNAs enriched in Wnt, Hedgehog, and Hippo signaling pathways during the formation of endometrial receptivity. LncRNAs 60,248.51 and 9,826.1 were screened as candidate lncRNAs, which can promote cell proliferation, cell migration capacity, secretion of ET-1, EGF, and CSF, and activation of Wnt and Hippo signaling pathways in endometrial epithelial cells, suggesting their positive role in endometrial receptivity in Tibetan Northwest Cashmere goats.

REFERENCES

Aljubran, F., & Nothnick, W. B. (2021). Long non-coding RNAs in endometrial physiology and pathophysiology. *Molecular and Cellular Endocrinology*, *525*, 111190. https://doi.org/10.1016/j.mce.2021.111190

Altmäe, S., Martinez-Conejero, J. A., Esteban, F. J., Ruiz-Alonso, M., Stavreus-Evers, A., Horcajadas, J. A., & Salumets, A. (2013). MicroRNAs miR-30b, miR-30d, and miR-494 regulate human endometrial receptivity. *Reproductive Sciences*, *20*(3), 308-317. https://doi.org/10.1177/1933719112453507

Ashary, N., Tiwari, A., & Modi, D. (2018). Embryo Implantation: War in Times of Love. *Endocrinology*, *159*(2), 1188-1198. https://doi.org/10.1210/en.2017-03082

Bouckenheimer, J., Assou, S., Riquier, S., Hou, C., Philippe, N., Sansac, C., Lavabre-Bertrand, T., Commes, T., Lemaitre, J.-M., Boureux, A., & De Vos, J. (2016). Long non-coding RNAs in human early embryonic development and their potential in ART. *Human Reproduction Update*, *23*(1), 19-40. https://doi.org/10.1093/humupd/dmw035

Critchley, H. O. D., Maybin, J. A., Armstrong, G. M., & Williams, A. R. W. (2020). Physiology of the Endometrium and Regulation of Menstruation. *Physiological Reviews*, *100*(3), 1149-1179. https://doi.org/10.1152/physrev.00031.2019

Cui, J., Liu, X., Yang, L., Che, S., Guo, H., Han, J., Zhu, Z., Cao, B., An, X., Zhang, L., & Song, Y. (2020). MiR-184 Combined with STC2 Promotes Endometrial Epithelial Cell Apoptosis in Dairy Goats via RAS/RAF/MEK/ERK Pathway. *Genes (Basel)*, *11*(9), 1052. https://doi.org/10.3390/genes11091052

Duan, C., Zhang, L., Gao, K., Guo, Y., Liu, Y., & Zhang, Y. (2022). Cashmere production, skin characteristics, and mutated genes in crimped cashmere fibre goats. *Animal*, *16*(7), 100565. https://doi.org/10.1016/j.animal.2022.100565

He, M., Li, L., Wei, X., Geng, D., Jiang, H., Xiangxiang, G., Zhang, Y., & Du, H. (2022). Xiaoyao powder improves endometrial receptivity via VEGFR-2-mediated angiogenesis through the activation of the JNK and P38 signaling pathways. *Journal of Ethnopharmacology*, *282*, 114580. https://doi.org/10.1016/j.jep.2021.114580

Heng, S., Samarajeewa, N., Wang, Y., Paule, S. G., Breen, J., & Nie, G. (2021). Podocalyxin promotes an impermeable epithelium and inhibits pro-implantation factors to negatively regulate endometrial receptivity. *Scientific Reports*, *11*(1), 24016. https://doi.org/10.1038/s41598-021-03425-2

Huang, K., Chen, G., Fan, W., & Hu, L. (2020). miR-23a-3p increases endometrial receptivity via CUL3 during embryo implantation. *Journal of Molecular Endocrinology*, *65*(2), 35-44. https://doi.org/10.1530/jme-20-0053

Jin, M., Lu, J., Fei, X., Lu, Z., Quan, K., Liu, Y., Chu, M., Di, R., Wang, H., & Wei, C. (2020). Genetic Signatures of Selection for Cashmere Traits in Chinese Goats. *Animals (Basel)*, *10*(10), 1905. https://doi.org/10.3390/ani10101905

Lessey, B. A., & Young, S. L. (2019). What exactly is endometrial receptivity? *Fertility and Sterility*, *111*(4), 611-617. https://doi.org/10.1016/j.fertnstert.2019.02.009

Li, C., Wu, Y., Chen, B., Cai, Y., Guo, J., Leonard, A. S., Kalds, P., Zhou, S., Zhang, J., Zhou, P., Gan, S., Jia, T., Pu, T., Suo, L., Li, Y., Zhang, K., Li, L., Purevdorj, M., Wang, X., Li, M., Wang, Y., Liu, Y., Huang, S., Sonstegard, T., Wang, M.-S., Kemp, S., Pausch, H., Chen, Y., Han, J.-L., Jiang, Y., & Wang, X. (2022). Markhor-derived Introgression of a Genomic Region Encompassing PAPSS2 Confers High-altitude Adaptability in Tibetan Goats. *Molecular Biology and Evolution*, *39*(12), msac253. https://doi.org/10.1093/molbev/msac253

Li, L., Jiang, H., Wei, X., Geng, D., He, M., & Du, H. (2019). Bu Shen Zhu Yun Decoction Improves Endometrial Receptivity via VEGFR-2-Mediated Angiogenesis. *Evidence-Based Complementary and Alternative Medicine*, *2019*, 3949824. https://doi.org/10.1155/2019/3949824

Lin, J., Ma, H., Li, H., Han, J., Guo, T., Qin, Z., Jia, L., & Zhang, Y. (2022). The Treatment of Complementary and Alternative Medicine on Female Infertility Caused by Endometrial Factors. *Evidence-Based Complementary and Alternative Medicine*, *2022*, 4624311. https://doi.org/10.1155/2022/4624311

Liu, H., Wang, C., Li, Z., Shang, C., Zhang, X., Zhang, R., Wang, A., Jin, Y., & Lin, P. (2021). Transcriptomic Analysis of STAT1/3 in the Goat Endometrium During Embryo Implantation. *Frontiers in Veterinary Science*, *8*, 757759. https://doi.org/10.3389/fvets.2021.757759

Liu, X., Zhang, L., Yang, L., Cui, J., Che, S., Liu, Y., Han, J., An, X., Cao, B., & Song, Y. (2020). miR-34a/c induce caprine endometrial epithelial cell apoptosis by regulating circ-8073/CEP55 via the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways. *Journal of Cellular Physiology*, *235*(12), 10051-10067. https://doi.org/10.1002/jcp.29821

Nishino, D., Kotake, A., Yun, C. S., Rahman, A. M. I., El-Sharawy, M., Yamanaka, K. I., Khandoker, M. A. M. Y., & Yamauchi, N. (2021). Gene expression of bovine endometrial epithelial cells cultured in matrigel. *Cell and Tissue Research*, *385*(1), 265-275. https://doi.org/10.1007/s00441-021-03418-7

Oghbaei, F., Zarezadeh, R., Jafari-Gharabaghlou, D., Ranjbar, M., Nouri, M., Fattahi, A., & Imakawa, K. (2022). Epithelial-mesenchymal transition process during embryo implantation. *Cell and Tissue Research*, *388*(1), 1-17. https://doi.org/10.1007/s00441-021-03574-w

Okada, C. T. C., Kaps, M., Reichart, U., Walter, I., Gautier, C., Aurich, J., & Aurich, C. (2022). Low plasma progesterone concentration during the early luteal phase delays endometrial development and the beginning of placentation in mares. *Animal Reproductive Science*, *247*, 107149. https://doi.org/10.1016/j.anireprosci.2022.107149

Shen, H. H., Zhang, T., Yang, H. L., Lai, Z. Z., Zhou, W. J., Mei, J., Shi, J.-W., Zhu, R., Xu, F.-Y., Li, D.-J., Ye, J.-F., Li, M.-Q. (2021). Ovarian hormones-autophagy-immunity axis in menstruation and endometriosis. *Theranostics*, *11*(7), 3512-3526. https://doi.org/10.7150/thno.55241

Song, Y., Han, J., Cao, F., Ma, H., Cao, B., & An, X. (2019). Endometrial genome-wide DNA methylation patterns of Guanzhong dairy goats at days 5 and 15 of the gestation period. *Animal Reproductive Science*, *208*, 106124. https://doi.org/10.1016/j.anireprosci.2019.106124

Tepekoy, F., Akkoyunlu, G., & Demir, R. (2015). The role of Wnt signaling members in the uterus and embryo during pre-implantation and implantation. *Journal of Assisted Reproductive and Genetics*, *32*(3), 337-346. https://doi.org/10.1007/s10815-014-0409-7

Yi, T., Liu, M., Li, X., Liu, X., Ding, Y., He, J., Xu, H., Gao, R., Mu, X., Geng, Y., Wang, Y., & Chen, X. (2019). Benzo(a)pyrene inhibits endometrial cell apoptosis in early pregnant mice via the WNT5A pathway. *Journal of Cellular Physiology*, *234*(7), 11119-11129. https://doi.org/10.1002/jcp.27762

Yue, C., Chen, A. C. H., Tian, S., Fong, S. W., Lee, K. C., Zhang, J., Ng, E. H., Y., Lee, K. F., & Lee, Y. L. (2020). Human embryonic stem cell-derived blastocyst-like spheroids resemble human trophectoderm during early implantation process. *Fertility and Sterility*, *114*(3), 653-664.E6. https://doi.org/10.1016/j.fertnstert.2020.01.009

Zhang, L., An, X. P., Liu, X. R., Fu, M. Z., Han, P., Peng, J. Y., Hou, J.-X., Zhou, Z.-Q., Cao, B.-Y., & Song, Y. X. (2015). Characterization of the Transcriptional Complexity of the Receptive and Pre-receptive Endometria of Dairy Goats. *Scientific Reports*, *5*, 14244. https://doi.org/10.1038/srep14244

Zhang, L., Liu, X., Cui, J., Che, S., Liu, Y., An, X., Cao, B., & Song, Y. (2019). LncRNA882 regulates leukemia inhibitory factor (LIF) by sponging miR-15b in the endometrial epithelium cells of dairy goat. *Journal of Cellular Physiology*, *234*(4), 4754-4767. https://doi.org/10.1002/jcp.27272

Zhang, L., Liu, X., Liu, J., Ma, X., Zhou, Z., Song, Y., & Cao, B. (2018). miR-26a promoted endometrial epithelium cells (EECs) proliferation and induced stromal cells (ESCs) apoptosis via the PTEN-PI3K/AKT pathway in dairy goats. *Journal of Cellular Physiology*, *233*(6), 4688-4706. https://doi.org/10.1002/jcp.26252

Zhou, S., Huang, C., Wang, W., & Liu, J. (2021). MiR-370-3p inhibits the development of human endometriosis by downregulating EDN1 expression in endometrial stromal cells. *Cellular Biology International*, *45*(6), 1183-1190. https://doi.org/10.1002/cbin.11552