Identification and characterization of circRNAs in the skin during the wool follicle development of Aohan fine wool sheep

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Abstract

Background Aohan fine wool sheep (AFWS) is an early fine wool variety breed cultivated in China. The wool has excellent quality and good textile performance. Investigating the molecular mechanisms that regulate wool growth is important for improving wool quality and yield. Circular RNAs (circRNAs) are non-coding RNAs which are widely expressed and can act as a competitive endogenous RNA (ceRNA) to bind to miRNA. Although circRNA has been studied in many fields, research in sheep wool follicles is limited. To understand the regulation of circRNA in the growth of fine wool sheep, we used RNA-seq to identify circRNAs in sheep shoulder skin at three stages, embryonic day 90 (E90d), embryonic day 120 (E120d), and Birth.

Results We identified 8,753circRNAs and found that 1,351 were differentially expressed. We also analyzed the classification and characteristic of the circRNAs in sheep shoulder skin. GO and KEGG were used for source genes of circRNAs, and these were mainly enriched in cellular component organization, regulation of primary metabolic process, tight junctions, and the cGMP-PKG and AMPK signaling pathways. In addition, we predicted interactions between 17 circRNAs and 8 miRNAs using miRanda (http://www.microrna.org/microrna/home.do). Based on the significant pathways, we speculate the circ-0005720, circ-0001754, circ-0008036, circ-0004032, circ-0005174, circ-0005519, circ-0007826 may play an important role in regulating wool follicle growth in AFWS. 5 circRNAs were randomly selected to validate the results of the RNA-seq by qRT-PCR.

Conclusion Our results provide more information about circRNAs in regulating wool follicle development in AFWS and provide a solid foundation for future experiments.

Background

Wool is a source of high-quality textile raw materials and animal products, and has a very important impact on the national economy. Improving the production of high-quality fine wool has become a hot topic in recent years. Wool growth is a very complex physiological and biochemical process which is influenced by genetics, the environment, and nutrition. Wool is developed from hair follicles (HF), and its yield and quality are closely related to the development of wool follicles. It is a complex organ of the skin and capable of self-regeneration. Its structure plays a very important role in its periodic growth process. Mammalian hair follicles are divided into primary hair follicles (PF) and secondary hair follicles (SF), with SF producing fine wool. Its morphogenesis involves the interaction of a series of signaling pathways between the epidermis and the dermis. The development of hair follicles is regulated by various signaling pathways, such as Wnt, Sonic hedgehog (SHH), Notch, Bone morphogenic protein (BMP) and Fibroblast growth factor (FGF). Various downstream signaling molecules, such as β-catenin, Msx1, and Msx2, are involved in hair follicle morphogenesis [1]. Many studies in recent years have identified that non-coding RNAs act as important post-transcriptional regulators of gene expression during hair follicle development, including miRNAs, circRNAs, and lncRNAs. LncRNA acts on the Wnt signaling pathway and affects hair

follicle growth and development [2]. Non-coding RNA has been shown to regulate wool fineness and growth of secondary hair follicles in cashmere goats [3].

Circular RNA (circRNA) is a novel type of noncoding RNA that regulates transcriptional and post transcriptional gene expression [4, 5], circRNAs are typically generated by back-splicing from exons of protein-coding genes and incorporation of the 5’ end and 3’ end. Because of the absence of 5’ and 3’ ends, they are more stable than linear RNAs and are resistant to RNase R [6, 7]. CircRNA is widely distributed in mammalian cells and endogenously regulates gene expression [8]. It has high stability, and specificity for tissue, developmental stage, and cell type [9, 10], circRNA molecules act as a miRNA molecule sponge [11], regulate gene transcription [12, 13], interact with RNA-binding proteins [14, 15], and translate proteins.

Recently, studies have found that melatonin exposure disturbs a key secretion signal of goat hair follicle stem cells, further regulating goat hair follicle development [16]. CircRNA participates in and regulates the regeneration of human skin tissue [17], has tissue-specific and stage-specific expression in chicken follicle granulosa cells, and is useful for investigating the regulatory mechanisms of follicular growth [18]. Research on the hair follicle cycle of the Angora rabbit revealed a lncRNA/circRNA-miRNA/mRNA network and showed that ncRNAs play an important role in regulating the HF cycle [19]. In a recent study of circRNA in the estrus and anestrus states of the sheep pituitary system, a total of 12,468 circRNAs and 9,231 differentially expressed circRNAs were identified [20]. However, there are few reports on the study of circRNA in development of sheep wool follicles.

AFWS is one of the fine wool sheep breeds in China. Its wool quality is excellent, and its textile process performance is good. Increased understanding of the function of the genes involved in wool follicle development could assist in the selective breeding of fine wool sheep traits and improve wool yield and quality [21]. Our laboratory studied the wool follicle development process of AFWS and found that a small amount of secondary wool follicles can be observed at E90d, a large number of secondary wool follicles were found at E120d, and secondary wool follicles at birth had mostly completed development [22].

Our understanding of circRNA expression in AFWS follicles to date is very limited. To study the relationship between circRNA and changes of wool follicle in sheep at different growth stages, RNA-Seq was used to detect the expression profiles of circRNA in skin of AFWS at E90d, E120d, and Birth. Our results indicate that circRNA plays an important role in the formation of sheep wool follicles.

Results

Secondary wool follicle growth procession

Hematoxylin and eosin H&E staining at E90d showed primary and early secondary stage wool follicles (Fig. 1a). The primary wool follicles occurred early, the hair bulbs were large, the wool follicles were long, and they had accessory structures such as sweat glands, sebaceous glands, and piloerection muscles.

The secondary wool follicle is smaller and grows near the epidermis of the primary wool follicle (Fig. 1b). At E120 days, the secondary wool follicle is separated from and arranged in parallel with the primary wool follicle (Fig. 1c, d). By birth, some of the secondary wool follicles have matured and passed through the body surface (Fig. 1e, f).

Sequencing and mapping of the sheep skin tissue transcriptome

To examine the circRNA expression profiles of the sheep skin during different developmental stages, we sequenced cDNA libraries of nine skin tissue samples from three sheep (A, B, and C) during three periods (embryonic day 90, embryonic day 120, and birth). Raw reads were acquired via Illumina sequencing, which were then processed to remove rRNA, low-quality sequences, and junction contamination, among other processing. All subsequent analysis is based on these processed clean reads. We obtained 87137921, 94904421, and 95385493 clean reads from the three shoulder tissue samples, which could be mapped to the sheep genome at E90d, E120d, and birth, respectively. A total of 8,753 candidate circRNAs were identified (Additional file 1: Table S1), 1,648of which (18.8%) were expressed in all periods (Fig. 2a). We list 30 highest-expressing circRNAs of each group (Table 1). Based on their location in the genome, the 8,753 circRNAs were classified into 6 types: classic (83.4%), alter-exon (8.6%), intron (1.2%), overlap-exon(5.5%), antisense(0.3%), and intergenic(1.0%) (Fig. 2b).

The circRNAs typically encoded 2 to 4 exons (Fig. 2c). In circRNAs with only one exon, the length of the exon was significantly longer than that of a cyclic RNA composed of multiple exons (Fig. 2d). According to the expression of circRNAs of all samples, the peak gene density is between 0.3 and 0.4 (Fig. 2e).

Identification of differentially expressed circRNAs

In this study, SRPBM values (spliced reads per billion mapping) were used to calculate circRNA expression levels, and a total of 8,753 circRNAs were detected, 1,351 differentially expressed circRNAs were screened after comparison in three different stages (Additional file 2: Table S2A,2B,2C). Volcano plots (Fig. 3a-c) and clustering maps (Fig. 3d) were used to illustrate the distribution of the DE circRNAs. CircRNAs with significant differential expression in the figure are represented by red (up-regulated expression) and green (down-regulated expression).

The number of significant differentially expressed circRNAs in the three comparison groups (Birth_E120d, Birth_E90d, and E120d_E90d) were respectively, 467, 377, and 507 (Fig. 3e). Among the DEGs (Differentially expressed genes), circ–0004932 and circ–0004936 correspond togene13410 (TRPS1), it has been reported that Trps1 is involved in the growth and development of hair follicle cells [23]. There are also some circRNAs similar to circ–0004932 and circ–0004936, such as circ–0000997 and circ–0000999 map to source gene851 (VAV3), and circ–0001520 and circ–0001524 map to source gene3008 (TMEFF1), which are all associated with hair follicle growth [24, 25].

GO and KEGG pathway enrichment analysis

The function of circRNA is reflected through their source genes, to determine the function of these genes, GO and KEGG analyses were performed. The function of circRNA can be further studied by analyzing which GO terms are significant enriched for the source gene. We obtained 2,104 source genes associated with the 8,753 differentially expressed circRNAs. 50 significant (P<0.05) GO terms were identified (Fig. 4 a-c,), the top 10 GO terms in each comparison groups were listed in Additional file 3: Table S3A, 3B, 3C, the most significantly enriched were: cellular component organization, regulation of primary metabolic process, intracellular part, intracellular organelle, membrane-bounded organelle, and protein binding.

Pathway analysis was performed based on the KEGG pathway database (Fig. 4d, Additional file 4: Table S4A, 4B, 4C), to predict significantly enriched pathways in the source genes, we identified 6 significantly enriched pathways (Endocytosis, Lysine degradation, Apoptosis, Human papillomavirus infection, Adherens junction, and Tight junction) enriching 55 source genes and their corresponding 255 circRNAs (Additional file 5: Table S5A). Among the 55 source genes, 7 are associated with hair follicle growth, and those 7 source genes have 35 circRNAs (Additional file 5: Table S5B). Of those 35 circRNAs, 7 are significantly expressed in our study: circ–0005720 source gene15869 (AKT3), circ–0001754 source gene3277 (TGFBR1), circ–0008036 source gene25354 (SMAD2), circ–0004032 source gene11746 (SOS2), circ–0005174 source gene13720 (RB1), circ–0005519 source gene15130 (EZH1), circ–0007826 source gene24949 (FGFR2). The network between the source genes and circRNAs were constructed (Fig. 5a).

Target miRNAs of differentially expressed circRNAs in different stage sheep

To further understand the functions of circRNAs, we used miRanda (http://www.microrna.org/microrna/home.do) to predict the interactions between circRNAs and miRNAs. A total of 17 circRNAs and 8 miRNAs were identified, the relationship between them were constructed by network and the sankey diagram (Fig. 6a, 6b, Table 2). For example, circ_0003042 is significantly differentially expressed in Birth_E120d, circ_0003042 is predicted to interact with miR–432, and may act as an “miRNA sponge,” binding all of the available miRNA to prevent its function.

Validation of circRNA expression by qRT-PCR

To validate the expression levels of differentially expressed circRNAs, we randomly selected 5 highly expressed circRNAs and detected their expression levels by qRT-PCR (Additional file 6: Table S6). These results were consistent with the trends observed in the RNA-Seq data, indicating that the RNA-Seq is reliable (Fig. 7a, b).

Discussion

In this study, we first investigated the expression of circRNAs in wool follicles at different growth stages of sheep skin. Using RNA-seq technology, we obtained 8,753 circRNAs in three development stages of sheep: 579 were found in Birth_E120d, 346 were found in Birth_E90d, and 579 were found in E120_E90d. Of the 1,351 identified differentially expressed genes, 684 were upregulated and 667 were downregulated.

In previous results of Angora rabbit hair follicle cycle determined via RNA sequencing, 247 differentially expressed circRNAs (128 upregulated and 119 downregulated) were found between the three HF cycle stages, and it was found that several circRNAs may play a role during the HF cycle, including novel_circ_0004876, novel_circ_0005177, and novel_circ_0026326 [19]. Many mammalian species have similar hair follicle growth patterns, and many animal models, such as goat [26], rat [27], and human [28] models, have been studied previously. The main purpose of analyzing sheep hair follicle circRNA sequencing is to reveal factors that may play a role in hair growth, thereby elucidating the underlying molecular mechanisms.

To further investigate the potential mechanisms of the action of the circRNAs in this study, we applied GO and KEGG analyses. In GO annotation, the number of DEGs between any two stages exhibits significant differences, reflecting a cumulative effect on phase traits. It was found that the source genes of the differentially expressed circRNAs function primarily in the Biological Processes, such as cellular component organization, the regulation of cellular processes, cellular macromolecule metabolic processes. The intracellular components, organelle and organelle part in Cellular Component; Binding, ion binding and heterocyclic compound binding in Molecular Function. These findings indicate that the different source genes of circRNAs in different growth periods play a significant role in the formation of wool follicle cells related to the GO terms, such as the regulation of metabolic processes. It has been found that most circRNA expression profiles follow their source gene expression profiles. This study suggests that circRNAs may be associated with these GO terms, but further validation is required.

KEGG is a pathway database for the systematic analysis of gene function, and the obtained results suggest that multiple signaling pathways form a complex regulatory network during hair follicle development. It has been reported that human papillomavirus infection [29], the adherens junction [30], and the tight junction [31] signaling pathways participate in the growth and development of hair follicles.

In our research, seven circRNAs (circ–0005720, circ–0001754, circ–0008036, circ–0004032, circ–0005174, circ–0005519 and circ–0007826) were identified from the significant KEGG pathways, and the source genes of the circRNAs (AKT3, TGFBR1,SMAD2,SOS2, RB1, EZH1, and FGFR2) have been reported to participate in the growth process of hair follicles [32–39]. Comparing these results with those of previous studies, the selected circRNAs may play an important role in the signaling pathways of different secondary wool follicle development stages of AFWS, therefore, further research is required.

There are some pathways that were not found to be significant in our study, but also enriched many source genes, and have been reported in previous studies to regulatethe development of skin and hair [40]. These pathways include the Hedgehog [41], MAPK [42], FoxO [43], TGF-beta [44], NF-kappa B [45], TNF[46], and Wnt [47] signaling pathways. In a previous study, the Wnt, Sonic hedgehog, and NF-kappaB/Edar pathways were found to be indispensable for the process of hair follicle growth [48]. The Edar signaling pathway is involved in controlling the development and circulation of the hair follicle (HF), the interaction between the ectodysplasin receptor (Edar) and bone morphogenetic protein (BMP) signaling and transcription is the core of the primary hair follicle model [49, 50]. Studies have shown that

Wnt/β-catenin signaling is important for NF-κB activation, and Edar can directly target Wnt.TheWnt/β-catenin and EDA/EDAR/NF-κB signaling pathways play an important role in the initiation and maintenance of primary hair follicle placodes [51].The current research on the relationship between these signaling pathways is not very clear, and the study of the molecular mechanisms of hair follicle development has primarily been conducted for mice and humans [52, 53].

Recent studies have found that some circRNAs (such as CDR1as and miR–7, SRY, and miR–138) have multiple binding sites for miRNAs [9, 54]. CircRNAs unable to regulate their target genes, therefore, function as an “miRNA sponge.” It has been reported that circRNA participates in many biological processes by acting as miRNA sponges, thereby releasing the inhibitory effect of the miRNA on the target gene [54]. In recent years, miRNAs have been researched in many aspects of hair follicle growth and cell cycling [55, 56]. A study has shown that circRNAs regulate gene expression through a circRNA-miRNA-mRNA pathway [57]. Another pioneering study has demonstrated that miRNAs are differentially expressed between the fat-tailed and short-tailed sheep breeds [58]. However, there remains a lack of research on circRNA in sheep secondary wool follicles at different growth stages. Based on the results of the KEGG pathway analysis, the circRNA-miRNA network, which contains 17 circRNAs and 8 miRNAs, was constructed to better understand the characteristics of sheep secondary wool follicles in different growth stages. In a previous study, miR–370–3p has been reported to act as a target of circRNA_NEK6 via the Wnt signaling pathway in the proliferation of thyroid carcinoma[59], and miR–432 has been reported to be associated with the formation of the curly hairs of Chinese tan sheep[60]. In addition, miR–27a regulates the cell cycle by inhibiting the TGF-β/smad pathway[61, 62]. The related circRNAs may play an important regulatory role in the growth and development of wool follicles in AFWS, and we will further verify this in future experiments.

In summary, our study detected a large number of circRNAs in the skin of AFWS, and these results provide a solid theoretical foundation for the investigation of circRNAs associated with sheep (secondary) wool follicle development. Furthermore, circ–0005720, circ–0001754, circ–0008036, circ–0004032, circ–0005174, circ–0005519, circ–0007826willbe chosen as candidate circRNAs for the further study of wool follicle regulation.

Conclusions

In this study, we investigated the expression of circRNA in the skin of different developmental stages of AFWS and identified 1,351 circRNAs differentially expressed across developmental times. Using miRanda (http://www.microrna.org/microrna/home.do)to predict the relationship between circRNAs and miRNAs, we obtained 17 pairs of circRNA-miRNA. miR–432 has been reported participate the wool formation of Chinese Tan sheep [60]. KEGG analysis of differentially expressed genes identified six significantly enriched pathways including 55 source genes, seven of which, corresponding to 35 circRNAs, are involved in the regulation of hair follicle growth, seven of those 35 circRNAs have significant differential expression, these findings may provide clues to aid further study of the molecular mechanisms of wool growth.

Methods

Sample preparation

The AFWS used in this study were fed in the AFWS Stud Farm of Inner Mongolia Autonomous Region. Nine healthy AFWS ewes of similar age (3–5 years old), body weight (55–60 kg), and body size were selected. The estrus of the 12 ewes was synchronized and artificial insemination was completed in September. Six fetuses were collected from two stages in utero by surgery under anesthetized: 90 and 120 days after mating, abbreviated below as E90d and E120d. Lambs were anesthetized after birth 0 day, abbreviated below as Birth. The ewes and lambs were anesthetized with sodium pentobarbital at a dose of 25 mg/kg by intravenous injection. After collecting samples, all ewes and birth 0 day lambs were released, the fetuses under anesthesia of E90d and E120d were placed in a carbon dioxide-containing gas chamber, and 20% of carbon dioxide was filled into the chamber every minute, the gas concentration reached 80%, the fetuses died. Operations according to the literatures [63,64].

The 2-cm-diameter skin tissue was collected from the shoulder position of three stages of wool follicle development: E90d, E120d, and Birth, for three individuals of each stage, nine sheep in all. All experiment sheep were fed in the same farm environment, and samples were stored in liquid nitrogen for total RNA extraction. All experimental and surgical procedures involved in this study followed the “Guidelines for Experimental Animals” of the Ministry of Science and Technology (Beijing, China). Operations and Animal Care were in accordance with the recommendations proposed by the European Commission (1997) and were sustained by the experimental animal ethics committee of QingDao Agricultural University. Skin samples were fixed in 4% formaldehyde, and paraffin sections were stained with hematoxylin-eosin (H&E) for histological observations.

RNA isolation and quality assessment

Skin tissue samples (about 0.5–1.0 g per sheep for each time period) were placed into clean RNAase-free Eppendorf tubes and stored inliquid nitrogen. We used TRIzol reagent (Life Technologies, CA, USA) to extract total RNA from the nine samples. RNase-free DNase (Tiangen, Beijing, China) was used to remove DNA contamination from the extracted RNA. RNA degradation and contamination was monitored on 1% agarose gels and RNA purity was measured at an OD260/280 using a NanoDrop ND–2000 instrument (Thermo Fisher Scientific, MA, USA).

CircRNA Sequencing

High-throughput whole transcriptome sequencing and subsequent bioinformatics analyses were performed by Annoroad Technologies (Beijing, China) as follows. A total of 3 μg RNA per sample was used for circRNA sample preparation. The Ribo-ZeroTM Gold Kit was used to remove rRNA from the sample, and different index tags were selected to build the library according to the NEB Next Ultra Directional RNA Library Prep Kit for Illumina (NEB, Ispawich, USA) specifications. The specific steps of library construction were as follows: Ribosomal RNA was removed using a kit, RNase R was addedto

remove linear RNA. Fragmentation Buffer was added to the reaction system to fragment the RNA, and then this fragmented RNA was used as a template for first strand cDNA synthesis with random primers (Random Hexamers). Second strand cDNA was synthesized by adding buffer, dNTPs, RNase H, and DNA Polymerase I. After purification by QiaQuick PCR kit and elution with EB buffer, the end of the repair, add adenine, add the sequencing linker, recover the target size fragment by agarose gel electrophoresis. Uracil N-glycosylase (UNG) was then added to digest the DNA strand prior to PCR amplification. Finally, agarose gel electrophoresis was used to recover the fragments of the target size. The constructed library was sequenced using Illumina, the sequencing strategy is PE150.

Sequencing analysis of circRNA

The raw reads generated by Illumina sequencing were processed to create clean reads by several processes, including de-junction contamination, removal of rRNA, and other processes. All subsequent analyses were based on the clean reads. The circRNA sequencing information analysis process of this study is divided into seven parts: (1) sequencing data quality control, (2) data alignment analysis, (3) circular RNA recognition and classification, (4) circular RNA characteristic analysis, (5) circular RNA differential analysis, (6) source gene function of differential circular RNA, and (7) analysis and miRNA molecular sponge analysis.

Identification of differentially expressed circRNAs

We used DEseq2 [65] to analyze the differential expression of circRNA, and the three sheep were used as biological replicates. Differentially expressed circRNAs were found by comparing one period with another. CircRNAs with $P< 0.05$ and absolute fold-change values of >1.5 in any of the pairwise comparisons were considered to be significantly differentially expressed, and up-regulated and down-regulated circRNA numbers were eventually obtained.

GO and KEGG pathway enrichment analysis

GO analysis and KEGG pathway analysis of the source genes of differentially expressed circRNAs were used for annotation. GO functional analysis were used Blast2GO method [66]. KOBAS software was used to test the statistical enrichment of differential gene expression in KEGG pathway [67]. GO term and KEGG path was with $P<0.05$ was considered significantly enriched.

Prediction of target miRNAs of circRNA

To explore the functions of circRNAs and predict which circRNA function as miRNA sponges, we used miRanda (3.3a) (http://www.microrna.org/microrna/home.do) [68] to predict the targeting relationship. In view of the known reports and the extractability of the sequences, we only selected CLASSIC and ANTISENSE circRNA types for the prediction of the miRNA targeting relationship.

Experimental validation of circRNAs

Quantitative real-time PCR (qRT-PCR) was used to validate circRNA expression. We selected 5 circRNAs for validation. The expression levels of the selected circRNAs were normalized against the housekeeping gene, GAPDH. The primers were designed using the Primer 3.0 software and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The qRT-qPCR was carried out in triplicate with the SYBR Green Master Mix(Roche, Australia) on a Bio-Rad CFX96 instrument (BioRad, CA, USA). The total 20 μL reaction mixture contained 10 μL of 2×iTaq^{TM}Universal SYBR$^{@}$ Green Supermix, 1 μL of cDNA, 8 μL of ddH_2O, and 0.5 μL of forward and reverse primers, and the following program was used: 95 °C for 10 min; 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s; 72 °C for 6 min. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression levels of relative quantification.

Abbreviations

“H&E staining”: hematoxylin and eosin staining;

AFWS: Aohan fine wool sheep;

E90d, E120d, Birth: Embryonic day 90, embryonic day 120, and Birth.

DEG: Differentially expressed gene;

GO: Gene Ontology;

KEGG: Kyoto Encyclopedia of Genes and Genomes;

SRPBM (spliced reads per billion mapping);

Declarations

Ethics approval and consent to participate

All experimental and surgery procedures involved in this study followed the “Guidelines for Experimental Animals” of the Ministry of Science and Technology of China(Beijing, China). The operations and animal care were in accordance with the recommendations proposed by the European Commission (1997), and were sustained by the Experimental Animal Ethics Committee of Qingdao Agricultural University. The written informed consent to participate was obtained from the AFWS Stud Farm of Inner Mongolia Autonomous Region.

Consent for publication

The written informed consent to publish was obtained from the AFWS Stud Farm of Inner Mongolia Autonomous Region.

Availability of data and materials

Additional data can be found in supplementary files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RRZ, JNH, and NL conceived and designed this study. RRZ, NL, JFL and GYW participated in sample collection. RRZ, FHH and LLL performed the experiments. RRZ, JNH and HGL analyzed the data and prepared the figures and tables. RRZ, NL, and JNH wrote the manuscript. All of the authors reviewed and approved the final manuscript.

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Tables

Table 1 The top 30 expressed circRNAs in three stages during the wool follicle development.

circRNA_ID	E90d	E120d	Birth	Source_Gene
oar_circ_0004116	1	1	1	gene11824
oar_circ_0007074	2	15	2	gene21688
oar_circ_0004478	3	4	5	gene12317
oar_circ_0005665	4	5	4	gene15784
oar_circ_0002449	5	2	3	gene5355
oar_circ_0000988	6	3	7	gene823
oar_circ_0006745	7	7	9	gene20657
oar_circ_0006895	8	12	24	gene20962
oar_circ_0005236	9	6	15	gene13823
oar_circ_0000584	10	11	10	gene2553
oar_circ_0006529	11	9	17	gene19832
oar_circ_0006098	12	10	11	gene16874
oar_circ_0004973	13	22	8	gene13488
oar_circ_0005612	14	18	16	gene14199
oar_circ_0006659	15	17	26	gene20411
oar_circ_0000745	16	8	6	gene67
oar_circ_0007371	17	13	12	gene22388
oar_circ_0005870	18	14	25	gene16336
oar_circ_0005692	19	16	20	gene15838
oar_circ_0000805	20	20	14	gene587
oar_circ_0004075	21	29	22	gene11790
oar_circ_0005100	22	19	19	gene13664
oar_circ_0000806	23	25	23	gene587
oar_circ_0004749	24	23	27	gene12991
oar_circ_0005915	25	27	13	gene16538
oar_circ_0007026	26	21	28	gene21585
oar_circ_0007070	27	26	18	gene21688
oar_circ_0000175	28	28	29	gene1821
oar_circ_0001881	29	24	21	gene3567
oar_circ_0007898	30	30	30	gene25089

Table 2 Prediction of targeting relationship between miRNA and circRNA.

miRNA_Name	circRNA_ID	Binding_Site_Number
oar-miR-27a	oar_circ_0002552	1
oar-miR-27a	oar_circ_0005874	1
oar-miR-329b-5p	oar_circ_0005112	1
oar-miR-329b-5p	oar_circ_0005113	1
oar-miR-329b-5p	oar_circ_0005367	1
oar-miR-329b-5p	oar_circ_0006525	1
oar-miR-370-3p	oar_circ_0002663	1
oar-miR-410-5p	oar_circ_0003475	1
oar-miR-410-5p	oar_circ_0003476	1
oar-miR-432	oar_circ_0003042	1
oar-miR-432	oar_circ_0003043	1
oar-miR-539-5p	oar_circ_0007183	1
oar-miR-539-5p	oar_circ_0007185	1
oar-miR-539-5p	oar_circ_0007199	1
oar-miR-539-5p	oar_circ_0007200	1
oar-miR-654-3p	oar_circ_0001220	1
oar-miR-665-3p	oar_circ_0000173	1

Figures

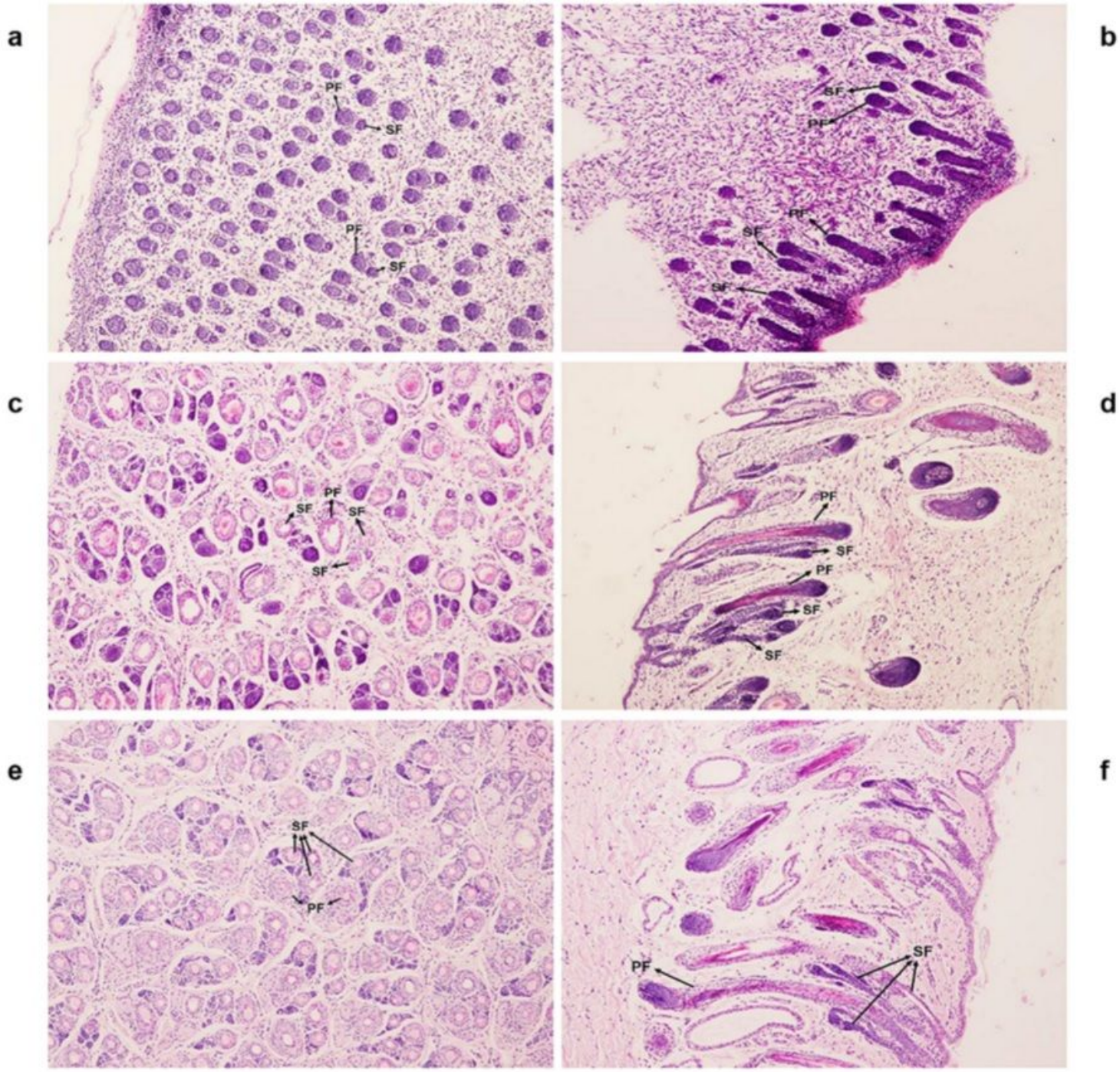

Figure 1

HE staining of wool follicle development of sheep. According to the staining results, the tissue morphology of secondary wool follicles at different stages was determined. (a,b), Horizontal slice and longitudinal slice of E90d. (c,d), Horizontal slice and longitudinal slice of E120d. (e,f), Horizontal slice and longitudinal slice of Birth stage.

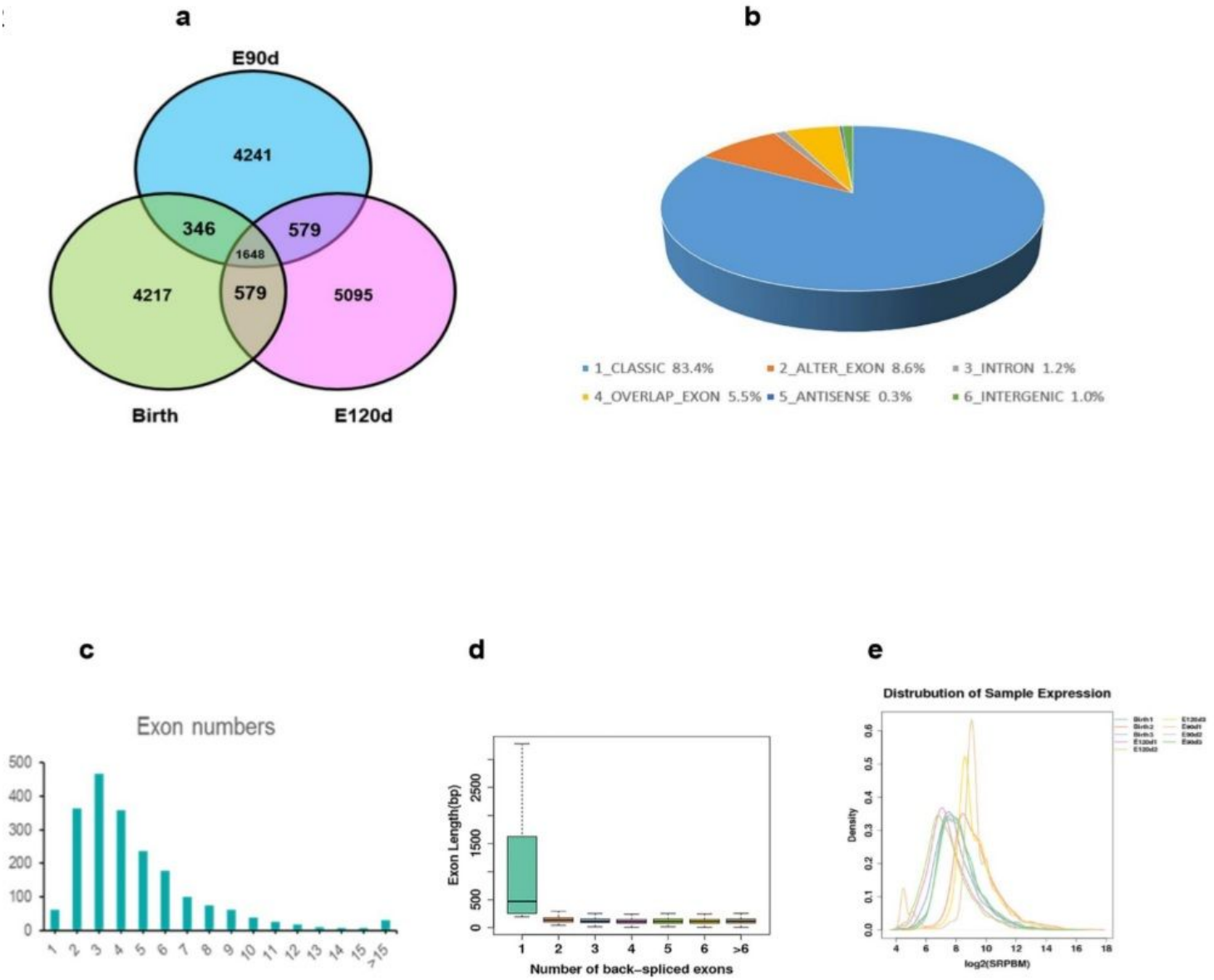

Figure 2

General characteristics of circRNAs in Aohan fine wool sheep skin. (a), Venn diagram shows circRNA annotated in sheep shoulder skin during three growth stages. (b), The classification of 4123 circRNAs in this study. Expression pattern of circRNAs at three growth stages, (c), Exon number, (d), Exon length, (e), Expression Density of Samples.

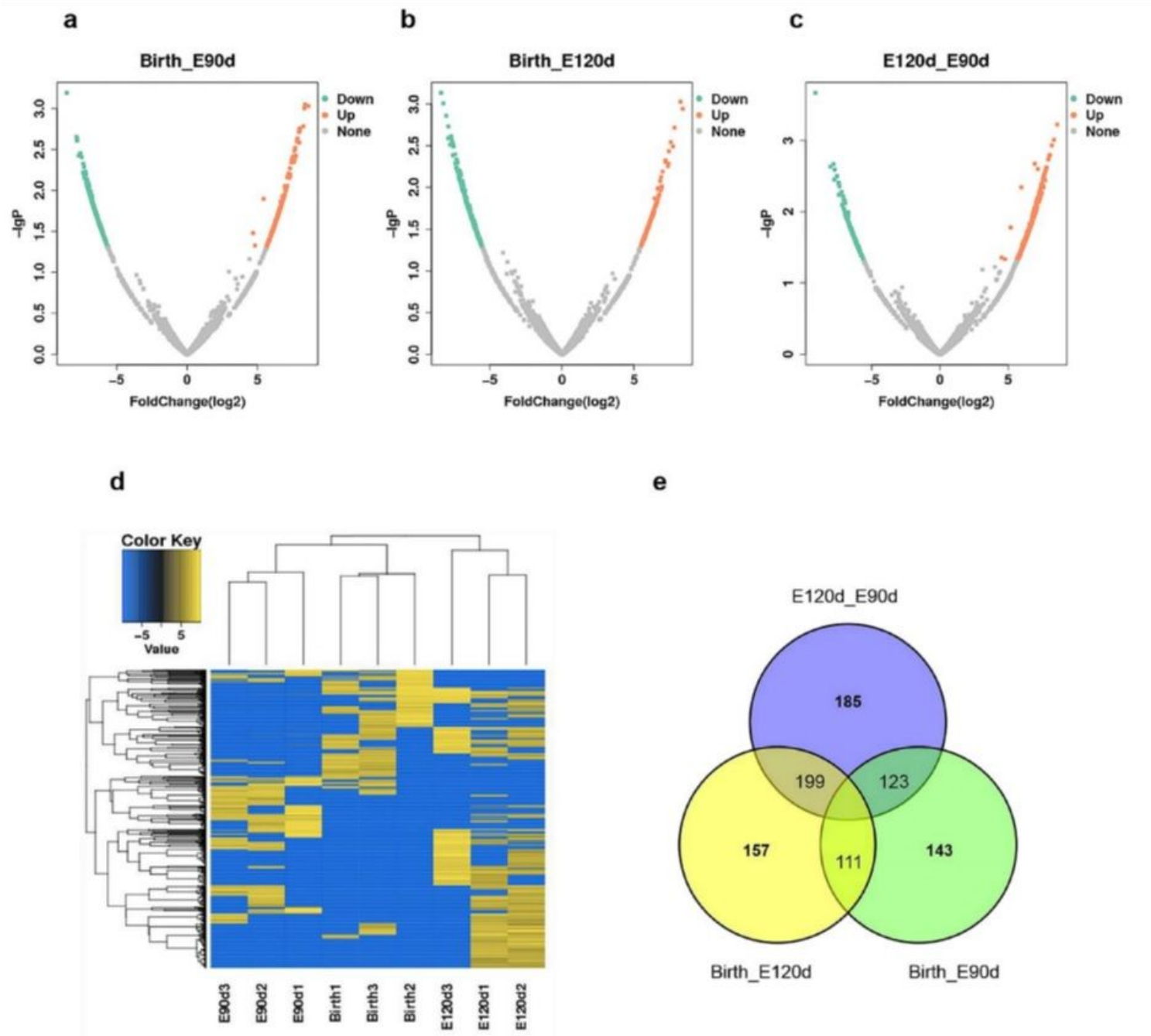

Figure 3

Identification of differentially expressed circRNAs. Volcano graphic analysis of differentially expressed circRNA in (a), Birth¬_E90d. (b), Birth_E120d. (c), E120d_E90d. The X axis is the change in expression fold in different samples, and the Y axis is the statistical significance of the change in expression. Red represents the up-regulated gene and green represents the down-regulated gene. (d), Heatmap of differentially expressed circRNA, Blue means lower expression, while yellow means higher expression. (e), The differentially expressed circRNAs in the pairwise comparisons groups.

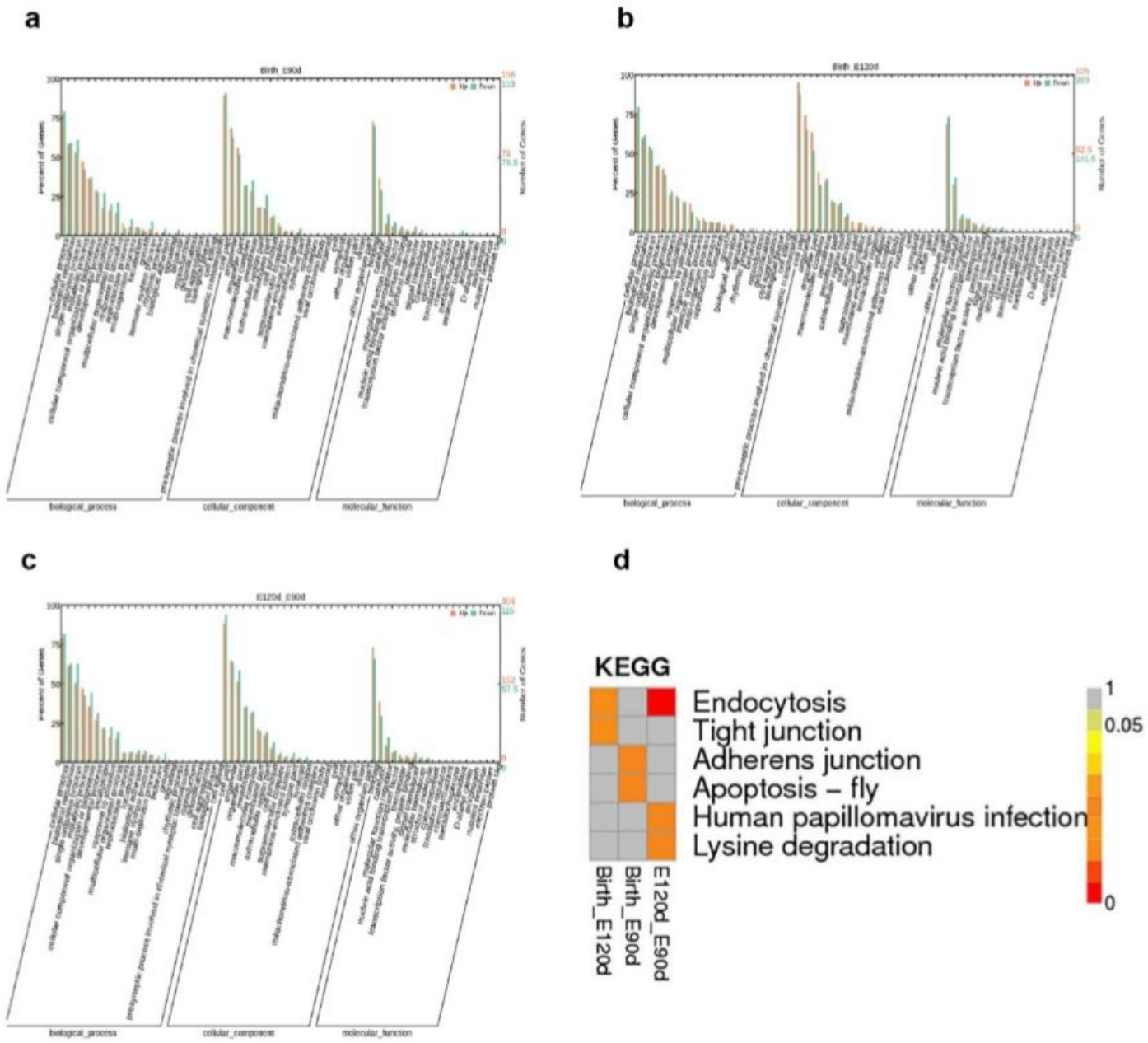

Figure 4

Function analysis of source genes of differentially expressed circRNAs. (a), GO analysis of circRNA host genes between birth and E90d. (b), GO analysis of circRNA host genes between birth and E120d. (c), GO analysis of circRNA host genes between E120d and E90d. (d), The KEGG heat of differentially expressed circRNAs.

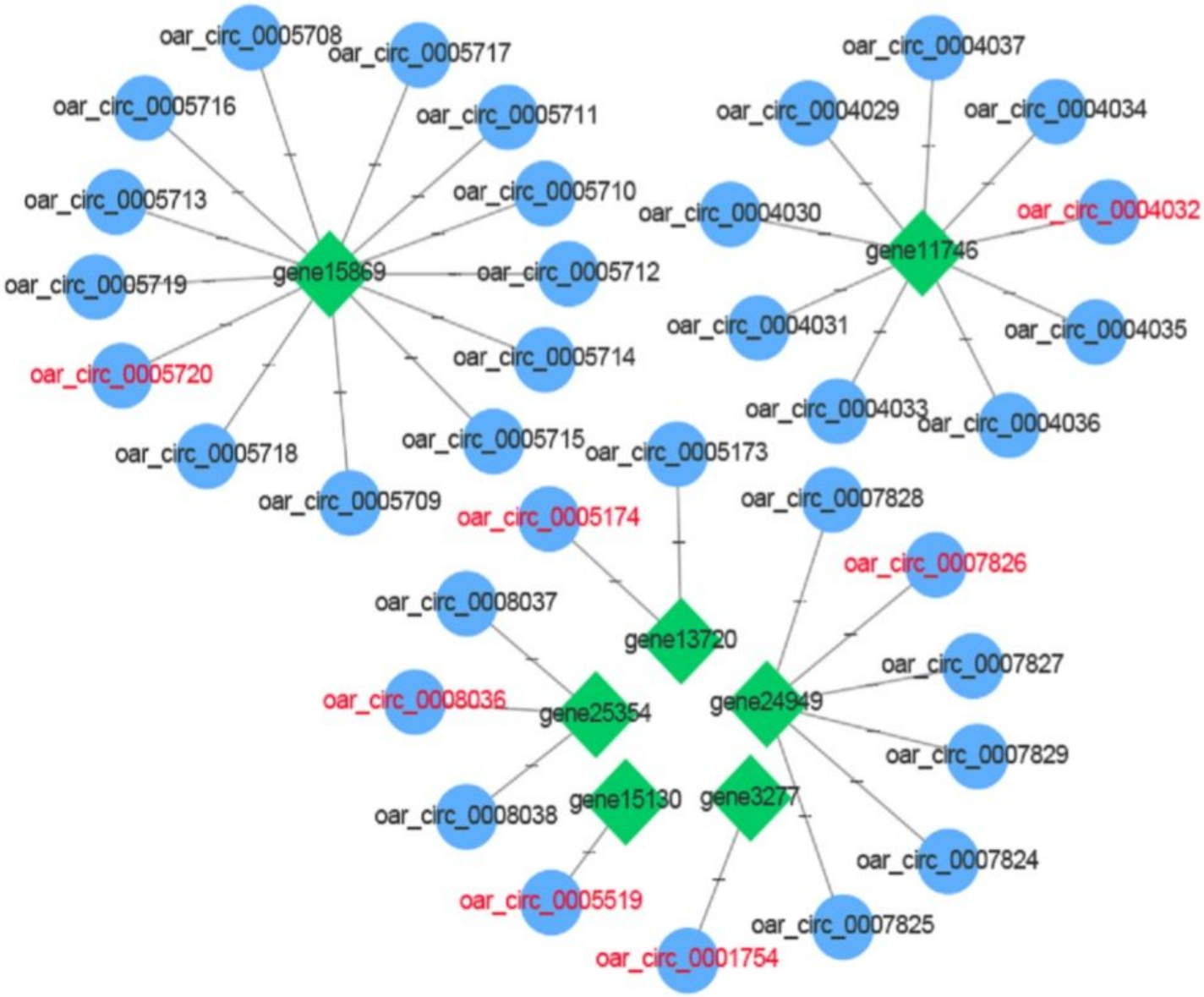

Figure 5

Interactions between circRNAs and source genes. The green diamonds represent source genes, the blue circle represent circRNAs. CircRNAs highlighted in red is our candidate genes.

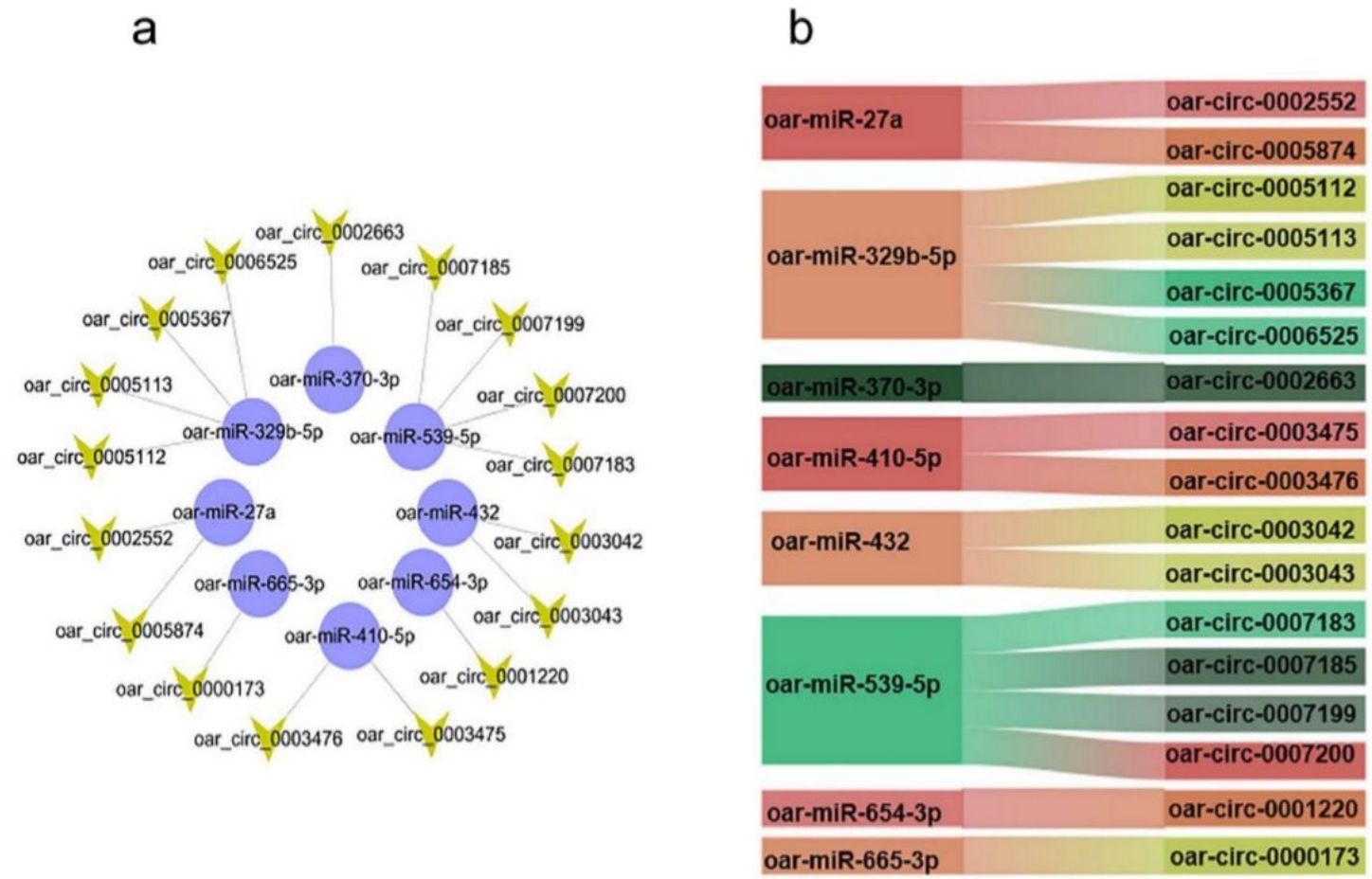

Figure 6

Interactions between circRNAs and miRNAs. (a), The yellow arrowheads represent circRNAs, the purple circle represent miRNAs. (b), The sankey diagram between circRNAs and miRNAs.

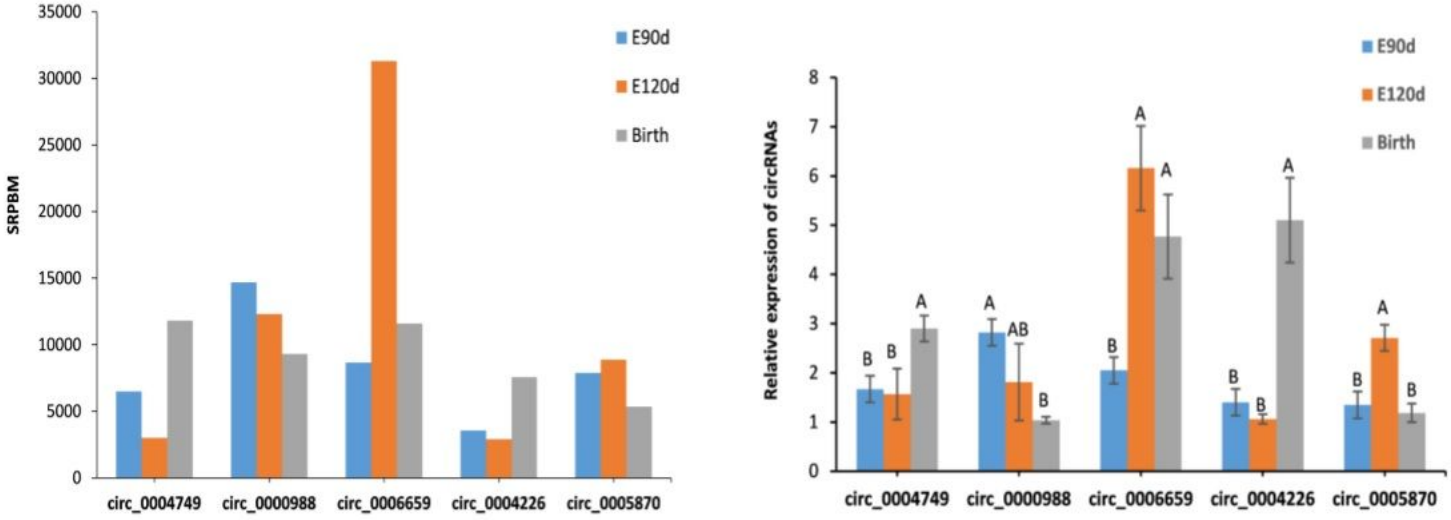

Figure 7

The validation of circRNA expression in skin samples among different wool growth stages. (a), The SRPBM of selected circRNAs at E90d, E120d and Birth stage. (b), qRT-PCR validation of differential expression levels of selected circRNAs at E90d, E120d and Birth stage. $P<0.05$ was considered to be significant, and $P<0.01$ was considered to be extremely significant.

Supplementary Files

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