TRIDHA
Journal of Clinical Cases & Reports
Volume 2022, Issue S4, 19 pages
https://doi.org/10.46619/joccr.2022.5-S4.1021

CLINICAL REVIEW

Transcriptome Analysis of Sheep IVF embryos based on single Cell RNA-Seq

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Received: 11 March 2022; Accepted: 22 March 2022; Published: 30 March 2022

ABSTRACT

OBJECTIVE

The purpose of the present study was to explore the transcriptome differences of sheep in *in vitro* fertilization (IVF). Embryos were fertilized *in vitro* at different developmental stages in order to assess the differences of the function, classification and metabolic pathway of differentially expressed genes and to provide a theoretical basis for revealing the regulatory mechanism of sheep early embryo development. The experiments aimed to promote the development of sheep embryo production technology *in vitro*.

METHODS

The single embryos derived from sheep 8-cell,16-cells and morulae were produced by IVF technology as samples and the sequencing library was constructed by the Smart-Seq 2 amplification technology. The transcriber was sequenced by Illumina HiSeqXten high-throughput sequencing technology and the effective sequences were analyzed by functional annotation and related bioinformatic analysis.

RESULTS

The results indicated that the clean reads of 8-cell to morula embryos were 53327656-67939390. There were 171170-211487 sites of single nucleotide polymorphisms (SNPs) and 8997-9624 sites of novel transcripts in sheep 8-cells to morulas respectively and 14013-15034 gene compared with the reference genome sequence of sheep. The following screening criteria were used: log2ratio ≥1 and Q-value <0.05. There were 190 DEGs were identified in the 8-cell embryos compared with those of the 16-cell, including 44 downregulated genes and 126 upregulated genes. 417 DEGs were identified in the morulae embryos compared with those of the16-cell, including 47 downregulated genes and 370 upregulated genes. Using the GO enrichment analysis, we explored the function of the DEGs.8-cell VS 16-cell GO analysis was significantly enriched in 94 term on BP. No significant difference

was found at CC and MF. were classified and annotated from the 16-cell stage to the morula development stage. These were enriched to 88 secondary items, a total of 78 second-level entries were noted in the BP classification. The 2 secondary items enriched in CC, the 8 secondary entries enriched in the MF. KEGG analysis of differentially expressed genes during sheep embryonic development indicated 126 pathways involved in the 16-cell developmental stage (from 8-cell to 16-cell stage), whereas the 11 main pathways with significant enrichment, 206 pathways involved in the morula developmental stage (from 16-cell to morula stage), whereas the two main pathways with significant enrichment.

CONCLUSION

The transcriptome of sheep IVF 8-cell to morula embryos was sequenced and analyzed by high-throughput sequencing. The number of differentially expressed genes was identified at different stages of sheep embryo development and the function, classification and metabolic pathway of differentially expressed genes were obtained. The current study offers substantial information on the identification of the sheep embryo transcriptome, revealing the molecular regulatory mechanism of sheep embryo development and perfecting the technology of embryo culture *in vitro*.

KEYWORD

In vitro fertilization; Embryo; Transcriptome; RNA-Seq

INTRODUCTION

Embryonic development is the result of the connection and cooperation of several genes expressed in time and space [1-4]. It is a dynamic process affected by specific parameters such as time and space. In the specific period of embryonic development, some differentially expressed genes participate in different biological functions and metabolic pathways, which can regulate the normal development of embryos [5,6].

In 2009 Tang et al. [7] initiated the single-cell RNA sequencing (scRNA-Seq) technology for the first time and analyzed the whole transcriptome of a single mouse blastocyst for the first time. In recent years, bioinformatics analysis methods, such as (PCA) or unsupervised clustering, have been used to analyze the composition of individual embryonic cells at different early stages of development. The dynamic changes of embryonic cell transcriptome before implantation were revealed. For example, the blastocysts of morula express different marker genes Sox2 and Id2, which can distinguish internal and external cells by marker genes. Even at the 2-cell stage, there are differences in gene expression between blastomeres [8,9]. Xue et al. [10] demonstrated that after fertilization, sperm cells from the same donor were injected into different oocytes, and 5% of gene transcripts were detected by paternal SNPs. Single cell transcriptome sequencing has become a revolutionary tool, which solves the problem of transcriptome sequencing of limited RNA samples. This technology can sequence single mammalian somatic cells and reveal the heterogeneity of single cells [11].

The RNA content of early embryos is low [12], which is difficult to meet the minimum RNA starting amount required for transcriptome sequencing. However, with the establishment and development of micro transcriptome sequencing technologies such as smart-seq2 [13], previous studies have successfully used RNA SEQ technology to analyze the regulatory mechanism of early embryonic development of human, pig and bovine species [9-11]. However, the number of studies on the expression of functional genes and their potential relationship with the

regulation of sheep embryonic development is very limited. The achievements of sheep genome sequencing technology and the development of high-throughput sequencing technology provide a fast and effective way to study the molecular mechanism of sheep embryonic development at the taxonomic level.

In this study, RNA from individual 8-cells, 16-cells and morula embryos was amplified using Smart-Seq2, and high-throughput sequencing analysis by IlluminaHiSeq Xten performed to reveal the regulatory mechanism of early embryonic development and lay the foundation for further improving *in vitro* fertilization.

MATERIAL AND METHODS

Ethics Statement

The study method was carried out in strict accordance with the relevant guidelines and regulations formulated by the Ministry of Agriculture and Rural Affairs of the People's Republic of China, and all the test protocols were approved by the by the Institutional Animal Care and Use Committee of Tarim University.

Chemicals

All chemicals were purchased from Sigma-Aldrich (Oakville,ON,Canada), unless otherwise specified.

Collection of Ovaries

Ovaries were collected from the Aksu slaughterhouse. Following slaughtering, the adult ewes had both their ovaries removed, which were sprayed with 75% ethanol. The samples were incubated at 38°C in the presence of sterilized normal saline [plus double antibody (green streptomycin mixture)] and transported back to the laboratory within 2 hours - 3 hours. The fallopian tubes, mesentery and other excess tissues on the ovary were cut off, washed once with normal saline and preheated at 38°C in advance. The samples were quickly washed 3 times with 75% ethanol and finally 3 times with normal saline at 37°C. The samples were placed in a 37°C water bath without passing through the ovarian beaker.

COCs Collection

The egg collection fluid was preheated in a 38°C water bath 2 hours in advance and approximately 10 ovaries were obtained. The water was removed on the filter paper and placed into petri dishes (60 mm) containing the egg collection fluid. Follicles that were 2 mm - 6 mm in diameter were scratched with a disposable surgical blade. A small amount of egg collection fluid was absorbed by a 5-ml syringe in order to rinse the ovary cutting site and facilitate the follicle fluid flow. The samples were left to precipitate for a few min and the eggs were visualized using a microscope. Grade A and B COCs were selected for mature culture.

COCs Culture

In the super-clean platform, the mature culture droplets were transferred to a 35 mm petri dish and 20 μl mature liquid was absorbed by the liquid transfer gun. The spacing was controlled at the bottom of the petri dish in order to produce microdroplets. 3 ml - 4 ml sterilized paraffin oil was added and 50 μl mature liquid was mixed with the original droplets after the paraffin oil was paved. The selected grade A and B COCs were washed with IVM solution 3 times and transferred into pre-balanced droplets. Each droplet was cultured in a carbon dioxide incubator (5% CO_2, 38.5°C, saturated humidity) for 24 hours. The diffusion of granulosa cells was observed. The loosely arranged COCs of the granulosa cells was transferred to PBS containing 0.1% hyaluronidase through an oral straw and 200 μl of the liquid was repeatedly blown to COCs. When 1 layer - 2 layers of the granulosa cells

were preserved, the blowing was terminated and the samples were rapidly transferred to the IVF solution. Following three times washing in the IVF solution, the first polar body excretion was observed. The oocytes excluded by the first polar body were used for *in vitro* fertilization.

The 0.5 ml diluted semen was slowly injected into the diluted semen from the bottom of the centrifuge containing 1 ml sperm capacitation solution. The diluted semen was incubated in a constant temperature incubator (38.5°C, 5% CO_2, 100% humidity) for 30 minutes. The supernatant was centrifuged for 5 minutes and discarded. This process was repeated. The mature oocytes with 1 layer - 2 layers of granulosa cells were transferred into 50 μl pre-balanced fertilized droplets following three times cleaning of the fertilized fluid, with a maximum of 15 COCs per droplet. The upstream semen was added to the fertilization droplets (the sperm density was approximately 1*106 /ml). The sperm and eggs were incubated in a CO_2 incubator for 18 h (38.5°C, 5% CO_2, 100% humidity).

Removal of Zona pellucida

When the embryo was transferred into 10 μl PE (*Streptomyces protease*) microdroplets with an oral straw and incubated at 50°C for 30 seconds at the final concentration of 5 mg PE, the gap between the zona pellucida and the blastomere was enlarged and slightly deformed. Washing of the embryo was performed in 2 ml aseptic PBS buffer (5% FBS + 2% PS). The zona pellucida was gently washed with an oral straw (smaller than the diameter of the embryo) 5 times in DPBS. The 8-cell,16-cell and morula embryos were collected and transferred to 10 μl lysates and liquid nitrogen was injected into the embryos within 2 minutes. Following freezing for several, 2 repeats were used for each group. The 8-cell,16-cells were marked as E801, E802, E1601 and E1602 and the morulae were marked as EM01 and EM02. The samples were transported by dry ice and sent to An Nuoda Genome Technology (Beijing) Co. Ltd.

RNA Extraction and Library Construction

The construction of the library was completed by Annuoda Genome Technology (Beijing) Co., Ltd. The single cell samples were collected in tubes containing cleavage components and ribonuclease inhibitors. Subsequently, smart seq2 method was used for amplification. The oligo DT primer was introduced into the reverse transcription reaction for the synthesis of the first strand cDNA, then the cDNA was amplified and enriched by PCR, and the production was cleaned up by magnetic bead purification. CDNA from qubit ® 3.0 fluorometer detection, using Agilent 2100 biological analyzer to ensure that the expected yield length is about 1-2kbp.Then, the cDNA was randomly cut by ultrasound for Illumina library preparation scheme, including DNA fragment, end repair, 3 'end a tail, adapter connection, PCR amplification and library verification. After the library was prepared, gene chip was introduced ® Gxtouch and step plus ™ The library quality was detected by real-time PCR system. Libraries meeting these criteria were then loaded onto the illuminaHiSeq platform for pe150 sequencing.

Data Analysis

In order to ensure the quality of the sequencing data, the raw reads obtained by the IlluminaHiSeqXten sequencing were filtered and the clean reads were obtained following removal of the joint sequence, the empty read sequence and the low-quality sequence (Phred quality < 5) [14,15]. The reference gene and genome annotation files were downloaded from the ENSEMBL website (ftp://ftp.ensembl.org/pub/release-100/fasta/ovis_aries/dna/Ovis_aries.Oar_v3.1.dna.toplevel.fa.gz). The reference genome library was constructed using bowtiev (1.0.1), and then the clean data was compared with the reference genome through hisat2 (v2.1.0)

[16]. The gene expression was calculated by FPKM (Fragments Per Kilobase Millon mapped reads). The comprehensive expression of two biological repeats in the (gc) morula, the (gb)16-cell and (ga)8-cell was obtained by calculating the expression amount of two biological repeats in the (gc) stage of the morula, (gb) stage of the16-cell and (ga) stage of the 8-cell by the base mean. The differentially expressed genes were selected according to the estimation of the gene expression levels by DESeq, which was achieved by the negative binomial distribution of the statistical methods. The P-value was corrected to control false positive according to Benjamini and Hochberg methods. The corrected P-value ≤0.05 was defined as the parameter required for the differentially expressed gene. DESeq2 was used to analyze the differential expression of biological repetitive samples between the two groups compared with the treatment group and the reference group. The genes with | log2Ratio | ≥1 and Q <0.05 were selected as differentially expressed genes and the number of up- and downregulated genes was obtained. The obtained DEGs were mapped to each entry in the GO database (http://www.geneontology.org/) and their number was calculated. Following correction of the P value by the Benjamini method [17], the GO entry with Q <0.05 was the GO entry used for significant enrichment of DEGs. The signaling pathways or metabolic pathways involved in the genes were analyzed by comparing their expression with the KEGG (Kyoto encyclopedia of genes and genomes) database (http://wego.genomics.org.cn) [18].

Analysis of Alternative Splicing and Single Nucleotide Polymorphism and Prediction of New Transcripts

The RPKM tool of AS profile (1.0.4) was used to analyze the structure and expression of alternative splicing events and Samtools (1.5) was used to analyze single nucleotide polymorphism (single nucleotide polymorphism, SNP) [19]. The sequenced sequences of the aligned genomes were assembled and spliced by the Cufflinks software (v2.2.1) [20]. Following filtering of the low-quality sequences (length ≤ 180 bp, Q value ≤ 10), the assembled transcript sequence was compared with the gene annotation information on the sheep genome. For example, the assembled transcript sequence was not aligned with the existing genes and was located on the genomes between the existing genes. The following conditions were satisfied: the distance from the existing annotated genes was more than 200 bp, the length was not shorter than 180 bp and the sequencing depth was not less than 2. These sequences were identified as potential new transcripts and new genes [21].

Validation by Real-Time Quantitative PCR (RT-qPCR)

Here, we selected 8 DEGs to verify the sequencing results via RT-qPCR. Primers were designed by Primer-BLAST in NCBI, as shown in Table 1. First strand cDNA was synthesized from 1μg total RNA using the reverse transcriptase Revert Aid (Takara) following manufacturers recommendations. PCR amplification was executed in reaction volumes of 10μL that included 1μL of cDNA,0.6μL of forward and reverse primers (10μM) for each gene, 5μL of 2×S6 Universal SYBR qPCR Mix (NovaBio. China), and 3.4μL of RNA-free double-distilled H2O. The cycling conditions were as follows: 95℃ for 30 seconds, 40 cycles of 95℃ for 5 seconds, and annealing temperature 60℃ for 30 seconds; a melt curve analysis was performed at 65℃-95℃.

Statistical Analysis

GAPDH were used as housekeeping genes for qPCR, and the mean expression levels of GAPDH were considered to be the expression levels of housekeeping genes. 8-cell was used to normalized the gene expression, and the relative gene expression was calculated by 2-ΔΔCT method.

GENE	Primer	Tm	Product length (bp)
GAPDH	F-GGTGATGCTGGTGCTGAG	58	184
	R-TGCTGACAATCTTGAGGGTAT		
KIT	F-TCTTCGTTCTGCTGCTCCTG	59	281
	R-GCTCAAGCCGCCTTTATTGG		
ZP4	F-CTTCAGGCTCCAAGTCCGTT	59	249
	R-TCGAGACTGGGGTCTGTTCT		
BMP15	F-GGGTTCTACGACTCCG	53	219
	R-TACTGCCTGCTTGACG		
GDF9	F-CACAGGGTCCTGACCAGAAG	59	259
	R-TTGTGTGGGGCCATAATCCA		
Hsf1	F-CCCTGGTTCGTGTCAAGGAG	60	265
	R-CTGAGCTCGGTCTTGTCCAG		
NANOG	F-ACCCGGGCTTCTATTCCTAC	58	151
	R-GCCTGACTGTTCCAGGAGTG		
MAPK13	F-CGTTACAACCAGACCGTGGA	59	153
	R-CTTCTGCACAAACTCGGCAC		
ACO2	F-TATGCCTACCCTGGGGTTCT	59	157
	R-TCACACCAATCACCTTGGGG		

Table 1: QT-PCR primers.

RESULTS

Sequencing Quality Evaluation and Basic Data Analysis

The embryo libraries of sheep 8-cell,16-cells and morulas constructed in this study met the requirements of transcriptome sequencing. The embryos were detected by the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The six sample peaks indicated apparent peaks of target products in the length range of 1-2 kb fragments, whereas some small fragments below 1 kb were noted. However, the proportion was small, indicating that the integrity of the original sample was optimal and that it met the requirements of database construction (Figure 1). The percentage range of Q30 of the six samples was estimated to 93.18%-94.02% (Figure 2), indicating that the sequencing quality and library construction quality were high and that the sequencing data were accurate and reliable. This could meet the needs of follow-up analysis. In the sequencing results, the contents of A-T and C-G of the six samples basically coincided with each other, indicating that the base composition was stable and balanced and that the sequencing quality was high (Figure 3).

	8-cell-1 (E801)	8-cell-2 (E802)	16-cell-1 (E1601)	16-cell-2 (E1602)	32-cell-1 (E3201)	32-cell-2 (E3202)
Clean Reads	56041526	56371414	67939390	58488860	53327656	53723168
Mapped Reads	52435031	52983454	64038390	55115234	49685950	50358084
Mapping Rate	93.56%	93.99%	94.26%	94.23%	93.17%	93.74%
UnMapped Reads	3606495	3387960	3901000	3373626	3641706	3365084
MultiMap Reads	2679213	2648094	3181456	2783301	2318186	2924575
MultiMap Rate	4.78%	4.70%	4.68%	4.76%	0.0435	5.44%
Total gene number	15034	14689	14850	14848	14013	14261

Table 2: RNA-Seq and mapping to the reference genome.

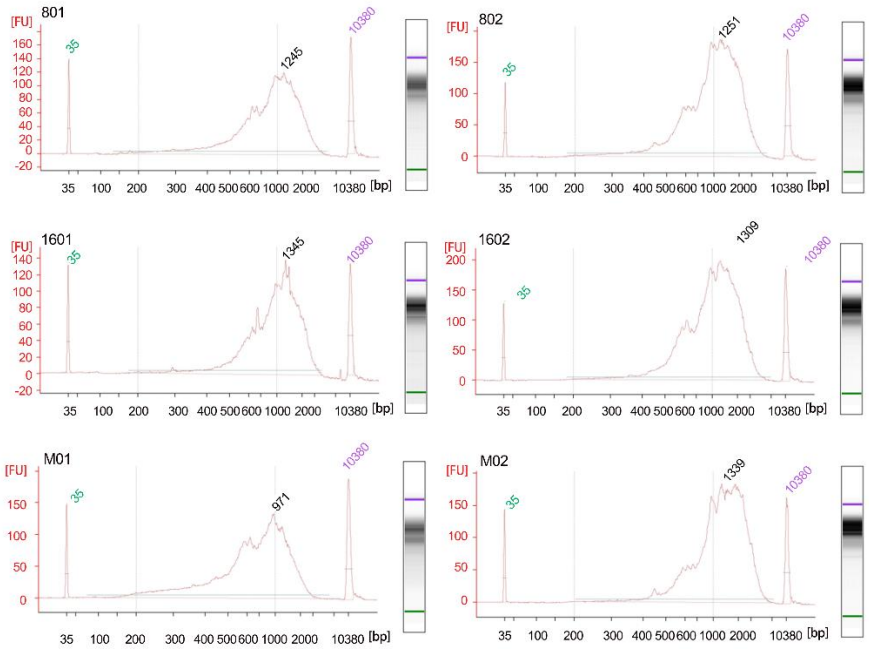

Figure 1: Peak figure of Agilent 2100 for cDNA integrity detection by Smart-Seq2 PCR amplification.
Note: Figure 801,802: 8-cell cDNA peak diagram;1601,1602:16-cell cDNA peak diagram; M01, M02: The cDNA peak diagram of morula.

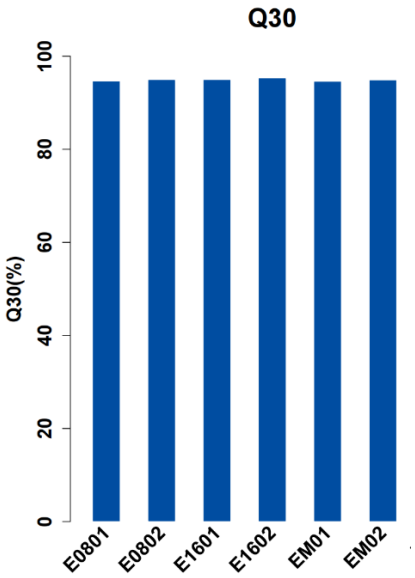

Figure 2: Base quality level of Q30 reactive sequencing. (The proportion of the bases with sequencing quality value was greater than 30 (error rate less than 0.1%) in the total RawReads.)

Embryos	No. of transcripts	No. of novel transcripts
8-cell-1（E801）	15034	9617
8-cell-2（E802）	14689	9146
16-cell-1（E1601）	14850	9297
16-cell-2（E1602）	14848	9624
32-cell-1（E3201）	14013	8997
32-cell-2（E3202）	14261	9243

Table 3: Detected transcripts and novel transcripts.

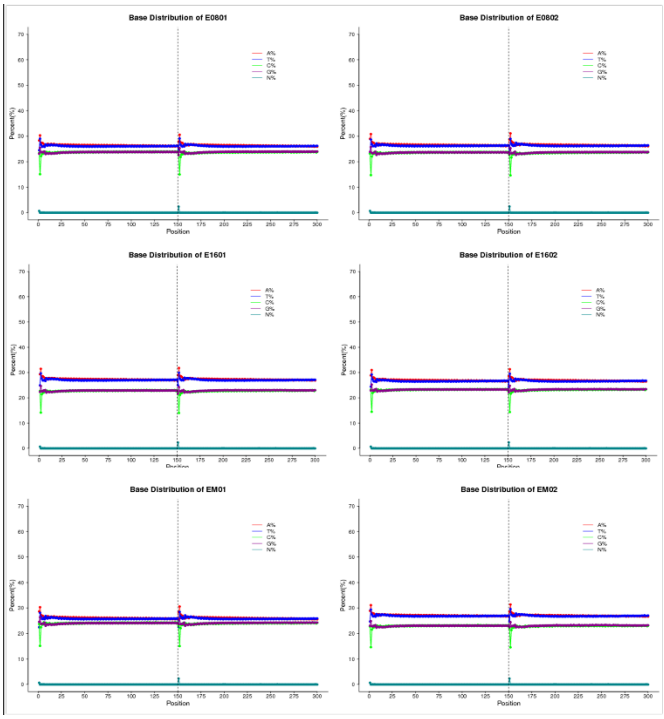

Figure 3: Content of A,T,G,C for reads of 8-cell (E0801,E0802), 16-cell (E1601,E1602) and Morula (EM01,EM02).

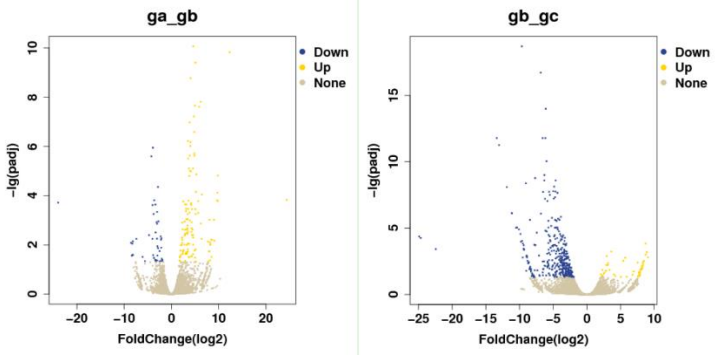

Figure 4: Volcanic diagram of sheep IVF 8-cell,16-cell and morula differentially expressed genes (ga_gb 8-cell *vs.* 16-cell gb_gc 16-cell *vs.* morula. Abscissa shows the changes in expression multiples in different experimental groups/samples, and the ordinate shows the statistically significant changes in expression levels. Different colors represent different classifications).

Analysis of Sequencing Results

Following filtration of the data of the original sequence, the filtered sequence of the sheep embryos at the three developmental stages of the 8-cell, 16-cell and morula embryos exhibited the following range: 53327656-67939390. The obtained clean reads were compared with the reference genome by the TopHat software [22-23]. The results indicated 93.17%-94.26% clean read alignment of the sheep reference genes in each stage and the sequence ratio of the multiple locations of the genome was 4.35%-5.44%, which satisfied the requirements (Table 2). The number of transcripts and predicted new transcripts compared at each stage of development is shown in Table 3. The novel transcripts were estimated to 9617-9243 (Table 3). Alternative splicing analysis by ASprofile software indicated mainly 10 types of splicing events in sheep embryos Table 4. SNP analysis by Samtools indicated the presence of 171170-211487 loci in each developmental stage of sheep embryos from the 8-cell to the morula embryos (Table 5). Count the number and proportion of unique alignment sequences (i.e. sequences that align only one position of the genome) on the three functional elements of genes (exon, intron and intergenic) (Table 6).

AS_type	8-cell-1 (E801)	8-cell-2 (E802)	16-cell-1 E1601)	16-cell-2 (E1602)	32-cell-1 (E3201)	32-cell-2 (E3202)
XAE	765	670	794	702	751	598
XIR	202	226	156	218	192	234
TTS	26468	25775	25390	25677	23184	24490
XMIR	8	10	10	6	12	2
XMSKIP	586	428	606	708	758	788
IR	762	748	584	778	876	806
XSKIP	1308	1110	1116	1472	1206	1530
SKIP	5838	5158	5028	6148	5770	7464
AE	2245	2263	1956	2438	2217	2662
MIR	22	42	16	24	48	28
TSS	27617	27367	27179	27452	23972	25903
MSKIP	1388	1130	1018	1286	2082	1976

Table 4: Alternative splicing events detected during embryonic development.

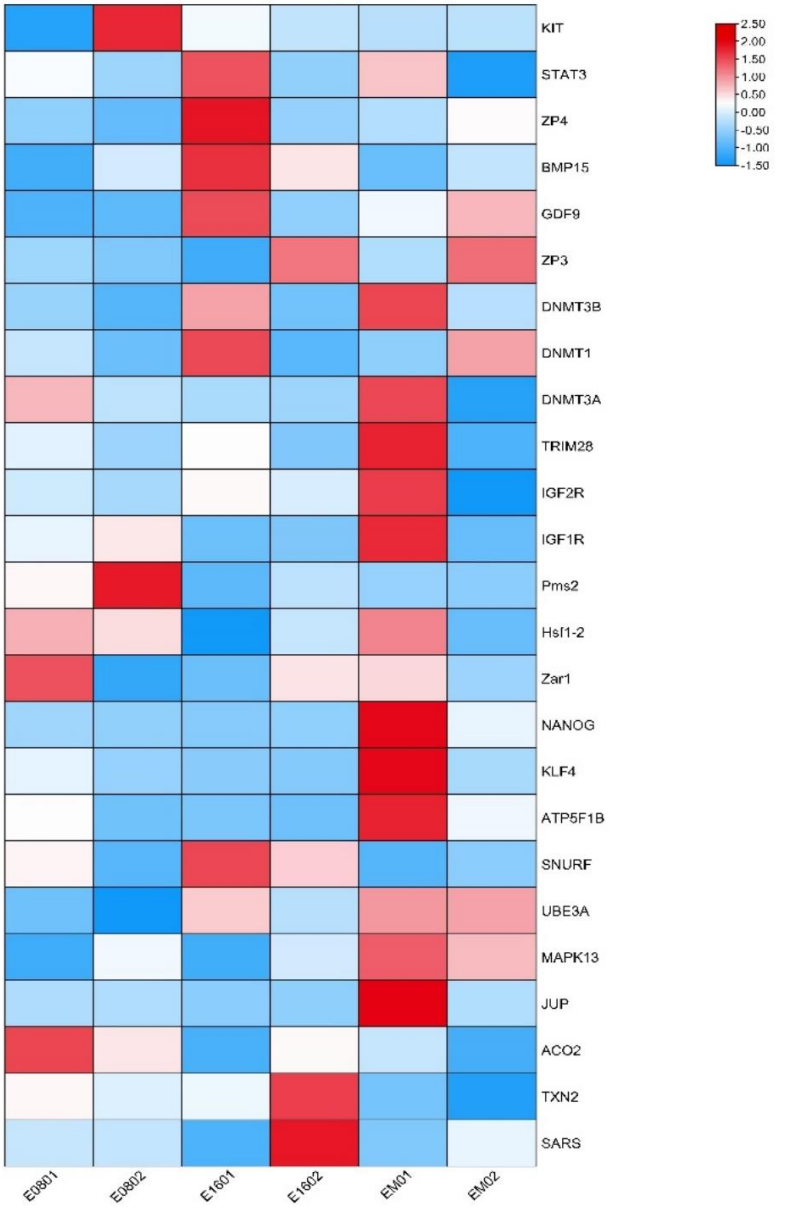

Figure 5: FPKM heat accumulation pattern of some genes in different stages of ovine cleavage (The maternally-associated genes: KIT, STAT3, ZP4, BMP15, GDF9, ZP3, Pms2, Hsf1-2 and Zar1. The methylation-associated genes were the following: DMNT3B, DMNT1 and DMNT3A. The proliferation-related genes were as follows: IGF2, TRIM28 and IGF1 and the stem cell-related genes were NANOG and KLF4. The FPKM values of ATP5F1B, SNURF, UBE3A, MAPK13, JUP, ACO2, TXN2 and SARS were obtained as the logarithm of 10 and the FPKM clustering heat map was drawn by the heml software).

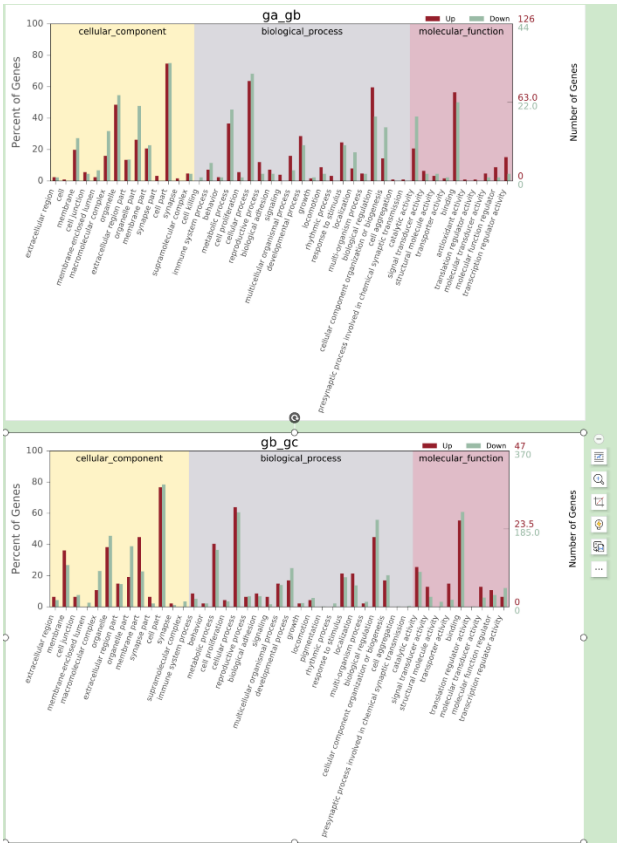

Figure 6: Gene Ontology classification of the DEGs during embryonic development. (ga_gb:8-cell *vs.* 16-cell, gb_gc:16-cell *vs.* morula.)

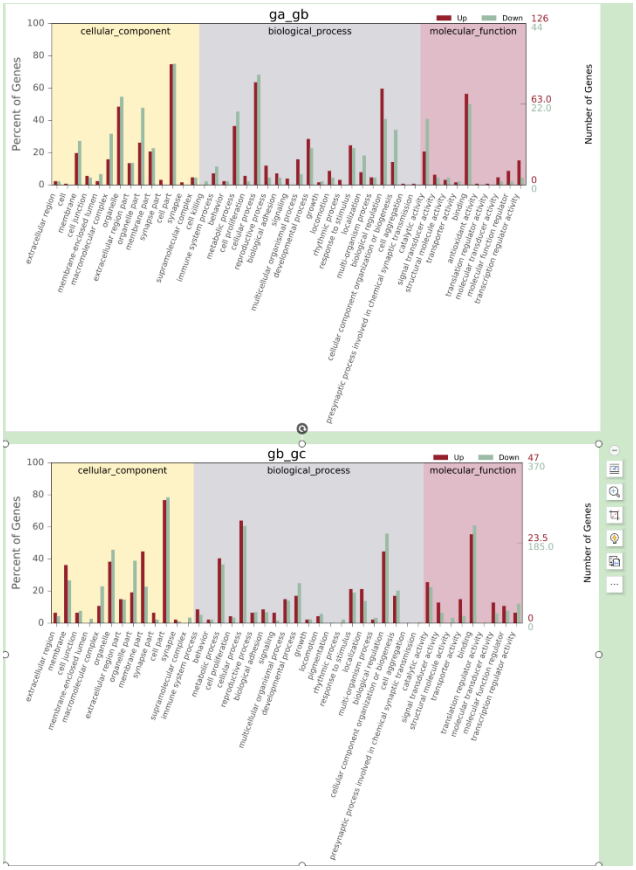

Figure 7: Gene Ontology classification of the Significant DEGs during embryonic development. (ga_gb:8-cell *vs.* 16-cell, gb_gc:16-cell *vs.* morula.)

Sample	8-cell-1 (E801)	8-cell-2 (E802)	16-cell-1 (E1601)	16-cell-2 (E1602)	32-cell-1 (E3201)	32-cell-2 (E3202)
SNP	190570	176889	199252	185622	159986	185523
InDel	12187	11133	12235	11558	11184	12468
Total	202757	188022	211487	197180	171170	197991

Table 5: Putative SNPs detected during embryonic development.

#Library	8-cell-1 (E801)	8-cell-2 (E802)	16-cell-1 (E1601)	16-cell-2 (E1602)	32-cell-1 (E3201)	32-cell-2 (E3202)
Exon	8331025 (35.81%)	8743717 (38.07%)	10012117 (36.60%)	8645583 (37.38%)	82422 (32.21%)	7670278 (35.62%)
Intron	3156227 (13.57%)	3077837 (13.40%)	3397301 (12.42%)	3377007 (14.60%)	41349 (16.16%)	3785084 (17.58%)
Intergenic	11776678 (50.62%)	11144496 (48.53%)	13947202 (50.98%)	11106048 (48.02%)	132148 (51.64%)	10075571 (46.80%)

Table 6: Distribution statistics of unique alignment sequences in reference genome region.

Gene Expression Characteristics and DEGs in Early Embryos

Based on the read value of the gene expression levels, the RPKM values of the genes at different stages of embryonic development were calculated by the RPKM method [24]. By comparison, 190 DEGs were identified in the 8-cell embryos compared with those of the 16-cell, including 44 downregulated genes and 126 upregulated genes. 417 DEGs were identified in the morulae embryos compared with those of the16-cell, including 47 downregulated genes and 370 upregulated genes. In order to directly reflect the difference in the multiple and significant levels of the differentially expressed genes, a volcano map was drawn (Figure 4). By passing through the FPKM value. A total of 30 key genes were found in every stages. The maternally-associated genes of sheep embryos were the following: KIT, STAT3, ZP4, BMP15, GDF9, ZP3, Pms2, Hsf1-2 and Zar1. The methylation-associated genes were the following: DMNT3B, DMNT1 and DMNT3A. The proliferation-related genes were as follows: IGF2R, TRIM28 and IGF1R. The stem cell-related genes were NANOG and KLF4 and the zygote related genes were ATP5F1B, SNURF, UBE3A, MAPK13, JUP, ACO2, TXN2 and SARS. They were obtained as the logarithm of 10 and the FPKM clustering heat map (Figure 5) was drawn by the heml software and the expression of 30 key genes was shown in Table 7.

GO and KEGG Analysis of DEGs

Using the GO enrichment analysis, we explored the function of the DEGs. 8-cell *vs.* 16-cell GO analysis was significantly enriched in Figure 6:94 term on BP, and the top 10 term functions with the highest enrichment were mainly: Negative regulation of cellular process, positive regulation of cellular process, regulation of gene express, regulation of nitrogen compound metabolic process, regulation of primary metabolic process, regulation of macromolecule metabolic process, positive regulation of biological process, regulation of cellular metabolic process, No significant difference was found at CC and MF. were classified and annotated from the 16-cell stage to the morula development stage, these were enriched to 88 secondary items (Figure 6). A total of 78 second-level entries were noted in the BP classification, of which the top 10 entries involved regulation of the metabolic process, nucleobase containing compound biosynthetic process, negative regulation of cellular process, organic cyclic compound biosynthetic process, RNA metabolic process, negative regulation of biological process, positive regulation of cellular process, positive regulation of biological process, regulation of cellular process and regulation of biological process. The two secondary items enriched in CC were the spliceosomal complex and the Intracellular component. The eight secondary entries enriched in the MF classification were the transcriptional

repressor activity, the RNA polymerase II proximal promoter sequence-specific DNA binding, the transcriptional repressor activity, the RNA polymerase II transcription regulatory region sequence-specific DNA binding, the transcription factor activity, the RNA polymerase II proximal promoter sequence-specific DNA binding, the transcription factor binding, the transcription factor activity, the protein binding, the transcription factor activity, the transcription factor binding, the RNA polymerase II transcription factor activity, the sequence-specific DNA binding and the transcription regulatory activity. According to the statistics of the secondary GO items, significant differences in the DEG annotation results were noted (Figure 6). It was found that the metabolic processes of BP, CC and MF were focused on biological functions, such as value-added, organic synthesis and nucleic acid synthesis. KEGG analysis of differentially expressed genes during sheep embryonic development indicated 126 pathways involved in the 16-cell developmental stage (from 8-cell to 16-cell stage), whereas the 11 main pathways with significant enrichment were the Basal cell carcinoma, Pathways in cancer, Transcriptional misregulation in cancer, HTLV-I infection, Cellular senescence, Gastric cancer, Hepatocellular carcinoma, Hippo signaling pathway, Non-small cell lung cancer, Glioma, Melanoma. 206 pathways involved in the morula developmental stage (from 16-cell to morula stage), whereas the two main pathways with significant enrichment were the Spliceosome and the signaling pathways regulating pluripotency of stem cells, respectively (Figure 7).

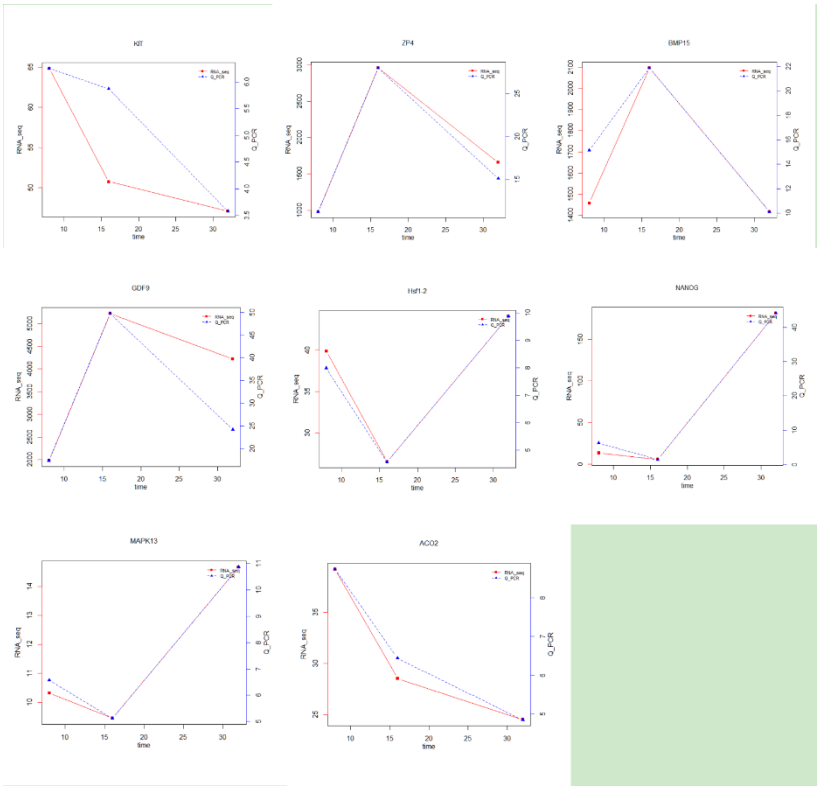

Figure 8: The validation of candidate genes. (Blue means Q-PCR, red means RNAseq, GAPDH were used as the reference gene for Q-PCR, RNA-seq relative expression was represent by FPKM).

Validation of Candidate Genes

To reveal the key genes associated with embryonic development, we screened several genes with higher expression levels among the 30 key differentially expressed genes, including KIT, ZP4, BMP15, GDF9, Hsf1, NANOG, MAPK13, ACO2. Then, the candidate genes were verified via RT-qPCR (Figure 8). Notably, similar results were reported as those obtained through sequencing, which confirmed the reliability of the sequencing data.

Gene	pfam ID	GO: Biological Process	GO: Cellular Component	GO: Molecular Function	KEGG:KO
ENSOARG00000001004	pfam09457	GO:0070164;GO:0015031;GO:0045055	GO:0005737;GO:0043231;GO:0030670;GO:0055037	GO:0017137	K12484
ENSOARG00000002647	pfam00418	GO:0000226;GO:0031175	GO:0005737;GO:0005874;GO:0005875;GO:0043005	GO:0008017	K10431
ENSOARG00000003764	pfam01553	GO:0016024;GO:0008544;GO:0006654;GO:0006644;GO:0001819;GO:0001961;GO:0019432	GO:0005783;GO:0005789;GO:0016021	GO:0003841	K13509
ENSOARG00000003855	pfam13964	GO:0042787	GO:0031463	.	.
ENSOARG00000006746	pfam02214	GO:0045742;GO:0051260	.	.	.
ENSOARG00000008413	pfam04811	GO:0006888;GO:0006886	GO:0030127;GO:0000139;GO:0030868	GO:0008270	K14006
ENSOARG00000009159	pfam00685	.	GO:0005737	GO:0047685	K16949
ENSOARG00000009580	pfam06008	GO:0007155;GO:0030198;GO:0030155;GO:0030334;GO:0045995	GO:0005605;GO:0005604;GO:0070062;GO:0005576	GO:0005201	K06241
ENSOARG00000009779	pfam00089	GO:0006957	GO:0005576	GO:0004252	K01334
ENSOARG00000010045	pfam00248	GO:0008209;GO:0006699;GO:0030573;GO:0008207;GO:0006707;GO:0007586;GO:0055114	GO:0005829;GO:0070062	GO:0004033;GO:0047787;GO:0005496	K00251
ENSOARG00000010052	pfam01393	GO:0016569;GO:0045892;GO:0048511;GO:0006351	GO:0000785;GO:0000779;GO:0000784;GO:0005635;GO:0005719;GO:0005720;GO:0031618;GO:0005819	GO:0019904	K11586
ENSOARG00000010368	pfam06733	GO:0006310;GO:0006281;GO:0006260;GO:0010569;GO:0000723	GO:0005634	GO:0051539;GO:0005524;GO:0004003;GO:0003677;GO:0046872	K11136
ENSOARG00000010925	pfam00241	GO:0050832	GO:0005737;GO:0005856;GO:0070062;GO:0005634	.	.
ENSOARG00000011187	pfam00596	GO:0030036;GO:0051017;GO:0051016;GO:0030097;GO:0032092;GO:0006461;GO:0055085	GO:0016023;GO:0005829;GO:0008290;GO:0005886	GO:0003779;GO:0051015;GO:0046982;GO:0042803;GO:0030507;GO:0005198	K18622
ENSOARG00000011927	pfam13833	.	.	GO:0005509	.
ENSOARG00000012281	pfam04727	GO:0050688	GO:0016020;GO:0043234	GO:0005096	.
ENSOARG00000013006	pfam00069	GO:0007049|cell cycle;GO:0006355;GO:0006351	GO:0005622	GO:0005524;GO:0004707	.
ENSOARG00000013238	pfam02535	GO:0070574;GO:0006882;GO:0071578;GO:0006829	GO:0005887;GO:0031090;GO:0005886	GO:0005385	K14714
ENSOARG00000013345	pfam03154	GO:0045666;GO:0006355	GO:0005938;GO:0005634;GO:0043234	.	.
ENSOARG00000013926	pfam12937	GO:0006508	.	GO:0004842	K10272
ENSOARG00000014599	pfam13414	.	GO:0005737;GO:0005783;GO:0072546;GO:0005739;GO:0005634	.	.
ENSOARG00000015213	pfam00083	GO:0007589;GO:0009437;GO:0015879;GO:0015695;GO:0015697;GO:0006814;GO:0006641	GO:0016324;GO:0005887;GO:0005739;GO:0005886	GO:0005524;GO:0015226;GO:0015491;GO:0000166;GO:0030165;GO:0015651;GO:0008513;GO:0015293	K08202
ENSOARG00000015685	pfam03359	GO:0023052	GO:0016020;GO:0045202	.	.
ENSOARG00000016705	pfam12171	.	.	GO:0046872	.
ENSOARG00000019263	pfam13561	.	GO:0005739;GO:0005777	GO:0004090	K11147
ENSOARG00000019315	pfam00069	GO:0006888;GO:0006468	GO:0005938;GO:0012505;GO:0030027;GO:0016020;GO:0048471	GO:0042803	K08875
ENSOARG00000019513	pfam02893	.	GO:0016021	.	.
ENSOARG00000020618	pfam00778	GO:0060070;GO:0032053;GO:0042384;GO:0035556;GO:0090179	GO:0005829	GO:0008013;GO:0005109	K02353
ENSOARG00000020758	pfam00995	GO:0007596;GO:0006886;GO:0006904	GO:0010008;GO:0005794;GO:0000139;GO:0016021	.	K12479
ENSOARG00000026747	pfam15498	.	GO:0005886	.	.

Table 7: The expression of 30 key genes.

DISCUSSION

In this study, RNA-seq technology was used for the first time to reveal the mechanism of early embryonic development of sheep from the perspective of monoembryonic transcriptome, which provided a new way to improve the production of sheep embryos *in vitro*. It provides a theoretical basis for further improving the information of sheep gene structure and identifying new genes related to embryonic development. Therefore, the samples were enriched by smart-seq2 amplification technology to construct sequencing library [25]. The transcriptome was sequenced by illuminaHisEqxten high-throughput sequencing technology. The results of

sequencing quality evaluation and data analysis showed that the sequencing quality and library construction quality were high, and the sequencing data were accurate and reliable.

Characteristics of Early Embryonic Development Recording Group of Sheep

Following filtration of the data of the original sequencing sequence obtained from Illumina HiSeqEqxten sequencing, the filtered sequence of sheep embryos at the two developmental stages of 8-cell,16-cell and morula was 53327656-67939390. Using the TopHat software to compare the obtained clean reads with the reference genome, the results indicated a range of 93.17-94.26% clean reads alignment on the sheep reference gene in each stage. The sequence ratio of (multi map rate) multiple locations of the genome was 4.35%-5.44%, which satisfied the requirements. The correlation between biological repeats was 90.86% and the repeatability between the samples was optimal. In the present study, a large number of alternative splicing in sheep 8-cell,16-cells and morulae was noted, indicating that alternative splicing is common in eukaryotes and that the splicing mode of genes is constantly changing at different stages of embryonic development to regulate cell proliferation, differentiation, migration and apoptosis. Certain genes are expressed at specific stages of embryonic development and play key regulatory roles at specific stages. It was found that the large number of novel transcripts was due to the lack of the Y chromosome sequence in the reference genome of the sheep. A total of 10 newly activated genes (including novel transcripts 2) were found in every stage, which were as follows: TMEM37, PLCD1, ITGB1BP1, CCND2, PROCR, C14orf28, PRMT2, KDM3A, ENSOARG00000009515 and ENSOARG00000002677. Cyclin D (CCNDs) plays an important role in the process of the cell cycle. It is a rate-limiting factor from the G1 phase to the S phase. It mainly mediates the transmission between extracellular signal molecules and the cell mitotic cycle. By binding to cyclin-dependent kinase 4/6 (CDK4/6) and activating its active complex to phosphorylate the downstream retinoblastoma protein (RB), cyclin D plays an important role in the process of the cell cycle. Subsequently, the release of the transcription factor E2F initiates DNA replication [26-28]. Previous studies have found that the positive regulation of the expression of CCND2 can promote the process of the cell cycle, thus accelerating the proliferation of granulosa cells. In contrast to these observations, the downregulation of the expression of CCND2 causes an inhibition in the proliferation of granulosa cells [29-30]. Previous studies on CCNDs were mainly performed on mammalian follicles.

Current studies have shown that PRMT2 participates in several biological processes, including immune response, inflammatory response, apoptosis and proliferation. This gene acts mainly through the synergistic action of different regulatory factors, which suggests that it can bind to different proteins and play a transcriptional regulatory role through a variety of mechanisms [31]. Endothelial protein C receptor, (EPCR) is encoded by the PROCR gene, which participates in the activating protein C pathway and plays a role in cell protection, anti-inflammation and anticoagulation.

Yagisawa et al. [32] confirmed for the first time that PLCD1 had a nuclear output (NES) and nuclear input (NLS) sequence, suggesting that it could shuttle between the cytoplasm and the nucleus. During the cell cycle, the Ca+2 influx stimulates the increase in the formation of Ca+2-dependent PLCD1-import in β1 nuclear input complexes, resulting in the accumulation of cell cycle-dependent PLCD1 in the nucleus [33]. The inhibition of PLCD1 activity leads to the increase of nuclear PIP2 levels and changes the transcriptional regulation and the degradation of cyclin E, thus affecting cell proliferation. In certain cases, overexpression of this protein leads to the prolongation of the G1/S and S phases. These studies suggest that PLCD1 has a specific and important function in cell cycle regulation

[34]. KIT, STAT3, ZP4, BMP15, GDF9, ZP3, Pms2, Hsf1-2 and Zar1 were confirmed to be maternal genes [36-36,]. The expression of these maternal genes from the 16-cell to the morula sheep embryos did not change significantly. The expression levels of the zygotic genome-related genes were not high. However, the marker gene NANOG of EGA was initially expressed in the morula stage. Therefore, it is concluded that sheep EGA may occur from the 16-cell to the morula stage. Methylation-related genes are stably and highly expressed, suggesting that the methylation of embryos is being reconstructed, which is consistent with previous studies.

GO Annotation and KEGG Analysis of Differentially Expressed Genes in Embryos

Using the GO enrichment analysis, we explored the function of the DEGs.8-cell *vs.* 16-cell GO analysis was significantly enriched in Figure 6:94 term on BP, no significant difference was found at CC and MF, indicating significant temporal differences in the developmental regulatory mechanism of sheep embryos at different developmental stages. The number of DEGs of the two developmental stages from the 16-cell to the morula stage was 16343, indicating significant temporal differences in the developmental regulatory mechanism of sheep embryos at different developmental stages. GO analysis indicated that 16343 DEGs were classified and annotated from the 16-cell stage to the morula developmental stage. All these genes involved BP, CC and MF that were enriched to 88 secondary items (Figure 6). A total of 78 second-level items were noted in the BP classification, among which the first 10 items were mainly associated with metabolic process regulation, cell process regulation and organic synthesis regulation, indicating that this period was the period of rapid embryo proliferation. Additional processes associated with the regulation of the metabolic process were the following: negative regulation of cellular process, positive regulation of cellular process, negative regulation of biological process and positive regulation of biological process. The data suggested that this mode of action could ensure the orderly and controlled proliferation.

Among the two secondary items enriched by CC, the eight secondary items enriched by the spliceosomal complex and the intracellular component in MF classification were concentrated in the DNA enzyme, RNA enzyme and transcription factor components, indicating that the gene expression was high at that time and that the embryo was proliferating rapidly. From these data, we can infer that during cleavage, the embryo proliferates rapidly. Concomitantly, a mechanism exists that regulates the proliferation rate in the embryo and ensures the prevention of cell cycle block or stall and the orderly proliferation of the embryonic cells. According to the statistics of the secondary GO items, significant differences were noted in the analysis of DEG annotation (Figure 6). It was found that BP, CC and MF3 were enriched in the functions of cell proliferation, immunity, stress, signal transduction and synapse, indicating that the embryos in this period were in a period of rapid proliferation. The enrichment of pigmentation was noted. It is considered that the enrichment of this process is related to the *in vitro* culture, which provides a basis for further optimization of the *in vitro* culture system.

KEGG analysis of differentially expressed genes during sheep embryonic development indicated 126 pathways involved in the 16-cell developmental stage (from 8-cell to 16-cell stage), whereas the 11 main pathways with significant and 206 pathways involved in the morula developmental stage (from 16-cell to morula stage), whereas the 2 main pathways with significant.

RNA-seq of pig SCNT embryos that were early-dividing (24 hours postactivation) and late-dividing (36 hours postactivation) was performed. In total, 3077genes were found to be differentially expressed between early-

dividing and later-dividing groups. GO and KEGG analyses revealed that early-dividing embryos have higher expressions of genes that participated in the meiotic cell cycle, while the genes in "ribosome", "mRNA surveillance pathway", "protein processing in endoplasmic reticulum", and "spliceosome" were excessively and highly expressed in late-dividing embryos [37]. Zhou et al. [38] found that highly expressed genes in 8-cell bovine SCNT embryos are enriched in “RNA processing”, “translation”, and “ribosome biogenesis” GO terms, which means that compared with 8-cell IVF embryos, the cloned embryos exhibit excessive transcription in RNA processing and translation-related genes and these genes are also significantly higher expressed in donor cells. However, Liu et al [39]. compared the transcriptome of mouse in vivo and SCNT embryos and found that 339 translation-initiation-related genes were downregulated and 1327 transcription-related genes were upregulated in mouse zygote to 2-cell stage SCNT embryos [31].

The discovery of the Nanog gene has recently become one of the greatest advances in maintaining the undifferentiated state of embryonic stem cells and identifying genes related to embryonic development. In the early stage of mouse embryonic development, the Nanog gene is not expressed in the early cleavage stage and trophoblast formation [40]. The earliest expression was in the morula stage and was limited to the inner cell mass. Some in vivo experiments have shown that the Nanog gene is not present in undifferentiated cell mass. Instead, it is responsible for the differentiation and production of endoderm cells. The Nanog gene is a gene necessary to maintain the totipotency of inner cell mass following October 4 initiation in the early stage of embryonic development [41]. In addition, the Nanog gene is highly expressed in embryonic stem cells. When embryonic stem cells differentiate, the expression of this gene is significantly downregulated. Genetic defects in embryonic stem cells are associated with abnormal Nanog expression. This can lead to the loss of pluripotency and the initiation of differentiation. The maintenance of totipotency and self-proliferation of embryonic stem cells by the Nanog gene is mainly facilitated via binding to the regulatory region of the target genes, selectively and by inhibiting the expression of differentiation genes or promoting the expression of pluripotent genes. Therefore, the high expression of the Nanog gene is closely associated with the maintenance of totipotency of embryonic stem cells, while cell differentiation is associated with the downregulation of the Nanog gene expression.

CONCLUSIONS

In the present study, the transcriptome of different stages of sheep single IVF embryo development was analyzed by single cell RNA-Seq for the first time and several differential genes and related pathways were obtained. Differentially expressed genes at different stages have their own specificity in quantity, function, classification and metabolic pathway. The results indicated that the period required from the 8-cell to the morula stage was the period of rapid embryo proliferation. Its metabolic pathway focused on the synthesis and regulation of substances related to cell proliferation and it was found that there was enrichment in both positive and negative aspects of the same process involved in metabolic regulation. This in turn prevented cleavage block or stall and ensured the order of embryo proliferation. Concomitantly, the present study provided a theoretical basis for improving the technology of sheep embryo production *in vitro*. This can provide additional information on sheep gene structure and on new genes related to embryonic development.

ACKNOWLEDGEMENTS

None.

FUNDING

This work was supported by National Natural Science Foundation of China "Study on the Molecular Mechanism of Bovine SRY Gene Promoting the Proliferation and Differentiation of Male Cleavage Embryos" (31660657); XPCC's young and middle-aged technological innovation leading talent team construction project (2015BC001); Graduate Scientific Research Innovation Project (TDBSCX201906).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Yan L, Yang M, Guo H, et al. (2003) Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. Nature Structural & Molecular Biology 20(9).
2. Cao S, Han J, Wu J, et al. (2014) Specific gene-regulation networks during the pre-implantation development of the pig embryo as revealed by deep sequencing. BMC Genomics 15(1): 4-4.
3. Graf A, Krebs S, Heininen-Brown M, et al. (2014) Genome activation in bovine embryos: Review of the literature and new insights from RNA sequencing experiments. Animal Reproduction Science 149(1-2).
4. Jiang Z, Sun J, Dong H, et al. (2014) Transcriptional profiles of bovine in vivo pre-implantation development. BMC Genomics 15(1): 756-756.
5. Yi Q, Liu Q, Cao M, et al. (2020) Transcriptional analysis and differentially expressed gene screening of spontaneous liver tumors in CBA/CaJ mice. Gene 725(C).
6. Zi XD, Luo B, Xia W, et al. (2018) Characterization of transcriptional complexity during pre-implantation development of the yak (Bos grunniens) using RNA-Seq. Reproduction in Domestic Animals 53(3): 759-768.
7. Tang F, Barbacioru C, Wang Y, et al. (2009) mRNA-Seq whole-transcriptome analysis of a single cell. Nature Methods 6(5): 377-382.
8. Deng M, Chen B, Liu Z, et al. (2020) YTHDF2 Regulates Maternal Transcriptome Degradation and Embryo Development in Goat. Frontiers in Cell and Developmental Biology 8: 580367.
9. Ji ZS, Liu QL, Zhang JF, et al. (2020) SUMOylation of spastin promotes the internalization of GluA1 and regulates dendritic spine morphology by targeting microtubule dynamics. Neurobiology of Disease 146: 105133.
10. Xue Z, Huang K, Cai C, et al. (2013) Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. Nature 500(7464): 593-597.
11. Tong L, Wei X, Jia P, et al. (2019) Application of single-cell transcriptional sequence in study of reproductive development. International Journal of Reproductive Health/Family Planning 38(03): 217-221.
12. Lan D, et al. (2014) RNA-Seq analysis of yak ovary: improving yak gene structure information and mining reproduction-related genes. Science China. Life sciences 57(9): 925-935.
13. Fast QC (2020) A quality control tool for high throughput sequence data. Babraham Bioinformatics.
14. Ewels P, Magnusson M, Lundin S, et al. (2016) MultiQC: Summarize analysis results for multiple tools and samples in a single report. Bioinformatics 32(19): 3047-3048.
15. Kim D, Langmead B, Salzberg SL (2015) HISAT: A fast spliced aligner with low memory requirements. Nature Methods 12(4): 357-360.

16. Benjamini Y, Hochberg Y, et al. (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological) 57(1): 289-300.
17. Reimand J, Isserlin R, Voisin V, et al. (2019) Pathway enrichment analysis and visualization of omics data using g: Profiler, GSEA, Cytoscape and Enrichment Map. Nature Protocols 14(2): 482-517.
18. Li H, Handsaker B, Wysoker A, et al. (2009) The Sequence alignment/map format and SAMtools. Bioinformatics (Oxford, England) 25(16) :2078-2079.
19. Trapnell C, Williams BA, Pertea G, et al. (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature Biotechnology 28(5): 511-515.
20. Roberts A, et al. (2011) Identification of novel transcripts in annotated genomes using RNA-Seq. Bioinformatics (Oxford, England) 27(17): 2325-2329.
21. Qiu Q, Zhang G, Ma T, et al. (2012) The yak genome and adaptation to life at high altitude. Nature Genetics 44(8): 946-949.
22. Trapnell C, Pachter L, Salzberg SL, et al. (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics (Oxford, England) 25(9): 1105-1111.
23. Wagner GP, Kin K, Lynch VJ (2012) Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples. Theory in Biosciences, Theorie in den Biowissenschaften 131(4): 281-285.
24. Picelli S, Bjorklund Ak, Faridani OR, et al. (2012) Smart-seq2 for sensitive full-length transcriptome profiling in single cells. Nature Methods 10(11): 1096-1098.
25. Malumbres M, Barbacid M (2009) Cell cycle, CDKs and cancer: A changing paradigm. Nature reviews. Cancer 9(3): 153-166.
26. Qian Z, Kazuhito S, Kay-Uwe W, et al. (2014) D-type Cyclins are important downstream effectors of cytokine signaling that regulate the proliferation of normal and neoplastic mammary epithelial cells. Molecular and Cellular Endocrinology 382(1): 583-592.
27. Xue-ying Y, Qin GQ, Sheng HH, et al. (2019) Transcriptome of bovine IVF embryos treated with Glutathione. Acta Veterinaria et Zootechnica Sinica 47(07): 1363-1372.
28. Olivier C (2002) Linking cyclins to transcriptional control. Gene 299(1-2): 35-55.
29. Summers AF, Pohlmeier WE, Sargent KM, et al. (2014) Altered theca and cumulus oocyte complex gene expression, follicular arrest and reduced fertility in cows with dominant follicle follicular fluid androgen excess. PloS ONE 9(10): e110683-e110683.
30. Zhang Q, Sun H, Jiang Y, et al. (2013) MicroRNA-181a suppresses mouse granulosa cell proliferation by targeting activin receptor IIA. PLoS ONE 8(3).
31. Belton A, Gabrovsky A, Bae YK, et al. (2017) HMGA1 induces intestinal polyposis in transgenic mice and drives tumor progression and stem cell properties in colon cancer cells. PLoS ONE 7(1): e30034.
32. Pendurthi UR, Rao LVM (2018) Endothelial cell protein C receptor-dependent signaling. Current Opinion in Hematology 25(3): 219-226.
33. Yagisawa H, Sakuma K, Paterson HF, et al. (1998) Replacements of single basic amino acids in the pleckstrin homology domain of Phospholipase C-δ1 alter the ligand binding, phospholipase activity, and interaction with the plasma membrane. Journal of Biological Chemistry 273(1): 417-424.

34. Wang X, Yao X, Zhao A, et al. (2021) Phosphoinositide-specific phospholipase C gene involved in heat and drought tolerance in wheat (*Triticum aestivum L.*). Genes & Genomics 43(10): 1167-1177.
35. Graf A, Krebs S, Zakhartchenko V, et al. (2014) Fine mapping of genome activation in bovine embryos by RNA sequencing. Proceedings of the National Academy of Sciences of the United States of America 111(11): 4139-4144.
36. Stallings JD, Zeng YX, Narvaez F, et al. (283) Phospholipase C-delta1 expression is linked to proliferation, DNA synthesis, and cyclin E levels. The Journal of Biological Chemistry 283(20): 13992-14001.
37. Liu Z, Xiang G, Xu K, et al. (2020) Transcriptome analyses reveal differential transcriptional profiles in early- and late-dividing porcine somatic cell nuclear transfer embryos. Genes 11(12): 1499.
38. Zhou C, Zhang J, Zhang M, et al. (2020) Transcriptional memory inherited from donor cells is a developmental defect of bovine cloned embryos. FASEB journal: Official Publication of the Federation of American Societies for Experimental Biology 34(1): 1637-1651.
39. Liu Y, Wu F, Zhang L, et al. (2018) Transcriptional defects and reprogramming barriers in somatic cell nuclear reprogramming as revealed by single-embryo RNA sequencing. BMC Genomics 19(1): 734-734.
40. Mitsui K, Tokuzawa Y, Itoh H, et al. (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES Cells. Cell 113(5): 631-642.
41. Yamaguchi S, Kimura H, Tada M, et al. (2005) Nanog expression in mouse germ cell development. Gene Expression Patterns 5(5): 639-646.