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Transcriptome Analysis of Cattle Embryos Based on Single Cell RNA-Seq

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ABSTRACT

Over the past few years, transcriptome sequencing has been applied to livestock and poultry, helping to select and investigate candidate genes associated with important traits. Yet, so far, only a few studies have reported differences in single-cell transcriptome between bovine embryos of different genders. In this study, we performed transcriptome analysis of cattle embryos based on a single Cell RNA-Seq. Bovine sex-controlled semen for artificial insemination were used to obtain different stage embryos: bovine 8 cell XX embryo, 8 cell XY embryo, 16 cell XX embryo, 16 cell XY embryo, morula XX embryo, morula XY embryo, blastocyst XX embryo, and blastocyst XY embryo. A sequencing library was constructed by the Smart-Seq2 amplification. The transcriptome was sequenced by Illumina HiSeqXten high-throughput sequence technology, and effective sequences were analyzed by functional annotation and related bioinformatics analysis. We found that Q30 percentage range of eight samples was 91.79-92.37%. The filtration sequence was 44106250-54234844. Compared with the reference genome by TopHat software, the net reading ratio of the bovine reference gene at each stage was 93.17-9 4.23%, the ratio of sequence numbers to multiple sites of the genome was 2.99-4.89. The DEG was identified by using the fold change ≥2 and FDR <0.01 as cut-off values. There were 525 differentially expressed genes. GO and KEGG analysis showed that "cell part", "organelle", and organelle part" were significantly enriched in cell composition categories. As for molecular functional categories, DEGs were significantly enriched in cellular process "," biological regulation "and metabolic process" during biological processes. Moreover, KEGG analysis showed that the most abundant pathways were oxidative phosphorylation and Wnt signaling pathway","MAPK signaling pathway "," Regulation of actin cytoskeleton "and VEGF signaling pathway". To conclude, these RNA-Seq results confirmed the differential expression of several genes in embryos of different genders during embryonic development. These DEGs partcipate in the transcriptional regulation of bovine embryonic development of different genders.

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Key words
Cattle, Early embryo, XX/XY embryo, RNA-Seq, Differential expression

INTRODUCTION

From birth to adulthood, mammalian males tend to develop faster than females (Erickson, 1997). Mouse experiments (Mittwoch, 1993; Silversides *et al*., 2012; Thornhill and Burgoyne, 1993) have suggested that pre-implantated XY male embryos develop faster than XX female embryos, which is closely associated with the haplotypes Y chromosomes of male embryos. Cassar *et al*. (1994, 1995) showed that male porcine embryos at 5, 6, and 10 days of fertilization developed faster than female embryos. Pergament *et al*. (1994) obtained the same conclusion examining epiphytic human embryos. Furthermore, Bernardi and Delouis (1996) examined *in vitro* fertilization of sheep and found that the rapidly developing embryos were mainly male. Xu *et al*. (1992) discovered that male embryos developed faster than female embryos by increasing the number of cells and mitotic index as early as the first cleavage. Somfai *et al*. (2006) found that the sex ratio of bovine embryos tends to be higher than that of females.

Single-cell RNA-Seq is a useful tool for measuring gene transcription and further understanding the physiology behind specific phenotypes. Over the past few years, transcriptome sequence has been applied to livestock and poultry and has helped select candidate genes associated with important traits by comparing global gene expression profiles among different animal populations in specific traits. This method has also been successfully tested in

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humans (Starostik *et al*., 2020), sheep (Yang *et al*., 2021), mouse (Huo *et al*., 2020), and other mammals.

While cattle are one of the most important sources of meat and milk for many people worldwide, only a few studies have reported on the single-cell transcriptome of bovine embryos of different genders. In this study, we used a single Cell RNA-Seq to analyze the transcriptome sequence of the early embryos identified by sex in holster bovine 8 cells, 16 cells, mulberry embryo cells, and blastocyst cells so as to further elucidate the differences in early embryonic development between bovine and males,

MATERIALS AND METHODS

Chemicals and culture media

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

Estrus synchronization

Eight 3-5 years old non-reproductive Holstein cattle were selected for concurrent oestrus superovulation treatment, and artificial insemination was conducted with frozen semen of gender-controlled Holstein cattle. The specific operation was put into CIDR on the first day, after which an intramuscular injection of 10ml vitamin ADE and 2ml estradiol was given. Then, 2ml FSH was intramuscularly injected in the morning and evening on day 9; 1.5 ml FSH in the morning and evening on day 10, and 1 ml FSH and 3 ml PG in the morning and evening on day 11. The silicone suppository was taken out on the 12th day, followed by intramuscular injection 0.5 mlFSH, and evening injection 0.5 ml FSH and 10 vitamins ADE, after which the estrus was observed; (first mating was conducted 10 - 12 h after stable estrus and at 12 h interval until 12 h after estrus). Sex-controlled X sperm artificial insemination and sex-controlled Y sperm artificial insemination were used in 8 cows (4 each).

Acquisition of early embryos

Embryos were washed on days 4, 5, 6, and 7 after mating by non-operative method (Taru *et al*., 2020), and one head of X, Y sperm recipient was operated daily. The embryos were detected under the microscope and stored in liquid nitrogen. XX and XY embryos from 8, 16, mulberry, and blastocyst stages were selected for later testing.

Embryo quality test

According to the 4th grade method, A and B embryos were used in later experiments.

Sex identification of embryos

We used sex-controlled semen for artificial insemination; thus, we expected embryos produced using X sperm to be females and embryos produced using Y sperm to be males.

The construction and sequencing of RNA extraction and sequencing

Libraries constructed by the library were completed by Anoda Genome Technology (Beijing) Co., Ltd. Single-cell samples were collected in test tubes containing lytic components and ribonuclease inhibitors. Subsequently, amplification was performed by Smart-Seq2 methods. Oligodeoxyribonucleic acid primers were introduced into reverse transcription to synthesize the first strand gene, followed by polymerase chain reaction amplification to enrich the gene, and magnetic beads were purified to clear the product. The gene was examined by a qubit 3.0 fluorometer, and the Agilent 2100 bioanalyzer was used to ensure about 1-2 kilobits/s Expected output. Then, the gene was randomly sheared with ultrasound for Illumina library preparation protocols, including DNA fragmentation, end-repair, 3' end trailing, joint ligation, polymerase chain reaction amplification, and library validation. The PerkinElmer laboratory chip touch and Step a real-time polymerase chain reaction system were used for library quality detection after library preparation. Libraries meeting these criteria were subsequently loaded onto the Illumina Hiseq platform for PE150 sequencing.

Transcriptome data analysis

To ensure the quality of the sequencing data, the original readings obtained by IlluminaHiSeqXten sequencing were filtered, and a clean reading was obtained after the combined sequence, the empty reading sequence, and the low-quality sequence (Phred quality <5) were removed. The reference genome library was constructed using the Bowtiev (1.0.1), and the clean data were subsequently compared with the reference genome by HISAT2 (v2.1.0). Gene expression was calculated using FPKM (mapping readings) per kilo-base million segment. Differentiation of differentially expressed genes was based on DESeq selection of genes. The estimation of expression level was realized by the negative binomial distribution of the statistical method. Calibration of P values according to Benjamin and Holberg methods was used to control false positives. The corrected P-value $\leq$0.05 was defined as the required parameters for differentially expressed genes. DESeq2 was used to analyze the differential expression of biological samples between the two groups and compared with the treatment group and the control group. The genes | log2Ratio |$\geq$1 and 0.05 were selected as differentially expressed genes to obtain up- and down-regulated genes. Map the obtained deg to each entry in the GO database and calculate their number. Using the Benjamin method GO entries with a mass fraction less

than 0.05 after correction for phosphorus values are GO entries for significant enrichment DEGs. by comparing the expression with the KEGG (Kyoto encyclopedia of genes and genomes) database, the signaling or metabolic pathways involved in genes were analyzed.

Analysis of alternative splicing and single nucleotide polymorphisms and prediction of new transcripts

The structure and expression of the RPKM tool from AS spectrum (1.0.4) for selective splicing events and the Samtoos (1.5) for single nucleotide polymorphism (single nucleotide polymorphism, SNP). The sequencing sequence of the genome was assembled and spliced by the Cufflinks Software (v2.2.1). After filtering the low mass sequences (length ≤ 180 bp, Q ≤ 10), the assembled transcription sequence was compared with gene annotated information on the bovine genome. For example, the assembled transcript sequence is not consistent with the existing genes and is located on the genome between the existing genes. Meet the following conditions: The distance from the annotated gene is more than 200 bp, the length is not less than 180 bp, and the sequencing depth is not less than 2. These sequences were identified as potential new transcripts and genes.

RESULTS

Sequencing quality assessment and basic data analysis

Bovine 8 cell XX embryo, 8 cell XY embryo, 16 cell XX embryo, 16 cells, morula embryo, mulberry embryo, mulberry XY embryo blastocyst stage XX embryo, and blastocyst stage embryo library constructed in this study met the requirements of transcriptome sequencing. All embryos were analyzed using Agilent 2100 bioanalyzer, high sensitivity DNA kit. An estimated Q30 percentage ranged from 91.79 to 92.37 % for eight samples, suggesting the high quality of sequencing and library construction and that the sequencing data were accurate and reliable (Fig. 1).

Analysis of the sequencing results filtered data from the original sequence, and the filtered sequences of eight bovine embryos of both sexes at the four developmental stages showed the range of 44106250-54234844. The obtained clean readings were then compared with the reference genome by TopHat software. The results showed that the net reading ratio of the bovine reference gene in each stage was 93.17-94.23 and the ratio of sequence number to multiple positions of the genome was 2.99-4.89 (Table I).

Analysis of differential expression genes (DEG) among female and male embryos

By folding changes ≥ 2 and FDR < 0.01 as cut-off, 7,369 DEGs were detected between bovine E8Y and E8X embryos, among which 5,197 were up reregulated and 2, 172 were downregulated genes. Moreover, there were 7,759 DEGs between E16Y_vs_E16X (3,806 upregulated genes and 3,953 downregulated genes), 9027 genes between MLY_vs_MLX (4381 upreregulated and 4646 downregulated genes), and 8004 between BLY_Total vs_BLX (3102 were upregulated and 4902 were downregulated genes) (Fig. 2). At all developmental points, a total of 525 genes were co-expressed in 4 periods (Fig. 3). We also identified 34 genes that were upregulated or down-regulated at the same time in four periods. FPKM to take the logarithm of 10. TBTools software was used to draw FPKM cluster heat map (Fig. 4).

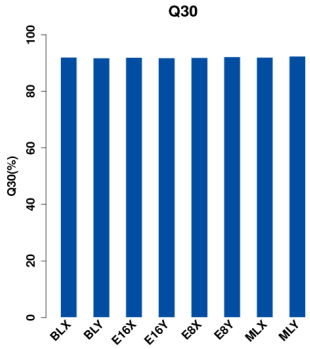

Fig. 1. Base quality level of Q30 reactive sequencing. The proportion of the bases with sequencing quality value was greater than 30 (error rate less than 0.1%) in the total Raw Reads.

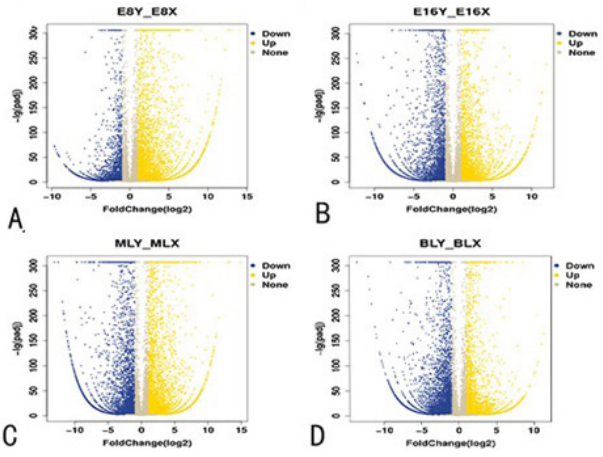

Fig. 2. Volcano map showing differential expression genes (DEG) among female and male embryos in each period. The number of DEGs in XX and XY embryos; yellow represents upregulated genes, blue represents the downregulated genes, gray shows the difference that is not significant.

Table I. RNA-Seq data from XX and XY embyros of cattle.

Sample	BLX	BLY	E16X	E16Y	E8X	E8Y	MLX	MLY
Clean reads number	48,724,936	44,106,250	47,144,886	51,965,704	54,234,844	48,524,512	48,155,058	50,922,978
Clean reads rate (%)	97.22	95.65	96.68	96.06	97.93	97.49	96.74	97.08
MultiMap reads	1,931,714	1,687,208	1,664,050	1,650,921	1,704,306	1,449,528	1,856,146	2,488,795
MultiMap rate	0.0396	0.0383	0.0353	0.0318	0.0314	0.0299	0.0385	0.0489

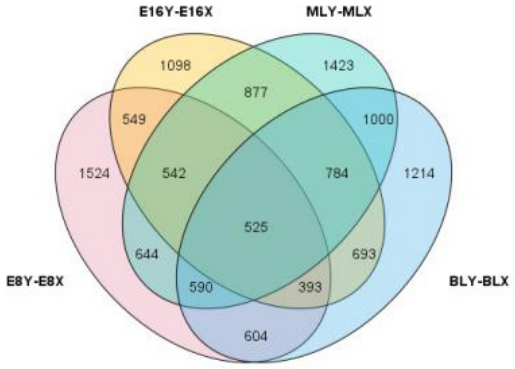

Fig. 3. Venn diagram of the DEGs of the four comparison groups.

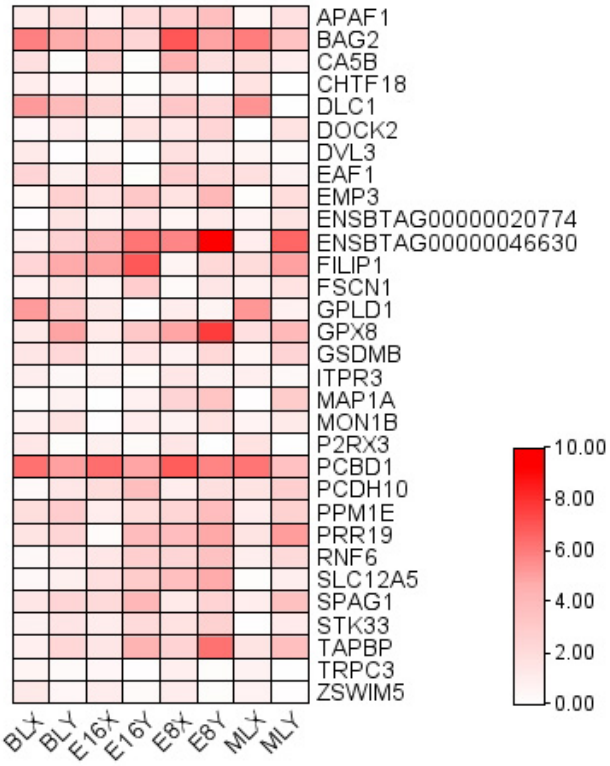

Fig. 4. Heatmap shows the gene expression of XX and XY embryos at different periods.

GO annotation and KEGG pathway analysis

GO and KEGG analysis was used to further understand the biological functions of genes in important gene expression profiles. DEGs were divided into three categories: Biological processes, cellular components, and molecular functions (Fig. 5). Cell part, organelle and organelle part were significantly enriched in cell composition categories. As for molecular functional categories, most DEGs were enriched in binding and catalytic activity. DEGs at the cellular level were significantly enriched in the following process: Biological regulation "and" metabolic process.

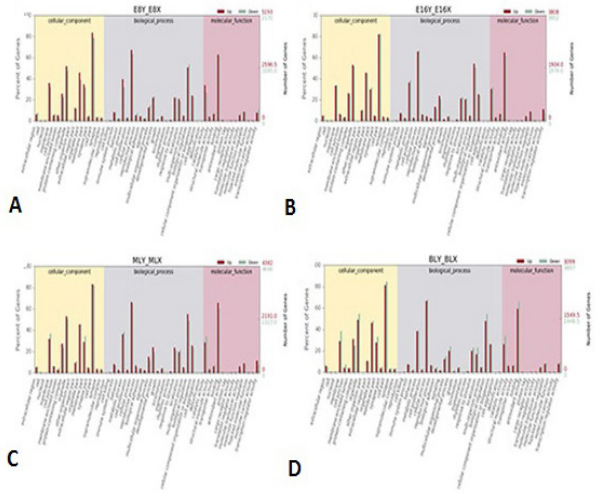

Fig. 5. GO analysis of DEGs. GO enrichment analysis of DEGs in cattle embryos. (A) E8Y_vs_E8X; (B) E16Y_vs_E16X; (C) MLY_vs_MLX; (D) BLY_vs_BLX. The abscissa represents GO terms, the ordinate on the left represents the percentage of genes in all genes annotated with GO, right was the number of genes.

The KEGG pathway of the DEGs is shown in Figure 6. KEGG analysis of differentially expressed genes in the development of bovine embryos of different genders indicated that the abundant pathways included oxidative phosphorylation, Wnt signaling pathway, MAPK signaling pathway, regulation of actin cytoskeleton, and VEGF signaling pathway.

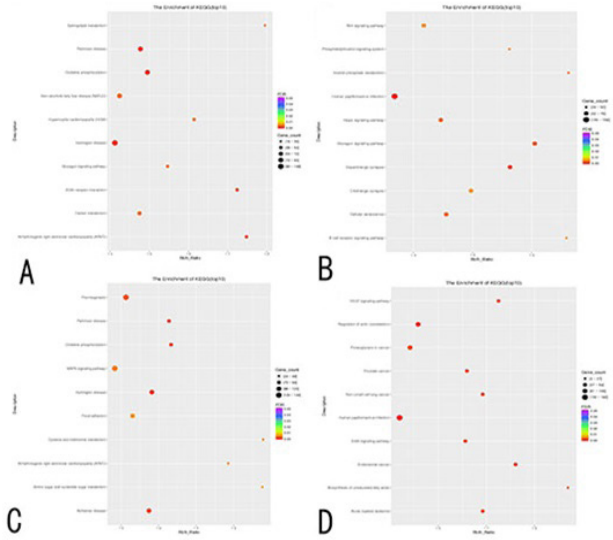

Fig. 6. Histogram (Top 10) of KEGG enrichment values in four groups. Annotation of DEGs in KEGG. (A) E8Y_vs_E8X; (B) E16Y_vs_E16X; (C) MLY_vs_MLX; (D) BLY_vs_BLX. In the figure, the path case of the differential top10 is collected, and each circle represents a KEGG path whose name appears on the left legend. The transverse coordinate is the enrichment factor, showing the ratio of the DEGs in the pathway to all DEGs in all pathways to the ratio of genes in the pathway to all genes in all pathways. The larger the enrichment factor, the more important the path is. The color of the circle represents the q value, the adjusted p-value by multiple hypothesis tests; the smaller the q value, the more important the path. The size of the circle represents the number of genes expressed with the differences annotated by the pathway; the larger the circle, the greater the number of genes.

DISCUSSION

In many mammals, the males tend to grow faster than the females, before and after birth. Previous studies have suggested that these differences are related to differences in genes and transcription regulators among males and females, which occur during embryogenesis. This phenomenon has been observed in mice, pigs, cattle, and sheep (Bermejo-Alvarez *et al*., 2010). Yet, previous studies were mainly based on *in vitro* fertilization methods. Embryos produced *in vivo* represent the basic and more realistic characteristics of embryo development compared to those produced by *in vitro* fertilization. In this study, we analyzed the transcriptome level of embryos produced in cattle with known sex at different stages so as to understand the dynamic changes in embryo development from the transcriptome level. An RNA-Seq technique was used to reveal the early embryo of cattle from the perspective of a single embryo transcriptome. A Smart-Seq2 amplification technique was used to enrich the samples and construct the sequencing library; Illumina Hi SeqXten high-throughput sequencing techniques were applied for transcriptome sequencing. The sequencing quality evaluation and data analysis results showed that the quality of sequencing and library construction was high, and the sequencing data were accurate and reliable.

The concentration range of single-cell RNA in mammals is 10-30 pg. The rRNA accounts for about 85% of the total RNA content. About 15-20% of the rRNA content is as low as 1-5% (Raj *et al*., 2006). The basic requirements for total RNA in general transcriptome sequencing are as follows: RNA sample concentration ≥400 ng/μl, total ≥20μg, A260/280 purity between 1.8 and 2.4, A260/230>1.8, Optimal integrity (RIN 7.0-8.0) and RNA28S/8S >1.0 mm. However, from breastfeeding, the total RNA concentration of each embryo from animal 2 cell stage to blastocyst stage is only 200-2. Therefore, trace RNA cannot meet the basic requirements of constructing a transcriptome sequencing library and high-throughput sequencing. With the continuous development of the new generation of high-throughput sequencing technology, RNA-Seq can be used to sequence the total transcriptional activity of any biological growth and development stage without the prior design of probes. Furthermore, it can accurately detect gene expression under various conditions and find several unknown molecular regulatory mechanisms (Mortazavi *et al*., 2008).

In this study, we used data from original sequencing sequences obtained from Illumina HiSeqXten sequencing. After filtration, the filtration sequences of bovine E8X, E8Y, E16X, E16Y, MLX, MLY, BLX, and BLY developmental embryos were 54234844, 48524512, 47144886, 51965704, 48155058, 50922978, 48724936, and 44106250, respectively. TopHat software was used to compare the obtained clean readings with the reference genome, and the results showed that the comparison range of clean readings of cattle reference genes at each stage was between 95.65% and 97.93%, respectively. For multiple loci in the genome, the sequence ratio was between 2.99% and 4.89%, thus meeting the requirements.

Embryonic development results from genetic information expressed in a certain time, space, and order. Several potential candidates for embryonic development were screened in this study. For example, *G6PC3* regulates cytoplasmic glucose utilization by hydrolysis of glucose-6-phosphate during glycolysis and glycogen decomposition. Mammalian embryos use glucose as the main source of nutrition (Goenka *et al*., 2020). Glucose is metabolized by oxidation or aerobic glycolysis to facilitate subsequent developmental stages. *ACOT7* is mainly involved in the

hydrolysis of arachidonic acid-coenzyme to arachidonic acid (AA) and coenzyme. It is an important precursor molecule of eicosanoic acid (arachidonic acid) that has an important role in cell signal transduction, cell proliferation regulation, and metabolic enzyme activation function (Feng and Liu, 2020). *TP53,* arginine methylation, is one of the most important post-translational modifications catalyzed by protein arginine methyltransferase (Frebourg *et al*., 2020). *Prmt7* is the only family member responsible for the single methylation of arginine residues (Chen *et al*., 2020). Studies have shown that *Prmt7* participates in male germ cell proliferation at the embryonic stage. *TMEM39* is a transmembrane protein located in the endoplasmic reticulum that regulates procollagen production and secretory cargo transport (Zhang *et al*., 2021). *DLC1* is another gene necessary for embryonic development (Zhang and Li, 2020). Previous studies have shown that *DLC1* can inhibit cell division, as demonstrated in gene trapping mouse models Real. In this study, the *DLC1* genes of male embryos in each stage were significantly down-regulated compared with female embryos, which may be related to the difference between male and female embryo development. *DVL3* genes have a certain relationship to developmental biology (Castro-Piedras *et al*., 2021).

Eaf family genes act in multiple cellular responses, such as tumor suppression and embryonic development. Analyses of gene expression profiles in *Eaf* deleted or over-expressed mammalian cells indicated that the roles of *Eaf1* and *Eaf2* in inhibiting TGF-β signals were conserved in mammals (Liu *et al*., 2017). Hua and his team reported that sperm-associated antigen 1 (SPAG1) was a direct target gene of miR-638, which, in turn, inhibits cell proliferation and cell cycle (Hu *et al*., 2017).

In the present study, we investigated the difference in developmental between X and Y sperm-sorted bovine embryos during the pre-implantation period. A total of 7,369 DEGs were detected between bovine E8Y and E8X embryos, among which 5,197 were upreregulated and 2, 172 were downregulated genes. Moreover, there were 7,759 DEGs between E16Y_vs_E16X (3,806 upregulated genes and 3,953 downregulated genes), 9027 genes between MLY_vs_MLX (4381 upreregulated and 4646 downregulated genes), and 8004 between BLY_ Total vs_BLX (3102 were upregulated and 4902 were downregulated genes) (Fig. 2). At all developmental points, a total of 525 genes were co-expressed in 4 periods (Fig. 3). We also identified 34 genes that were upregulated or downregulated at the same time in four periods. This data indicated significant differences in the developmental regulation mechanism of bovine with a different sex. In the preimplantation stage, and that the effective activity of mitochondria is a determinant of the rate of intersex development (Bermejo-Alvarez *et al*., 2011).

GO and KEGG analyses were conducted to further understand the biology of genes in important gene expression profiles function. DEGs were divided into three categories: biological processes, cellular composition, and molecular function. The results showed that “cell part”, “organelle”, and organelle part” were significantly enriched in cell composition categories. As for molecular functional categories, DEGs were significantly enriched in cellular process, biological regulation and metabolic process during biological processes (Fig. 3). These RNA-Seq results further confirm the differential expression of some genes in embryos of different genders during embryonic development. DEGs identified may play a role in the transcriptional regulation of bovine embryonic development of different genders.

Furthermore, the KEGG analysis of differentially expressed genes showed that the abundant pathways included “oxidative phosphorylation”, “Wnt signaling pathway”, “MAPK signaling pathway”, “Regulation of actin cytoskeleton”, and “VEGF signaling pathway”. The KEGG pathways play crucial roles in embryo development. Oxidative phosphorylation is a cellular process that harnesses the reduction of oxygen to generate high-energy phosphate bonds in the form of adenosine triphosphate (ATP). It is a series of oxidation-reduction reactions involving electrons from NADH and FADH2 to oxygen across several protein, metal, and lipid complexes in the mitochondria, known as the electron transport chain (ETC) (Deshpande and Mohiuddin, 2021). The development of genomic procedures such as transgenesis and microarray analysis have allowed the discovery of nearly 600 differentially expressed genes between male and female mouse blastocysts (Kobayashi *et al*., 2006). These results confirm differences previously reported in cattle (Gutiérrez-Adán *et al*., 1996).

CONCLUSIONS

Transcriptome sequencing of embryos from different developmental stages and different sex of cattle was carried out in this study. Several potential candidates for embryonic development, such as SPAG1, MAP1A, GDF6, GSDMB, GPX8, FILIP1, EAF1, DVL3, and CHTF18, were screened and discussed. Moreover, our data suggested that key regulatory pathways, such as oxidative phosphorylation, and glucose metabolism, have been shown to play a crucial regulatory role in embryonic development. Thus, this study is helpful to understand the genetic structure of bovine embryonic transcriptome and provides useful resources and markers for future functional genomics research. At the same time, this study is the

theoretical basis for the regulation of the mechanism of developmental differences between different genders and different periods of bovine embryos. This could provide additional information on bovine gene structure and new genes associated with embryonic sex differences.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article/as supplementary information files.

Ethical approval and consent to participate

All animal experiments were conducted according to the Regulations and Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China, revised in 2004). The present study was approved by the Institutional Animal Care and Use Committee of Tarim University (SYXK 2020-009).

Consent for publication

Not applicable.

Statement of conflict of interest

The authors declare that they have no conflict of interest.

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