WHOLE TRANSCRIPTOME SEQUENCING REVEALS NON-CODING RNAs RELATED TO EMBRYO MORPHOGENESIS AND DEVELOPMENT IN RABBIT

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ABSTRACT

The roles of Long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) in embryonic development remain unclear. We performed a comprehensive analysis of lncRNA and circRNA profiles in different stages of rabbit embryos by whole transcriptome sequencing. We identified 719 lncRNAs and 744 circRNAs that were differently expressed between stage S1, S2 and S3. A total of 241 differently expressed lncRNAs and 166 differently expressed circRNAs were significantly involved in function of embryonic morphogenesis and development. A RNA network was established and among the embryonic development-associated RNAs, lncRNA TCONS_00009253 and TCONS_00010436 were persistently downregulated, while circRNA circRNA_07129, circRNA_15209 and circRNA_12526 were continuously upregulated, and their co-expressed mRNAs TBX1, WNT3 and FGFR2 were continuously downregulated during the embryo development. These candidate RNAs were mainly involved in the Wnt, PI3K-Akt and calcium signaling pathways. This work provides candidate lncRNAs and circRNAs that may be indispensable for the morphogenesis and development of rabbit embryos.
Key words: circRNAs, lncRNAs, embryo morphogenesis, embryo development, rabbit, RNA-seq.

INTRODUCTION

Rabbit meat have long been consumed in China due to its delectable taste and flavor. In spite of animal welfare and other ethical issues, Europe is the world's second largest rabbit meat producer as the production of rabbit meat have gradually became industrialize since 1970 (Cullere *et al.*, 2018). Emerging research found that rabbit meat has the characteristics of high protein content, low fat content and low cholesterol compared with other animals (Hermida *et al.*, 2006). Therefore, improving the production and quality of rabbit meat may be an important task.

Non-coding RNAs, including microRNA, long non-coding RNA (lncRNAs) and circular RNAs (circRNAs), are named for lacking the ability to encode proteins or peptides. Studies show that lncRNA can develop its biological function by binding DNA, RNA, or protein though its specific sequences or structural (Zhan *et al.*, 2017; Wei *et al.*, 2017). Our early research found that lncRNA TCONS_00013557 and XR_518424.2 might play an important roles in rabbit's skeletal muscle development by analyzing their co-expressed mRNAs (Kuang *et al.*, 2018). CircRNAs have become a new topic in noncoding RNA. Unlike other noncoding RNA, circRNAs have a covalently closed loop structure which makes it more difficult to be cleaved by exonuclease than linear transcript and thus more stable in cells (Chen *et al.*, 2015). In this study, we detected the expression patterns of mRNAs, lncRNAs and circRNAs in rabbit embryo at three growth stages. Furthermore, we predicted the lncRNA and circRNAs that might affect the

development of rabbit's embryo. This study will uncover the potential roles of lncRNAs and circRNAs in the development of rabbit's embryo, and further provide a potential way for the increase of rabbit yield.

MATERIALS AND METHODS

Animals and experimental design

Qixing rabbits were obtained from the Sichuan Animal Sciences Academy in Chengdu, Sichuan, China. Fifty broods of Qixing rabbits from the same family was bred at the same time. Skeletal muscle samples were collected at the embryonic age of days 18 (S1), 22 (S2), and 26 (S3), and each stage collect three individuals. Samples were saved in liquid nitrogen immediately. The animal experiment in this study was approved via the animal care and ethical committee of Sichuan Animal Science Academy. This study was performed according to the National Institutes of Health NIH Guidelines and National Research Council's publication "Guide for Care and Use of Laboratory Animals".

Chemical Analyses

RNA isolation, library construction and sequencing

The total RNA was extracted with Trizol regent (Invitrogen, USA) from the embryo. TruSeq Stranded Total RNA with Ribo-Zero Gold kit (Illumina, San Diego,CA, USA) were used to eliminate the ribosomal RNA in the quality qualified RNA. Illumina Standard RNA sample library preparation kit (Illumina, San Diego,CA, USA) were used to prepare the Strand-specific RNA-seq (ssRNA-seq) libraries according the manufacturer's instructions. After qualified by Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA, USA). The strand-specific libraries were sequenced on an Illumina HiSeq X ten instrument. Library construction and Illumina sequencing were performed by OE Biotech CO., LTD (Shanghai, China).

Identification of lncRNA and circRNA

We adopted rigorous steps to identify credible lncRNA: (1) reconstructed the mapped reads with Stringtie software; (2) align the spliced transcripts and the reference transcripts using cuff compare software; (3) screening the transcripts longer than 200 bp and containing two or more exons out from the alignment; (4) finally, elect the transcripts with non-coding ability using CPC, CNCI, Pfam and PLEK. The normalization of the expression values were performed by FPKM. The primary identification of circRNA was performed with CIRC.

Expression level analysis and differential expression analysis of lncRNA, circRNA and mRNA

CircRNA expression level were analysed according to RPM while FPKM was used to quantify lncRNA and mRNA. According to the result of FPKM and RPM, PCA was used to investigate the distribution and the correlation among samples. The differential expression analysis of lncRNA, circRNA and mRNA were performed with the DESeq (version 1.18.0), respectively. The filter condition was set as $p\text{-value} < 0.05$ and $\text{FoldChange} > 2$.

GO enrichment analysis and KEGG enrichment analysis

To explore the main function of lncRNA/circRNA in the development of rabbits' embryo, all the mRNA co-express with differentially expressed lncRNA/circRNA were annotated by GO. The top 10 lncRNA/circRNA whose co-expressed mRNAs had the most GO terms and the enriched mRNA ≥ 5 were screened. The GO enrichment graphs were draw for the co-expressed mRNA. We also analysed the enrichment of co-expressed mRNA annotated by KEGG and screened the top 10 lncRNA/circRNA whose co-expressed mRNAs had the most KEGG terms and the enriched mRNA ≥ 2 for further study.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from the embryonic cells with Trizol regent (Invitrogen, USA) and cDNA was transcribed from corresponding RNA using a reverse transcription kit. RT-PCR was began on an ABI QuantStudio™6 Flex System with SYBR-Green PCR master mix kit (Applied Biosystems, Inc. Foster City, CA, USA). The amplification conditions is 95℃ for 10min, followed by 45 cycles of 95℃ for 15s, 60℃ for 60s and 95℃ for 15s. $2^{-\triangle\triangle Ct}$ method was used to calculate the results.

Statistical Analysis

Data analysis were performed by SPSS 21.0 (IBM Corp Armonk, NY, USA). All values were presented as mean ± S.D. and one-way analysis of variance (ANOVO) was used to analysis the difference between groups. the statistically significant difference (*) was $P < 0.05$ while $P < 0.001$ refer to the extremely significant difference (**).

RESULTS AND DISCUSSION

Characterization of lncRNAs and circRNAs

CPC, CNCI, Pfam and PLEK was used to screen out transcripts with coding potential, and finally identified 2589 lncRNA transcripts, including 1407 novel lncRNAs. There were 585 lncRNAs sized 201~300 bp, which occupied the most proportion, following with 629 lncRNAs length >=1000 bp. Four types of lncRNAs were identified, among which intergenic lncRNA has the largest number (980), followed by sense-overlapping lncRNA (939), intronic lncRNA (337) and anti-sense lncRNA (333). A total of 20458 circRNAs were identified, with average length 1975.17. There were 3575 circRNAs sized 201~300 bp, 3379 circRNAs sized 301~400 and 2546 circRNAs length > 2000 bp.

Differently Expressed RNAs Between Different Developing Embryos

A total of 7158 mRNAs were differently expressed between S1, S2 and S3. The expression of 794 mRNAs were uniquely different between S2 and S1, 2070 mRNAs were uniquely altered between S3 and S1, and 770 mRNAs were uniquely alterled between S3 and S2(Fig. 1A). Especially, 342 mRNAs were persistently upregulated and 110 mRNAs were persistently downregulated during the development of embryo. Moreover, a total of 719 lncRNAs were differently expressed between S1, S2 and S3, including 95, 202, and 112 lncRNAs uniquely changed in the comparison of S2 versus S1, S3 versus S1, and S3 versus S2 (Fig. 1B). Importantly, 18 and 5 lncRNAs were persistently upregulated and downregulated, respectively, during the development of embryo. Additionally, 744 circRNAs were differently expressed between S1, S2 and S3, including 101, 322, and 112 circRNAs uniquely changed in the comparison of S2 versus S1, S3 versus S1, and S3 versus S2 (Fig. 1C). Interestingly, 7 circRNAs were persistently upregulated during the development of embryo.

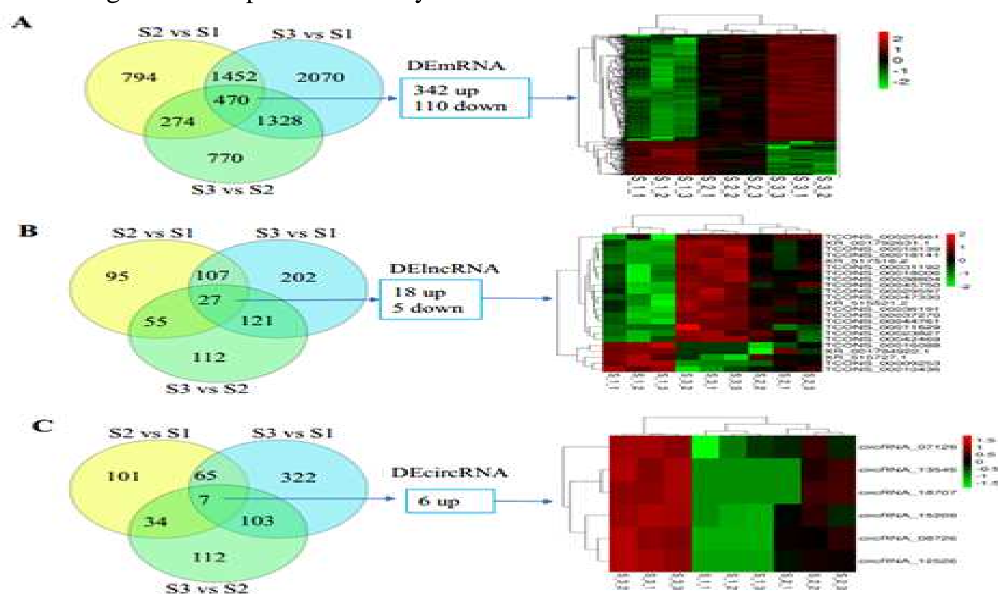

Figure 1: Differently Expressed RNAs Between Different Developing Embryos

Pathway of lncRNAs and circRNAs Involved in Embryonic Development

KEGG analysis was performed to analyze the signaling pathway of lncRNAs and circRNAs that was associated with embryonic morphogenesis and development. TCONS_00009253 was mainly involved in the pathway of PI3K-Akt signaling pathway, Wnt signaling pathway and EGFR tyrosine kinase inhibitor resistance. While circRNA_12526 was primarily involved in the signaling pathway of PI3K-Akt signaling pathway, extracellular matrix (ECM)-receptor interaction, and Calcium signaling pathway .

Regulated Network of lncRNAs, circRNAs and mRNAs Involved in Embryonic Development

To investigate the molecular regulatory network of lncRNAs, circRNAs and mRNAs in the development of embryo, we establish a network using co-expressed mRNAs that were involved in the embryonic morphogenesis and development, and were persistently upregulated or downregulated during embryonic development. The results showed the regulated network of TCONS_00009253, TCONS_00010436, XR_001792631.1, circRNA_07129, circRNA_15209 and circRNA_12526. All these three circRNAs targeted TBX1, and all these circRNA and lncRNAs targeted WNT 3 and FGFR2.

Validation of the Selected lncRNAs and Co-expression mRNAs

To verify the sequencing results, real time PCR was used to measure the lncRNAs and circRNAs that were involved in the embryonic morphogenesis and development. In accordant with the sequencing results, real time PCR showed the expression of lncRNA TCONS_00009253 and TCONS_00010436 was persistently decreased with the development of rabbit embryo. While lncRNA XR_001792631.1 displayed no significant difference among three stages. Moreover, circRNAs including circRNA_07129, circRNA_15209 and circRNA_12526 was persistently increased during embryonic development. The expression of co-expressed mRNAs TBX1, WNT 3 and FGFR2 was persistently decreased with the development of embryo in rabbits. Correlation analysis displayed fold change data between real time PCR and RNA-Seq was significantly positive correlation (correlation coefficient R2 = 0.8214, $p < 0.0001$), indicating the reliability of our transcriptome sequencing analysis.

CONCLUSIONS

We identified the rabbit embryo development related lncRNAs and circRNAs, and revealed their co-expressed genes and potential signaling pathway. lncRNA TCONS_00009253 and TCONS_00010436 were persistently downregulated during the rabbit embryo development, while circRNA circRNA_07129, circRNA_15209 and circRNA_12526 were continuously upregulated. Moreover, their co-expressed mRNAs TBX1, WNT 3 and FGFR2 were continuously downregulated during the rabbit embryo development. Overall, this work provides candidate lncRNAs and circRNAs that may be indispensable for the development of rabbit embryos.

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