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Identification of gene expression changes in rabbit uterus during embryo implantation

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ABSTRACT

Embryo implantation in the rabbit is unique in that a typical fusion type of implantation is employed, in which trophoblast cells adhere and fuse to the apical surface of uterine epithelial cells. In the present study, we analyzed global gene expression changes in the rabbit uterus during embryo implantation by using RNA-seq. We identified a total of 536 differentially expressed genes (fold change >2 and adjusted p-value <0.01), of which 266 genes were down-regulated and 270 genes were up-regulated at the implantation site compared to the inter-implantation site. Functional clustering revealed that cell adhesion is among top ranked enriched terms from both gene ontology and pathway analysis, highlighting the importance of cell adhesion during embryo implantation in rabbits. Through gene network analysis, we prioritized 9 genes using the hub gene method. Our study provides a valuable resource for in-depth understanding of the mechanism underlying embryo implantation in rabbits.

1. Introduction

Embryo implantation into the uterus is an essential process for mammalian reproduction [1]. However, this process varies among mammals [2]. The rabbit is unique in that a typical fusion type of embryo implantation is employed, in which trophoblast cells adhere and fuse to the apical surface of uterine epithelial cells to create large knob-like structures [3]. The rabbit is a good model to study molecular events of embryo implantation because it is an obligate ovulator and the pregnancy can be precisely timed [4]. A number of genes have been implicated in rabbit embryo implantation. These include integrins (ITGAV and ITGB3) [5], cell surface mucin 1 (MUC1) [4], vascular endothelial growth factor (VEGF) [6], heparin-binding epidermal growth factor (HB-EGF) [7], proprotein convertase 5/6 (PC5/6) [8] and osteopontin (SPP1) [9].

Despite these discoveries, all these genes were first identified in mouse implantation and then extrapolated to the rabbit in a straightforward way [10]. So far, genuine candidate genes with regard to rabbit embryo implantation remain undefined. In recent years, high-throughput transcriptomic approaches such as microarray and RNA-seq have been developed, making it possible for studying genome-wide gene expression simultaneously. By using these approaches, several studies have reported altered uterine gene expression profiles associated with embryo implantation in humans, mice, rhesus monkeys, cows and pigs, respectively [11].

RNA-seq, which utilizes the latest massively parallel sequencing, has been shown to be a highly accurate tool for quantifying gene expression levels [12]. In contrast to the microarray, RNA-seq has several advantages. Firstly, it is an unbiased method which is not limited to detecting pre-designed sequences [12]. Secondly, it does not suffer from cross-hybridization since DNA sequences can be mapped to unique regions of the genome [13]. Lastly, it has no upper limit for quantification [14]. In the present study, we took advantage of the RNA-seq approache to investigate the global gene expression changes in the rabbit uterus during embryo implantation. Our study contributes to an increase in the knowledge on molecular mechanisms underlying embryo implantation in the rabbit.

2. Materials and methods

2.1. Sample collection

Adult New Zealand White rabbits, aged 3–4 mo and weighted 2.5–3.1 kg, were used for this study. Female rabbits were mated with males and the time of mating was designated as day 0 of pregnancy. Animals (n = 3) were euthanased by an overdose of pentobarbital on day 7.25 (6 h after day 7) of pregnancy. Uterine fragments from the implantation site (IS) and the inter-implantation site (IIS) were collected separately. The IS fragments were opened anti-mesometrially and embryonic tissues were removed and discarded under a stereomicroscope. In order to ensure a complete removal, the implantation site was cut into halves and one half was examined in sequential frozen sections. All samples were flash-frozen in liquid nitrogen and stored at −80 °C for further analysis. All the rabbit experimental procedures were

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approved by the Institutional Animal Care and Use Committee of South China Agricultural University.

2.2. RNA-seq analysis

Total RNA from uterine samples was extracted using TRIzol reagent (Invitrogen). RNA purity was assessed using the ND-1000 Nanodrop and RNA integrity was evaluated using the Agilent 2200 TapeStation. The following RNA quality control parameters were used: A260/A280 ratio ≥1.8, A260/A230 ratio ≥2.0 and RIN (RNA integrity number) [15] value ≥7.0. The mRNA-seq sample preparation kit (Illumina) was used for the preparation of RNA-seq libraries. Briefly, poly-$(A)^+$ mRNA was purified with oligo-(dT) magnetic beads and fragmented to approximately 200 bp in fragmentation buffer. The obtained cleaved RNA fragments were reverse transcribed to first-strand cDNA, followed by second-strand cDNA synthesis. The double-stranded cDNA fragments were purified, end-repaired and then ligated to sequencing adapters. After purification, suitable fragments were amplified through 15 cycles of PCR to generate the final sequencing libraries. Finally, PCR products were purified and quantified for high-throughput sequencing using the Illumina HiSeq™ 2500. After sequencing, a computational pipeline was used to process RNA-seq data. Raw sequence data in fastq format were filtered to remove reads with >10% unknown nucleotides. Clean reads were mapped to rabbit reference genome OryCun2 with Tophat v1.4.0 [16] allowing no more than two mismatches. Transcript isoforms were assembled using Cufflinks v1.3.0 [17] and combined with the gene annotations from ENSEMBL database release 81 [18]. Gene expression levels were measured using fragments per kilobase of transcript per million mapped fragments (FPKM). To compare profile differences between two groups, a *t*-test with Benjamini-Hochberg multiple test correction was employed. Differentially expressed genes were chosen according to the criteria of fold change >2 and adjusted p-value <0.01.

2.3. Validation by quantitative RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen). Before amplification, RNA samples were treated with DNase I (Invitrogen) to eliminate potential genomic DNA contamination. The PrimeScript reverse transcriptase reagent kit (TaKaRa) was used for reverse transcription. Quantitative PCR was performed using the SYBR Premix Ex Taq™ kit (TaKaRa) on the Rotor-Gene 3000 A system (Corbett Research). The ACTB gene was amplified as a reference gene for normalization. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were listed in Supplementary Table 1.

2.4. Gene ontology (GO), pathway and network analysis

GO term enrichment analysis was performed by using the DAVID tool [19]. The significance cutoff for adjusted p-value (Benjamini-Hochberg multiple test correction method) was set at 0.01. The R package wordcloud was used to generate word cloud for significantly enriched GO terms. The font sizes in the word cloud were proportional to -log10 of adjusted p-value for each enriched GO terms. The DAVID tool was also employed for pathway enrichment analysis. The same significance cutoff as GO analysis was adopted. The STRING database v10.0 [20] was used to create gene network. The minimum combined score was set to 0.900 (highest confidence). Cytoscape software was applied for visualization and analysis of the gene network. Connectivity for each gene was analyzed by NetworkAnalyzer [21]. The connectivity threshold value for hub genes was the mean plus two standard deviations.

3. Results

3.1. Identification of transcriptomic differences

To systematically investigate gene expression changes in the rabbit uterus upon embryo implantation, RNA-seq data were generated from the implantation site (IS) and the inter-implantation site (IIS) on day 7.25 of pregnancy, with three biological replicates respectively. The RNA-seq raw data were deposited in Gene Expression Omnibus database (GSE76115). Following a computational pipeline (illustrated in Fig. 1A), data analysis revealed that, of all 33.4 million reads obtained in this study, 82.5% were mappable to the rabbit genome. Mapped reads were used to estimate normalized transcription level as fragments per kilobase of transcript per million mapped fragments (FPKM). It has been estimated that a gene with FPKM value of 1 is approximately equivalent to one copy per cell [13]. Transcriptome clustering based on Pearson correlation distances showed that the IS samples were readily separated from the IIS ones, confirming global gene expression changes in the rabbit uterus during embryo implantation (Fig. 1B).

Using a fold change cutoff of 2 and an adjusted p-value cutoff of 0.01, we identified a total of 536 differentially expressed genes (Fig. 1C). Among them, 266 genes were down-regulated and 270 genes were up-regulated at IS in comparison to IIS (Supplementary Table 2). The range of fold change values is illustrated in Fig. 1D.

In order to validate the RNA-seq data, a total of 6 genes with various fold changes were randomly selected and quantified by quantitative RT-PCR (qRT-PCR). This analysis was done on RNA samples isolated from an independent set of biological replicates. In general, the expression trend of these genes measured by qRT-PCR was consistent with RNA-seq data and statistical significance was reached at $p < 0.05$ for all tested genes (Fig. 1E). Furthermore, Pearson correlation analysis showed a statistically significant correlation ($r = 0.9973$, $p = 0.000000706$) between the qRT-PCR and RNA-seq results, indicating that our RNA-seq data were of high quality.

3.2. Gene ontology and pathway analysis

For gene ontology (GO) analysis, all differentially expressed genes were functionally categorized based on GO annotation terms using the DAVID tool. Enriched GO terms are classified according to biological process (BP), cellular component (CC) and molecular function (MF) (Fig. 2A and Supplementary Table 3). In the BP category, 17 terms were significantly enriched, including response to wounding, skeletal system development, cell adhesion, response to hypoxia, vasculature development, response to steroid hormone stimulus, inflammatory response, response to endogenous stimulus, regulation of cell proliferation, cell migration, angiogenesis, positive regulation of immune system process, response to extracellular stimulus, protein processing, regulation of cell shape, translational elongation, and protein maturation. The enriched CC categories were proteinaceous extracellular matrix, extracellular matrix, cell surface, basement membrane, external side of plasma membrane, plasma membrane, cell projection, cell-substrate junction, membrane-bounded vesicle, and focal adhesion. With respect to molecular function, the enriched terms were integrin binding, protein complex binding, polysaccharide binding, pattern binding, metalloendopeptidase activity, glycosaminoglycan binding, endopeptidase activity, carbohydrate binding, growth factor binding, peptidase activity, and calcium ion binding. Interestingly, of all the top ranked enriched GO terms, cell adhesion from BP, extracellular matrix from CC and integrin binding from MF consensually pointed to cell adhesion. This result indicates that cell adhesion is likely the most important process during embryo implantation in rabbits. We next extracted 46 genes associated with cell adhesion from all the 536 differentially expressed genes. Among them, 16 genes were down-regulated and 30 genes were up-regulated (Fig. 2B). Notably, the number of up-regulated genes is significantly higher than down-regulated genes