

# Circular RNAs Expression Profiles and Bioinformatics Analysis in Bronchopulmonary Dysplasia

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## Research Article

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# Abstract

**Background:** Bronchopulmonary dysplasia (BPD) is a congenital chronic lung illness characterised by alveolar simplification and abnormal pulmonary microvasculature angiogenesis. Circular RNAs (circRNAs) have recently attracted significant interest due to their function in developing many disorders, but their role in BPD remains largely untapped. The purpose of this work was to discover a profile of circRNA expression related to severe BPD and to anticipate its possible biological activities.

**Methods:** PBMCs were extracted from peripheral blood, and the circRNAs expression profiles in the PBMCs of severe BPD group (n = 3) and control group (n = 3). We identified and grouped significantly differently expressed circRNAs between the two groups. Following that, we used bioinformatics to design a circRNA-miRNA-mRNA network. Gene ontology and the Kyoto Encyclopedia of Genes and Genomes were performed.

**Results:** Among hundreds of circRNAs, 171 were upregulated, and 81 were downregulated (fold change > 1.5, P < 0.05). Using GO and KEGG analyses revealed that circRNA target genes were considerably enriched. These genes are involved in transcription regulation, protein phosphorylation, and cell adhesion. A crosstalk network was developed to investigate the interactions of several components, including four circRNAs, sixteen microRNAs and thirty-seven messenger RNAs. A pathway network identified several critical pathways involved in the pathogenesis and development of BPD. These include the Wnt signalling pathway, the Hippo signalling pathway, and signalling pathways regulating stem cell pluripotency.

**Conclusions:** CircRNAs in the peripheral blood of severe BPD children were considerably changed in the early post-birth period, which may be critical in developing this disease.

## 1 Introduction

In neonatal care, bronchopulmonary dysplasia (BPD) is a critical disorder that seriously affects the survival rate and neurodevelopmental outcomes 1. BPD was first described in 1967 by Northway and is now one of the most common congenital disabilities globally 2. During that era, the mortality of even more significant prematurity-meaning the baby was born prematurely-was 60%. Preterm newborns' survival rates have dramatically increased due to remarkable progress in perinatal treatment 3. Preterm babies delivered at 28 weeks had a 94% survival rate, with a higher speed at 23 and 24 weeks 4. At 28 weeks' gestational age (GA) 5, roughly 20% of survivors develop BPD, affecting up to 80% of infants less than 24 weeks of gestation 6, 7. The frequency continues to climb 4. These infants suffer from many potential complications of BPD, including lifelong pulmonary problems and abnormal neurodevelopment outcomes 8.

Initially, BPD was defined as requiring a persistent oxygen supplement for 28 days postnatally, as determined by a typical chest radiograph and marked by severe airway inflammation and lung fibrosis 9, 10. Since the 1970s, extensive application of antenatal corticosteroids, exogenous surfactants, and rational ventilatory support has evolved into a new pattern of chronic lung disease, as well as the

invention of the phrase "new bronchopulmonary dysplasia"<sup>11</sup>. The condition is characterised by alveolar simplification and pulmonary dysangiogenesis<sup>12</sup>. Oxygen requirements for babies born with bronchopulmonary dysplasia were first defined at 28 days of life and 36 weeks postmenstrual age, rather than a fixed time of continuous oxygen treatment, as had previously been thought to be the case<sup>13</sup>.

Despite many risk factors that have been shown to interact directly with the aberrant development of injured lung, including chorioamnionitis, barotrauma or volutrauma, oxygen toxicity, fluid overload, genetic factor, and inflammation, the mechanism is still perplexing and complex<sup>9, 14</sup>. There is growing recognition that genetic and epigenetic factors also potentially impact development and evolution towards BPD<sup>15</sup>. Recent advances in high-resolution microarray and genome-wide sequencing technology enable researchers to identify better various diseases' genetic mechanisms<sup>10, 16</sup>. A new family of molecules, circRNA, has lately attracted significant attention among researchers<sup>17</sup>.

Uncountable eukaryotic cells contain considerable non-coding RNAs known as circular RNAs (circRNAs)<sup>18, 19</sup>. In contrast to linear RNAs<sup>20</sup>, circRNAs undergo non-canonical splicing, resulting in a longer half-life and more excellent resistance to RNase R<sup>21, 22</sup>. Several studies confirmed multiple functions of circRNAs, including serving as miRNA sponge<sup>23</sup>, binding RBPs<sup>24</sup>, and translating peptides<sup>25</sup>, while as miRNA sponge was the most extensively studied form of mechanism<sup>26</sup>. Evidence suggests their remarkable expression profiles and critical roles across several biological processes, including the control of cell proliferation; the protection of cells from oxidative stress; and tumour growth in various cancer types<sup>27, 28</sup>. However, the processes driving circRNAs' aberrant landscape and their physiological or pathogenic functions in BPD remain little understood<sup>29</sup>.

Microarray gene expression analysis is a novel approach for studying genes, biological pathways, and their connections in disease states or exposure factors. Understanding the molecular processes that drive disease progression, such as chronic lung disease, is now mainly attributable to this new study tool<sup>30</sup>. Investigations of gene expression in animal models have been extensive. Still, analogous studies with primary fetal human lung cells or clinical samples are advised since they may directly represent the specific molecular changes in human neonates exposed to hyperoxia<sup>31</sup>.

Our study aimed to identify different circRNAs expressions in severe bronchopulmonary dysplasia and explore the potential biomarkers for accurate and early disease prediction. The results indicate that abnormal circRNA expression patterns may be critical in the molecular mechanisms behind BPD and that unravelling the signalling and transcriptional network would give more insights into the processes of lung development and regeneration after damage.

## **2 Methods And Patients**

### **2.1 Ethical compliance**

The Jiangsu Commission of Health funded this study. Before any samples were collected, the ethical office of the Suzhou Municipal Hospital gave their blessing to this research and required both parents to

sign an informed permission form.

## 2.2 Specimen collection

CircRNA microarray analysis was performed on three pairs of venous blood samples from the severe BPD and control groups. All infants were born at a gestational age less than 28 weeks and or with a birth weight less than 1,250g in the Suzhou Municipal Hospital from May to December 2019. We collected 2–3 ml of peripheral blood specimens from preterm infants 7–10 days after birth, applied lymphocyte isolation solution to separate PMBC within 2 hours, transferred them into enzyme-free 1.5 ml EP tubes, and added the appropriate amount of TRizol for freezing at -80°C for subsequent experiments immediately. The severe BPD was defined using the physiologic definition (requiring oxygen saturation of more than 30% or continuous positive airway pressure (CPAP)/mechanical ventilation) at 36 weeks' GA 13, 32. Three preterm infants without BPD were chosen as controls with gestational age-matched. Infants were excluded if they had chromosomal abnormalities or significant congenital deformities or took corticosteroids in the first week of birth to improve lung function. All the samples remained after routine tests during the hospitalisation of the premature infants. Table 1 contains basic information about the six newborns.

Table 1  
Clinical characteristics of the control and severe BPD group

|                     | Control group (n = 3) |    |        | BPD group (n = 3) |    |        | P-value |
|---------------------|-----------------------|----|--------|-------------------|----|--------|---------|
|                     | 1#                    | 2# | 3#     | 1#                | 2# | 3#     |         |
| Gestational age (w) | 27 + 1                | 28 | 28 + 5 | 27 + 6            | 26 | 27 + 2 | 0.206   |

*PROM: premature rupture of membranes; RDS: respiratory distress syndrome; PDA: patent ductus arteriosus; NEC: necrotising enterocolitis; EOS: early-onset neonatal sepsis; LOS: late-onset neonatal sepsis; IVH: intraventricular haemorrhage; VAP: ventilator-associated pneumonia; ROP: retinopathy of prematurity.*

|                          | Control   | group     | (n = 3)   | BPD       | group | (n = 3)   | P-value |
|--------------------------|-----------|-----------|-----------|-----------|-------|-----------|---------|
| Birth weight (g)         | 1050      | 970       | 850       | 770       | 680   | 950       | 0.186   |
| Sex gender               | male      | female    | female    | female    | male  | female    | -       |
| PROM                     | +         | -         | +         | +         | -     | -         | -       |
| Preeclampsia             | -         | +         | +         | -         | -     | +         | -       |
| Antenatal steroids       | +         | +         | +         | +         | +     | +         | -       |
| Apgar 1 min              | 8         | 6         | 7         | 4         | 8     | 8         | 0.830   |
| Apgar 5 min              | 9         | 9         | 9         | 8         | 8     | 9         | 0.116   |
| RDS > stage 2            | +         | -         | -         | +         | -     | +         | -       |
| Surfactant treatment     | +         | +         | +         | +         | +     | +         | -       |
| Days with ventilator (d) | 2         | 0         | 1         | 21        | 36    | 19        | 0.044   |
| Days with CPAP (d)       | 10        | 21        | 12        | 14        | 18    | 22        | 0.421   |
| Hospitalization days     | 60        | 73        | 82        | 92        | 84    | 74        | 0.230   |
| PDA                      | +         | -         | -         | -         | -     | -         | -       |
| IVH > stage 2            | -         | -         | +         | -         | +     | +         | -       |
| NEC > II B               | -         | -         | -         | -         | +     | +         | -       |
| EOS                      | -         | +         | -         | -         | +     | -         | -       |
| LOS                      | 1950      | 2010      | 2030      | 1980      | 1670  | 2000      | 0.401   |
| VAP                      | discharge | discharge | discharge | discharge | dead  | discharge | -       |
| ROP                      |           |           |           |           |       |           |         |
| Discharge weight (g)     |           |           |           |           |       |           |         |
| Outcome                  |           |           |           |           |       |           |         |

*PROM: premature rupture of membranes; RDS: respiratory distress syndrome; PDA: patent ductus arteriosus; NEC: necrotising enterocolitis; EOS: early-onset neonatal sepsis; LOS: late-onset neonatal sepsis; IVH: intraventricular haemorrhage; VAP: ventilator-associated pneumonia; ROP: retinopathy of prematurity.*

## 2.3 RNA preparation and circular RNA microarray analysis

According to the manufacturer's protocol, total RNA was extracted separately using TRIzol reagent (Invitrogen, United States). As an extra precaution to ensure that only circular RNAs remained after digestion, total RNAs were first digested using the Rnase R enzyme from Epicentre, Inc. After isolating these enriched circular RNAs. They were amplified and converted into fluorescent circular RNAs utilising a random priming technique (Arraystar Super RNA Labeling Kit; Arraystar). Using the RNeasy Mini Kit, the tagged cRNAs were isolated (Qiagen). With the NanoDrop ND-1000, we could determine the cRNA concentrations and activities (pmol Cy3/ $\mu$ g cRNA). Only samples with OD260/280 ratios between 1.60–1.80 were included. After fragmenting 1  $\mu$ g of each labelled cRNA with five  $\mu$ l ten  $\times$  Blocking Agent and one  $\mu$ l 25  $\times$  Fragmentation Buffer, the mixture was heated to 60°C for 30 minutes before being diluted with 25  $\mu$ l two  $\times$  Hybridization buffer. A total of 50  $\mu$ l hybridisation solution was put into the gasket slide, which was then attached to the circRNAs expression microarray slide. Using an Agilent Hybridization Oven, the slides were incubated for 17 hours at 65°C. The cleaned, fixed and scanned hybridisation arrays were performed using the Agilent Scanner G2505C.

## 2.4 RNA microarray data analysis

Data from microarrays were obtained through Agilent Feature Extraction software. GeneSpring V12.0 (Agilent, Santa Clara, CA) was used to sum up, data from the two groups. The data were quantile standardised and further processed using R's limma function. The final normalised intensity in the analysis was derived using log<sub>2</sub>-transformed normalised data. Using Benjamini and Hochberg's technique, P-values were adjusted to decrease false discovery rates. CircRNAs with a fold change of more than 1.5 and a P value of less than 0.05 were different.

## 2.5 Quantitative Reverse transcription-polymerase Chain Reaction Validation Assay

Candidates for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were selected when they satisfied all of the following criteria: (a) Increased expression in the BPD group, (b) FC > 2.0, P adj < 0.01. Following that, five circRNAs were selected (has\_circ\_0007054, has\_circ\_0057950, has\_circ\_0050386, has\_circ\_0057953, and has\_circ\_0120151). According to the manufacturer's instructions, total RNA samples were reverse transcribed into cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, United States). The expression of circRNAs was confirmed using a QuantStudio5 Real-time PCR System and an SYBR Green Master Mix (2, Arraystar, United States) for quantitative polymerase chain reaction (qPCR) (Applied Biosystems, United States). Real-Time RT-qPCR experiments were performed using 40 ng cDNA in a final volume of 10  $\mu$ l. We utilised NCBI Primer-BLAST and Genaray Biotech to design and manufacture all primers in this work (Shanghai, China). Instead of convergent primers, RT-qPCR validation of circRNA was performed using divergent primers rather than convergent ones. Circular RNA validation sequences were obtained from the circBase database. For each PCR run, a 10  $\mu$ l reaction volume was prepared as follows: 2 litres of water, 0.5 litres each primer (10 mM), 5 litres Power SYBRV2 Green PCR Master Mix (2), and 2 litres RT product The Real-Time RT-qPCR cycling protocol was initiated at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 1 minute, and finally 95°C for 10 seconds, 60°C for 1 minute, and 95°C for 15 seconds. All measurements

were performed in triplicate and standardised to the levels of  $\beta$ -actin. We used the  $2^{-\Delta\Delta Ct}$  technique to determine the expression levels. The presence of a single peak in the melt curve indicated the PCR products' specificity. Additionally, electrophoresis was utilised to validate the PCR results using a 1.5 per cent agarose gel. This study's primer sequences, both forward and reverse are included in Table 2.

Table 2  
Sequences of forwarding and reverse primers

| Gene  | Sequence (5'-3')   | Length(bp) |
|---|--|------------|
| $\beta$ -actin  | F: 5'GTGGCCGAGGACTTTGATTG3'<br>R: 5'CCTGTAACAACGCATCTCATATT3'    | 73         |
| hsa_circRNA_0007054   | F:5'GACAAGTCCGAGGTGATAGTTACA3'<br>R: 5'CTTTTCCCCCAGTCAATG3'      | 118        |
| hsa_circRNA_0057953   | F:5'GGTCCGTCTAATGAAGAACAGATTT3'<br>R: 5'CGCTTTTGGGAATCAGTTACAC3' | 89         |
| hsa_circRNA_0050386   | F: 5'TCCACCGAGATCCAGAACAGA3'<br>R: 5'GAGTGGCGTATTCCCATTGTT3'     | 123        |
| hsa_circRNA_0057950   | F: 5'GTCCGTCTAATGAAGAACAGGTAA3'<br>R: 5'CATCTCCACTCTGCTGGTTCTC3' | 71         |
| hsa_circRNA_0120151   | F: 5'GGAGCCAGAAGGAAGAGTGTAT3'<br>R: 5'CCCAAGAGTCCATGATTTTA3'     | 140        |
| <i>The circRNA ID is in circBase (<a href="http://circbase.org">http://circbase.org</a>).</i> |  |            |

## 2.6 Construction of ceRNA network and enrichment analysis

A circRNA-miRNA-mRNA network based on the ceRNA hypothesis was constructed in Cytoscape 3.6.1 to investigate these relationships. To identify the downstream target genes for each circRNA, we used the MiRWalk v3.0 (<http://mirwa.lk.umm.uni-heidelberg.de/>) and three prediction algorithms (Targets can, MiRDB, and mirTarbase). Finally, we used the four confirmed circRNAs and predicted miRNAs/mRNAs to show the ceRNA network graphically. These genes' roles and signalling pathways were further elucidated using GO and KEGG analyses.

## 2.7 Statistical analysis

There were two groups: the BPD group and the control group. If the data distribution was expected, a 2-tailed Student's t-test or Mann-Whitney U test was used to compare the expression levels of circRNAs in each group. Nonparametric Mann-Whitney U-tests were employed instead if they were not available. GraphPad Prism7 was used to perform the above-described statistical studies (GraphPad Software, USA). A P value of less than 0.05 was deemed statistically significant.

## 3 Result

### 3.1 CircRNA expression profiling

A total of 252 circRNAs were identified to be differentially expressed between the severe BPD and control groups (FC > 1.5 and Padj 0.05), with 171 being upregulated and 81 being downregulated. We compared the samples' post-normalisation expression value distributions using a box plot. Each sample's log2 ratio followed the same pattern, as shown in Fig. 1a. We noticed that most dysregulated circRNAs originated in protein-coding exons, whereas some developed in introns and intergenic regions (Fig. 1b). The bar chart also showed the distribution of circRNAs on human chromosomes (Fig. 1c). Hierarchical clustering analysis (Fig. 1d) and volcano and scatter plots (Fig. 1e) were used to illustrate the differences in circRNA expression between the two groups (Fig. 1e and Fig. 1f). Tables 3 and 4 summarises the top 10 circRNAs that have been up-or down-regulated.

Table 3  
The top 10 upregulated circRNAs

| circRNA ID         | chromosome | circRNA type | GeneSymbol | Fold change | P-value |
|--------------------|------------|--------------|------------|-------------|---------|
| hsa_circRNA_003574 | chr20      | exonic       | GID8       | 2.354       | <0.0001 |
| hsa_circRNA_103332 | chr3       | exonic       | NKTR       | 2.093       | 0.0035  |
| hsa_circRNA_006752 | chr22      | exonic       | NF2        | 2.045       | 0.0042  |
| hsa_circRNA_102504 | chr19      | exonic       | ANKRD27    | 3.19        | 0.0048  |
| hsa_circRNA_102039 | chr17      | exonic       | TAF15      | 3.065       | 0.0069  |
| hsa_circRNA_030448 | chr13      | exonic       | LMO7       | 2.089       | 0.0072  |
| hsa_circRNA_102900 | chr2       | exonic       | CREB1      | 2.266       | 0.0105  |
| hsa_circRNA_007054 | chr1       | exonic       | TMEM50A    | 4.853       | 0.0155  |
| hsa_circRNA_045234 | chr17      | exonic       | DDX42      | 2.052       | 0.019   |
| hsa_circRNA_043820 | chr17      | exonic       | PTRF       | 2.125       | 0.022   |

Table 4  
The top 10 downregulated circRNAs

| circRNA ID         | chromosome | circRNA type | GeneSymbol | Fold change | P-value |
|--------------------|------------|--------------|------------|-------------|---------|
| hsa_circRNA_002210 | chr8       | exonic       | ZNF706     | 2.847       | 0.03623 |
| hsa_circRNA_103332 | chr3       | exonic       | CASC15     | 3.505       | 0.0318  |
| hsa_circRNA_006752 | chr22      | antisense    | ARID1B     | 2.152       | 0.0295  |
| hsa_circRNA_102504 | chr19      | exonic       | CEP192     | 2.518       | 0.0456  |
| hsa_circRNA_102039 | chr17      | exonic       | YY1AP1     | 2.596       | 0.0485  |
| hsa_circRNA_030448 | chr13      | exonic       | CSPP1      | 2.022       | 0.0432  |
| hsa_circRNA_102900 | chr2       | exonic       | ABLIM1     | 2.32        | 0.0234  |
| hsa_circRNA_007054 | chr1       | exonic       | STAT3      | 2.963       | <0.0001 |
| hsa_circRNA_045234 | chr17      | exonic       | NETO2      | 2.698       | 0.0377  |
| hsa_circRNA_043820 | chr17      | exonic       | RFWD3      | 2.48        | 0.0136  |

## 3.2 qRT-PCR validation for the selected circRNAs

Previously, results indicated that the BPD group had more up-regulated circRNAs than down-regulated circRNAs. Thus, to confirm the findings of the circRNA microarray study, qRT-PCR was utilised to identify five elevated circRNAs selected using the criteria indicated before. In the BPD group samples, four out of five circRNAs (hsa\_circ\_0007054, hsa\_circ\_0057950, hsa\_circ\_0050386, and hsa\_circ\_0120151) were considerably overexpressed, which was supported by RNA microarray analysis; nevertheless, hsa\_circ\_0057953 could not be amplified by qPCR (Fig. 2).

## 3.3 Enrichment analysis of circular RNA-targeted genes

GO, and KEGG pathway analyses were used to annotate the function of circRNA-targeted genes. We retrieved 423 GO terms, and the top 20 most enriched terms are shown in Fig. 3(a, b and c). As confirmed by the data collected, these target genes were predominantly involved in biological activities, including transcriptional control by the RNA polymerase II promoter, positive and negative regulation of DNA-templated transcription, signal transmission, and protein phosphorylation. Moreover, KEGG pathway analysis revealed 94 signalling pathways, the most enriched and significant of which included the Wnt signalling pathway, the Hippo signalling pathway, signalling pathways regulating stem cell pluripotency, the calcium signalling pathway, invasion of epithelial cells by bacteria, the MAPK signalling pathway, and the cGMP-PKG signalling pathway (Fig. 3d).

## 3.4 Construction of ceRNA network

According to the mechanism referred to as "competing for endogenous RNA (ceRNA)," investigations have revealed that circRNAs may behave as ceRNA molecules, interacting with miRNA performance and

synthesis by competing for miRNA binding sites 26. TargetScan and miRanda assisted us in identifying the miRNAs and target genes downstream of the four differently expressed circRNAs. Cytoscape created the ceRNA network diagram, including circRNAs, miRNAs, and downstream target genes. As shown in Fig. 4, the ceRNA network contains fifteen miRNAs and one hundred and fifty-seven mRNAs.

## 4 Discussion

Over the last several decades, numerous treatments have been incrementally improved and developed, including increased prenatal corticosteroids, surfactant replacement, and less intrusive breathing procedures. Unfortunately, none of these techniques significantly reduced the incidence of bronchopulmonary dysplasia (BPD) 33. Most fatalities were caused by abrupt respiratory distress, with computational and postmortem evidence of airway diseases including distal inflammatory responses, extensive fibroproliferative alterations, and hypertensive pulmonary vascular remodelling. Notably, Dr Northway and colleagues uncovered additional characteristics of BPD in their investigation, including prematurity-related retinopathy and brain injury 3. Bronchopulmonary dysplasia, the newborn form of chronic lung disease, has contributed to substantial increases in morbidity and mortality in recent years. However, the failure to unravel the molecular mechanisms behind the pathogenesis is a primary reason for the lack of specific therapies. With the recent discovery of several non-coding RNAs, including microRNAs, long non-coding RNAs (lncRNAs), and circular RNAs, more spotlights have been focused on their gene regulation. Previous research has indicated the probable relationship between lung injury and non-coding RNAs 34, revealing the essential roles in lung injury. Numerous studies have shown a link between miRNAs and the advancement of lung injury and fibrosis, cell proliferation, and cell apoptosis, including miR-29a-3p 34, miR-34a 35, and miR-184 36 in BPD mice models. Besides, lncRNAs are not only involved in lung maturation 37 but are also regarded as crucial regulators of biological activities and BPD progression 10, 34, 38. According to research, lncRNA Rian rescued lung cells from hyperoxia-induced damage by targeting miR-421 39. Along with the tangible expression of lncRNA H19 in the lung tissues of BPD model mice 40, it was shown that lncRNA CASC2 protects against lung damage caused by hyperoxia exposure, with CASC2 decreasing aberrant cell apoptosis in both animal and cell models 41. However, owing to the challenges of computational prediction in the context of poor sequence conservation and low homology, lncRNAs remain relatively unexplored and need more additional studies to elucidate. This study examined the abnormal patterns of circRNAs expression in the peripheral blood of severe BPD patients throughout their early life (7–10 days after birth). Microarray profiling detected 252 differentially expressed genes, including 171 upregulated and 81 downregulated genes in the BPD group compared to the control group, with a fold change of more than 1.5, a P-value less than 0.05, and an FDR less than 0.05. Following that, we began screening potential genes for additional investigation.

CircRNAs were initially discovered in 1979 via electron microscopy in human Hela cells. Mounting evidence demonstrated the widespread of circRNAs in various species 42. CircRNA could be categorised into three groups, exonic (ecircRNA), exon-intron (elcircRNA), and intronic (ciRNA) 43, with lengths varying from 100 to several kilobases, depending on the amount of back-spliced exons and introns. The median size is typically between 500 and 700 nucleotides and consists of 2–3 exons 44. Most circRNAs are

ecircRNAs, which reside mainly in the cytoplasm but may also be expelled into the extracellular environment through exosomes. Additionally, certain exonic circRNAs are found in the nucleus, enhancing protein retention or attracting proteins to chromatin 45. In contrast, elcircRNAs and ciRNAs are most often detected inside of nucleus 24.

Until recently, circular RNAs (circRNAs) were not expected to participate in various biological activities. Researchers have found evidence to support the hypothesis that the miRNA-binding protein (RBP) circRNAs regulate biological and pathological processes by sponging miRNA, modulating the expression of parental genes, and even monitoring protein translation or being translated into peptides 28, 46. In the cell nucleus, circRNAs serve as decoys for miRNAs, preventing them from binding and inhibiting their target mRNAs 47, 48. CircABCC4 was uncovered by sponging miR-663a and upregulating PLA2G6 expression to promote BPD evolution 49. However, on the other, certain circRNAs have the potential to sponge proteins due to their propensity to bind proteins. Circular RNAs are crucial in transcription, alternative splicing, and chromatin looping when locked in the nucleus 50. For instance, the binding of circPABPN1 to HuR inhibits HuR from binding to PABPN1 mRNA and hinders PABPN1 translation 51. It has been established that circMBL, for instance, dominates the expression of its parent gene, MBL, by interfacing with RBPs and creating a negative feedback loop between MBL and the creation of circMBL 52. CircHuR blocked the binding of the CCHC-type zinc finger nucleic acid-binding protein (CNBP) binding to the HuR promoter, reducing HuR production and tumour development 53.

We carefully filtered critical circRNAs for the following criteria: differential circRNAs with P values of less than 0.05 and a fold change of more than 2, acceptable lengths between 200 and 1000 bp, and exonic type. Then Real-time Q-PCR validated candidate genes picked by microarray analysis. The hypergeometric distribution test was used to perform differential RNA GO (<http://www.geneontology.org/>) and KEGG (<https://www.genome.jp>) analyses.

In our study, four of five upregulated circRNAs (hsa\_circ\_0007054, hsa\_circ\_0057950, hsa\_circ\_0050386, hsa\_circ\_0120151) were confirmed by qRT-PCR, which was consistent with microarray assays. Hsa\_circ\_0007054 is derived from the TMEM50A/SMP1 gene. This gene is situated between the RHD and RHCE genes in the RH gene locus 54, with a sequence of potential transmembrane domains, yet its protein product's function is elusive. The gene hsa\_circ\_0057950 is generated from parental genes that encode multiple functional proteins – CREB1. This gene encodes several transcription factors through alternation splicing, acting as homodimer binding to the cAMP-responsive element or stimulating hormone-induced gene transcription through the cAMP pathway. CREB1 is one of the pivotal transcription factors (TFs) in human malignancies, believed to function effectively as oncogenes or tumour suppressors like miRNAs, as shown by the broader range of cancer-related papers 55–57. Furthermore, CREB1 could modify the VE-cadherin expression of endothelial cells in mediating sepsis-induced inflammatory lung injury 56. Another circRNA was hsa\_circ\_0050386, derived from VARP, also known as ANKRD27, which codes VPS9-ankyrin-repeat protein. Numerous molecules interacting with VARP have been identified during the past years, and considerable research focuses on VARP's multiple activities in endosomal trafficking, including positive regulators of neurite outgrowth 58 and retromer-mediated

sorting transmembrane proteins from endosomes to plasma membranes 59. Calcium and the second messenger diacylglycerol could activate the protein kinase C (PKC) family of serine and threonine-specific kinases encoded by the PRKCE parent gene has\_circ\_0120151 60. This kinase might influence cellular energy metabolism and, consequently, cell function, from phosphorylating many protein targets, participating in diverse cellular signalling pathways, neuron channel activation 61, apoptosis, and cardioprotection from ischemia 60, as well as insulin exocytosis. Additionally, a group of tumour promoters called phorbol esters are crucial receptors for PKCs 62–64. Furthermore, preeclampsia's aetiology is linked to the process of apoptosis in the placental syncytiotrophoblast, as shown in the most recent publication on PRKCE 65.

Depending on bioinformatics techniques, the circRNA-miRNA-mRNA network of the four differently expressed circRNAs was ascertained. Has-miR-57887, hsa-miR-6842-3p, hsa-miR4505, hsa-miR-4638-5p, and hsa-miR-6071 were anticipated to be adversely regulated by the upregulated has\_circ\_0057950.

The hsa-miR-5787 has previously been shown to limit fibroblast cell proliferation by binding to the eIF5 gene. However, no direct evidence indicates their function in BPD. Yoo H et al. have previously validated this 66. Similarly, Bao Z et al. 67 revealed that hsa-miR-5787 might attenuate the inflammatory response mediated by LPS/TLR4 in macrophages through the NF- $\kappa$ B signalling pathway downregulating its expression and producing pro-inflammatory cytokines such as IL-6 and TNF- $\alpha$ . Due to the increased expression of has\_circ\_0057950 under this study, we hypothesised that hsa-miR-5787 is downregulated, which stimulates fibroblast cell proliferation while boosting LPS/TLR4-mediated inflammatory response via NF- $\kappa$ B in macrophages, leading to increased release of inflammatory factors such as IL-6 and TNF- $\alpha$ . These actions may further exacerbate the progression in bronchopulmonary dysplasia, but more investigation is required.

To get a clear grasp of the interaction of the ceRNA network's target genes, we conducted function and pathway analysis to provide more underlying mechanisms of BPD. Gene Ontology (GO) terminology from three categories (GO: biological process, GO: cellular component, and GO: molecular function) was employed for route enrichment analysis and biological interpretation. According to GO analysis, these target genes were most enriched in the categories "negative control of transcription from the RNA polymerase II promoter" (GO:0000122) and "transcription DNA-templated" (GO:00006351). Non-coding RNAs are so numerous in eukaryotes as a significant regulator of the transcriptional process at multiple levels, function through controlling transcription factor occupancy and epigenetic modifications, to direct interference with RNAP II function 68, 69. Although transcription of a lncRNA may indirectly regulate nearby mRNA genes, either positively or negatively 69, these circRNAs were novel ones and different from previous results in this field. Nevertheless, the exact role and underlying mechanism are still in progress.

Additionally, we employed KEGG analysis to elucidate the function of the ceRNA network's target genes and acquired notable signalling pathways, some of which were congruent with the present understanding of BPD and others that were entirely novel. BPD formation and progression are modulated by diverse signalling pathways, including the p53 signalling pathways 31, TGF- $\beta$  signalling pathway 70, NF- $\kappa$ B

signalling 71 and MAPK signalling pathways 40. Both human and animal-derived tissues or cells are suitable for microarray and high-throughput sequencing of non-coding RNA expression patterns. More in-depth bioinformatics research has uncovered a complex network of molecular connections. For example, researchers once used deep Illumina sequencing to reveal variable expression of long non-coding RNAs in hyperoxia-induced bronchopulmonary dysplasia rat models. The result indicated that enriched KEGG terms are tied directly to ECM-receptor interaction, the cell cycle, and cytokine-cytokine receptor interaction 38. Moreover, significant pathways enriched in VEGF signalling, vascular smooth muscle contraction, thyroid hormone signalling, platelet activation, and oxytocin signalling were identified in another hyperoxia-induced bronchopulmonary dysplasia rat model 72, 73. The authors of another study applied RNA deep sequencing and bioinformatics analysis to identify circRNAs that were substantially changed by HCMV productive infection in human embryonic lung fibroblasts (HELFL). According to their investigations, the host genes of different circRNAs were linked to focal adhesion, ECM-receptor interaction, amoebiasis, and the PI3K-Akt signalling pathway 74. The circRNA expression patterns from the peripheral blood of BPD patients and normal preterms were obtained through bioinformatics, conforming critical metabolic processes including ECM-receptor interactions, protein digestion and absorption, RNA transport, and bacterial invasion of epithelial cells 49.

In our study, the KEGG pathway analysis revealed several lung developments, aberrant injury, and repair-associated pathways that have recently been confirmed 75, 76. Hippo, bacterial invasion of epithelial cells, cGMP-PKG, and MAPK are all inextricably linked with the pathogenesis of BPD 77. For instance, the Hippo signalling pathway, crucial for embryonic lung maturation and postnatal airway homeostasis, could lose function due to lung injury 78. Also, in complicated pregnancies, including preeclampsia (PE) and chorioamnionitis (CA) 79, a postal bacterial infection of the lungs may increase the chance of preterm newborns developing bronchopulmonary dysplasia (BPD) 80, 81.

Furthermore, we observed another signalling pathway that may play an essential role in lung agenesis, namely the Wnt signalling pathway. The Wnt gene family contains 19 glycoproteins interacting with cell surface receptors 82, 83. Canonical Wnt/ $\beta$ -catenin signalling pathway, which initially appears in the anterior foregut region, forms the trachea and two lung buds (E9.5). As a result, mice lacking endoderm-specific catenin expression or Wnt2/2b<sup>-/-</sup> mutants have an increased risk of lung agenesis 84, 85. Inactivation of  $\beta$ -catenin at the epithelial level leads to decreased branching and anomalous proximal-distal patterning 86, 87. As an additional bonus, non-canonical Wnt pathways govern stem cell maintenance and modulate activating protein kinase C and CaMKII signalling pathways 88. Conversely, the inflammatory response plays another vital role in lung injury, and activation of the Wnt/catenin pathway in AEC2 leads to increased production of IL-1, which promotes the inflammatory pro-fibrotic response 89. These findings reveal that circRNAs may modulate molecular mechanisms of BPD, and in the future, more specific circRNAs could come into light function as prospective diagnostic and therapeutic targets.

The current research has certain limitations. To begin, differentially expressed circRNAs were found in a limited number of patients and should be confirmed in a larger cohort of patients with severe BPD.

Second, using bioinformatics methods, the roles of differentially expressed circRNAs were hypothesised. We validated two circular RNAs using human embryonic lung epithelial cells and animal BPD models while collecting further clinical samples. Moreover, we received municipal funds (Item ID: SKJYD2021112) to support our future projects. In short, our future research project will focus on the functional impact of their interplay.

## Conclusion

To summarise, our results demonstrated that abnormalities in lung development and damage arise early after the birth of preterm infants and inevitably result in a bunch of differentially expressed circRNAs. Transcriptional control and post-transcriptional regulation may both benefit from these new circRNAs. The specific biological activities, signalling routes, and molecular processes of these unique circRNAs need to be further explored, even though most of these circRNAs have been confirmed in BPD development.

## Declarations

Ethics approval and consent to participate

On March 23rd, 2019, the ethical office of the Suzhou Municipal Hospital authorised this research with reference number F201936. Both parents were fully informed and signed an informed consent form before collecting peripheral blood and the microchip test. The waiver will have no harmful effect on the patients' rights or welfare, and all patients' data will be kept anonymous. The authors affirm that this research was done in line with the 2013 revision of the Helsinki Declaration.

Consent for publication

All informed consent forms have been completed.

Availability of data and materials

Due to the sensitivity of human data, the data that support the findings of this study are not publicly available, but are available from the corresponding author upon reasonable request.

Competing interests

For this study, Yang Zuming was fortunate to get financial assistance from the Jiangsu Commission of Health and Yu Lun got fund from the Suzhou Commission of health. Yu Lun and Yang Zuming have no conflicts of interest to declare.

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## Authors' contributions

Yu Lun and Yang Zuming made equal contributions to the work; they conceived the research, gathered and analysed data, and wrote the report. The final paper has been reviewed and approved by all the writers.

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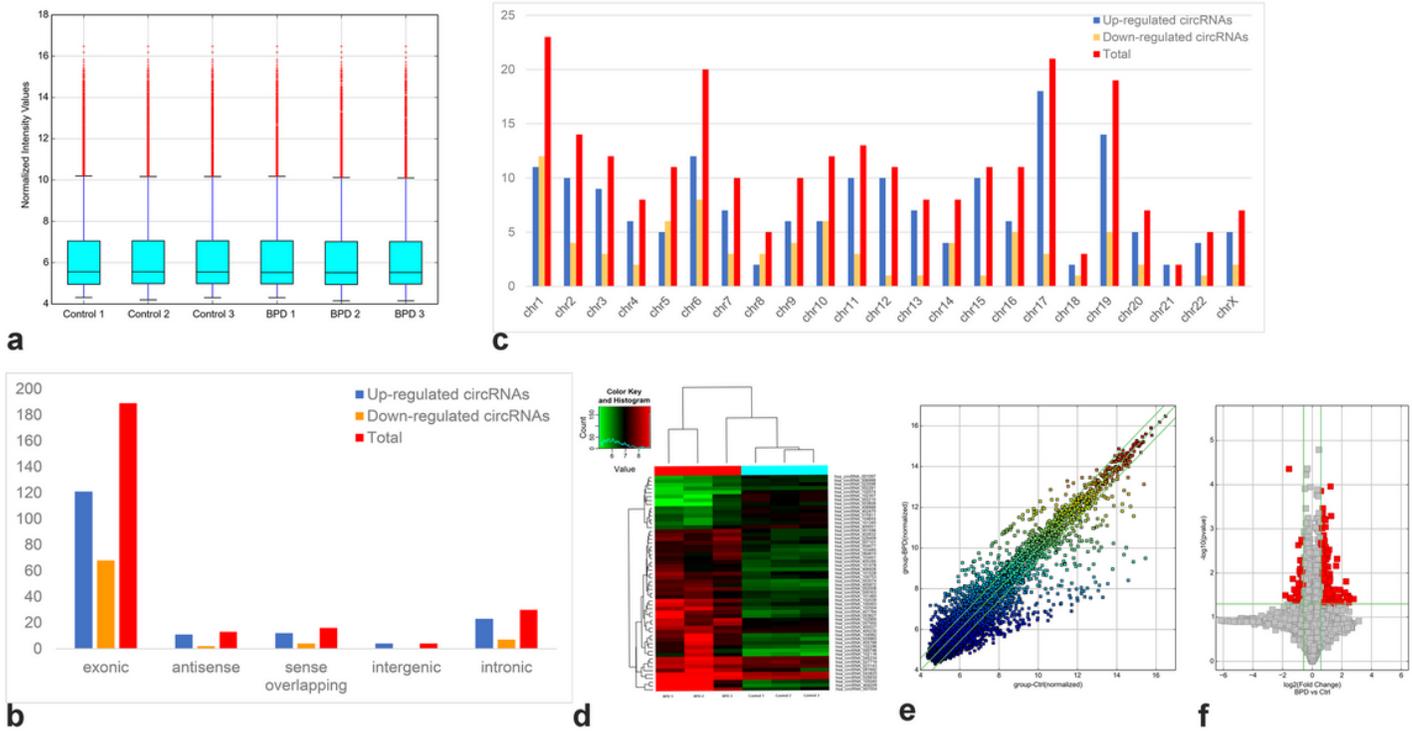
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## Figures



**Figure 1**

1a: Box plot. Six blood samples were used in this experiment, and the Y-axis shows the normalised intensity levels for each sample. In the databases, all six samples were standardised. All samples had an equal distribution of circRNAs.

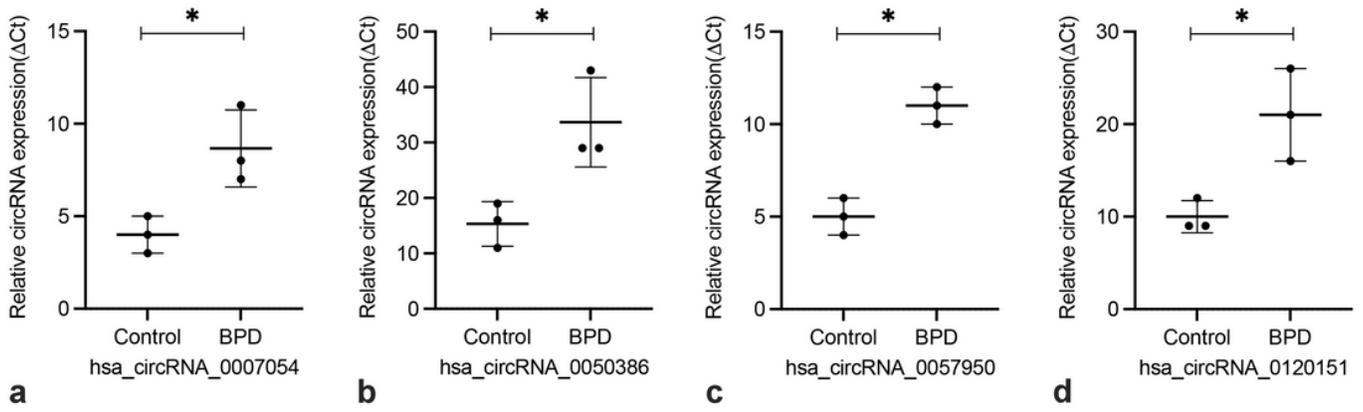
1b: Shows the five kinds of circRNAs that have been identified. Exonic circRNA is circRNA derived from the exons of the linear transcript; Intronic circRNA is circRNA that originates from an intron of the linear transcript; Antisense circRNA is circRNA derived from the same gene locus as the linear RNA but transcribed from the opposite strand, and Intergenic circRNA is circRNA that arises from a gene locus other than the linear transcript.

1c: chrDistribution of CircRNA. The differently expressed circRNAs' chromosomal distributions.

1d: CircRNAs with differential expression between the two groups were analysed via hierarchical clustering.

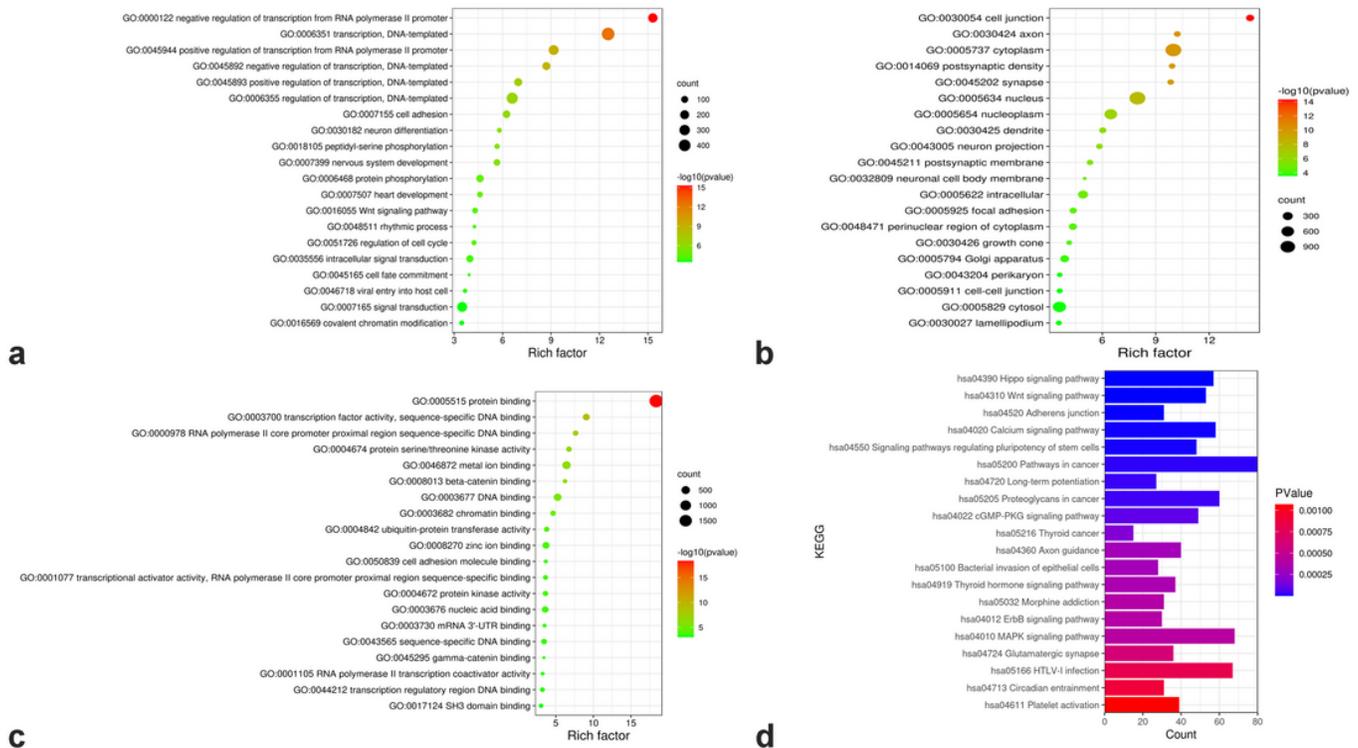
1e: A scatter plot was employed to compare the amounts of circRNA expression between control and BPD samples (log2 scaled).

1f: Fold-change data and p-values generated the volcano figure. The circRNAs of statistical significance was shown with the red dots.



**Figure 2**

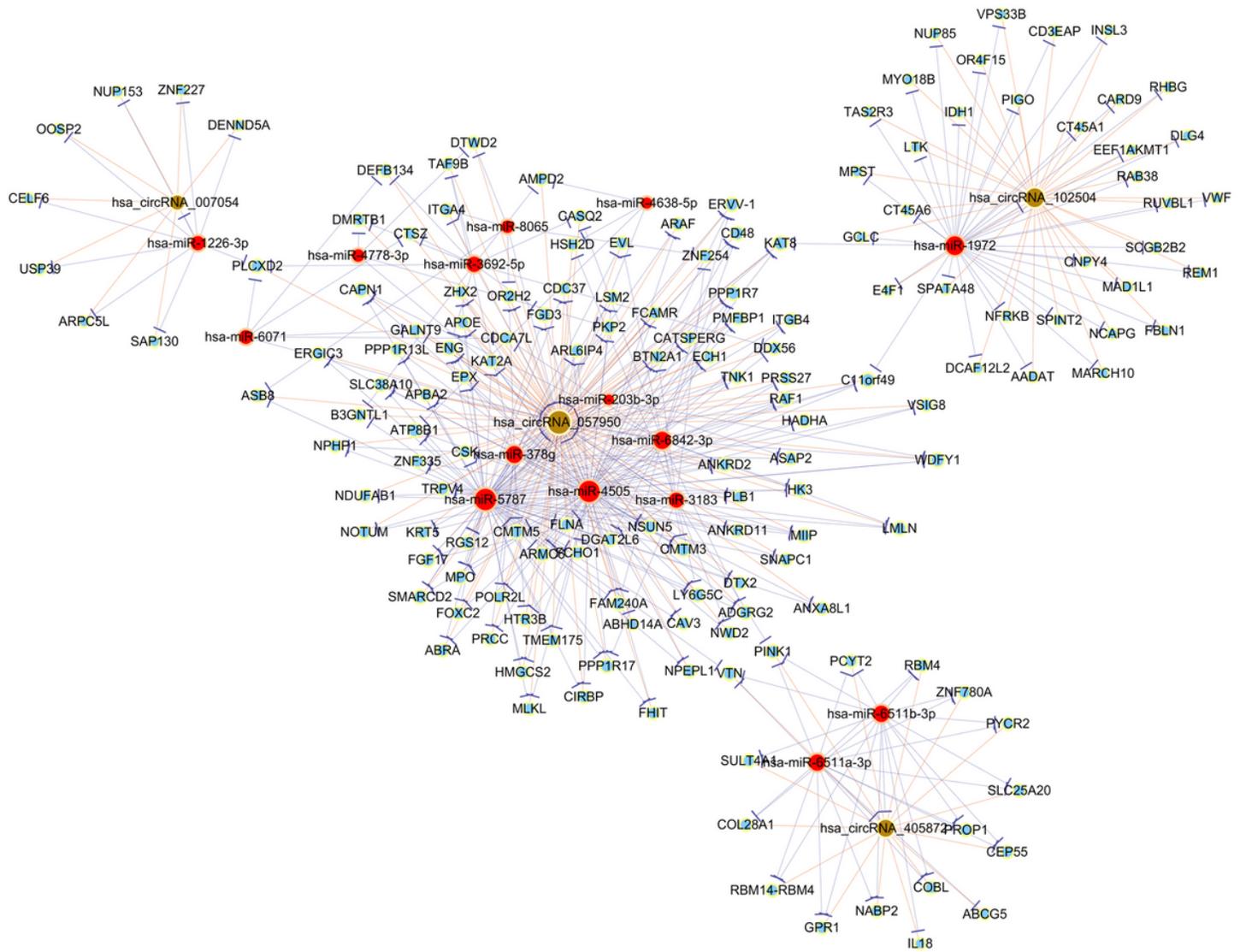
qPCR was used to quantify the expression levels of the circRNAs of interest. Gene expression was assessed using  $\Delta C_t$  values, which were then normalised to  $\beta$ -actin levels of expression. Means  $\pm$  SEM was presented in the table. \* P < 0.05.



**Figure 3**

a, b and c: GO enrichment analysis for the target genes of the four validated circRNAs.

d: KEGG pathway analysis for the target genes of the four validated circRNAs.



**Figure 4**

With the four differently expressed circRNAs, a ceRNA network was built.