Identification of a lncRNA-related Competing Endogenous RNA Network in Recurrent Implantation Failure

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Research Article

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Abstract

Background: Impaired endometrial receptivity is supposed to be a major element leading to recurrent implantation failure (RIF). Numerous studies have identified that the lncRNAs-miRNAs-mRNAs regulation network functions in the generation of receptive uterus. Long non-coding RNAs could act as competing endogenous RNAs in the pathogenesis of RIF. However, our understanding of the underlying mechanism is still limited.

Results: Based on the RNA-Seq results, 617 DEmRNAs, 69 DElncRNAs and 107 DEmiRNAs were identified in the RIF group compared with the control group. To investigate the role of lncRNAs in RIF, we constructed a lncRNA related ceRNA network. A total of 3 lncRNAs, 8 miRNAs and 69 genes were identified. Above all, our study obtained 120 lncRNAs-miRNAs-mRNAs relationships in the ceRNA network. Among three hub lncRNAs, PART1 and PWRN1 were upregulated whereas PGM5P3-AS1 was downregulated in RIF endometrium. Meanwhile, three down-regulated miRNAs (hsa-miR-1207-5p, hsa-miR-134-5p, hsa-miR-1225-5p) and five up-regulated miRNAs (hsa-miR-30c-5p, hsa-miR-30b-5p, hsa-miR-145-5p, hsa-miR-21-5p, hsa-miR-196b-5p) were shown.

Conclusions: We constructed a lncRNA-related ceRNA network and identified three hub lncRNAs in recurrent implantation failure. The results may provide further understanding in the pathogenesis of RIF as well as potential diagnostic and therapeutic targets.

Introduction

Embryo implantation is a complex process requiring a favorable environment in the endometrium. During the process, trophoblasts in embryo adhere to endometrial epithelial cells and a synchronized dialogue is occurred between viable embryo and receptive endometrium [1]. Despite impressive progress in human assisted reproductive technology, numerous couples fail to conceive even after several attempts. Recurrent implantation failure (RIF) is defined as the absence of clinical pregnancy after placement of four good quality embryos in at least three fresh or frozen cycles, and in women under 40 years old [2,3]. Apparently, endometrial receptivity defects and desynchronization of embryo and endometrium represent an important contributor to RIF [4,5]. However, the underlying molecular changes in the endometrium remains unclear.

Long non-coding RNAs (lncRNAs) are characterized as a class of non-coding RNAs with more than 200 nucleotides in length, which can directly regulate expression of protein-coding mRNAs [6]. In recent years, increasing attention has been paid to the potential gene regulation function of lncRNAs. Several lncRNAs are identified as important components of regulatory networks in mammalian reproduction, including spermatogenesis, oocyte maturation, trophoblast cells migration, placenta development, and establishment of endometrial receptivity [6-9].

MiRNAs are defined as non-coding RNAs about 22 nucleotides longer, which can bind to 3’-UTR region of mRNAs and inhibit gene expression at post-transcriptional level [10]. MiRNAs are reported to function in

multiple biological processes, such as cell proliferation, differentiation and apoptosis [11,12]. In particular, Dong et al showed that miR-223-3p can suppress LIF expression and pinopode formation in mice endometrium, which lead to embryo implantation defects [13]. Li et al indicated that miR-429 and miR-451 may be pivotal in murine embryo implantation [14,15]. Our previous study revealed that miR-23a-3p could inhibit CUL-3 expression during embryo implantation, thus increase endometrial receptivity via the modulation of β-catenin ubiquitination [16]. Taken together, these studies propose that miRNAs play important role during embryo implantation.

The ceRNA hypothesis suppose that lncRNAs could function as competitive endogenous RNA, which interact with miRNA via miRNA response elements (MRE) of target gene [17]. Although increasing experimental evidences confirmed the theory [18-20], studies related to RIF are still limited. The present study was aimed to construct a RIF related lncRNA-miRNA-mRNA network and provide new insights in the pathogenesis of RIF.

Results

Identification of DEGs, DElncRs, and DEmiRs

After data preprocessing, a total of 617 DEGs were identified, of which 381 genes were significantly up-regulated and 236 genes were significantly down-regulated. 69 DElncRs were identified, which were made up of 35 significantly up-regulated lncRNAs and 34 significantly down-regulated lncRNAs. Additionally, the differential expression analysis identified 107 DEmiRs, including 66 significantly up-regulated miRNAs and 34 significantly down-regulated miRNAs. Heatmaps and volcano plots representing the expression of DEGs, DElncRs, and DEmiRs were shown in Fig.1.

Functional enrichment analysis of DEGs

Gene ontology (GO) and KEGG pathway enrichment analysis were performed and the biological processes and pathways regulated by DEGs were displayed in Fig.2.up-regulated genes enriched in regulation of transcription, DNA-templated and morphogenesis of an epithelium, whereas down-regulated genes enriched in cellular response to retinoic acid and canonical Wnt signaling pathway. The enriched pathways included fatty acid degradation and inositol phosphate metabolism.

Construction of PPI network

The PPI network was constructed with the PPI pairs of proteins encoded by DEGs. 172 codes and 298 interaction pairs were involved in the network. Cytoscape software was used to visualize the constructed network as shown in Fig.3. Four high degree hub genes (GNG11, BDKRB2, PLCB1, THBS1) were identified.

Functional enrichment analysis of DElncRs and DEGs

According to the co-expression analysis, 944 co-expression DElncRs-DEGs pairs involved 54 lncRNAs and 341 mRNAs were obtained. Of which 596 pairs were positive correlation. lncRNA functional enrichment analysis was performed based on the co-expression mRNAs. The results of KEGG pathway analysis were shown in Fig.4.

Prediction of DEmiRs target genes

Predictions using miRWalk2.0 revealed a total of 121 pairs of DEmiRs-mRNA interactions. The DEmiRs-mRNA regulation network was shown in Fig.5, consisting of 12 miRNAs and 77 mRNAs. The KEGG pathways enriched were represented in Fig.6.

ceRNA network construction

The ceRNA network was displayed in Fig.7. A total of 120 relationships were obtained, including 8 miRNAs, 3 lncRNAs and 69 genes. Three hub lncRNAs were PART1, PWRN1 and PGM5P3-AS1. Three down-regulated miRNAs (hsa-miR-1207-5p, hsa-miR-134-5p, hsa-miR-1225-5p) and five up-regulated miRNAs (hsa-miR-30c-5p, hsa-miR-30b-5p, hsa-miR-145-5p, hsa-miR-21-5p, hsa-miR-196b-5p) were shown.

Discussion

Although the clinical pregnancy rate of in vitro fertilization-embryo transfer (IVF-ET) has reached approximately 50-60%, a portion of couples still suffer from recurrent implantation failure. Studies have demonstrated the pivotal role of lncRNAs in embryo implantation in mammals [7,9,20]. Based on the RNA-Seq results that have been obtained, we constructed a RIF related ceRNA network to propose potential diagnostic biomarkers or therapeutic targets. In total, 617 DEmRNAs, 69 DElncRNAs and 107 DEmiRNAs were identified, among which 381 mRNAs, 35 lncRNAs, and 66 miRNAs were upregulated, and 236 mRNAs, 34 lncRNAs, and 34 miRNAs were downregulated.

In general, the GO and KEGG analyses revealed that up-regulated genes enriched in regulation of transcription, DNA-templated and morphogenesis of an epithelium. Meanwhile, down-regulated genes enriched in cellular response to retinoic acid and canonical Wnt signaling pathway. The enriched pathways included fatty acid degradation and inositol phosphate metabolism. These biological events were involved in embryo implantation and identified by previous studies [21]. The canonical Wnt signaling pathway, known as Wnt/β-catenin signaling pathway, is identified as a major signaling branch involved in the maintenance of endometrial receptivity [22]. Moreover, the fatty acid degradation pathway is confirmed to be essential in endometrial stroma cell decidualization [23,24]. Inositol phosphate metabolism pathway may play an important role in regulating the estrogen-dependent activation of

cellular events in the rat endometrium [25]. Besides, the significant enrichment pathways of DElncRNAs were Inositol phosphate metabolism and Phosphatidylinositol signaling system, suggesting that inositol phosphate related pathways might be pivotal in the pathogenesis of RIF.

In addition, the PPI network was constructed and four high degree hub genes (GNG11, BDKRB2, PLCB1, THBS1) were identified. GNG11, also known as G protein subunit gamma 11, is an important component of the transmembrane signaling system [26]. Ayusawa and colleagues has revealed that GNG11 modulates cellular senescence in normal human fibroblasts by oxidative stress [27]. Their further study showed that increased GNG11 expression can induce reactive oxygen species (ROS) generation and abnormal nuclear morphology, thus inhibiting cell growth in SUSM-1 cells [28]. Previous studies have demonstrated that oxidative stress and reactive oxygen species system is crucial in maintenance of endometrial receptivity [29,30]. Taken together, GNG11 may be a potential regulator in acquiring of endometrial receptivity. Bradykinin (BK) is a vasoactive peptide which participates in a variety of biological processes [31-33]. There are two subtypes of bradykinin receptor, BDKRB1 and BDKRB2, of which BDKRB2 has a high affinity with bradykinin. BDKRB2 has been reported to be involved in the epithelial-mesenchymal transition (EMT) process which is critical for multiple endometrial functions [34]. PLCB1 has been identified to be essential for uterine preparation during embryo implantation and impaired expression of PLCB1 lead to embryo implantation failure in mice [23]. Ramhorst et al displayed that vasoactive intestinal peptide (VIP) induces endometrial stromal cells decidualization and promotes angiogenesis by down-regulating thrombospondin-1 (THBS1) [35].

To investigate the role of lncRNAs in RIF, we constructed a lncRNA related ceRNA network. Three hub lncRNAs were PART1, PWRN1 and PGM5P3-AS1. PART1 and PWRN1 were confirmed to influence cell proliferation, apoptosis, migration and invasion in many malignant tumors [36,37]. These basic biological and pathological processes have also been proved to affect endometrial receptivity. Meanwhile, a study using bioinformatics approaches proposed that PGM5P3-AS1 may be associated with the progression of hepatocellular carcinoma [38]. Furthermore, present study indicated three down-regulated miRNAs (hsa-miR-1207-5p, hsa-miR-134-5p, hsa-miR-1225-5p) and five up-regulated miRNAs (hsa-miR-30c-5p, hsa-miR-30b-5p, hsa-miR-145-5p, hsa-miR-21-5p, hsa-miR-196b-5p) in this ceRNA network. Hsa-miR-145 overexpression was found during the decidualization period in endometrium of recurrent implantation failure patients. Overexpressed hsa-miR-145 suppresses endometrial stromal cells decidualization through inhibition of Smad1 [39]. Moreover, hsa-miR-145 affects embryo attachment by reducing the expression of IGF1R and PAI-1 in endometrial epithelial cells [40,41]. Additionally, miR-21 is highly expressed in mouse endometrial stromal cells at implantation sites and plays a key role in embryo implantation via regulating the target gene RECK [10]. Referring to adenomyosis, overexpressed miR-21 can resume impaired decidualization through modulation of KLF12 and NR4A1 expression [42]. Besides, the high level of miR-30 family in endometrial epithelial cells is essential for establishment of endometrial receptivity [43]. These hub lncRNAs and miRNAs may regulate the development of RIF, whereas further investigations are still needed to validate the underlying mechanisms.

Above all, our study obtained 120 lncRNAs-miRNAs-mRNAs relationships in the ceRNA network. Numerous studies have identified that the lncRNAs-miRNAs-mRNAs regulation network functions in the pathogenesis of RIF and generation of receptive uterus [44-48]. The ceRNA network deserves more attention since it could contribute more to our understanding of embryo implantation. However, given that these results proposed in the study are only based on bioinformatic methods, in vitro experiments should be established to validate potential diagnostic and therapeutic targets for RIF.

Conclusions

In the present study, we constructed a lncRNA-related ceRNA network and identified three hub lncRNAs in recurrent implantation failure. The results may provide further understanding in the pathogenesis of RIF as well as potential diagnostic and therapeutic targets. However, further studies should be conducted to validate the function of above lncRNAs.

Materials And Methods

Acquisition of microarray data

In this study, lncRNA-mRNA and miRNA microarray datasets were downloaded from the public Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/). The accession number was GSE71331 and GSE71332, respectively. GSE71331 was conducted through GPL19072 platforms (Agilent-052909 CBC_lncRNAmRNA_V3). GSE71332 was conducted through GPL18402 (Agilent-046064 Unrestricted_Human_miRNA_V19.0_Microarray). Both microarray datasets contained profiles on the same set of samples, including seven RIF (recurrent implantation failure) samples and five normal control samples.

Data preprocessing and DEGs, DElncRs, and DEmiRs screening

Raw data were received and read into the R statistical environment (version 3.4.3, http://www.r-project.org), followed by data preprocessing using the Robust Multiarray Average (RMA) algorithm, including background adjustment, quantile normalization and finally summarization□log base 2 scale.

The differential expression of mRNAs, lncRNAs, and miRNAs was analyzed using the Linear Models for Microarray (limma) package (version 3.34.9, http://bioconductor.org/packages/release/bioc/html/limma.html). The false discovery rate (FDR) was applied for multiple testing corrections based on the Benjamini and Hochberg method. Only RNAs with *P*-values < 0.05 and $|\log_2 FC(\text{fold change})| > 0.585$ were considered as differentially expressed genes (DEGs), lncRNAs (DElncRs), or miRNAs (DEmiRs).

Functional enrichment analysis

Functional enrichment analysis was performed with the commonly used DAVID (version 6.8, https://david.ncifcrf.gov/). Gene ontology (GO) and KEGG pathway enrichment analysis were conducted on the up- and down-regulated genes to identify the significantly overrepresented biological functional categories. *P*-Value < 0.05 and gene count ≥ 2 were chosen as the threshold.

PPI network of the DEGs

The STRING database (version 10.5, https://string-db.org) was used to predict the PPI pairs of proteins encoded by DEGs. The parameter of PPI score was set as 0.9 (indicating highest confidence). The PPI network was illustrated by Cytoscape software (version 3.6.1, http://www.cytoscape.org/).

Pathway enrichment analysis of DElncRs and DEGs

The co-expression relationships between the DElncRs and DEGs were calculated using Pearson correlation coefficients. The threshold was set as |r| >0.9 and adjust *P* value < 0.05. KEGG functional enrichment analysis of lncRNAs involved in the DElncR-DEG interaction was performed using R clusterProfiler package. Benjamini–Hochberg (BH) adjusted *P*-value < 0.05 was chosen as the threshold.

Prediction of DEmiRs target genes

miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/miRretsys-self.html) was applied to predict the target genes of the DEmiRs. The miRNA-target gene interaction pairs from at least 6 of the 7 commonly used databases (miRWalk, Microt4, miRanda, mirbridge, PITA, RNA22, Targetscan) were collected. The miRNA-gene regulatory network was constructed using Cytoscape software. The KEGG pathway enrichment analysis of the miRNAs involved in the regulatory pairs was performed through R clusterProfiler package. Benjamini–Hochberg (BH) adjusted *P*-value < 0.05 was chosen as the threshold.

Construction of ceRNA network

Prediction module of DIANA-LncBase v.2 database (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex) was used to obtain DEmiRs-lncRNA interaction pairs with screening score > 0.75. Based on the predicted DEmiRs-lncRNA interaction pairs, DEmiRs-mRNA interaction pairs and DElncRs-DEGs positive regulatory relationships obtained above, a comprehensive lncRNA-miRNA-mRNA network (ceRNA network) was constructed.

Abbreviations

BK: Bradykinin; ceRNA: Competing endogenous RNA; DAVID: Database for Annotation, Visualization and Integrated Discovery; DElncRs: Differentially expressed long non-coding RNAs; DEmiRs: Differentially expressed microRNAs; DEGs: Differentially expressed Genes; FDR: false discovery rate ; GEO: Gene Expression Omnibus; GO: Gene Ontology; IVF-ET: In vitro fertilization-embryo transfer; LncRNA: Long non-coding RNA; mRNA: Messenger RNA; miRNA: MicroRNA; RIF: Recurrent implantation failure; ROS: reactive oxygen species; VIP: vasoactive intestinal peptide;

Declarations

Acknowledgements

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Conflict of Interest

The authors declare no conflicts of interest.

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Consent for Publication

Not applicable

Availability of Data and Materials

Not applicable

Authors Contribution

GC and KH: methodology. YS: software. GC: supervision. KH and YS: writing—original draft preparation.KH: writing—review and editing.

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Figures

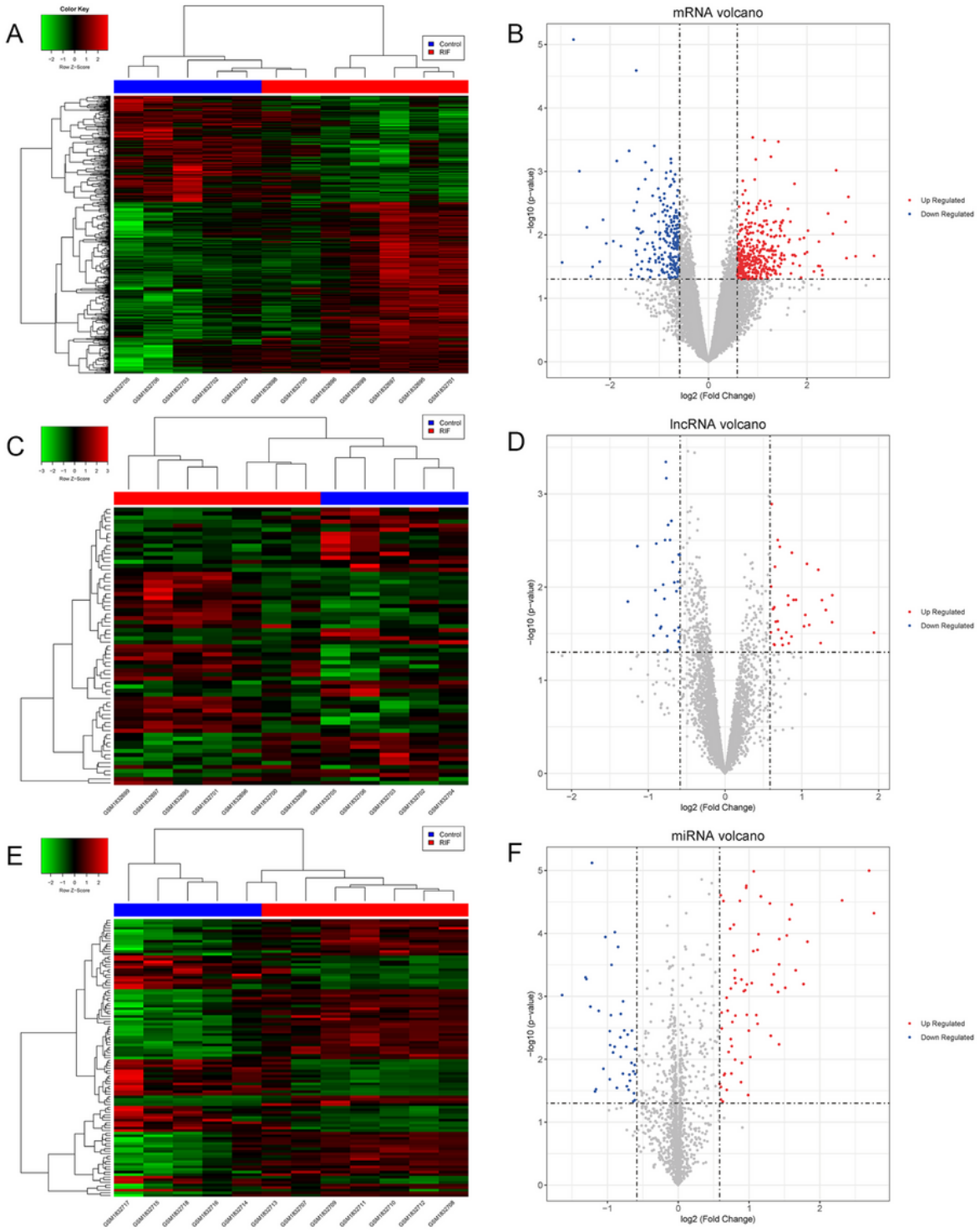

Figure 1

RNA-Seq results of samples from patients with repeated implantation failure (RIF) and controls. (A, C, E) Hierarchical clustering of differentially expressed mRNAs (DEGs), lncRNAs (DELncRs), and miRNAs (DEmiRs). RIF groups (n = 7) and Control groups (n = 5) clustered in different groups. (B, D, F) The volcano plot of all expressed mRNAs, lncRNAs, and miRNAs. Up- and down-regulated DEGs with $P < 0.05$ and FC > 2 and < 0.5 are highlighted in dark red and dark green, respectively.

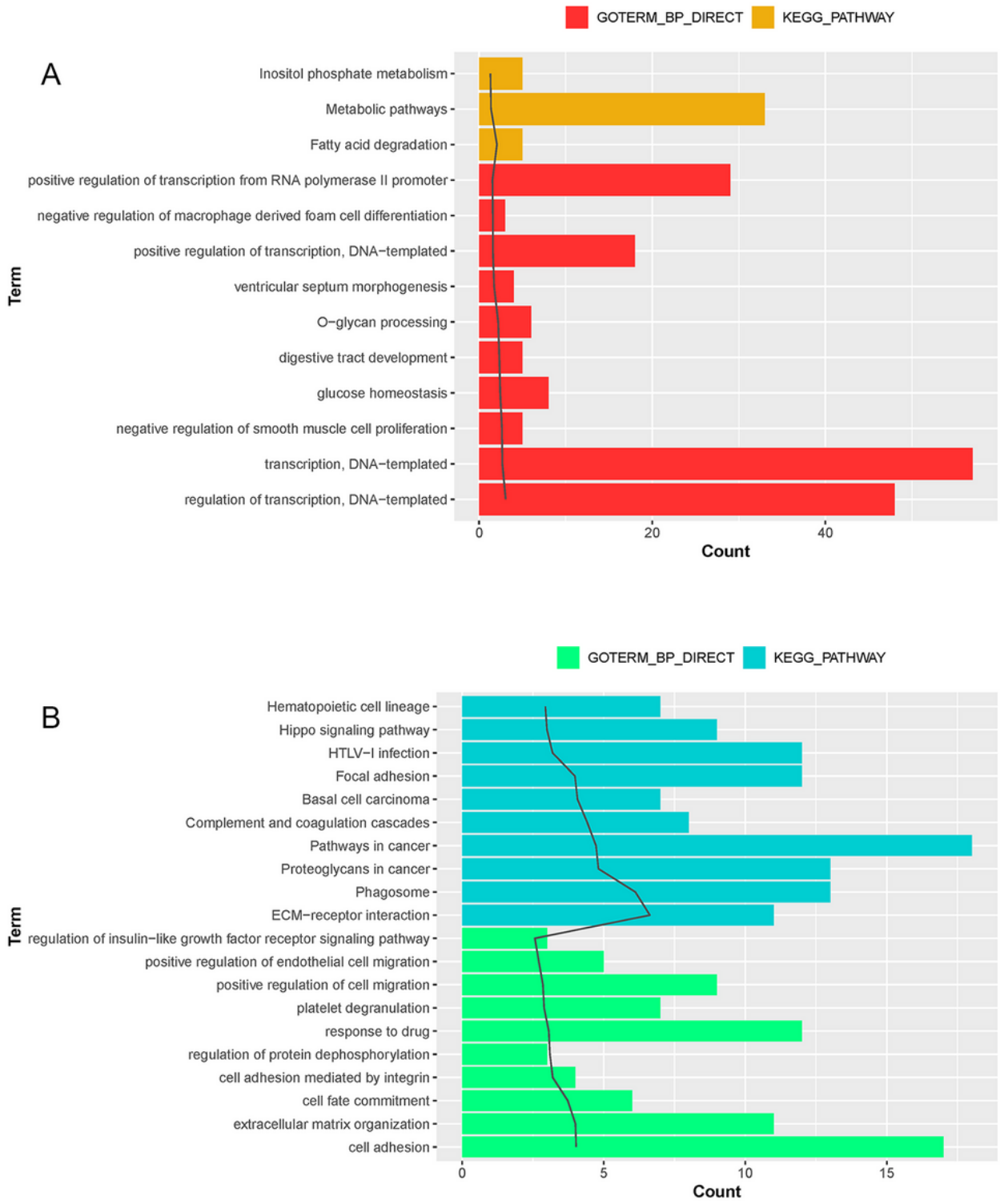

Figure 2

GO and KEGG pathway enrichment of DEGs. (A) Enrichment of up-regulated DEGs. (B) Enrichment of down-regulated DEGs. The horizontal axis is the number of enriched genes, and the vertical axis is the term of GO and KEGG pathway.The grey line represents the P value as the significance threshold.

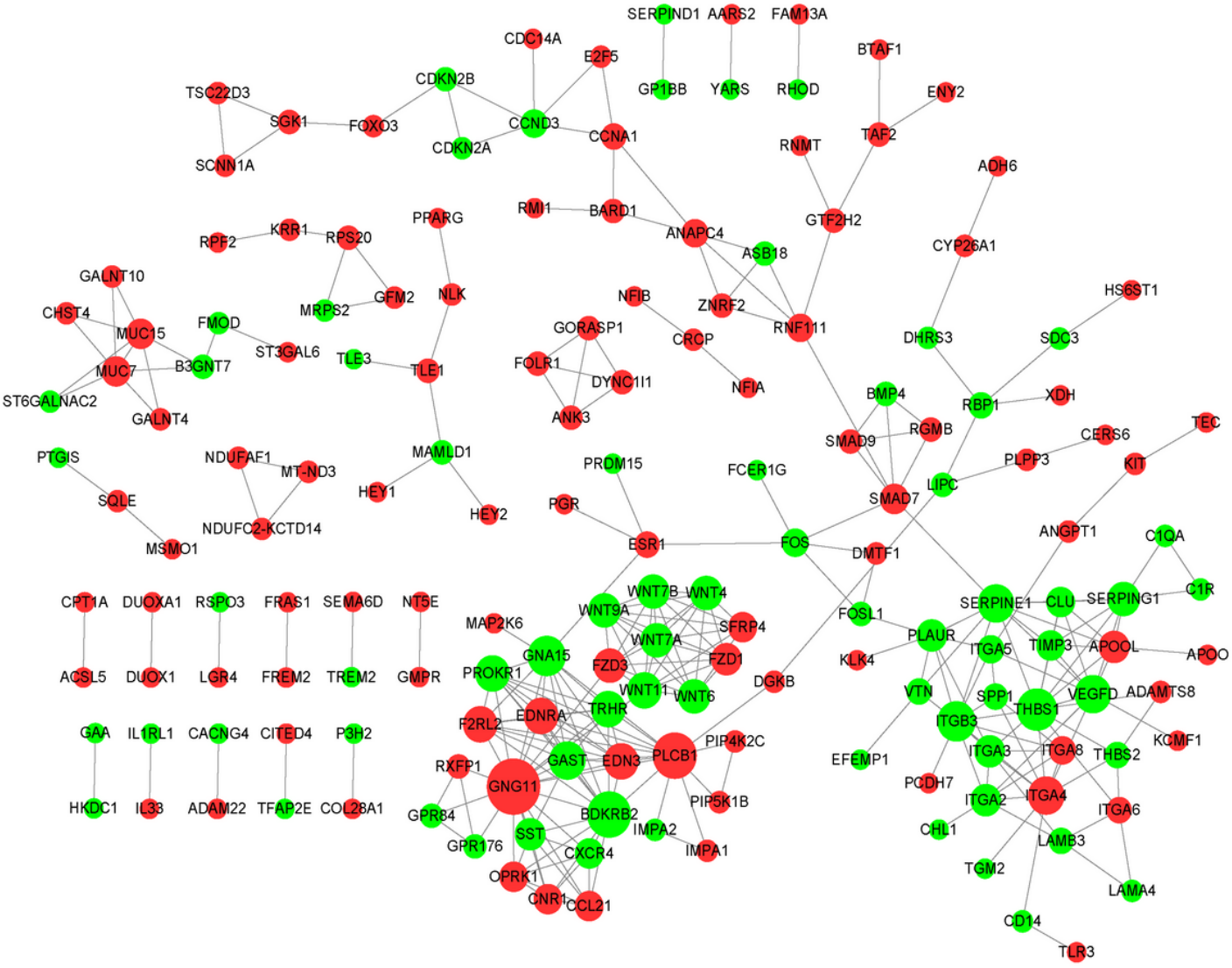

Figure 3

The PPI network of DEGs. Red represents up-regulated DEGs, green represents down-regulated DEGs, node size represents degree value.

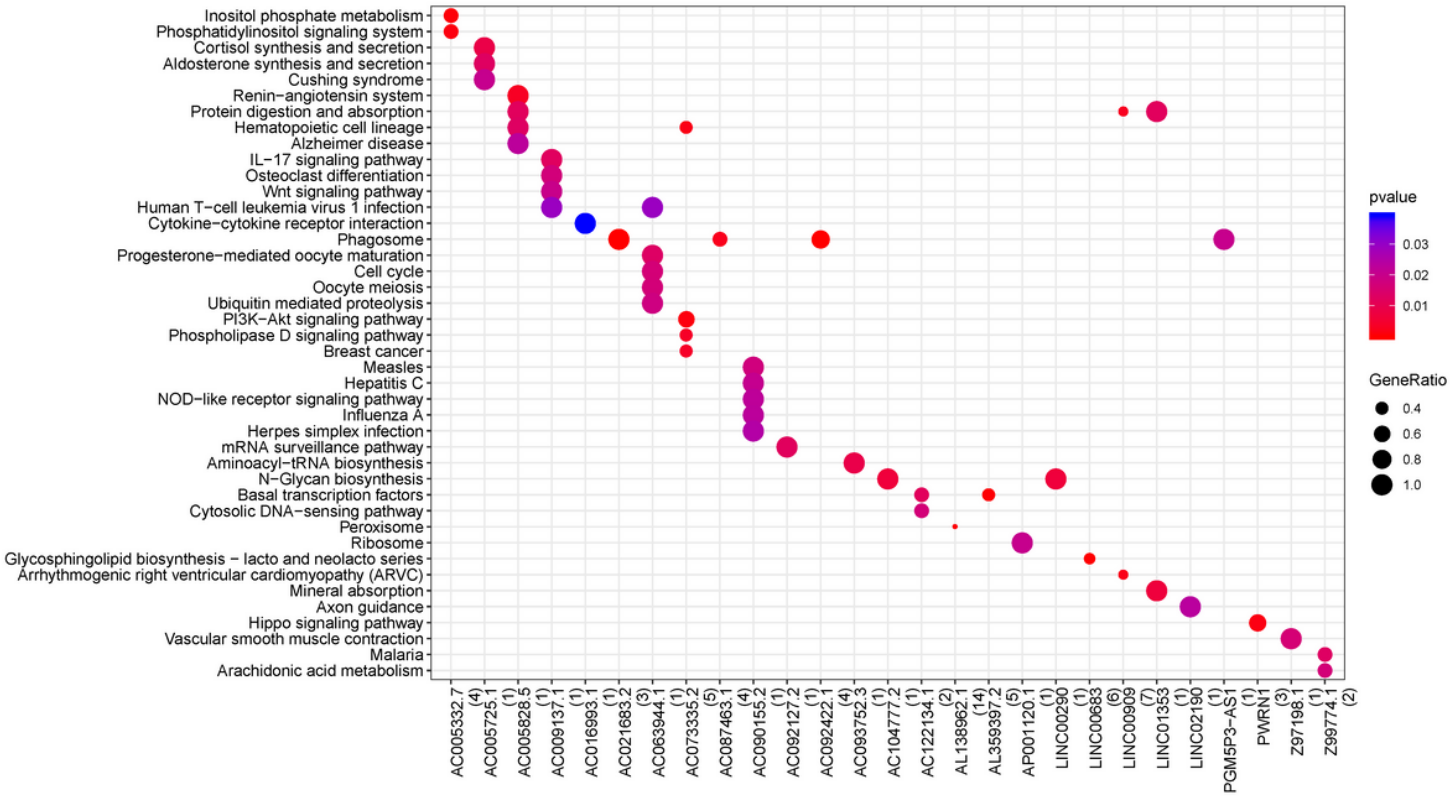

Figure 4

Bubble map of lncRNA KEGG pathway enrichment. The horizontal axis represents the differentially correlated lncRNAs, the vertical axis represents the significantly correlated KEGG pathway names, and the change of bubble color from blue to red indicates the correlation significance from low to high. The dot size represents GeneRatio.

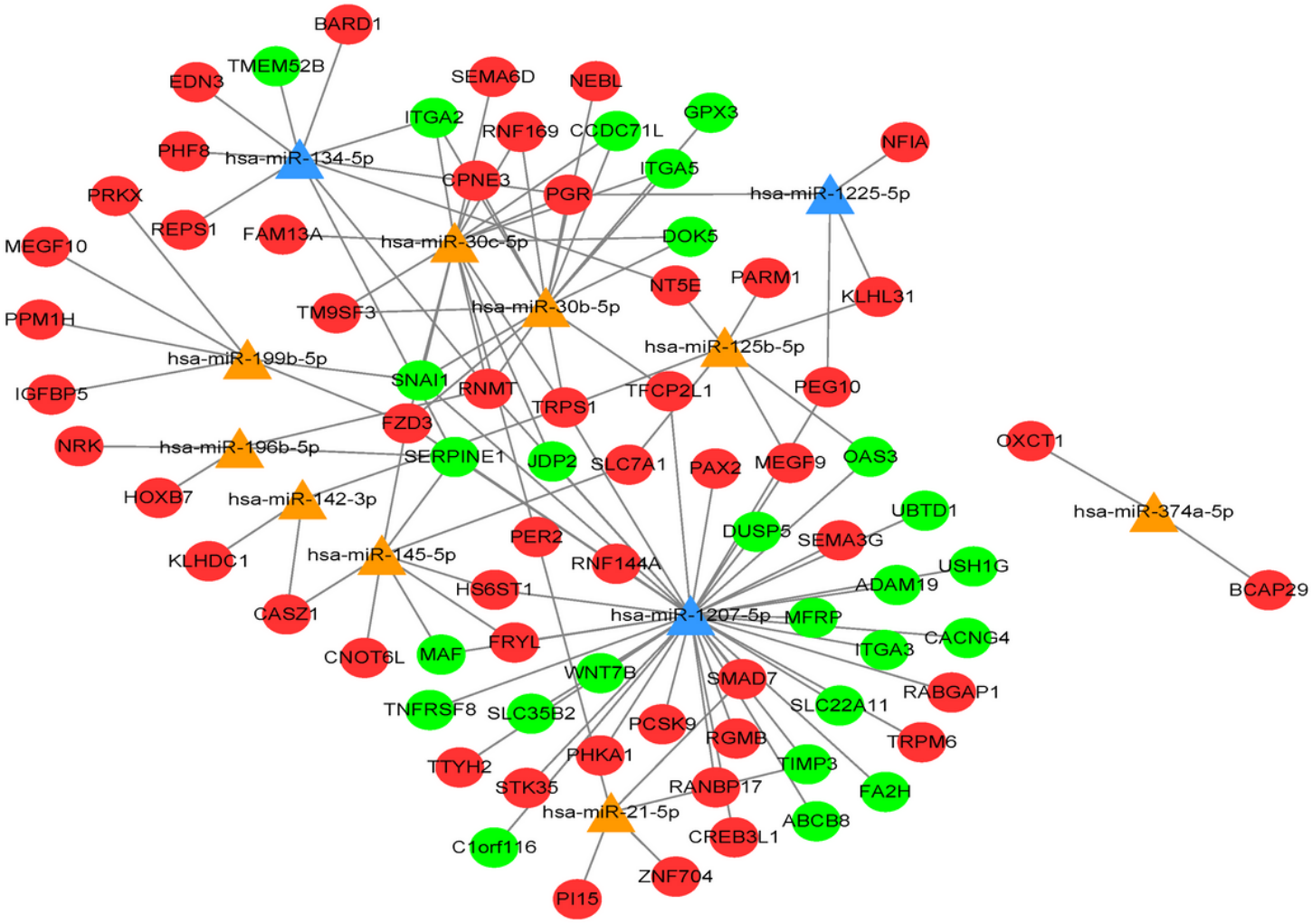

Figure 5

The miRNA-mRNA regulation network. The red circle represents the up-regulated DEGs, the green circle represents the down-regulated DEGs, the orange triangle represents the up-regulated miRNAs, and the blue triangle represents the down-regulated miRNAs.

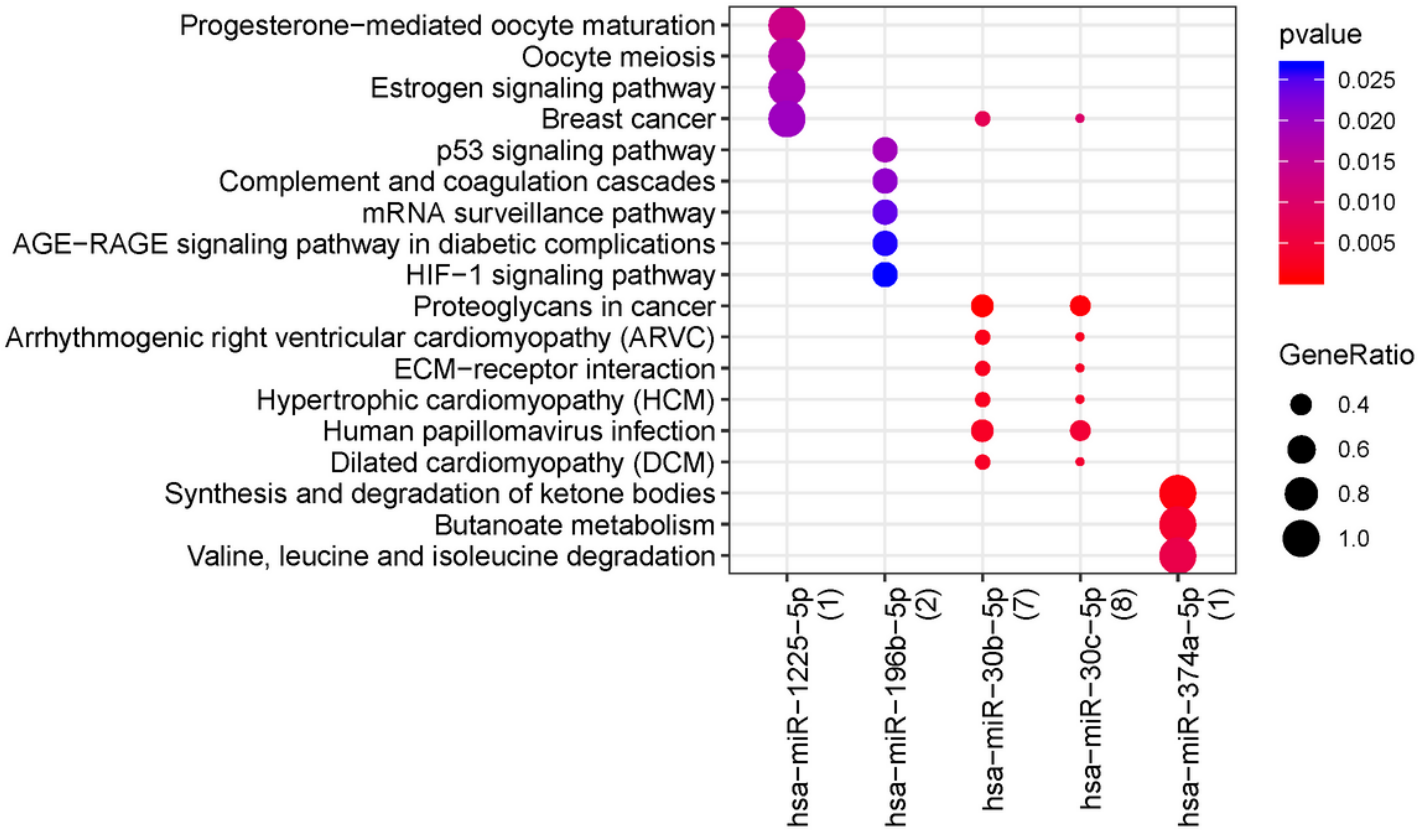

Figure 6

Bubble map of miRNA KEGG pathway enrichment. The horizontal axis represents the differentially correlated miRNAs, the vertical axis represents the significantly correlated GO/KEGG pathway names. The change of bubble color from blue to red indicates the correlation significance from low to high. The dot size represents GeneRatio.

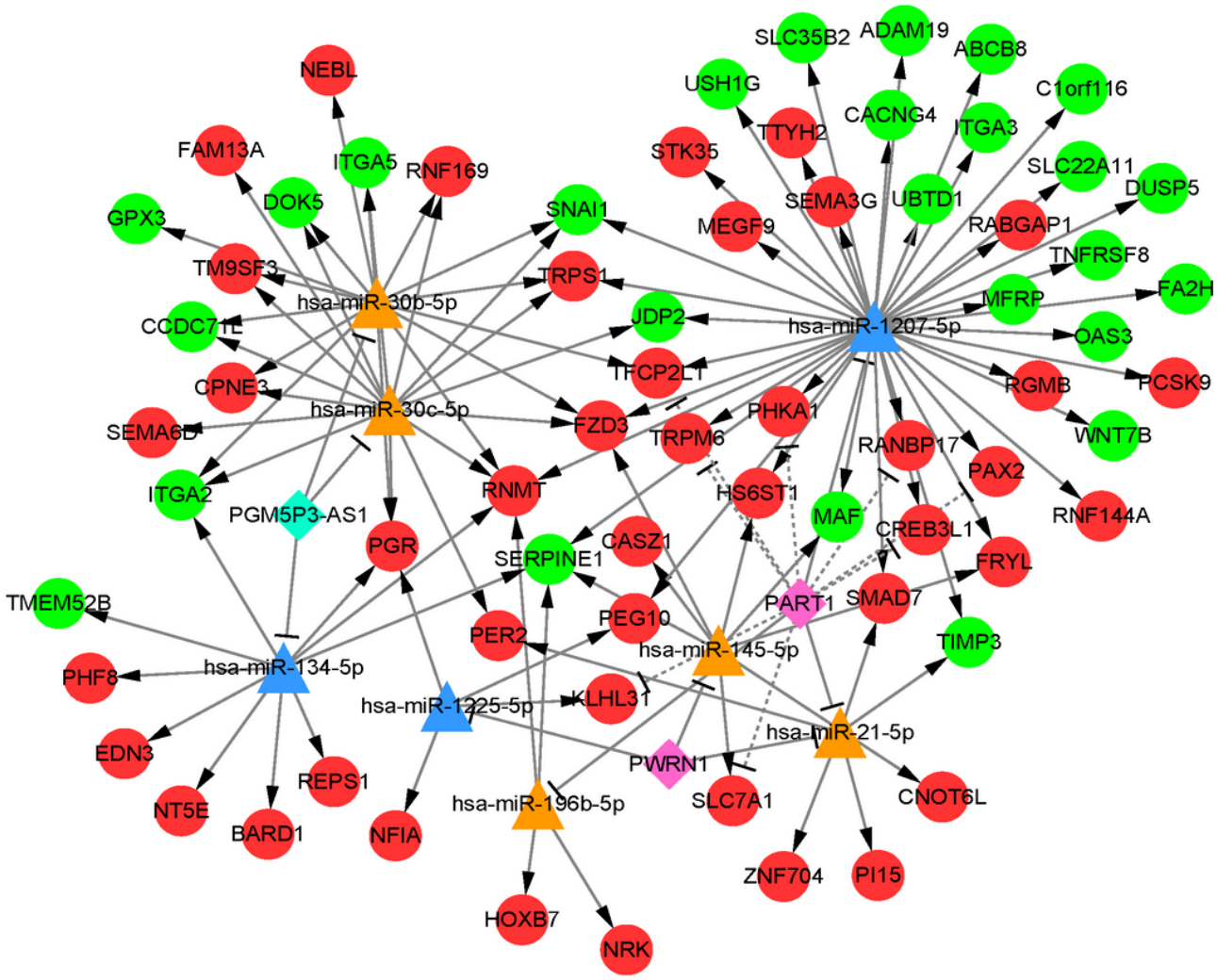

Figure 7

The ceRNA regulation network. The red circle represents the up-regulated DEGs, the green circle represents the down-regulated DEGs. The orange triangle represents the up-regulated miRNAs, the blue triangle represents the down-regulated miRNAs. The pink diamond represents the up-regulated lncRNAs, the bright blue diamond represents the down-regulated lncRNAs. The solid line represents the ceRNA regulatory relationship, and the dotted line represents the co-expression regulatory relationship. The arrow lines represent the regulation of miRNA-mRNA, and the T-shape represents the regulation of lncRNA-miRNA.