MicroRNA-mRNA regulatory network related to lipid metabolism in bovine mammary epithelial cells

Lixin Xia
Jilin University
Zhihui Zhao
Jilin University
Chunyan Lu
Jilin University
Ping Jiang
Guangdong Ocean University
Haibin Yu
Jilin University
Xiaohui Li
Jilin University
Xiang Yu
Jilin University
Juan Liu
Guangdong Ocean University
Xibi Fang
Jilin University
Runjun Yang (yrj@jlu.edu.cn)
Jilin University https://orcid.org/0000-0003-1401-4318

Research article

Keywords: Milk fat percentage, Bovine mammary epithelial cells, microRNA, Conjoint analysis, Dairy cattle

Posted Date: January 3rd, 2020

DOI: https://doi.org/10.21203/rs.2.20029/v1

Abstract

Milk fat percentage is an important factor of milk quality in dairy cattle. Functional microRNAs and genes can affect lipid synthesis and metabolism through differential expression in bovine mammary epithelial cells (BMECs). It is necessary to screening the crucial candidate gene and miRNA on milk fat percentage. In this study, we extract total RNA of BMECs isolated from Chinese Holstein cows with high and low milk fat percentages for the conjoint analysis of RNA-seq and Solexa sequencing data. 190 differentially expressed genes and 33 differentially expressed microRNAs (DERs) were enriched in 488 GO terms and 12 KEGG pathways significantly ($p < 0.05$) based on the conjoint analysis. The detection of triglyceride production in BMECs showed that bta-miR-21-3p and bta-miR-148a promote triglyceride synthesis, whereas bta-miR-124a, bta-miR-877, bta-miR-2382-5p and bta-miR-2425-5p inhibit triglyceride synthesis. Meanwhile, the target relationships between PDE4D and bta-miR-148a, PEG10 and bta-miR-877, SOD3 and bta-miR-2382-5p, and ADAMTS1 and bta-miR-2425-5p were verified using luciferase reporter assays and quantitative RT-PCR. The conjoint analysis can more accurately screen candidate regulator related to milk fat percentage at the molecular level, which provided a scientific research method screening functional miRNA and gene for the breeding of new high-quality dairy cows.

Background

Milk is rich in vitamins, fats, proteins and other nutrients, serving as an important nutrient in people's daily lives. Milk fat percentage is one of the most important factors affecting milk quality which mainly affected by heredity, feeding conditions, environment and other factors; thus, studying the regulation of milk fat metabolism is of great significance to improve the milk quality of dairy cattle, making it a hot and difficult subject in the research area of dairy cattle genetics and breeding[1, 2]. The mammary gland is composed of lactation tissue and connective tissue, has the physiological function of synthesizing and secreting milk in dairy cattle[3]. Bovine mammary epithelial cells (BMECs) cultured in vitro can still maintain the ability to synthesize and secrete milk and can be used as an experimental cell model. Understanding the mechanism of fat biosynthesis and metabolism in BMECs can provide a theoretical basis for further studies on the genetic improvement and breeding of new dairy cattle.

MicroRNA (miRNA) is a class of single-stranded RNA molecules encoded by endogenous genes with a length of approximately 22 nucleotides[4]. MiRNAs cannot encode proteins themselves, but they can regulate a variety of biological processes by recognizing the 3'-untranslated region (UTR) of downstream target genes to degrade the mRNA or obstruct protein translation[5, 6]. The differential expression of miRNAs in the mammary gland at different developmental stages of different cattle species indicates that it may play an important role in regulating fat deposition in milk[7, 8]. It has been found that most mRNAs in milk whey are present in exosomes, whereas miRNAs in milk whey are present in the supernatant as well as exosomes according to miRNA and mRNA microarray analyses of bovine raw milk and total RNAs purified from exosomes (prepared by ultracentrifugation) and ultracentrifuged supernatants[9]. To screen the regulatory factors that influence traits more accurately, the conjoint analysis of miRNAs and mRNAs can obtain candidate miRNAs and target genes more effectively to

clarify the regulatory mechanism of traits more clearly[10]. For example, to understand the molecular mechanism underlying intramuscular fat (IMF) deposition after castration in beef cattle, there was an investigation of differentially expressed miRNAs (DERs) and mRNAs in the IMF of bulls and steers in Qinchuan cattle using next-generation sequencing, which showed that 580 upregulated genes were mainly associated with lipid metabolism, lipogenesis, and fatty acid transportation signaling pathways, and 52 DERs were identified[11].

At present, there are article on the miRNA-mRNA regulatory network fine-tuning the porcine muscle fiber, but no artical on the conjoint analysis of functional genes and miRNAs related to milk fat metabolism in BMECs. Therefore, we extracted total RNA from BMECs with high and low milk fat percentages for a conjoint analysis of RNA-seq and Solexa sequencing data to screen candidate DERs and their target differentially expressed genes (DEGs) related to milk fat percentage and verify the regulatory mechanism of DERs on milk lipid metabolism to provide a molecular basis for future gene and miRNA studies of milk fat percentage and the marker-assisted selection of milk fat percentage trait.

Results

1. Functional DEGs on milk fat percentage screened by RNA-seq analysis

Among all expressed genes, a total of 17,771 genes were identified as differentially expressed in the BMECs of Chinese Holstein cows with high and low milk fat percentages, with 8505 upregulated DEGs and 9267 downregulated DEGs, of which 829 genes (525 upregulated DEGs and 304 downregulated DEGs) were significantly differentially expressed.

Functional enrichments of the DEGs by Gene Ontology (GO) analysis showed that 4132 GO terms were enriched, of which 2725, 397 and 1010 were enriched in biological process, cellular component and molecular function GO terms, respectively. A total of 531 biological process GO terms were significantly enriched ($p<0.05$), 54 cellular component GO terms were significantly enriched ($p<0.05$), and 134 molecular function GO terms were significantly enriched ($p<0.05$). The most significant GO terms are listed in ascending order of the p value (Fig 1A). To verify the relationship and connection of significant GO terms, a GO-Tree (Fig 1B) was constructed by exploring the regulatory networks of significant biological process GO terms ($p<0.01$), which suggested that most GO terms were upregulated, and the GO terms of the upregulated genes were related to the unsaturated fatty acid biosynthetic process and the fatty acid biosynthetic process, which are related to the regulation of lipid metabolism.

Functional enrichments on the DEGs by Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that 228 KEGG pathways were enriched, of which 45 KEGG pathways were significantly enriched ($p<0.05$). The most significant KEGG pathways are listed in ascending order of the p value (Fig 2A). A Pathway-Act-Network (Fig 2B) was constructed by analyzing the regulatory network of significant pathways of both the up- and downregulated genes ($p<0.05$), which suggested that most KEGG pathways were downregulated, and the downregulated genes are related to fat digestion and absorption and the P-53 signaling pathway.

To systematically study the regulatory relationship between different genes, all DEGs were used to construct an intergene signal transduction network, and the intergene interaction network was integrated to obtain the interaction network between genes (Fig 3), such as secreted frizzled related protein 1 (SFRP1) gene inhibits the expression of Wnt family member 5B (WNT5B), and SH3 domain containing GRB2 like 3, endophilin A3 (SH3GL3) gene binds fibroblast growth factor receptor 3 (FGFR3) gene.

2. Functional DERs on milk fat percentage screened by Solexa sequencing

Among all expressed miRNAs, a total of 347 miRNAs were identified as differentially expressed in the BMECs of Chinese Holstein cows with high and low milk fat percentages, with 166 upregulated DERs and 181 downregulated DERs, of which 40 miRNAs (17 upregulated DERs and 23 downregulated DERs) were significantly differentially expressed, The top 10 upregulated and downregulated DERs were listed in ascending order of the p value (Table 3).

All the target genes regulated by the DERs were predicted by the combination of the miRbase and the TargetScan. Functional enrichments by GO analysis showed that 2240 GO terms were enriched, of which 1513, 213 and 514 were enriched in biological process, cellular component and molecular function GO terms, respectively. A total of 386 biological process GO terms were significantly enriched ($p<0.05$), 45 cellular component GO terms were significantly enriched ($p<0.05$), and 120 molecular function GO terms were significantly enriched ($p<0.05$). The most significant GO terms are listed in ascending order of the p value (Fig 4A). Functional enrichments by KEGG analysis showed that 152 KEGG pathways were enriched, of which 21 KEGG pathways were significantly enriched ($p<0.05$). The most significant KEGG pathways are listed in ascending order of the p value (Fig 4B).

3. Functional DEGs and DERs on milk fat percentage screened by multi-omics Analysis

After the conjoint analysis of RNA-seq and Solexa sequencing data in BMECs with high and low milk fat percentages (The RNA-seq and Solexa sequencing data have been submitted to the GenBank databases under accession number GSE137488. Addresses are as follows: GenBank: www.ncbi.nlm.nih.gov/genbank), a total of 190 DEGs (159 upregulated DEGs and 31 downregulated DEGs) were screened, including thrombospondin 1 (THBS1), Kruppel-like factor 6 (KLF6), low-density lipoprotein receptor (LDLR), and aspartate beta-hydroxylase domain-containing 2 (ASPHD2), which are regulated by 33 DERs (11 miRNAs upregulated and 22 miRNAs downregulated), including bta-miR-127, bta-miR-222, bta-miR-193a and bta-miR-29b (Fig 5).

Different ontologies were used to depict molecular function, cellular component and biological process. Functional enrichments by GO analysis showed that 190 DEGs screened by the conjoint analysis were significantly enriched ($p<0.05$) in 488 GO terms, in which 359, 37 and 92 were significantly enriched in biological process, cellular component and molecular function GO terms, respectively. The most significant functional biological process GO terms included the response to glucocorticoid (GO:0051384) and positive regulation of the transforming growth factor beta receptor signaling pathway (GO:0030511); the most significant functional cellular component GO terms included the extracellular matrix

(GO:0031012) and cell surface (GO:0009986); the most significant functional molecular function GO terms included heparin binding (GO:0008201) and very-low-density lipoprotein particle receptor activity (GO:0030229) (Fig 6A).

The KEGG database contains information on molecular interactions and reaction networks. A pathway-based analysis would facilitate our understanding of the interactions between the target genes obtained from the conjoint analysis. Functional enrichments by KEGG analysis showed that 190 DEGs screened by the conjoint analysis were distributed in 119 KEGG pathways, in which 12 KEGG pathways were significantly enriched ($p<0.05$), such as phenylalanine metabolism (PATH:00360) and the cytokine-cytokine receptor interaction (PATH:04060). The top 15 pathways are listed in ascending order of the p value including tyrosine metabolism and fat digestion and absorption (Fig 6B).

4. The detection of triglyceride production regulated by miRNAs in BMECs

To confirm the effect of miRNAs on triglyceride content, we collected BMECs 48 h post-transfection with the mimics, inhibitor and negative control plasmids of 6 DERs. Compared with the negative control (Nc): triglyceride content was significantly increased in the miR-21-3p mimics group but significantly decreased in the miR-21-3p inhibitor group ($p<0.05$); there was no significant change in the miR-124a mimics group but a significant increase in the miR-124a inhibitor group ($p<0.05$); triglyceride content was significantly increased in the miR-148a mimics group but significantly decreased in the miR-148a inhibitor group ($p<0.05$); there was no significant change in the miR-877 mimics group but a significant increase in the miR-877 inhibitor group ($p<0.05$); triglyceride content was significantly decreased in the miR-2382-5p mimics group ($p<0.05$), but there was no significant change in the miR-2382-5p inhibitor group; and triglyceride content was significantly decreased in the miR-2425-5p mimics group but significantly increased in the miR-2425-5p inhibitor group ($p<0.05$) (Fig 7), suggesting that bta-miR-21-3p and bta-miR-148a promote triglyceride production, whereas bta-miR-124a, bta-miR-877, bta-miR-2382-5p and bta-miR-2425-5p inhibit triglyceride production.

5. Quantitative RT-PCR analysis of the DERs and their target DEGs

Compared with the negative control: the expression of the 6 DERs was significantly increased in the mimics group and significantly decreased in the inhibitor group ($p<0.05$); the expression of glutamic-oxaloacetic transaminase 2 (GOT2) and SH3 domain binding protein 5 (SH3BP5) was upregulated in both the miR-21-3p mimics and miR-21-3p inhibitor groups ($p<0.05$); the expression of peroxiredoxin 6 (PRDX6) was significantly upregulated in the miR-124a inhibitor group ($p<0.05$); the expression of immunoglobulin like domain containing receptor 2 (ILDR2) was upregulated in both the miR-124a mimics and miR-124a inhibitor groups; the expression of phosphodiesterase 4D (PDE4D) was significantly upregulated in the miR-148a inhibitor group but downregulated in the miR-148a mimics group ($p<0.05$); the expression of paternally expressed 10 (PEG10) was significantly downregulated in the miR-148a mimics group but upregulated in the miR-148a inhibitor group ($p<0.05$); the expression of superoxide dismutase 3 (SOD3) was significantly downregulated in the miR-2382-5p mimics group but upregulated in the miR-2382-5p inhibitor group ($p<0.05$); and the expression of ADAM metallopeptidase with

thrombospondin type 1 motif 1 (ADAMTS1) was significantly downregulated in the miR-2425-5p mimics group but upregulated in the miR-2425-5p inhibitor group ($p<0.05$) (Fig 8). The quantitative RT-PCR results showed that PRDX6, PDE4D, PEG10, SOD3 and ADAMTS1 were negatively regulated by DERs.

6. Target verification of miRNAs and target genes by luciferase reporter assay

Compared with the DERs mimics+pmiR-BR-REPORT (si) groups: luciferase activity was increased in both the miR-21-3p mimics+GOT2-wild type (WT) and miR-21-3p mimics+GOT2-mutant type (mut) groups; luciferase activity was increased in both the miR-21-3p mimics+SH3BP5-WT and miR-21-3p mimics+SH3BP5-mut groups; luciferase activity was increased in both the miR-124a mimics+PRDX6-WT and miR-124a mimics+PRDX6-mut groups; luciferase activity showed no difference between the miR-124a mimics+ILDR2-WT and miR-124a mimics+ILDR2-mut groups; luciferase activity was significantly decreased in the miR-148a mimics+PDE4D-mut group ($p<0.05$); luciferase activity was significantly decreased in the miR-148a mimics+PEG10-mut group ($p<0.05$); luciferase activity was significantly decreased in the miR-2382-5p mimics+SOD3-mut group ($p<0.05$); and luciferase activity was significantly decreased in the miR-2425-5p mimics+ADAMTS1-mut group ($p<0.05$) (Fig 9). The results revealed that no targeted binding sequence exists in the 3'-UTR of genes which we predicted to be located between GOT2, SH3BP5 and bta-miR-21-3p, PRDX6, ILDR2 and bta-miR-124a. However, the targeted binding sequence exists in the 3'-UTR of genes which we predicted to be located between PDE4D and bta-miR-148a, PEG10 and bta-miR-877, SOD3 and bta-miR-2382-5p, and ADAMTS1 and bta-miR-2425-5p.

Discussion

By RNA-seq analysis of BMECs isolated from Chinese Holstein cows with low and high milk fat percentages, 829 DEGs (525 upregulated DEGs and 304 downregulated DEGs) were obtained; by Solexa analysis of BMECs isolated from Chinese Holstein cows with low and high milk fat percentages, 40 DERs (17 upregulated DERs and 23 downregulated DERs) were obtainedand; and a total of 190 DEGs (159 upregulated DEGs and 31 downregulated DEGs) were screened by the conjoint analysis of RNA-seq and Solexa sequencing data. Functional enrichments by GO analysis revealed 154 significantly enriched biological process GO terms, 25 cellular component GO terms and 46 molecular function GO terms ($p < 0.05$), including the oxidation-reduction process, the extracellular vesicular exosome and oxidoreductase activity, acting on the CH-OH group of donors in both the RNA-seq and conjoint analyses. Functional enrichments by KEGG analysis showed that the DEGs were distributed in 118 KEGG pathways, in which 7 KEGG pathways were significantly enriched ($p < 0.05$), such as phenylalanine metabolism, the cytokine-cytokine receptor interaction and tyrosine metabolism in both the RNA-seq and conjoint analyses. Overall, a conjoint analysis can reduce errors caused by other factors to define candidate functional DERs and DEGs associated with milk fat metabolism more accurately, which contributes to a more precise understanding of the molecular mechanism of the regulation of milk fat metabolism.

By analyzing the regulatory network of target genes and miRNAs, we found that the mechanism of miRNAs on milk fat percentage is very complex. A single miRNA regulates a single gene[12], a single miRNA regulates multiple target genes at the same time[13], or a single target gene is regulated by multiple miRNAs simultaneously[14, 15], thus affecting the lipid metabolism of BMECs. In our experiments, 6 miRNAs (miR-21-3p, miR-124a, miR-148a, miR-877, miR-2382-5p, and miR-2425-5p) and 8 predicted target genes (GOT2, SH3BP5, PRDX6, ILDR2, PDE4D, PEG10, SOD3, and ADAMTS1) were selected to verify the target relationship. The following may explain why some did not have a significant regulatory relationship. The DEGs were screened according to the targeted regulatory relationships predicted by TargetScan and miRBase in the conjoint analysis of RNA-seq and Solexa sequencing data; however, the regulation of target genes by miRNAs in cells is complex. In our luciferase reporter assay, we inserted only approximately 60 bp of the DNA sequence before and after the predicted target DNA sites rather than the entire 3’-UTR of the gene. Therefore, there may be a site effect between the binding of miRNAs to the predicted target DNA sequence. We will verify this speculation in a future study.

Triglyceride is a fatty molecule formed by the polymerization of three molecules of long-chain fatty acids and one molecule of glycerol that can be transported in the blood in the form of lipoproteins[16]. The metabolic regulation of triglyceride in mammals plays an important role in the ability and balance of the mammalian body. Triglyceride has the highest lipid content in the human body. Excessive triglyceride can lead to obesity, cardiovascular, fatty liver, hyperlipidemia and other diseases, and the abnormal state of triglyceride synthesis can also lead to lipid metabolism disorders and other diseases[17, 18]. Triglyceride is also an important part of milk, accounting for 90% of milk fat, and the synthesis of triglyceride in cattle mammary cells can affect milk fat percentage[19, 20]. MiR-148a and miR-17-5p can promote the synthesis of triglyceride and fatty acid metabolism in goat mammary epithelial cells by negatively regulating peroxisome proliferator activated receptor alpha (PPARa) and PPARG coactivator 1 alpha (PPARGC1a), and interference with the expression of PPARa and PPARGC1a can promote the synthesis of milk fat by regulating the genes related to lipid metabolism[21]. In our study, conjoint analysis showed that bta-miR-148a was significantly downregulated in BMECs with high milk fat percentage, but miR-148a can significantly increase the content of triglyceride ($p < 0.05$). Studies have shown that miR-124a can inhibit the expression of patatin like phospholipase domain containing 2 (ATGL) and abhydrolase domain containing 5 (CGI-58) to reduce fat decomposition, leading to an increase in triglyceride content and the accumulation of lipids[22], which is contrary to our conjoint analysis data showing that bta-miR-124a was significantly downregulated in BMECs with high milk fat percentage and triglyceride content was significantly increased in the miR-124a inhibitor group ($p < 0.05$). Previous research has shown that miR-21-3p plays an important role in triglyceride production by regulating the expression of the target gene ELOVL fatty acid elongase 5 (Elovl5)[23], miR-29b can promote triglyceride production and suppress apoptosis in BMECs by regulating the expression of the target genes lipoprotein lipase (LPL) and thymine DNA glycosylase (TDG), which are consistent with our conjoint analysis data showing that bta-miR-21-3p and bta-miR-29b are significantly upregulated in BMECs with high milk fat percentage. In summary, a large number of known and novel DERs were screened by conjoint analysis are mainly related to

triglyceride production and milk fat metabolism, suggesting that DERs and target DEGs can assist in the candidate marker-assisted selection of milk fat percentage.

In our experiment, the conjoint analysis accurately screened a larger number of novel candidate miRNAs and target genes related to milk fat metabolism. Moreover, the effects of 6 DERs (bta-miR-21-3p, bta-miR-124a, bta-miR-148a, bta-miR-877, bta-miR-2382-5p and bta-miR-2425-5p) on triglyceride content in BMECs were detected, and the target relationship between 6 DERs and 8 DEGs (GOT2, SH3BP5, PRDX6, ILDR2, PDE4D, PEG10, SOD3 and ADAMTS1) was verified, which provides a theoretical basis for studying the regulatory mechanism of lipid metabolism in BMECs and regulators for future dairy cattle breeding.

Conclusion

Together, in the present study provides a scientific and effective research method screening functional miRNA and gene related to milk fat percentage by conjoint analysis of RNA-seq and Solexa sequencing of BMECs with high and low milk fat percentages. We identified the regulation of 6 DERs on lipid metabolism, which showed that bta-miR-21-3p and bta-miR-148a promote triglyceride synthesis, whereas bta-miR-124a, bta-miR-877, bta-miR-2382-5p and bta-miR-2425-5p inhibit triglyceride synthesis. Furthermore, the quantitative RT-PCR results showed that the expression level of PRDX6, PDE4D, PEG10, SOD3 and ADAMTS1 were negatively regulated by DERs, and luciferase reporter assay showed that the target relationships existed between PDE4D and bta-miR-148a, PEG10 and bta-miR-877, SOD3 and bta-miR-2382-5p, and ADAMTS1 and bta-miR-2425-5p, which means miRNA affect the expression of gene via not only target relationship but also other regulatory factors. Our findings identified miRNA-mRNA regulatory network involved in lipid metabolism in bovine mammary epithelial cells and provided a foundation for the study of the molecular mechanisms related to milk fat percentage.

Materials And Methods

Cell culture and transfection

The experimental BMECs were previously constructed in our laboratory[24]. The basal medium consisted of DMEM/F12 (HyClone, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Pasching, Austria) and 1% (v/v) penicillin and streptomycin (PAA, Pasching, Austria). The cell culture plates (Thermo, Suzhou, China) were then incubated in an incubator (Thermo, Marietta, OH, USA) containing 5% CO2 at 37°C. The medium for transfection consisted of DMEM/F12 supplemented with 10% (v/v) fetal bovine serum.

Total RNA extraction

Total RNA from BMECs with high and low milk fat percentages was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA was further treated with RNase-free DNase I (NEB, Beijing, China), and RNA longer than 18 nucleotides was recovered. RNA

concentration was measured by NanoDrop 2000 (Thermo Scientific, USA). The quality of RNA was assessed by the Agilent2200 (Agilent, USA).

Conjoint analysis of RNA-seq and Solexa sequencing

(1) RNA-seq analysis: The sequencing library of each RNA sample was prepared using Ion Total RNA-Seq Kit v2 according to the protocol provided by manufacturer (Life technologies, USA). The transcript with poly (A)+ RNA of Cattle were analyzed using high-throughput Life technologies Ion Proton Sequencer. Reads sequenced were filtered and mapped to Cattle genome (download from NCBI) using Mapsplice software. The mapped reads was counted to achieve the expression of each gene based on the gene annotation information from NCBI database. In order to achieve the best quality of the RNA Sequencing, Novelbio applied the reads filtration to filter the reads with lower quality and short sequence under following criteria: read length > 50; over 30% base quality >13. Using the internationally recognized algorithm EBSeq based on the fold change (FC) and false discovery rate (FDR) thresholds to screen the DEGs in the BMECs of Chinese Holstein cows with high and low milk fat percentages[25, 26]. Genes were considered differentially expressed according to $\log_2$FC >1 or $\log_2$FC <-1and FDR <0.05.

(2) Solexa sequencing analysis: We performed a differential expression analysis according to the data of Solexa sequencing obtained by a previous study in our laboratory and screened the DERs in the BMECs of Chinese Holstein cows with high and low milk fat percentages using the internationally recognized EBSeq algorithm based on the FC and FDR thresholds[27]. MiRNAs were considered differentially expressed according to $\log_2$FC >1 or $\log_2$FC <-1and FDR <0.05.

(3) Conjoint analysis of RNA-seq and Solexa: All target genes regulated by DERs were predicted by the combination of miRbase (http://www.mirbase.org) and TargetScan (http://www.targetscan.org). All the predicted target genes were intersected with the DEGs obtained by RNA-seq, and then the DEGs and DERs were obtained by a combined analysis.

GO and KEGG pathway enrichment analyses

GO analysis was applied to analyze the main functions of the DEGs according to GO, which is the functional classification of NCBI (http://www.ncbi.nlm.nih.gov). The GO category was classified by Fisher's exact test and the χ^2 test, and the FDR was calculated to correct the P value: the smaller the FDR was, the smaller the error in judging the P value[28]. We computed the P values for the GO terms of all the DEGs. Enrichment provides a measure of the significance of the function: as the enrichment increases, the corresponding function is more specific, which helps us identify those GO terms with concrete functional descriptions in the experiment.

Pathway analysis was used to determine the significant pathways of the DEGs. Pathway annotations of microarray genes were downloaded from KEGG (http://www.genome.jp/Wegg/). Fisher’s exact test was used to find the significant enrichment pathways. The resulting P values were adjusted using the BH FDR algorithm (Benjamini and Hochberg 1995). Pathway categories with an FDR < 0.05 were reported.

Vector construction of candidate miRNAs and luciferase reporter

(1)Construction vector of six candidate DERs: The mimics, inhibitor and negative control vectors of 6 miRNAs (bta-miR-21-3p, bta-miR-124a, bta-miR-148a, bta-miR-877, bta-miR-2382-5p and bta-miR-2425-5p) were constructed by GenePharma Company (Suzhou, China).

(2)Construction of luciferase reporter vectors: We predicted the DNA-binding sites of miRNAs to the target gene's 3'-UTR with TargetScan. For the luciferase reporter assay, the WT and mut DNA sequences were obtained by annealing performed in a 10 μL reaction with 0.5 μL forward primer, 0.5 μL reverse primer, 1 μL 10 × Ex Taq buffer and 8 μL ddH_2O under the following procedure: 95°C for 5 minutes, then dropped at a rate of 1.5°C per minute to 22°C. The DNA fragment was then ligated with the linearized pmiR-BR-REPORT plasmid to construct the WT and mut vectors. All DNA oligo nucleotides used for annealing were synthesized by Sangon (Shanghai, China), and the sequence details are shown in Table 1.

Determination of triglyceride content

The Triglyceride Extraction Kit (APPLYGEN, China) was used to separate the cells following the manufacturer's instructions. Then, the cell lysates were collected in 1.5 mL centrifuge tubes, heated at 70°C for 10 minutes, centrifuged at 2000 g for 5 minutes at room temperature, and the supernatant was extracted to detect the contents of triglyceride and protein on a SpectraMax M5 Microplate Reader. The protein content was used as a reference to correct the variation in cell number, each sample was analyzed in triplicate.

Quantitative RT-PCR of DEGs and DERs

Total RNA was extracted and reverse transcribed to cDNA using the PrimeScript^{TM} RT Reagent Kit with gDNA Eraser (Takara Biological Company, Japan) for the quantitative RT-PCR analysis of 8 DEGs (GOT2, SH3BP5, PRDX6, ILDR2, PDE4D, PEG10, SOD3 and ADAMTS1) and 6 DERs (bta-miR-2382-5p, bta-miR-124a, bta-miR-21-3p, bta-miR-148a, bta-miR-877 and bta-miR-2425-5p). Quantitative RT-PCR was performed in a 10 μL reaction with 0.5 μL forward primer, 0.5 μL reverse primer, 5 μL SYBR Green Real-Time PCR Master Mix (Roche, Germany), 1 μL cDNA and 3 μL ddH_2O according to the manufacturer's instructions under the following procedure: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 30 s on a PCRmax (Eco, United Kingdom). Each sample was analyzed in triplicate, and the average threshold cycle (Ct) was calculated with the $2^{-\triangle\triangle Ct}$ method. The reverse transcription primers of the miRNAs and primers for the quantitative RT-PCR analysis of DERs and target DEGs were designed using Primer 6.0 and synthesized by Sangon (Table 2).

Luciferase reporter assay

The luciferase reporter experiments were performed with BMECs at a density of 1×10^5 cells in 24-well plates (BIOFIL, Guangzhou, China). To validate the target relationship of the candidate miRNAs and genes, 1.5 μg miRNA mimic plasmid was cotransfected with 1.5 μg WT, 1.5 μg mut or 1.5 μg pmiR-BR-

REPORT plasmid into BMECs. Forty-eight hours after transfection, the firefly (hluc+) and Renilla (hRluc) luciferase activities were measured with the Dual-Glo luciferase assay system (Promega) according to the manufacturer's instructions. The hluc+ gene was used as a reference gene to correct the variation in transfection efficiency, and the relative luciferase activity was calculated as hRluc/hluc+. All experiments for the luciferase reporter assay were performed in triplicate.

Statistical analysis

The data were analyzed by GraphPad 6.0 statistical software with a completely random design of Student's t test for the comparison of two independent groups. The mean and standard deviation (SD) or standard error of mean (SEM) among triplicate samples were calculated, and $p<0.05$ was considered to indicate statistical significance.

Abbreviations

BMECs, bovine mammary epithelial cells; DERs, differentially expressed microRNAs; 3'-UTR, 3'-untranslated region; IMF, intramuscular fat; DEGs, differentially expressed genes; SFRP1, secreted frizzled related protein 1; WNT5B, Wnt family member 5B; FGFR3, fibroblast growth factor receptor 3; Nc, negative control; THBS1, thrombospondin 1; KLF6, kruppel-like factor 6; LDLR, low-density lipoprotein receptor; GOT2, glutamic-oxaloacetic transaminase 2; SH3BP5, SH3 domain binding protein 5; PRDX6, peroxiredoxin 6; ILDR2, immunoglobulin like domain containing receptor 2; PDE4D, phosphodiesterase 4D; PEG10, paternally expressed 10; SOD3, superoxide dismutase 3; WT, wild type; mut, mutant type; PPARa, peroxisome proliferator activated receptor alpha; PPARGC1a, PPARG coactivator 1 alpha; ATGL, patatin like phospholipase domain containing 2; Elovl5, ELOVL fatty acid elongase 5; LPL, lipoprotein lipase; TDG, thymine DNA glycosylase; FDR, false discovery rate; hRluc, Renilla luciferase activity; SD, standard deviation; SEM, standard error of mean.

Declarations

Ethics approval and consent to participate

All animal experiments in this study abided strictly by the ordinance for the care and use of laboratory animals of the Jilin University Animal Care and Use Committee (permit number:SYXK (Ji) pzpx20181227083).

Consent for publication

Not applicable

Availability of data and materials

The data sets used and analyzed during the current study are available. The

RNA-seq and Solexa sequencing data have been submitted to the GenBank databases

under accession number GSE137488.

Competing interests

The authors declare that they have no competing interests

Funding

This work was supported by the National Major Special Project on New Varieties Cultivation for Transgenic Organisms (2016ZX08009003-006), the National Natural Science Foundation of China (no. 31672389, 31772562, 31802034 and 31972993) and the Key Laboratory of Animal (Poultry) Genetics Breeding and Reproduction, Ministry of Agriculture(Poultrylab2018-4).

Authors' contributions

In our current research, Lixin Xia and Zhihui Zhao were mainly to complete the experiments and writing works. Xibi Fang and Runjun Yang were proposed experimental design and ideas. And then Ping Jiang, Haibin Yu and Juan Liu were given help during the experiment process; Runjun Yang, Xiaohui Li and Xiang Yu were assisting in data analysis.

Acknowledgements

The authors gratefully thank Dr.Binglei Sheng at Heilongjiang Bayi Agricultural University for taking technical support.

References

1. Allen NN: The fat percentage of milk as affected by feeding fats to dairy cows. Journal of Dairy Science 1934, 17(5):379-395.
2. Bernabucci U, Basiricò L, Morera P: Impact of hot environment on colostrum and milk composition. Cellular and molecular biology 2013, 59(1):67-83.
3. Peaker M: The mammary gland in mammalian evolution: a brief commentary on some of the concepts. Journal of mammary gland biology and neoplasia 2002, 7(3):347-353.
4. Felekkis K, Touvana E, Stefanou C, Deltas C: microRNAs: a newly described class of encoded molecules that play a role in health and disease. Hippokratia 2010, 14(4):236.
5. Ying S-Y, Chang DC, Lin S-L: The microRNA (miRNA): overview of the RNA genes that modulate gene function. Molecular biotechnology 2008, 38(3):257-268.
6. Bruscella P, Bottini S, Baudesson C, Pawlotsky J-M, Feray C, Trabucchi M: Viruses and miRNAs: More friends than foes. Frontiers in microbiology 2017, 8:824.

7. Peng J, Zhao J-S, Shen Y-F, Mao H-G, Xu N-Y: MicroRNA expression profiling of lactating mammary gland in divergent phenotype swine breeds. International journal of molecular sciences 2015, 16(1):1448-1465.
8. Cai X, Liu Q, Zhang X, Ren Y, Lei X, Li S, Chen Q, Deng K, Wang P, Zhang H: Identification and analysis of the expression of microRNA from lactating and nonlactating mammary glands of the Chinese swamp buffalo. Journal of dairy science 2017, 100(3):1971-1986.
9. Izumi H, Tsuda M, Sato Y, Kosaka N, Ochiya T, Iwamoto H, Namba K, Takeda Y: Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. Journal of dairy science 2015, 98(5):2920-2933.
10. Li Z, Yan M, Yu Y, Wang Y, Lei G, Pan Y, Li N, Gobin R, Yu J: LncRNA H19 promotes the committed differentiation of stem cells from apical papilla via miR-141/SPAG9 pathway. Cell death & disease 2019, 10(2):130.
11. Zhang Y-Y, Wang H-B, Wang Y-N, Wang H-C, Zhang S, Hong J-Y, Guo H-F, Chen D, Yang Y, Zan L-S: Transcriptome analysis of mRNA and microRNAs in intramuscular fat tissues of castrated and intact male Chinese Qinchuan cattle. PloS one 2017, 12(10):e0185961.
12. Wang H, Luo J, Chen Z, Cao WT, Xu HF, Gou DM, Zhu JJ: MicroRNA-24 can control triacylglycerol synthesis in goat mammary epithelial cells by targeting the fatty acid synthase gene. Journal of Dairy Science 2015, 98(12):9001-9014.
13. Yang Y, Pan Q, Sun B, Yang R, Fang X, Liu X, Yu X, Zhao Z: miR-29b Targets LPL and TDG Genes and Regulates Apoptosis and Triglyceride Production in MECs. Dna & Cell Biology 2016, 35(12).
14. Wang H, Moyano AL, Ma Z, Deng Y, Lin Y, Zhao C, Zhang L, Jiang M, He X, Ma Z: miR-219 Cooperates with miR-338 in Myelination and Promotes Myelin Repair in the CNS. Developmental Cell 2017, 40(6):566.
15. Gu X, Fu C, Lin L, Liu S, Su X, Li A, Wu Q, Jia C, Zhang P, Chen L: miR-124 and miR-9 mediated downregulation of HDAC5 promotes neurite development through activating MEF2C-GPM6A pathway. Journal of Cellular Physiology 2017, 233(1):673.
16. Du X, Herrfurth C, Gottlieb T, Kawelke S, Feussner K, Rühling H, Feussner I, Maniak M: Dictyostelium discoideum Dgat2 can substitute for the essential function of Dgat1 in triglyceride production but not in ether lipid synthesis. Eukaryotic Cell 2014, 13(4):517-526.
17. Nordestgaard BG, Varbo A: Triglycerides and cardiovascular disease. Lancet 2014, 384(9943):626-635.
18. Ong KT: The role of adipose triglyceride lipase in hepatic lipid metabolism, non-alcoholic fatty liver disease and insulin resistance. Dissertations & Theses - Gradworks 2013.
19. Kuksis A, Marai L, Myher JJ: Triglyceride structure of milk fats. Journal of the American Oil Chemists Society 1973, 50(6):193.
20. Mendelson CR, Zinder O, ., Blanchette-Mackie EJ, Chernick SS, Scow RO: Lipoprotein lipase and lipid metabolism in mammary gland. Journal of Dairy Science 1977, 60(4):666-676.

21. Chen Z, Luo J, Sun S, Cao D, Shi H, Loor JJ: miR-148a and miR-17–5p synergistically regulate milk TAG synthesis via PPARGC1A and PPARA in goat mammary epithelial cells. RNA biology 2017, 14(3):326-338.
22. Das S, Stadelmeyer E, Schauer S, Schwarz A, Strohmaier H, Claudel T, Zechner R, Hoefler G, Vesely P: Micro RNA-124a regulates lipolysis via adipose triglyceride lipase and comparative gene identification 58. International journal of molecular sciences 2015, 16(4):8555-8568.
23. Li X, Jiang P, Yu H, Yang Y, Xia L, Yang R, Fang X, Zhao Z: miR-21-3p Targets Elovl5 and Regulates Triglyceride Production in Mammary Epithelial Cells of Cow. DNA and cell biology 2019, 38(4):352-357.
24. Lu C, Yang R, Liu B, Li Z, Shen B, Yan S, Zhang Y, Zhang L, Zhao Z: Lines from Chinese Holstein Dairy Cow. Journal of Animal and Veterinary Advances 2012, 11(8):1166-1172.
25. Benjamini Y, Hochberg Y: Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal statistical society: series B (Methodological) 1995, 57(1):289-300.
26. Javanmard A, Montanari A: Online rules for control of false discovery rate and false discovery exceedance. The Annals of statistics 2018, 46(2):526-554.
27. Shen B, Zhang L, Lian C, Lu C, Zhang Y, Pan Q, Yang R, Zhao Z: Deep sequencing and screening of differentially expressed MicroRNAs related to milk fat metabolism in Bovine primary mammary epithelial cells. International journal of molecular sciences 2016, 17(2):200.
28. Rivals I, Personnaz L, Taing L, Potier M: Enrichment or depletion of a GO category within a class of genes: which test? Bioinformatics 2007, 23(4):401-407.

Tables

Table 1. DNA oligo for luciferase reporter assay

Primers	DNA sequences(5’-3’)
GOT2-WT-F	GGCCACAACCTTCCTGTCTTCAGCCTCTGCTGTTATGAAATCCACATGGAGGAAGAGGG
GOT2-WT-R	TCGACCCTCTTCCTCCATGTGGATTTCATAACAGCAGAGGCTGAAGACAGGAAGGTTGT
GOT2-mut-F	GGCCACAACCTTCCTGTCTTCAGCCTTTCGTCGTATGAAATCCACATGGAGGAAGAGGG
GOT2-mut-R	TCGACCCTCTTCCTCCATGTGGATTTCATACGACGAAAGGCTGAAGACAGGAAGGTTGT
SH3BP5-WT-F	GGCCTGTCTATGGGGTGAGTGCTCCTGGGCTGCTGTGTTTTCCGGTGCAGAGTCAGTTG
SH3BP5-WT-R	TCGACAACTGACTCTGCACCGGAAAACACAGCAGCCCAGGAGCACTCACCCCATAGACA
SH3BP5-mut-F	GGCCTGTCTATGGGGTGAGTGCTCCTGGGTTCGTCGGTTTTCCGGTGCAGAGTCAGTTG
SH3BP5-mut-R	TCGACAACTGACTCTGCACCGGAAAACCGACGAACCCAGGAGCACTCACCCCATAGACA
ILDR2-WT-F	GGCCGCACTCTTGATTAGGTTGTCATCAGGTGCCTTTTGATAAATGTTTAAAATACAC
ILDR2-WT-R	TCGAGTGTATTTTAAACATTTATCAAAAGGCACCTGATGACAACCTAATCAAGAGTGC
ILDR2-mut-F	GGCCGCACTCTTGATTAGGTTGTCATCAGGCTTCTGTTGATAAATGTTTAAAATACAC
ILDR2-mut-R	TCGAGTGTATTTTAAACATTTATCAACAGAAGCCTGATGACAACCTAATCAAGAGTGC
PRDX6-WT-F	GGCCCACTAAAAATTTCTTGGGATCCTTTGTGCCTTCAGCAGCTTTCTCCTCTGTTCGT
PRDX6-WT-R	TCGAACGAACAGAGGAGAAAGCTGCTGAAGGCACAAAGGATCCCAAGAAATTTTTAGTG
PRDX6-mut-F	GGCCCACTAAAAATTTCTTGGGATCCTTTGCTTCTGCAGCAGCTTTCTCCTCTGTTCGT
PRDX6-mut-R	TCGAACGAACAGAGGAGAAAGCTGCTGCAGAAGCAAAGGATCCCAAGAAATTTTTAGTG
PDE4D-WT-F	GGCCTGAATAGTGTGGTTCGGTGAGCTGCACTGACCTCTACATTTTGTATGATATGTAA
PDE4D-WT-R	TCGATTACATATCATACAAAATGTAGAGGTCAGTGCAGCTCACCGAACCACACTATTCA
PDE4D-mut-F	GGCCTGAATAGTGTGGTTCGGTGAGCGAACGTTCCCTCTACATTTTGTATGATATGTAA
PDE4D-mut-R	TCGATTACATATCATACAAAATGTAGAGGGAACGTTCGCTCACCGAACCACACTATTCA
PEG10-WT-F	GGCCTGCACCTCCTTGTGATTCTTTCCTCTATAATTGCTACCCCTAAAGATTTCAGATA
PEG10-WT-R	TCGATATCTGAAATCTTTAGGGGTAGCAATTATAGAGGAAAGAATCACAAGGAGGTGCA
PEG10-mut-F	GGCCTGCACCTCCTTGTGATTCTTCTTTCCATAATTGCTACCCCTAAAGATTTCAGATA
PEG10-mut-R	TCGATATCTGAAATCTTTAGGGGTAGCAATTATGGAAAGAAGAATCACAAGGAGGTGCA
SOD3-WT-F	GGCCGGAGCCTTTCCATGCCCCGACTCCCCTCTAAGTGCCCCTAGACCGCTCCAC
SOD3-WT-R	TCGAGTGGAGCGGTCTAGGGGCACTTAGAGGGGAGTCGGGGCATGGAAAGGCTCC
SOD3-mut-F	GGCCGGAGCCTTTCCATGCCCCGACTCTTCTCTAAGTGCCCCTAGACCGCTCCAC
SOD3-mut-R	TCGAGTGGAGCGGTCTAGGGGCACTTAGAGAAGAGTCGGGGCATGGAAAGGCTCC
ADAMTS1-WT-F	GGCCCTGGAGGAGTCCAGGACGCCTTGCCCTGGTGATCAACCAGGGGTGGCGATG
ADAMTS1-WT-R	TCGACATCGCCACCCCTGGTTGATCACCAGGGCAAGGCGTCCTGGACTCCTCCAG
ADAMTS1-mut-F	GGCCCTGGAGGAGTCCAGGACGCCTTGTTCTGGTGATCAACCAGGGGTGGCGATG
ADAMTS1-mut-R	TCGACATCGCCACCCCTGGTTGATCACCAGAACAAGGCGTCCTGGACTCCTCCAG

Table 2. Primers for quantitative RT-PCR

Primers	DNA sequences(5’-3’)
bta-miR-21-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGACAGCC
bta-miR-124a-RT	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACCTTGGCAT
bta-miR-148a-RT	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACACAAAGTT
bta-miR-877-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCTGCGC
bta-miR-2382-5p-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACAGCTTC
bta-miR-2425-5p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACACAGCTT
U6-RT/R	AACGCTTCACGAATTTGCGT
bta-miR-21-3p-F	TGCGGTAACAGCAGTCGATGG
bta-miR-21-3p-R	CAGTGCAGGGTCCGAGGT
bta-miR-124a-F	TGCGGTTAAGGCACGCGGTGAAT
bta-miR-124a-R	CAGTGCAGGGTCCGAGGT
bta-miR-148a-F	TGCGGTTCAGTGCACTACAGAA
bta-miR-148a-R	CAGTGCAGGGTCCGAGGT
bta-miR-877-F	TGCGGGTAGAGGAGATGGC
bta-miR-877-R	CAGTGCAGGGTCCGAGGT
bta-miR-2382-5p-F	ACACTCCAGCTGGGAGGGGAGTGCCTGGGA
bta-miR-2382-5p-R	TGGTGTCGTGGAGTCG
bta-miR-2425-5p-F	TGCGGAGGGGAGTGCCTGGG
bta-miR-2425-5p-R	CAGTGCAGGGTCCGAGGT
U6-F	CTCGCTTCGGCAGCACA
GOT2-F	CGTGCGGCTTTGACTTC
GOT2-R	CACTGTCGCCATCTCCTTC
SH3BP5-F	TCCGCTCTGTCCTTGTGG
SH3BP5-R	GGAAGTCCTGCGTGGCT
ILDR2-F	GGACGGATGTTATCAACGAAG
ILDR2-R	GGCAGACACAGGTACTGGCA
PRDX6-F	ATGGGGCATTCTCTTCTCC
PRDX6-R	GATGGCAAGGTCCCGATTC
PDE4D-F	ACCTGAGCAACCCAACCAAG
PDE4D-R	CGTCTCCCACAGAGGATGAAC
PEG10-F	CTGAACCTGTGCCTCTATTGT
PEG10-R	GACGCAGCATCATCTTGG
SOD3-F	ACGCCAAGGTGACGGAGATC
SOD3-R	GGAAGCCCTCAAGGTGGAAGAA
ADAMTS1-F	TGACTTCCAACGCTGCCCT
ADAMTS1-R	GCCTGTAAGCCATCATCCTCT
ACTB-F	GCAGGTCATCACCATCGG
ACTB-R	CCGTGTTGGCGTAGAGGT

Table 3. The top 10 up-regulated and down-regulated DERs

AccID	HH_Normalized	LL_Normalized	Log2FC(HH/LL)	Style
bta-miR-144	12.7382	0.0000	20.0000	up
bta-miR-1277	92.3518	2.8261	5.0302	up
bta-miR-33a	343.9308	18.8410	4.1902	up
bta-miR-21-3p	99.7824	6.5943	3.9195	up
bta-miR-193a-3p	2056.1544	153.5541	3.7431	up
bta-miR-32	54.1373	4.7102	3.5227	up
bta-miR-30b-5p	225.0412	21.6671	3.3766	up
bta-miR-193a	693.1692	92.3209	2.9085	up
bta-miR-29b	4620.7744	818.6414	2.4968	up
bta-miR-196b	169.8424	32.0297	2.4067	up
bta-miR-124a	0.0000	117.7562	-20.0000	down
bta-miR-127	0.0000	22.6092	-20.0000	down
bta-miR-219-3p	0.0000	20.7251	-20.0000	down
bta-miR-485	0.0000	13.1887	-20.0000	down
bta-miR-124b	1.0615	77.2481	-6.1853	down
bta-miR-2454-5p	1.0615	21.6671	-4.3513	down
bta-miR-2425-5p	2.1230	35.7979	-4.0757	down
bta-miR-224	18.0458	187.4679	-3.3769	down
bta-miR-2393	5.3076	48.0445	-3.1782	down
bta-miR-320b	4.2461	32.9717	-2.9570	down

Figures

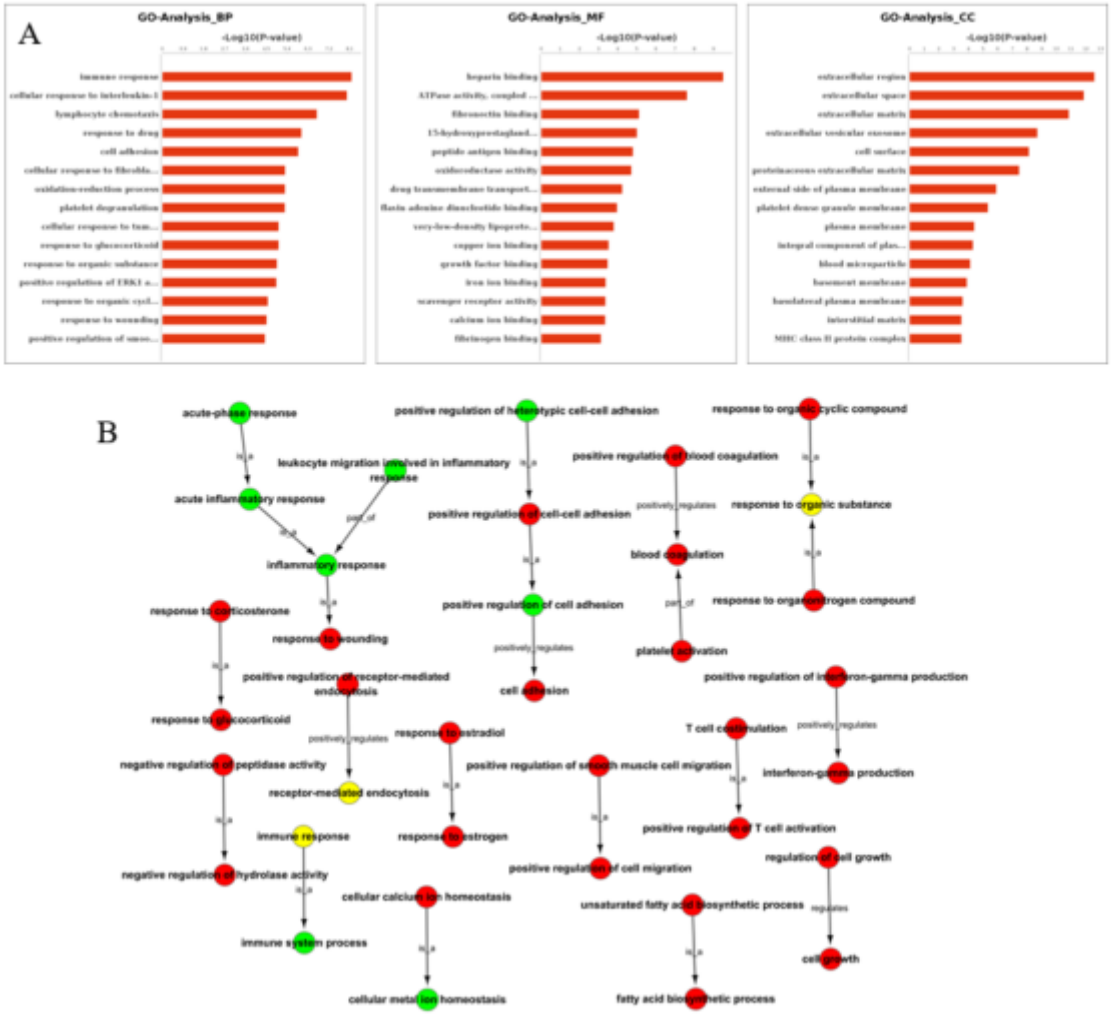
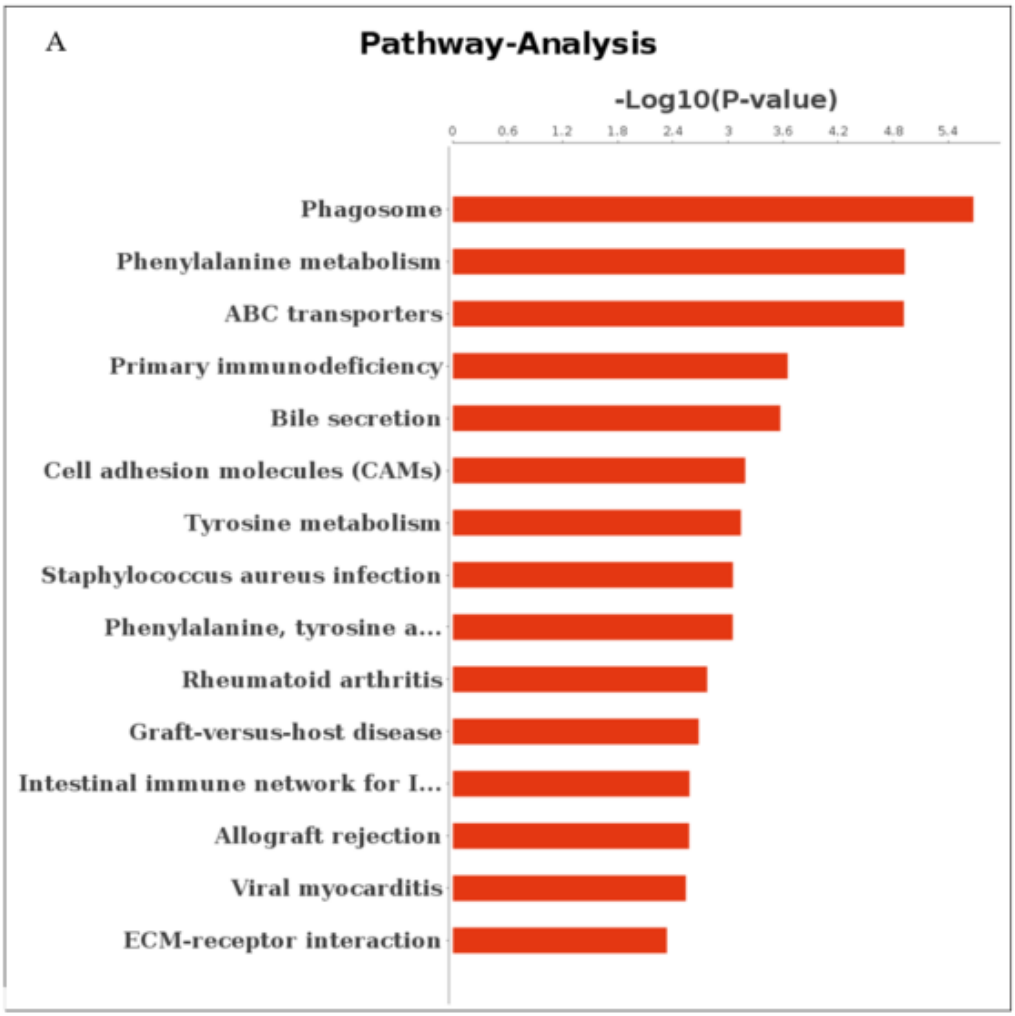


Figure 1

GO terms of RNA-seq analysis A GO term enrichment of DEGs. Horizontal axis: functional significance level -log10(P value), longitudinal axis: name of the entry in the Gene Ontology database corresponding to GO. BP: Biological Process; CC: Cellular Component, MF: Molecular Function. B GO-Tree of DEGs. RED: GO terms of upregulated genes, GREEN: GO terms of downregulated genes.



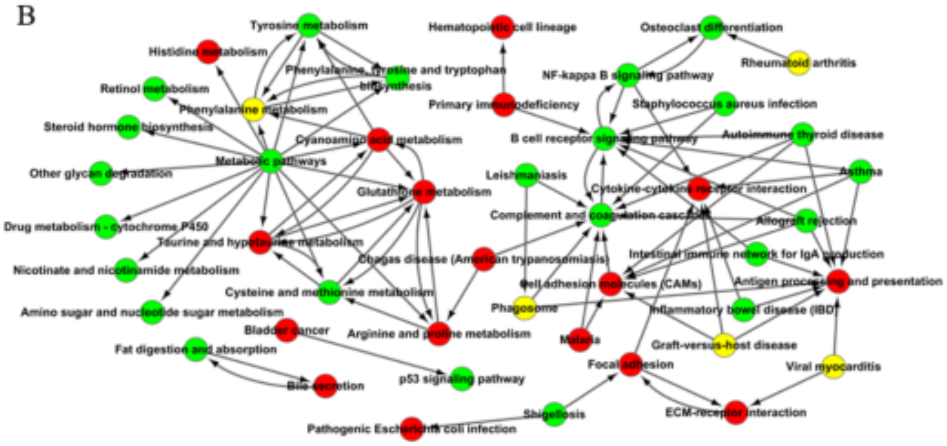


Figure 2

KEGG pathways of RNA-seq analysis A KEGG pathway enrichment of DEGs. Horizontal axis: functional significance level -log10(P value), longitudinal axis: name of the pathway in the KEGG database. B Pathway-Act-Network of DEGs. RED: pathway terms of upregulated genes, GREEN: pathway terms of downregulated genes.

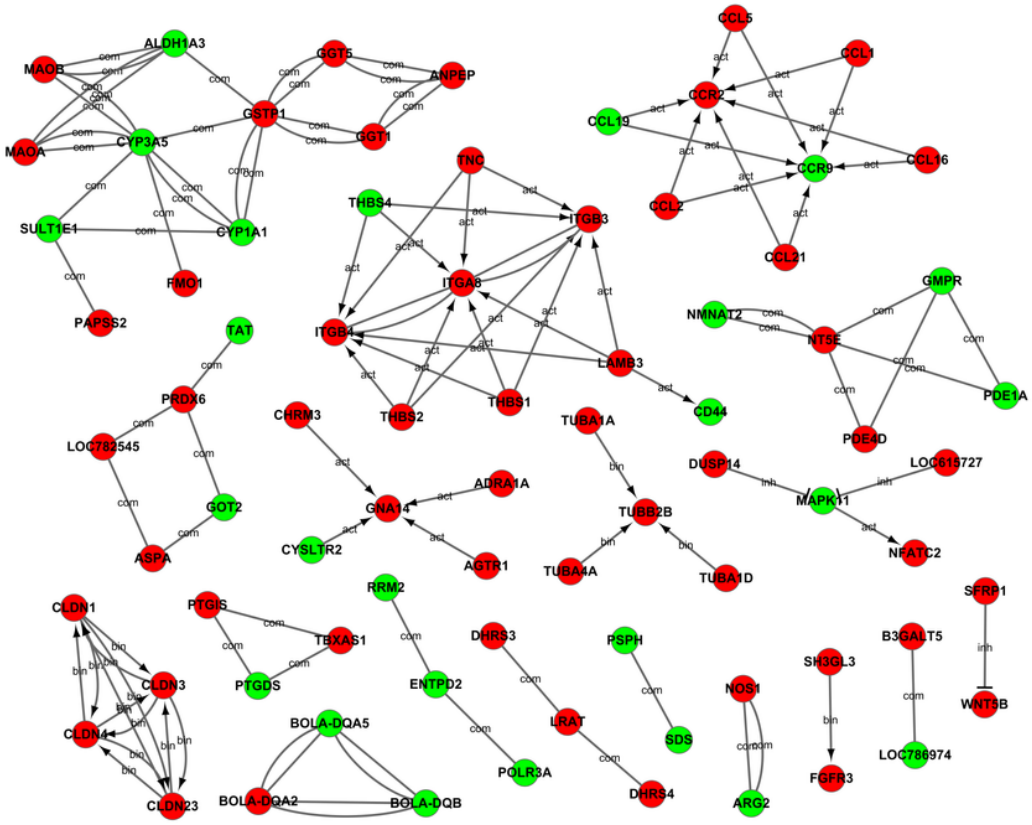


Figure 3

Gene-Act-Network of RNA-seq analysis RED: upregulated DEGs, GREEN: downregulated DEGs; act: activation, com: compound, int: inbibit, bin: binding.

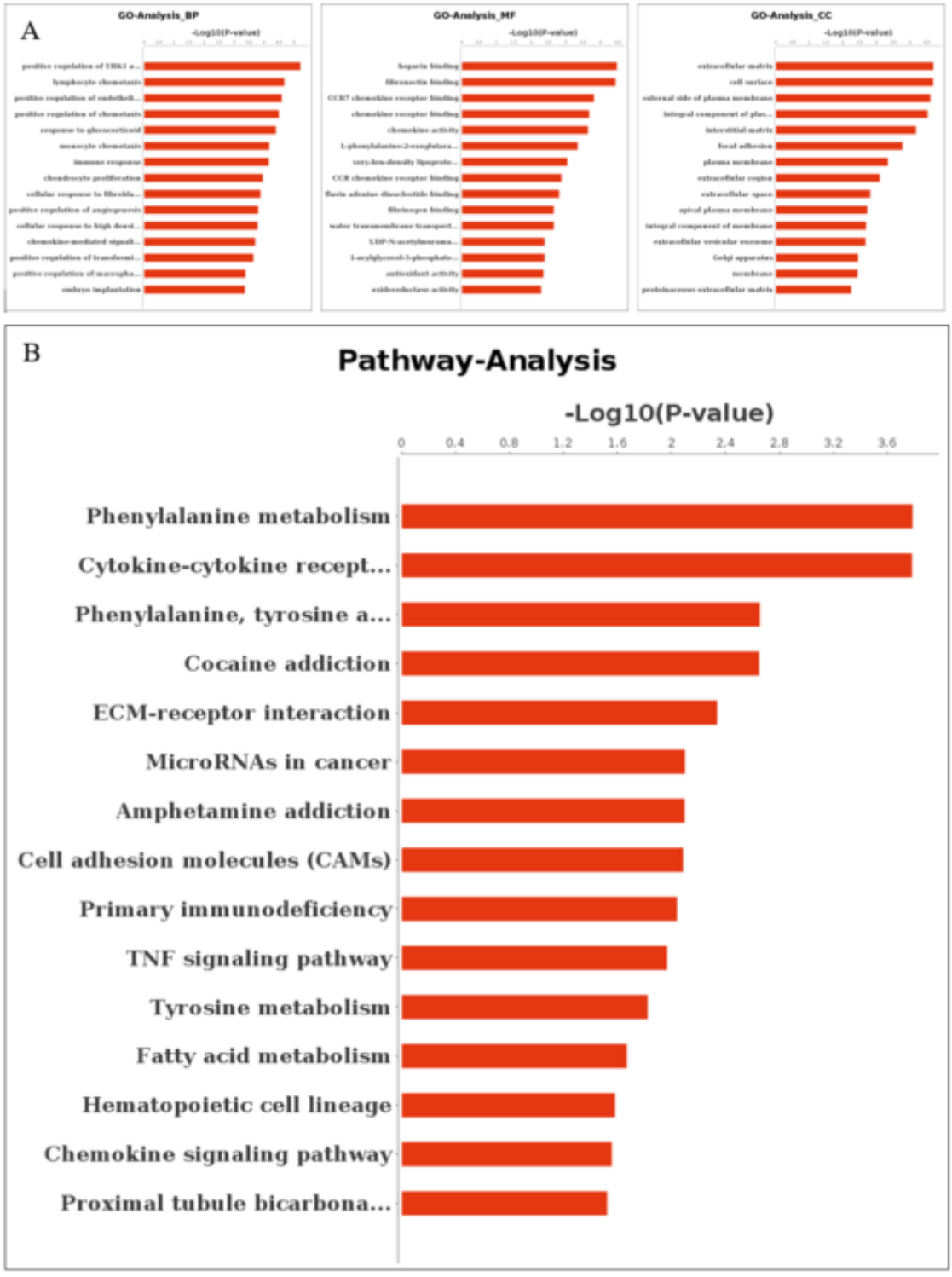


Figure 4

GO terms and KEGG pathway of Solexa analysis A GO term enrichment of all target genes regulated by the DERs. Horizontal axis: functional significance level -log10(P value), longitudinal axis: name of the entry in the Gene Ontology database corresponding to GO. BP: Biological Process; CC: Cellular Component, MF: Molecular Function. B KEGG pathway enrichment of all target genes regulated by the DERs. Horizontal axis: functional significance level -log10(P value), longitudinal axis: name of the pathway in the KEGG database.

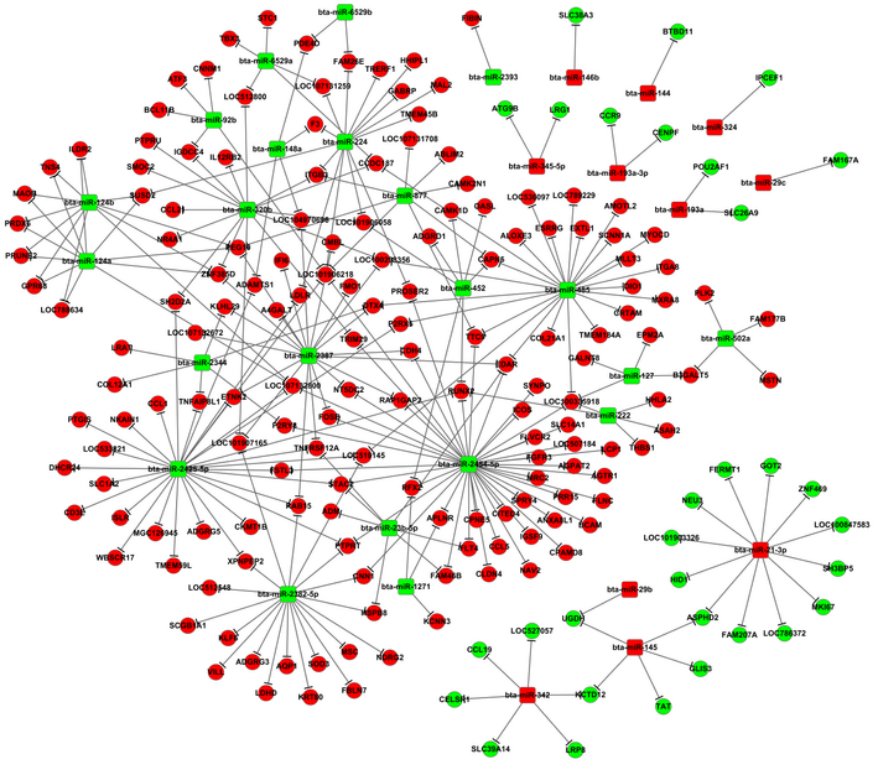


Figure 5

MicroRNA-mRNA regulatory network in BMECs A Regulatory network of DERs on DEGs. RED: upregulated DEGs and DERs, GREEN: downregulated DEGs and DERs.

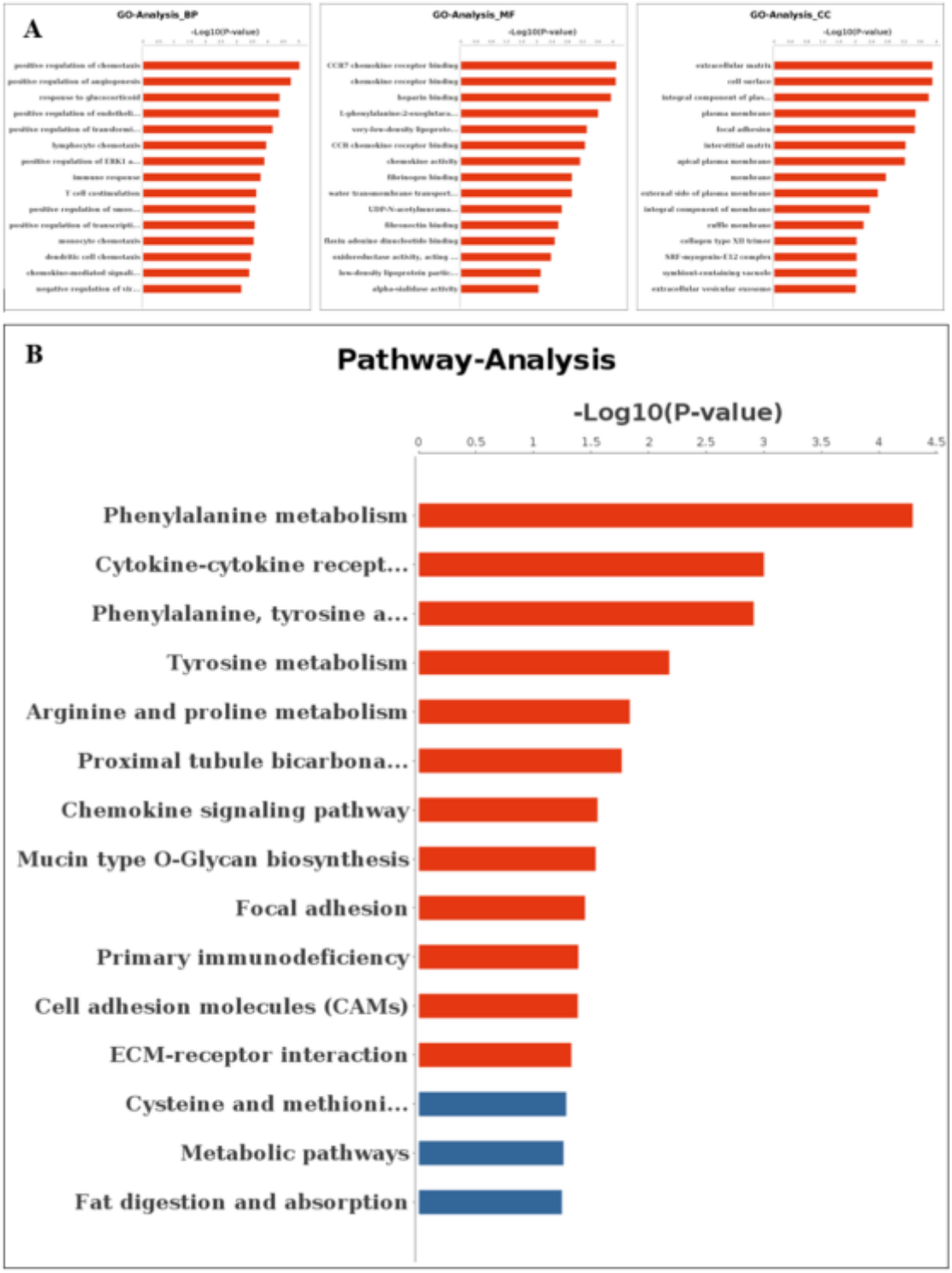


Figure 6

GO terms and KEGG pathway of conjoint analysis A GO analysis of DEGs screened by the conjoint analysis of RNA-seq and Solexa sequencing data. Horizontal axis: functional significance level -log10(P value), longitudinal axis: name of the entry in the Gene Ontology database corresponding to GO. BP: Biological Process; CC: Cellular Component, MF: Molecular Function. B KEGG pathway analysis of DEGs screened by the conjoint analysis of RNA-seq and Solexa sequencing data. Horizontal axis: functional significance level -log10(P value), longitudinal axis: name of the pathway in the KEGG database.

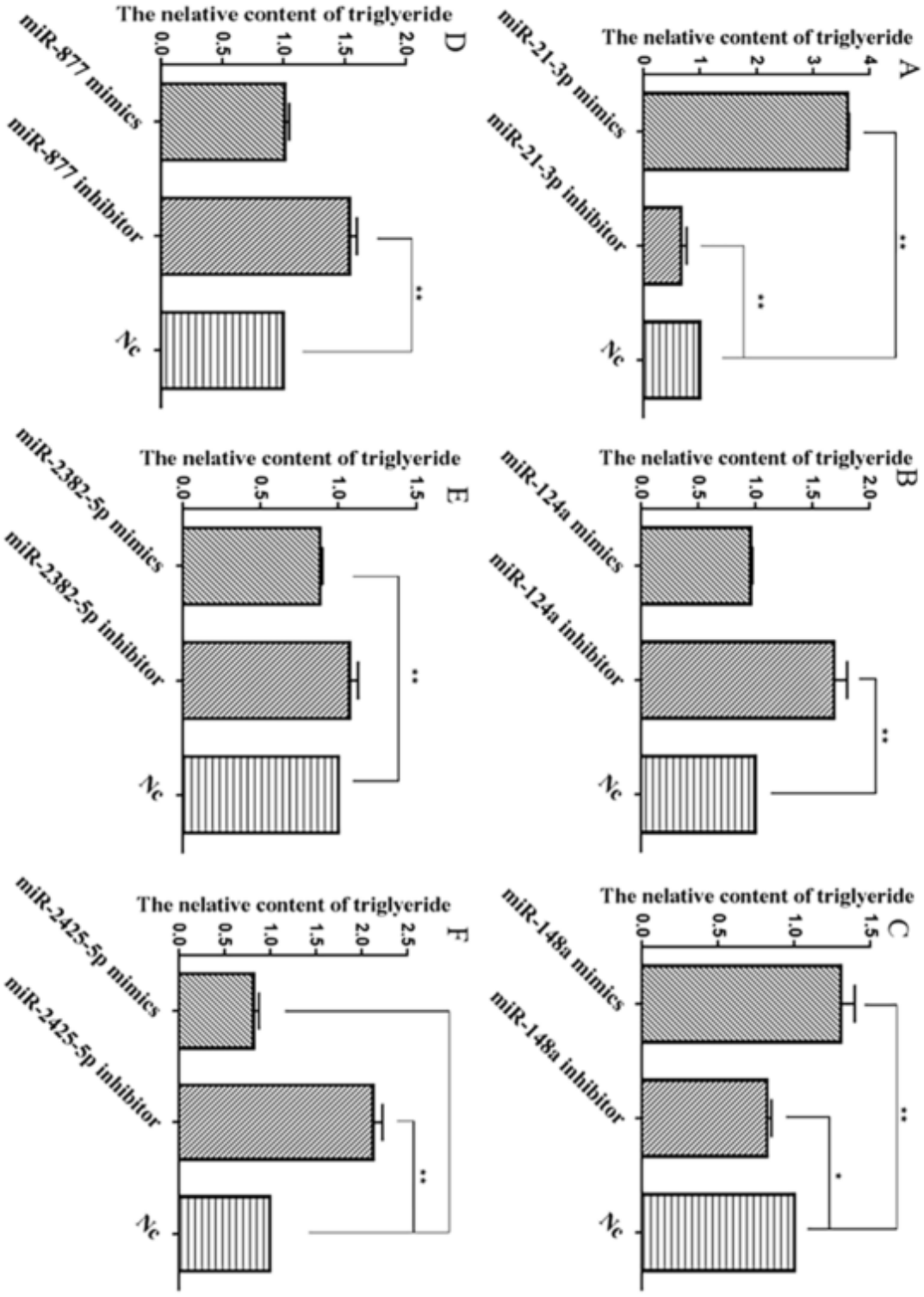


Figure 7

The detection of triglyceride production regulated by DERs in BMECs A Relative content of triglyceride in BMECs transfected with miR-21-3p mimics, inhibitor and Nc. B Relative content of triglyceride in BMECs transfected with miR-124a mimics, inhibitor and Nc. C Relative content of triglyceride in BMECs transfected with miR-148a mimics, inhibitor and Nc. D Relative content of triglyceride in BMECs transfected with miR-877 mimics, inhibitor and Nc. E Relative content of triglyceride in BMECs transfected

with miR-2382-5p mimics, inhibitor and Nc. F Relative content of triglyceride in BMECs transfected with miR-2425-5p mimics, inhibitor and Nc.

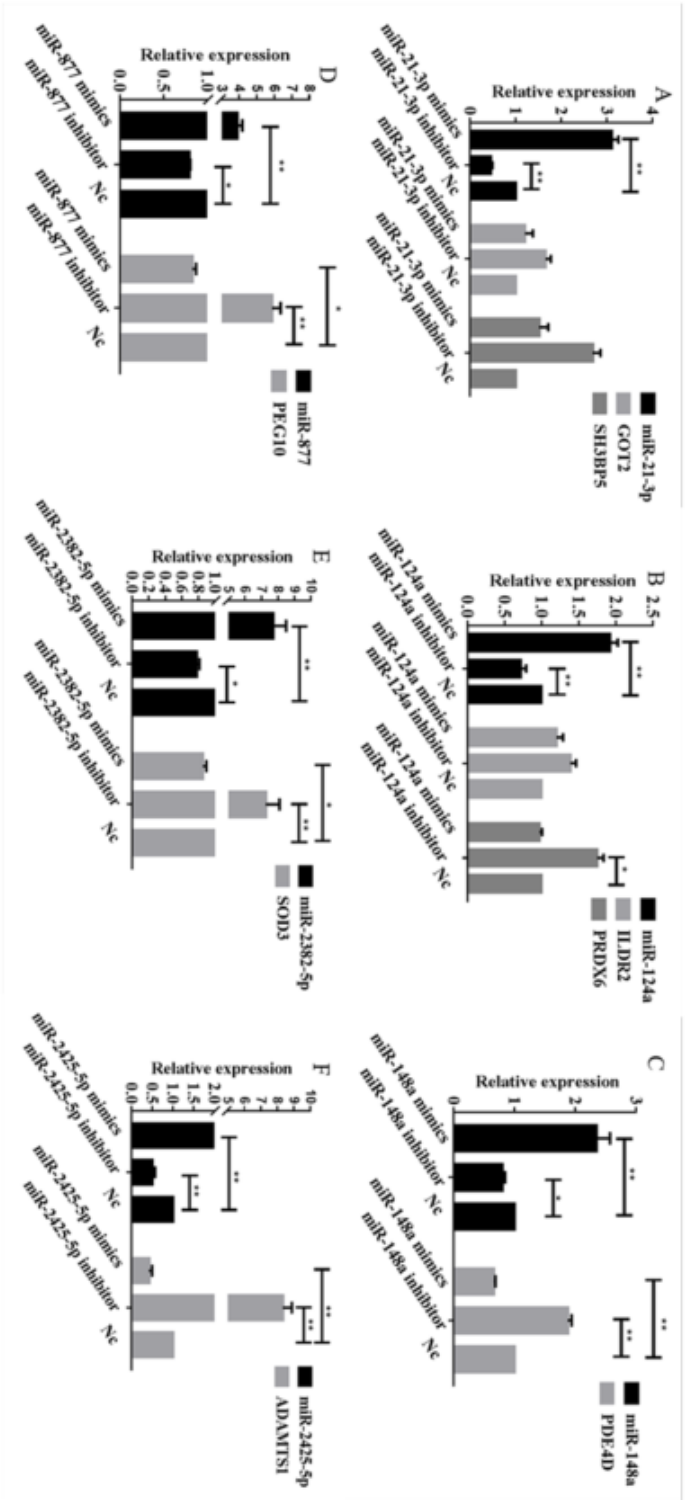


Figure 8

Quantitative RT-PCR for detection the mRNA expression of target DEGs and DERs A Relative expression of miR-21-3p and target genes GOT2 and SH3BP5 following transfection with miR-21-3p mimics, inhibitor and Nc. B Relative expression of miR-124a and target genes ILDR2 and PRDX6 following transfection

with miR-124a mimics, inhibitor and Nc. C Relative expression of miR-148a and target gene PDE4D following transfection with miR-148a mimics, inhibitor and Nc. D Relative expression of miR-877 and target gene PEG10 following transfection with the miR-877 mimics, inhibitor and Nc. E Relative expression of miR-2382-5p and target gene SOD3 following transfection with miR-2382-5p mimics, inhibitor and Nc. F Relative expression of miR-2425-5p and target gene ADAMTS1 following transfection with miR-2425-5p mimics, inhibitor and Nc.

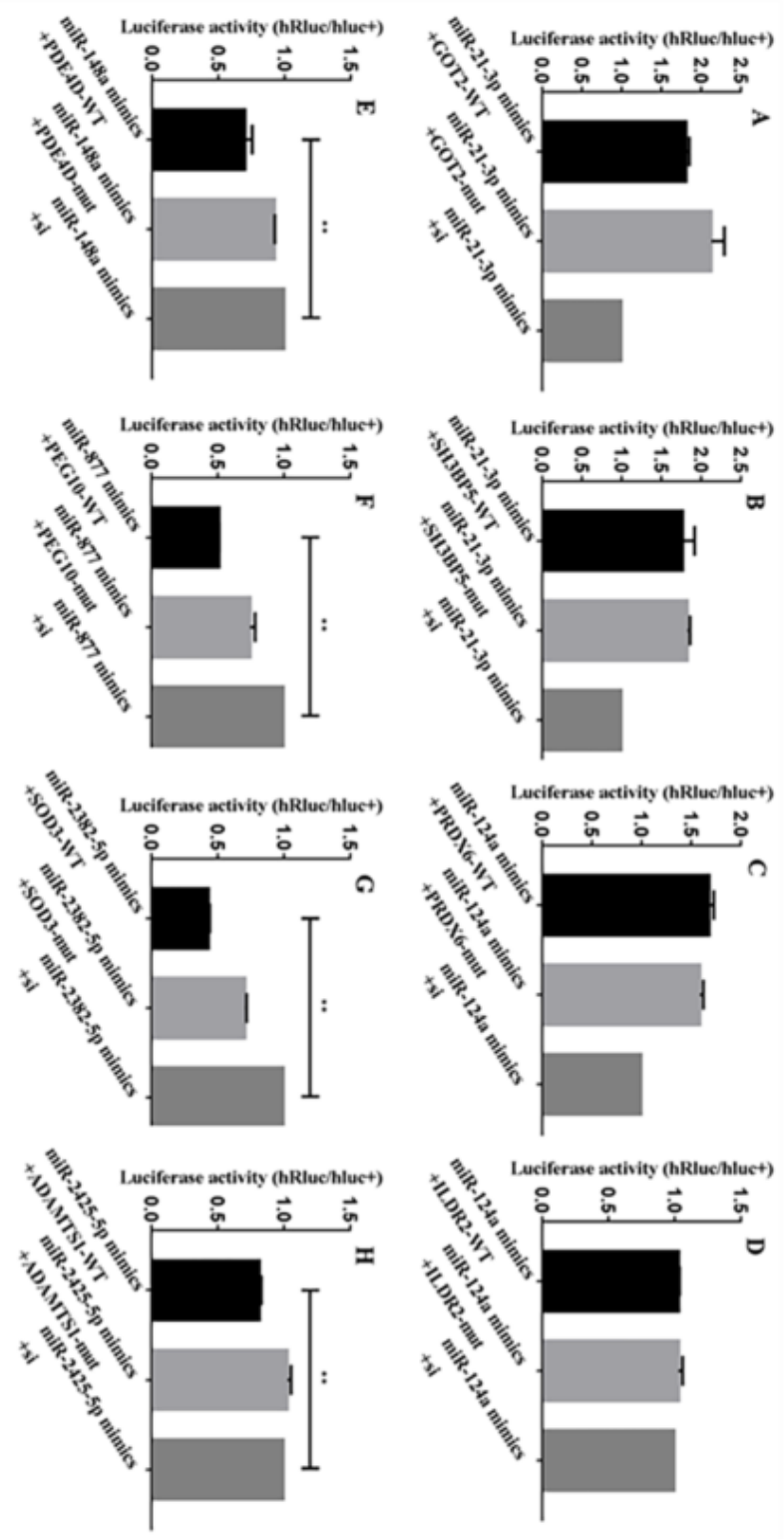


Figure 9

Validation the target relationship of DERs and DEGs Luciferase activities were detected in BMECs cotransfected with miR-21-3p mimics and pmiR-RB-REPORT-GOT2-WT/mut/si vectors (A) or pmiR-RB-REPORT-SH3BP5- WT/mut/si vectors (B). Luciferase activities were detected in BMECs cotransfected with miR-124a mimics and pmiR-RB-REPORT-ILDR2-WT/mut/si vectors (C) or pmiR-RB-REPORT-PRDX6-WT/mut/si vectors (D). Luciferase activities were detected in BMECs cotransfected with miR-148a mimics and pmiR-RB-REPORT-PED4D-WT/mut/si vectors (E). Luciferase activities were detected in BMECs cotransfected with miR-877 mimics and pmiR-RB-REPORT-PEG10-WT/mut/si vectors (F). Luciferase activities were detected in BMECs cotransfected with miR-2382-5p mimics and pmiR-RB-REPORT- SOD3-WT/mut/si vectors (G). Luciferase activities were detected in BMECs cotransfected with miR-2425-5p mimics and pmiR-RB-REPORT-ADAMTS1-WT/mut/si vectors (H).