



Construction of hub transcription factor–microRNAs–messenger RNA regulatory network in recurrent implantation failure

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Abstract

Purpose Recurrent implantation failure (RIF) affects up to 10% of in vitro fertilization (IVF) patients worldwide. However, the pathogenesis of RIF remains unclear. This study was aimed at identifying hub transcription factors (TFs) of RIF in bioinformatics approaches.

Methods The GSE111974 (mRNA), GSE71332 (miRNA), and GSE103465 (mRNA) datasets were downloaded from the Gene Expression Omnibus database from human endometrial tissue using R version 4.2.1 and used to identify differentially expressed TFs (DETFs), differentially expressed miRNAs, and differentially expressed genes for RIF, respectively. DETFs were subjected to functional enrichment analysis and the protein–protein interaction network analysis using the Search Tool for the Retrieval of Interacting Genes (version 11.5) database. Hub TFs were identified using the cytoHubb plug-in, after which a hub TF–miRNA–mRNA network was constructed using Cytoscape v3.8.2.

Results Fifty-seven DETFs were identified, in which Gene Ontology analysis revealed to be mainly involved in the regulation of transcription. Kyoto Encyclopedia of Genes and Genomes pathway analysis suggested that DETFs were enriched in transcriptional misregulation in cancer, aldosterone synthesis and secretion, AMPK signaling pathway, and cGMP-PKG signaling pathway. *EOMES*, *NKX2-1*, and *POU5F1* were identified as hub TFs, and a hub TF–miRNA–mRNA regulatory network was constructed using these three hub TFs, four miRNAs, and four genes.

Conclusion Collectively, we identified three promising molecular biomarkers for the diagnosis of RIF, which may further be potential therapeutic targets. This study provides novel insights into the molecular mechanisms underlying RIF. However, further experiments are required to verify these results.

Keywords Recurrent implantation failure · Transcription factor · MicroRNA · Regulatory network · Bioinformatics analysis

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Introduction

Approximately 10% of infertile patients younger than 40 years of age who undergo at least three in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) cycles and undergo transfer of four or more high-quality embryos worldwide experience embryo implantation failure [1, 2]. Recurrent implantation failure (RIF) inflicts a severe psychological burden as well as economic stress on infertile couples. Despite significant advancements in infertility treatment, the etiology of RIF remains poorly understood, and few effective treatments are available.

Transcription factors (TFs) can bind to specific DNA sequences to increase or block the recruitment of RNA polymerase, thereby regulating messenger RNA (mRNA) expression [3, 4]. Extensive research has shown that TFs can directly regulate the expression of microRNAs (miRNA) in addition to mRNAs [5]. miRNAs are a subset of non-coding RNAs (ncRNAs), comprising 18–25 nucleotides, that can recognize and bind to the three untranslated regions of mRNAs and regulate translation at the post-transcriptional level by inhibiting or degrading target mRNAs [6]. Therefore, TFs, miRNAs, and mRNAs exert synergistic effects on genetic regulatory networks by forming multiple feed-forward and feedback loops. Recently, there has been a surge in research focused on analyzing miRNA expression profiles to explore the mechanisms underlying RIF and to identify potential biomarkers. For example, the detection of *hsa-miR-199a-5p* and *hsa-miR-4306* in the RIF endometrium could be used to clinically assess the probability of successful embryo transfer [7]. Additionally, predictive models based on circulating miRNAs have been proposed, which have indicated that *hsa-miR-96-5p* and *hsa-miR-378e* have the potential to serve as predictive markers of RIF [8]. Moreover, *has-miR-145*, *has-miR-23b*, *has-miR-31*, and *has-miR-30b* have been identified as potential biomarkers for the early diagnosis of RIF [9]. Furthermore, network analysis has been employed to reveal the mechanisms of RIF. Ahmadi et al. and our team both used transcriptome sequencing of fertile women and women with RIF to identify related functional ncRNAs and to construct a circRNA–miRNA–mRNA network [10, 11]. A prior study further constructed a TF–mRNA regulatory network, discovering that transcription factors *SUZ12*, *AR*, *TP63*, *NANOG*, and *TCF3* are involved in the major regulation of RIF [11]. However, a systematic analysis of the TF–miRNA–mRNA network in the RIF endometrium with DETFs as the key element to provide a new insight into the mechanisms of RIF has yet to be reported.

In the present study, we aimed to identify hub TFs and to construct a hub TF–miRNA–mRNA regulatory network of RIF using bioinformatics approaches to provide a systematic

perspective on the molecular mechanism of recurrent implantation failure and to identify novel potential therapeutic targets.

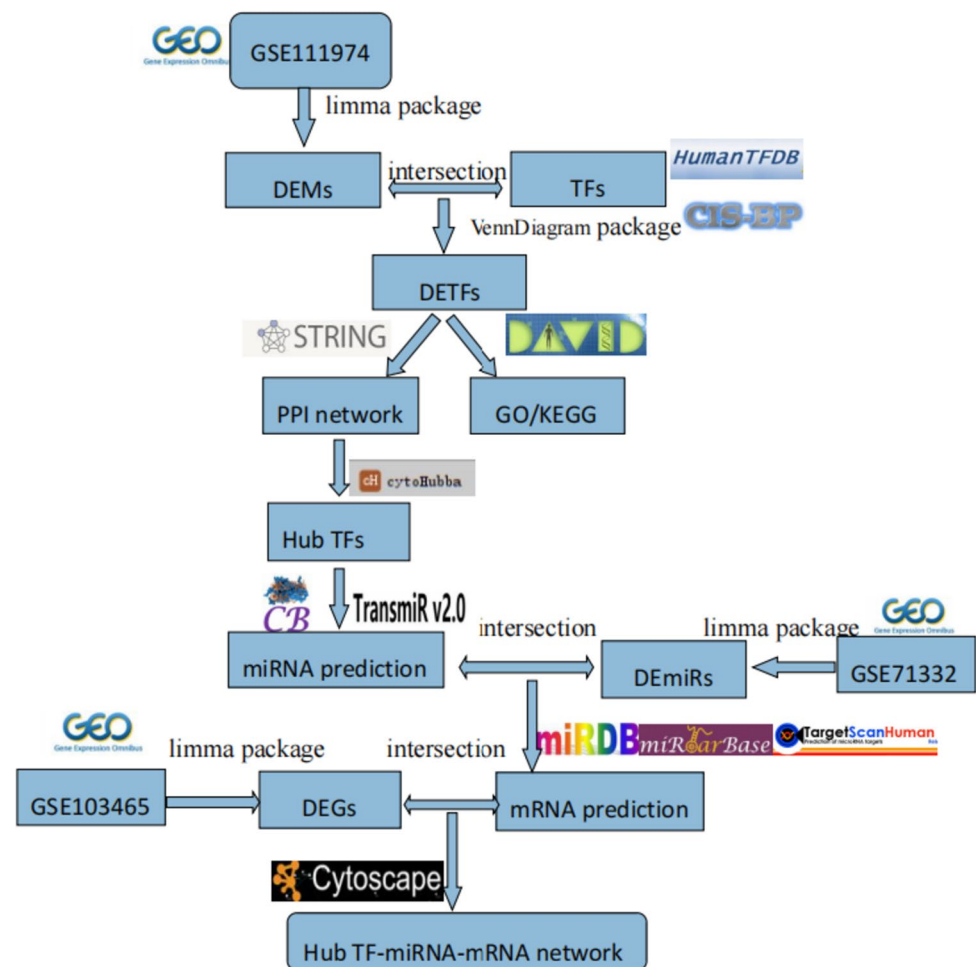
In the current study, the GSE111974 [12], GSE71332 [13], and GSE103465 [14] microarray datasets related to RIF were downloaded from the Gene Expression Omnibus (GEO) database, and differentially expressed mRNAs (DEMs), miRNAs (DEmiRs), and genes (DEGs) were identified using R version 4.2.1. Subsequently, we analyzed the interactions between DEMs and TFs from the Human Transcription Factor Database (Human TFDB) (version 3.0) [15] and Catalog of Inferred Sequence Binding Preferences (CISBP) (version 2.0) [16] database to identify DETFs of RIF. To understand the molecular mechanisms underlying the action of TFs in RIF, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analyses of DETFs were performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8. Furthermore, we constructed a protein–protein interaction (PPI) network using the Search Tool for the Retrieval of Interacting Genes (STRING) (version 11.5) [17] database and identified hub TFs using the cytoHubba plug-in. To understand the regulation of hub TFs in RIF, ChIPBase v3.0 and TransmiR v2.0 [18] online tools were applied to predict target miRNAs of DETFs. The predicted miRNAs and DEmiRs were intersected to obtain overlapping miRNAs. Similarly, the intersection part of predicted genes of the overlapping miRNAs obtained by TargetScan v7.2, miRDB, and miRTarBase online tools and DEGs was taken as the overlapping genes. Finally, a hub TF–miRNA–mRNA regulatory network was constructed by Cytoscape v3.8.2 software. This flowchart is shown in Fig. 1.

Methods

Data resource

Gene expression profiles of endometrium in RIF were searched in the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>). Three expression profile datasets (GSE111974 [12], GSE71332 [13], and GSE103465 [14]) were identified and downloaded. Microarray data of GSE111974 [12] was based on the platform GPL17077 (Agilent-039494 SurePrint G3 Human GE v2 8 × 60K Microarray 039381) and contained 48 samples of endometrial tissue during the window of implantation, including samples from 24 RIF patients and 24 fertile controls. The fertile control patients had a history of at least 1 clinical pregnancy or live birth. GSE71332 [13] contained 6 RIF samples and 5 fertile control samples during the same period of the menstrual cycle, and the detection platform for miRNA extraction was GPL18402 Agilent-046064 Unrestricted_Human_miRNA_V19.0_Microarray (miRNA ID version). Similarly, GSE103465 [14] microarray and the corresponding GPL16043 GeneChip® PrimeView™ Human Gene Expression Array (with

Fig. 1 The flow diagram of the bioinformatics analysis. GEO, Gene Expression Omnibus; Human TFDB, Human Transcription Factor Database; CISBP, Catalog of Inferred Sequence Binding Preferences; DAVID, Database for Annotation, Visualization and Integrated Discovery; STRING, the Search Tool for the Retrieval of Interacting Genes; TFs, transcription factors; DETFs, differentially expressed TFs; DEMs, differentially expressed messenger RNAs; DEmiRs, differentially expressed microRNAs; DEGs, differentially expressed genes; PPI, protein–protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes



External spike-in RNAs) were used to extract genes, which included 3 RIF patients and 3 fertile women during the window of implantation. The characteristics of these three microarrays are summarized in Table 1. There were no significant differences in the general data between the cases and controls. TFs were searched and downloaded from Human TFDB (version 3.0) Database [17] and CISBP (version 2.0) Database [18]; the intersection of TFs from the two online databases via the Venn Diagram v1.6.20 package of R version 4.2.1 was used for subsequent studies.

Identification of DETFs, DEMs, and DEGs

GSE111974 [12] microarray data were extracted and normalized by R version 4.2.1, and DEMs between the RIF and control groups were obtained using the limma v3.46.0 package of R version 4.2.1, followed by intersecting DEMs with TFs from 2 online databases using the Venn Diagram v1.6.20 package to obtain differentially expressed TFs (DETFs). Similarly, we obtained DEmiRs from GSE71332 [13] microarrays and differentially expressed genes (DEGs) from GSE103465

Table 1 Essential characteristics of the three gene expression profiling datasets

| GEO ID | Platform | Detected RNA type | Tissue | Sample (n) |
|----------------|----------|-------------------|--------------------|---|
| GSE111974 [12] | GPL17077 | Messenger RNA | Endometrial tissue | RIF patients (n = 24) Fertile controls (n = 24) |
| GSE71332 [13] | GPL18402 | MicroRNA | Endometrial tissue | RIF patients (n = 6) Patients with male infertility, tubal factors, or unexplained infertility (n = 5) |
| GSE103465 [14] | GPL16043 | Messenger RNA | Endometrial tissue | RIF patients (n = 3) Patients with tubal infertility (n = 3) |

[14] microarrays. The selection criteria for DEMs were a false discovery rate (FDR) < 0.05 , $\log_2FC > 0.7$, and those for DEmiRs and DEGs were a FDR < 0.05 , $\log_2FC > 0.5$.

Functional enrichment analysis of DETFs

To understand the main functions of DETFs, GO and KEGG pathway analyses were applied through the DAVID v6.8 (<https://david.ncifcrf.gov/tools.jsp>) online tool and visualized by ggplot2 package (version 3.3.5) in the R version 4.2.1. Significant enrichment was defined as $p < 0.05$ and counts > 2 . GO included biological process (BP), cellular component (CC), and molecular function (MF).

Construction of PPI networks of DETFs and identification of hub TFs

STRING (version 11.5) [17] online database, which is a tool used to identify known protein–protein interactions and predict genes at the protein level, was used to build a PPI network for DETFs. Cytoscape v3.8.2 was used to visualize the PPI network, where protein entities were represented by nodes and their interconnections were symbolized by edges. To identify the hub transcription factors (TFs) in this network, we used the Maximal Clique Centrality (MCC) algorithm in the cytoHubba plug-in of Cytoscape v3.8.2, considering the nodes with higher degrees (top 5%) as hub TFs.

Prediction of hub TF–miRNA pairs

ChIPBase v3.0 (<https://rna.sysu.edu.cn/chipbase3/index.php>) and TransmiR v2.0 [18] online tools were used to predict target miRNAs of hub TFs. The predicted miRNAs and DEmiRs were intersected using the Venn Diagram v1.6.20 package, resulting in overlapping miRNAs. The hub TF–miRNA pairs were formed by the overlapping miRNAs and upstream hub TFs for further analysis.

Prediction of the target genes of miRNAs

The target genes of the overlapping miRNAs were predicted by TargetScan v7.2 (http://www.targetscan.org/vert_72/), miRDB (<http://www.mirdb.org/>), and miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/php/search.php>) online tools. The intersection obtained using the Venn Diagram v1.6.20 package was used as the predicted gene list, as described previously [10]. Similarly, the intersection part of predicted genes and DEGs was considered as the list of overlapping genes for subsequent analysis.

Construction of a hub TF–miRNA–mRNA network

The hub TF–miRNA–mRNA network was structured and visualized by integrating hub TF–miRNA pairs and miRNA–target genes through shared miRNAs in Cytoscape v3.8.2.

Results

Identification of DETFs, DEMs, and DEGs

A total of 913 DEMs were identified from the GSE111974 [12] microarray, of which 482 were downregulated and 431 were upregulated (Online Resource 1). The relative expression levels of these DEMs were shown in volcano plots (Fig. 2a). Similarly, 160 DEmiRs were extracted from GSE71332 [13] microarray, of which 56 were downregulated and 104 were upregulated (Fig. 2b, Online Resource 2), while 2056 DEGs (Fig. 2c, Online Resource 3) were identified from GSE103465 [14], of which 1399 were downregulated and 657 were upregulated. Subsequently, 1665 TFs were downloaded from the Human TFDB and 1639 TFs from the CISBP. A total of 1507 intersection TFs were obtained from the two online databases using the Venn Diagram v1.6.20 package (Fig. 2d), after which DEMs were intersected with 1507 intersection TFs to obtain the final list of DETFs. A total of 57 DETFs were identified (Fig. 2e), the gene names of which listed in Table 2.

Enrichment analyses of DETFs

The GO terms and KEGG pathways were analyzed through the DAVID v6.8 online tool (Online Resource 4), and the top 10 results were visualized by ggplot2 package (version 3.3.5) in the R version 4.2.1 (Fig. 3). BP analysis revealed that DETFs were significantly enriched in the regulation of transcription, DNA-templated transcription, and transcription of the RNA polymerase II promoter. CC analysis further indicated that DETFs were primarily involved in the nucleus, nucleoplasm, and transcription factor complexes. In MF analysis, DETFs were predominantly enriched in transcription factor activity and DNA binding. KEGG pathway analysis suggested that DETFs were enriched in transcriptional misregulation in cancer, aldosterone synthesis and secretion, adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) signaling pathway, and cyclic guanosine 3',5'-monophosphate (cGMP)-protein kinase G (cGMP-PKG) signaling pathway.

Construction of the PPI network of DETFs and identification of hub TFs

The PPI network of the 57 proteins encoded by the DETFs was constructed using the STRING [17] database

Fig. 2 Volcano plots for each microarray and Venn diagram of TFs. **a** Volcano plots of DEMs based on GSE111974 [12]. **b** Volcano plots of DEmiRs based on GSE71332 [13]. **c** Volcano plots of DEGs based on GSE103465 [14]. **d** Venn diagram of TFs from Human TFDB and CISBP. **e** Venn diagram of DEMs and TFs. TFs, transcription factors; Human TFDB, Human Transcription Factor Database; CISBP, Catalog of Inferred Sequence Binding Preferences; DEMs, differentially expressed mRNAs; DEmiRs, differentially expressed microRNAs; DEGs, differentially expressed genes

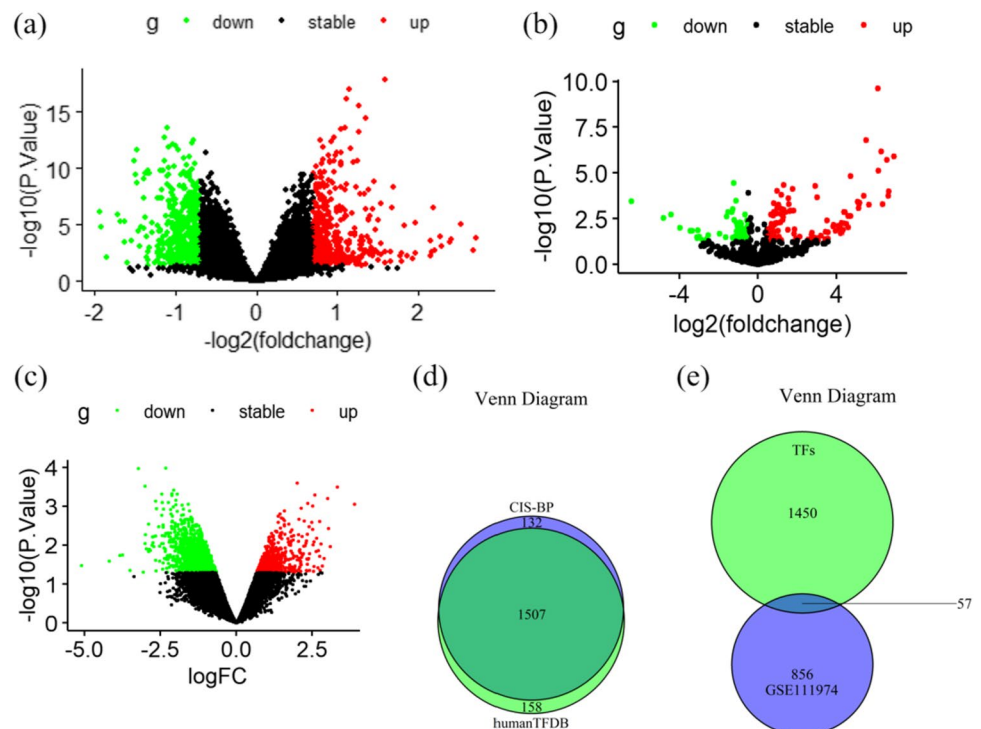


Table 2 Gene names and regulation of 57 intersecting DETFs

| Intersection DETFs | Regulation | Number of DETFs |
|---|------------|-----------------|
| <i>EVX1, ZNF518A, HOXA6, ZNF532, PBX2, ZBTB48, HIF3A, HOXB3, ATF6B, ZEB2, SOX6, AHR, PLAGL2, IRF6, SOX13, CREB3L2, TFCP2L1, FOXO3, ZHX3, GATAD2B, TFEB, HES5, CSRNP1, ZNF750, ZNF589, MAFF, IKZF2, NR4A2, HES2, OTX1, POU5F1, ZBTB16, PPARG</i> | Up | 33 |
| <i>ZNF205, ZNF329, SP8, ZNF768, ZNF337, ZNF121, ZNF222, BCL11B, L3MBTL3, WT1, ZNF214, SRF, AHRR, ME0X2, BNC2, TWIST2, E0MES, FOXS1, NKX2-1, FOXF2, MAF, BATF3, HOXC6, CENPA</i> | Down | 24 |

and visualized by Cytoscape v3.8.2, which included 28 nodes and 37 edges (Fig. 4a). Three hub TFs (*E0MES*, *NKX2-1*, and *POU5F1*) were selected from 28 nodes using the MCC algorithm in cytoHubba plug-in (Fig. 4b).

Prediction of hub TF-miRNA pairs

To further investigate the hub DETF-miRNA regulatory network of RIF, five overlapping miRNAs (*has-miR-1290*,

has-miR-6132, *has-miR-2278*, *has-miR-1246*, and *has-miR-760*) were obtained by intersecting 407 and 325 predicted miRNAs of the three hub DETFs from TransmiR and ChIPBase online tools, respectively, and 160 DEmiRs (Fig. 5). Three hub DETFs and five overlapping miRNAs constituted six hub TF-miRNA pairs, which is listed in Online Resource 5.

Prediction of the target genes of miRNAs

In total, 9802 predicted genes were obtained from TargetScan and 1424 predicted genes from miRDB and 565 predicted genes from miRTarBase, resulting in 64 overlapping predicted genes through Venn Diagram package in R version 4.2.1 (Fig. 6a). Similarly, 5 overlapping genes were identified from intersection of the 64 predicted genes and DEGs (Fig. 6b). Eleven miRNA-gene pairs were constructed using five miRNAs (*has-miR-1290*, *has-miR-6132*, *has-miR-2278*, *has-miR-1246*, and *has-miR-760*) and five genes (*FOXA1*, *LARPI*, *CAPZAI*, *CDK6*, and *CKS2*); the regulatory relationships of these genes are listed in Online Resource 6. Because mature miRNAs regulate translation at the post-transcriptional level by recognizing and binding to the 3' untranslated region of mRNAs to inhibit or degrade target mRNAs [6], five miRNA-mRNA pairs were identified using four miRNAs (*has-miR-1290*, *has-miR-6132*, *has-miR-2278*, and *has-miR-1246*) and four genes (*LARPI*, *CAPZAI*, *CDK6*, and *CKS2*), which are listed in Online Resource 6.

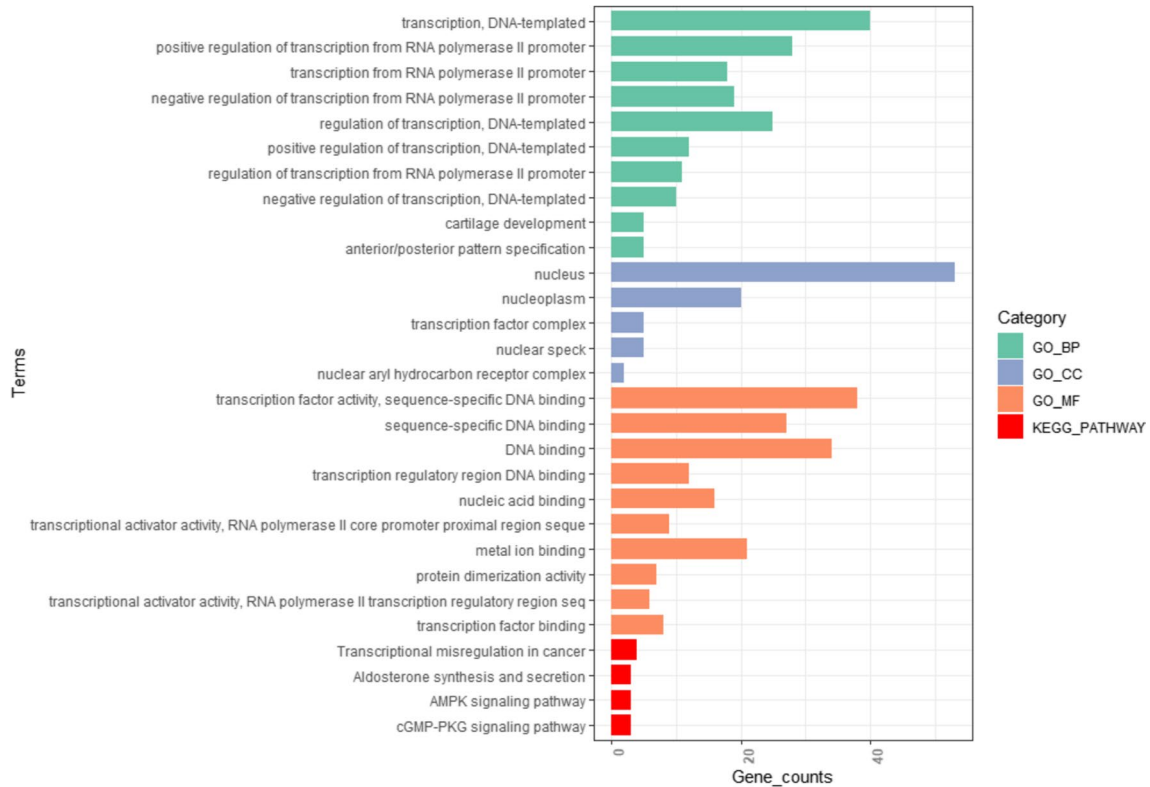


Fig. 3 The top 10 GO terms and KEGG pathways of 57 DETFs. DETFs, differentially expressed transcription factors; GO, Gene ontology; BP, biological processes; CC, cellular components; MF, molecular functions; KEGG, Kyoto Encyclopedia of Genes and

Genomes; AMPK, adenosine 5'-monophosphate (AMP)-activated protein kinase; cGMP-PKG, cyclic guanosine 3',5'-monophosphate (cGMP)-protein kinase G

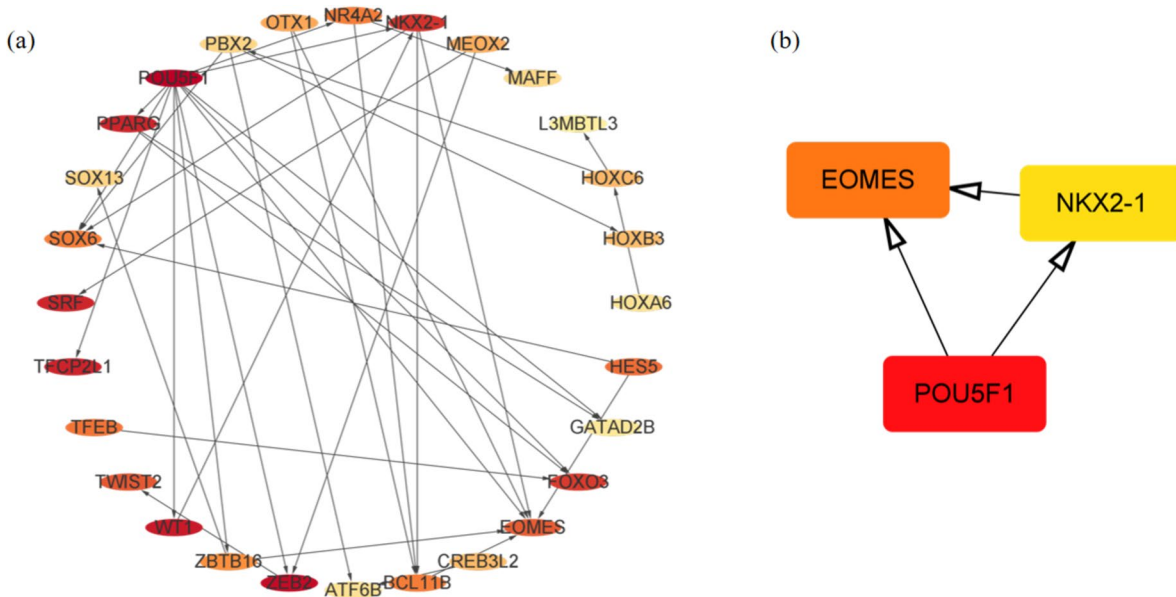


Fig. 4 A PPI network and hub gene regulatory network. **a** A PPI network of the 57 DETFs in RIF. This network consists of 28 nodes and 37 edges. **b** Three hub TFs (*EOMES*, *NKX2-1* and *POU5F1*)

extracted by cytoHubba plug-in. PPI, protein-protein interaction; DETFs, differentially expressed transcription factors; RIF, recurrent implantation failure

Venn Diagram

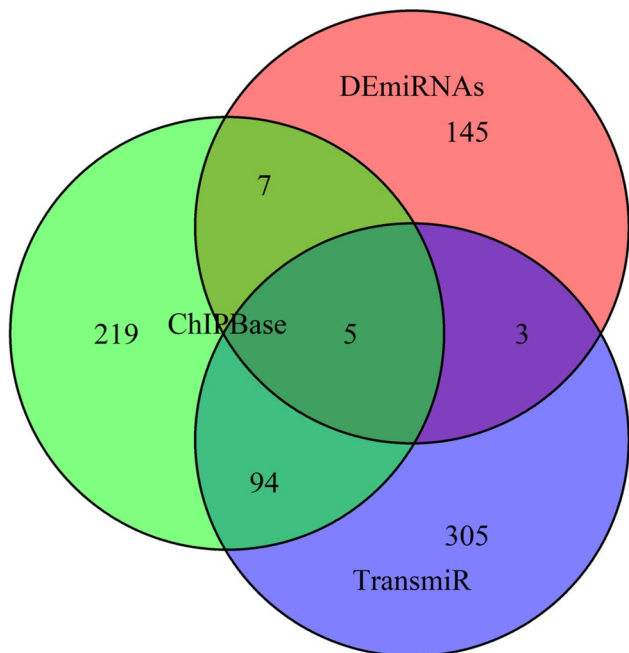


Fig. 5 Venn diagram of miRNA predicted by TranmiR, ChIPBase online tools, and DEmiRs from GSE71332

Construction of a TF–miRNA–mRNA network

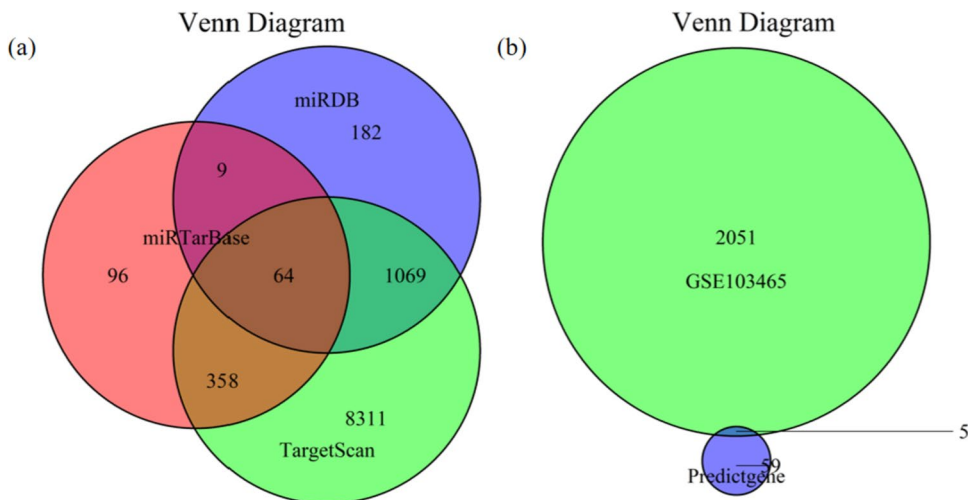
After integrating 6 TF–miRNA pairs and 5 miRNA–target genes interactions, a hub TF–miRNA–mRNA regulatory network with 6 interactions containing 3 TFs (*EOMES*, *NKX2-1*, and *POU5F1*), 4 miRNAs (*has-miR-1290*, *has-miR-6132*, *has-miR-2278*, and *has-miR-1246*) and 4 genes (*LARP1*, *CAPZA1*, *CDK6*, and *CKS2*) was constructed and visualized by Cytoscape v3.8.0, including the following regulatory axes: *EOMES/has-miR-1290/CAPZA1*,

EOMES/has-miR-6132/LARP1, *NKX2-1/has-miR-6132/LARP1*, *POU5F1/has-miR-2278/CDK6*, *POU5F1/has-miR-1246/CKS2*, and *POU5F1/has-miR-1246/CAPZA1* (Fig. 7).

Discussion

The etiology and pathogenesis of RIF remain unclear, and its treatment is limited. Therefore, identifying relevant molecules to elucidate the molecular mechanism of RIF is of great significance for exploring its etiology and therapeutic targets. At present, there are many studies on mRNA and miRNAs in RIF; however, only a few studies have been conducted on TFs that can regulate the expression of mRNA and miRNAs. Therefore, there is an urgent need to investigate the role of transcription factors and their related pathways in RIF. The results of such an analysis will not only be beneficial for exploring the pathogenesis of RIF but will also help to provide targets for the treatment of RIF. In the present study, 57 DETFs were identified between the RIF and fertile control groups during the window of implantation using a bioinformatics method. GO enrichment analysis of DETFs revealed that DETFs were significantly enriched in the regulation of transcription, DNA template, and transcription from the RNA polymerase II promoter. KEGG pathway analysis further suggested that DETFs were enriched in transcriptional misregulation in cancer, aldosterone synthesis and secretion, and the AMPK and cGMP-PKG signaling pathways. PPI analysis of the DETFs revealed that *EOMES*, *NKX2-1*, and *POU5F1* were hub TFs of RIF. Finally, a hub TF–miRNA–mRNA regulatory network consisting of the six follow regulatory axes was formed: *EOMES/has-miR-1290/CAPZA1*, *EOMES/has-miR-6132/LARP1*, *NKX2-1/has-miR-6132/LARP1*,

Fig. 6 Venn diagram of genes. **a** Venn diagram of genes predicted by TargetScan, miRDB, and miRTarBase through Venn Diagram package. **b** Venn diagram of 2056 DEGs and 64 predicted genes through Venn Diagram package. DEGs, differentially expressed genes



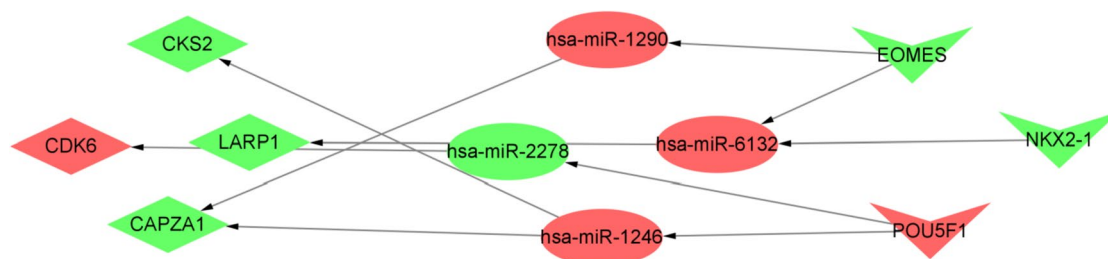


Fig. 7 Hub TF–miRNA–mRNA regulatory network of RIF, consisting of 3 TFs, 4 miRNAs, and 4 mRNAs. Red represents upregulation; green represents downregulation. The down arrow represents TFs, the

ellipse represents miRNAs, and the diamond represents mRNAs. PPI, protein–protein interaction; TF, transcription factor; miRNA, microRNA; mRNA, messenger RNA; RIF, recurrent implantation failure

POU5F1/has-miR-2278/CDK6, *POU5F1/has-miR-1246/CKS2*, and *POU5F1/has-miR-1246/CAPZA1*.

EOMES (eomesodermin) is closely related to immune function and participates in the regulation of the functions of immune cells, including natural killer (NK) cells, CD8 (+) T cells, and decidual (dCD4) (+) T cells. A good maternal-fetal immune balance is key to a successful pregnancy. NK cells are abundant at the maternal-fetal interface during the first trimester, and *EOMES* is overexpressed in NK cells in the human and mouse uterus and decidua during early pregnancy [19]. *EOMES* is the primary regulator of NK cell development, maturation and function [20]. *EOMES* is involved in the regulation of dCD4 (+) T and dCD8 (+) T cells during early pregnancy and plays a vital role in the induction and maintenance of maternal fetal tolerance [21, 22]. *EOMES* not only affects endometrium/decidua, but also influences embryo self-development, metabolism, and trophoblast cell function [23]. A single-cell sequencing study of endometrium of RIF patients in WOI found that *EOMES* is expressed in tissue-resident NK1 and NK2 cells, which also implies that *EOMES* mainly plays a role through NK cells during the window of implantation in RIF patients [24]. However, the effect of *EOMES* on RIF has not previously been reported. This study found that *EOMES* plays a role in RIF by regulating *miR-1290* and *miR-6132*, which has not yet been confirmed in clinical samples.

POU5F1 (POU class 5 homeobox 1), also known as Octamer-4 (*Oct4*), is a homologous domain transcription factor of the Pit-Oct-Unc (POU) family involved in mammalian embryonic development, cell lineage specification, germ cell pluripotency maintenance, and regulation of somatic cell reprogramming into induced pluripotent stem cells by binding through the POU domain to specific octamer sequence motifs (ATGCAAT) on the enhancer and promoter regions of target genes to activate or inhibit gene expression [25]. A thin endometrial model was constructed by bilateral uterine artery ligation in rats and *POU5F1* expression in endometrium was decreased [26]. Contrary to the results of our study, this indicates that the mechanism of *POU5F1* expression in patients with RIF is different from

that in patients with a thin endometrium. Studies have found that *POU5F1* binds to the *miR-302* promoter in P19 mouse embryonic cancer cells to activate the expression of *miR-302*, which is involved in the self-renewal and maintenance of pluripotency in P19 cells [27]. Wang et al. [28] showed that *POU5F1* transactivates *miR-125b* via interaction with the *miR-125b* promoter and inhibits the expression of *BAK1*, thereby promoting tumorigenesis and inhibiting the apoptosis of cervical cancer cells. This study showed that *POU5F1* in RIF primarily affected the expression of *CAPZA1*, *CKS2*, and *CDK6* by regulating the miRNAs *miR-1246* and *miR-2278*. However, there have been no other reports on the impact of *POU5F1* on RIF through miRNAs; therefore, validation experiments are needed.

NKX2-1 (*NK2* homeobox 1), also known as thyroid transcription factor-1 (*TTF-1*), is a homeodomain transcription factor considered to be specific to the thyroid, lung, and central nervous systems. *NKX2-1* controls the differentiation of telencephalic GABAergic interneurons and oligodendrocytes, and regulates the generation of telencephalic astrocytes during embryonic development [29]. *NKX2-1* gene deletion during embryonic development also results in the absence of peripheral lung and thyroid tissue [30]. Prior studies have suggested that *NKX2-1* inhibits the expression of *miR-200c* in mouse lung epithelial cells by controlling the transcriptional activity of *miR-200c* 5' flanking regions, and promotes the expression of nuclear factor I/B and myeloblastic oncogenes to affect lung development and tumorigenesis [31]. There have been no prior reports on the expression of *NKX2-1* in the endometrium of patients with RIF. This study found that low expression of *NKX2-1* stimulates the expression of *miR-6132* in RIF; however, further in-depth experimental studies are warranted to verify these results.

The results of the present study further indicated that *miR-1246* affects embryo implantation by inhibiting the expression of *CAPZA1* and *CKS2*. Ponsuksili et al. [32] found that the expression of *miR-1246* in the endometrium on the 3rd day of the bovine estrous cycle was lower than that on the 7th day, and the expression of *miR-1246* may be regulated by hormones. This indirectly shows that high expression of *miR-1246* is not

conducive to implantation, although no relevant verification has been performed in the human implantation window. Our results further showed that *miR-2278* is weakly expressed and regulates the expression of *CDK6* in the endometrium of patients with RIF, while *miR-6132* is overexpressed and plays a role in RIF by inhibiting the expression of *LARPI* (La-related Protein 1). No previous studies have confirmed the relationship between *miR-2278*, *miR-6132*, and RIF. Therefore, further studies on this topic are urgently required.

The expression level of *miR-1290* in the endometrium on the 7th day is higher than that on the 3rd day in the estrous cycle in bovine [32]. A study on embryo implantation found that *miR-1290*, an exosome derived from placental trophoblast cells, promotes the epithelial-mesenchymal transition process of endometrial epithelial cells by targeting *LHX6*, and further promotes the interaction between the endometrium and the embryo. In addition, exosomal *miR-1290* is involved in the embryo implantation by promoting inflammation and angiogenesis, thereby enhancing the interaction between the embryo and uterus [33]. In current study, *miR-1290* affected the adhesion of embryos through targeted regulation of *CAPZA1*. Conversely, our study did not involve embryos, analyzing only miRNA expression in the endometrium during the luteal phase. Thus, we temporarily excluded the possibility that *miR-1290* was secreted by trophoblast exosomes and acted on the endometrium.

The study revealed that *LARPI* expression is decreased in the endometrial lining of patients with RIF. *LARPI* was initially shown to be involved in spermatogenesis, embryogenesis, and cell cycle progression in *Drosophila* [34]. Recent studies have shown that the mechanisms of *LARPI* are different under different stress conditions. When nutrients are abundant and mammalian target of rapamycin complex 1 is active, *LARPI* immobilizes and stabilizes terminal oligopyrimidine (*TOP*) mRNA via the N-terminal La module. In contrast, *LARPI* binds to and inhibits *TOP* mRNA translation through the C-terminal DM15 region, but its overall effect is to reduce cell growth and proliferation [35]. However, additional research is required to ascertain whether the role of *LARPI* varies in the endometrium of patients with RIF under different stress conditions.

Our findings further suggested that patients with RIF exhibit a noticeable decrease in *CAPZA1* expression within the endometrial lining. *CAPZA1* (capping actin protein of muscle Z-line alpha subunit 1) encodes the α subunit of F-actin capping protein, which regulates the dynamic assembly of actin filaments and cell motility by binding to the barbed end of actin filaments [36]. Accumulating evidence has demonstrated that aberrant expression of *CAPZA1* is strongly associated with the development of some cancers, with a tumor suppressor role in breast cancer [37] and complete hydatidiform mole [38]. Given the lack of research

on the role of *CAPZA1* in the endometrium, it is crucial to conduct in-depth meticulous studies to ensure a more comprehensive understanding of its potential role and impact.

CKS2 (cyclin-dependent kinase subunit 2) belongs to a highly conserved cyclin dependent kinase (*CDK*) binding protein family and plays a critical role in somatic cell division and early embryonic development [39]. Prior research has shown that *CKS2*^{-/-} oocytes exhibit reduced and delayed activity of maturation-promoting factor during meiosis, resulting in defects in germinal vesicle breakdown, anaphase-promoting complex/cyclosome activation, and meiotic spindle assembly. The expression of *CDK1* and *cyclin A1/B1* of *CKS2*^{-/-} germ cells was significantly reduced [40]. *CKS2* can further increase the expression of *cyclin*, *cyclin A*, *cyclin B1*, and *CDK1*, thereby promoting cell proliferation, and is also strongly related to mitochondrial caspase-dependent cell apoptosis mediated by *bax* [41, 42]. These findings are primarily consistent with our results, suggesting that decreased *CKS2* expression may contribute to RIF by affecting the cellular cycle and proliferation of endometrial cells. However, further studies are required to verify this hypothesis.

In the present study, *CDK6* was highly expressed in the endometria of patients with RIF. *CDK6*, a serine/threonine protein kinase, is a member of the cyclin-dependent kinase family that functions as a cell cycle kinase and transcription regulator [43]. *CDK6* affects the biological behavior of endometrial cells by promoting cell transformation from the G0/G1 phase to the S phase, thereby promoting the progression of membranous ectopic disease [44]. This is consistent with the results of *CDK6* expression in this study. However, whether the mechanism of action of RIF is similar requires further verification.

Currently, only a few studies have investigated the mechanisms of the action of TFs in RIF. To the best of our knowledge, this study is the first to construct a hub TF–miRNA–mRNA network using the GEO database. However, given that these results are only based on bioinformatic analysis, further in-depth experimental studies are essential to validate the possible roles of these six axes in RIF.

Conclusions

Overall, 57 DETFs were identified in patients with RIF, of which *EOMES*, *NKX2-1*, and *POU5F1* were identified as the hub TFs. KEGG pathway analysis further showed that these DETFs are involved in the regulation of transcriptional misregulation in cancer, aldosterone synthesis and secretion, and the AMPK and cGMP-PKG signaling pathway. Finally, we constructed a hub TF–miRNA–mRNA regulatory network for RIF. Overall, the results of the present study show

that *EOMES*, *NKX2-1*, and *POU5F1* may have the potential to serve as molecular biomarkers for the diagnosis and potential therapeutic targets of RIF. This study provides novel insights into the molecular mechanisms underlying RIF. However, further experiments are required to verify these results.

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Data availability The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Declarations

Ethics approval Publicly available datasets were analyzed in this study, so ethical review, approval, and written informed consent for participation were not required for the study.

Competing interests The authors declare no competing interests.

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