



# Identification of Differentially Expressed mRNAs and lncRNAs Contributes to Elucidation of Underlying Pathogenesis and Therapeutic Strategy of Recurrent Implantation Failure

Lin Liu<sup>1,2,3,4</sup> · Yidan Liu<sup>2</sup> · Yu Tian<sup>1</sup> · Ying Cao<sup>5</sup> · Ting Wang<sup>1</sup> · Shengyan Mi<sup>1</sup> · Run Yang<sup>6</sup> · Simin Liu<sup>6</sup> · Xiaoling Ma<sup>1,2,3,4</sup> · Jing Wang<sup>6</sup>

Received: 3 March 2024 / Accepted: 22 June 2024  
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## Abstract

Recurrent implantation failure (RIF) is a complex and poorly understood clinical disorder characterized by failure to conceive after repeated embryo transfers. Endometrial receptivity (ER) is a prerequisite for implantation, and ER disorders are associated with RIF. However, little is known regarding the molecular mechanisms underlying ER in RIF. In the present study, RNA sequencing data from the mid-secretory endometrium of patients with and without RIF were analyzed to explore the potential long non-coding RNAs (lncRNAs) and messenger RNAs (mRNAs) involved in RIF. The analysis revealed 213 and 1485 differentially expressed mRNAs and lncRNAs, respectively (fold change  $\geq 2$  and  $p < 0.05$ ). Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses indicated that these genes were mostly involved in processes related to immunity or inflammation. 5 key genes (*TTR*, *ALB*, *TF*, *AFP*, and *CFTR*) and a key module including 14 hub genes (*AFP*, *ALB*, *APOA1*, *APOA2*, *APOB*, *APOH*, *FABP1*, *FGA*, *FGG*, *GC*, *ITIH2*, *SERPIND1*, *TF* and *TTR*) were identified in the protein–protein interaction (PPI) network. The 5 key genes were used to further explore the lncRNA–miRNA–mRNA regulatory network. Finally, the drug ML-193 based on the 14 hub genes was identified through the CMap. After ML-193 treatment, endometrial cell proliferation was increased, the hub genes were mostly down-regulated, and the ER marker *HOXA10* was up-regulated. These results offer insights into the regulatory mechanisms of lncRNAs and mRNAs and suggest ML-193 as a therapeutic agent for RIF by enhancing ER.

**Keywords** Recurrent implantation failure · Messenger RNA · Long non-coding RNA · Competing endogenous RNA · Endometrial receptivity · ML-193

## Introduction

Infertility poses a significant challenge to human reproduction. Approximately 15% of patients undergoing infertility treatment experience recurrent implantation failure (RIF)

Lin Liu, Yidan Liu, Yu Tian, Ying Cao and Ting Wang contributed equally to this work.

✉ Lin Liu  
ldyy\_liul@lzu.edu.cn

✉ Xiaoling Ma  
maxl2005@163.com

✉ Jing Wang  
jwang\_2017@hotmail.com

<sup>1</sup> The First Clinical Medical College of Lanzhou University, Lanzhou, Gansu, China

<sup>2</sup> The Basic Medical Sciences College of Lanzhou University, Lanzhou, Gansu, China

<sup>3</sup> The reproductive center, the First Hospital of Lanzhou University, Lanzhou, Gansu, China

<sup>4</sup> Clinical Research Center for Reproductive Diseases of Gansu Province, Lanzhou, Gansu, China

<sup>5</sup> Reproductive Medicine Center, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei, China

<sup>6</sup> School of Public Health, Gansu University of Chinese Medicine, Lanzhou, Gansu, China

[1]. Coughlan et al. [2] proposed classical diagnostic criteria for RIF and defined this condition as a failure to achieve a clinical pregnancy after transfer of at least 4 high-quality embryos in a minimum of 3 fresh or frozen cycles in women under the age of 40 years. Successful embryo implantation occurring during the implantation window is a process that requires a reciprocal interaction between blastocyst and the endometrium [3]. The window of implantation usually spans 3 to 6 days within the secretory phase. During this time span, the endometrium attains the temporal differentiation and reaches receptivity status, allowing embryo adhesion and invasion [4]. The changes that occur during this process are regulated by multiple molecules, including noncoding RNA, cytokines, growth factors, and cell adhesion molecules [5, 6]. Genetic polymorphism of the genes involved in embryo implantation, immune responses and endometrial receptivity (ER) are likely to be involved in the pathophysiology of RIF [7]. RIF places substantial psychological and economic burdens on patients and their families as well as healthcare associations. However, the precise etiology and pathogenesis of RIF are complex and remain unclear.

With the recent advances in high-throughput sequencing technology, it is now well-established that non-coding RNAs, such as microRNA (miRNA), circular RNA and long non-coding RNA (lncRNA), play distinct regulatory roles in biological processes [8]. lncRNA, defined as a kind of non-coding RNA longer than 200 bp, has attracted considerable attention because of its aberrant expression in various diseases [9]. Some lncRNAs can act as competitive endogenous RNAs (ceRNAs) to regulate the expression of downstream messenger RNA (mRNA) by sponging shared miRNA activity, thereby sequestering miRNAs and regulating the function of miRNA-targeted mRNAs [10]. Furthermore, the contributions of dysregulated expression of lncRNAs to the occurrence of RIF have been reported. A study showed that the expression of lncRNA H19 was down-regulated in the endometrium of patients with RIF and acted as a sponge that regulated integrin  $\beta$ 3 through its antagonistic effect on

let-7, which might affect ER and embryo implantation [11]. Nevertheless, with respect to the clinical value of lncRNA in RIF, evidence regarding the differences in lncRNA expression between patients with and without RIF is still limited.

Therefore, the aim of our study was to identify mRNA and lncRNA related regulatory networks in RIF through RNA sequencing (RNA-seq) and bioinformatics analysis. And drug ML-193 was predicted based on the 14 hub genes identified in the protein–protein interaction (PPI) network, the effect of ML-193 on endometrial cells was partly verified. ML-193 should be further investigated as a potential drug for the treatment of RIF patients in the future.

## Materials and Methods

### Patients Selection and Sample Collection

The inclusion and exclusion criteria of patients who provided mid-secretory phase endometrium are shown in Table 1. In total, 18 tissue specimens from the mid-secretory phase endometrium (RIF group,  $n = 10$ ; control group,  $n = 8$ ) and 10 normal late proliferative endometrial tissue specimens were included in this study. The 18 endometrial samples were obtained during the mid-secretory phase as confirmed by histological diagnoses. Among these specimens, 6 (RIF group,  $n = 3$ ; control group,  $n = 3$ ) were randomly selected for RNA-seq, and the remaining 12 (RIF group,  $n = 7$ ; control group,  $n = 5$ ) underwent RT-qPCR. The eligibility criteria for the 10 patients who provided the late proliferative endometrial tissue was consistent with our previous study [12]. The 10 endometrium samples were used for primary cell culture. The CCK8 was used in 4 specimens and RT-qPCR was performed in 6.

This study was approved by the Research Ethics Committee of the First Hospital of Lanzhou University (Approval No. LDYYLL2019-45). Informed consent was obtained from all participants.

**Table 1** Inclusion and exclusion criteria of patients who provided mid-secretory proliferative phase endometrium

	Control Group ( $n=3$ )	RIF ( $n=3$ )
Inclusion criteria	Age under 40 years	Age under 40 years
	Underwent IVF/ICSI-ET due to fallopian tube or male factors and conceived in the next cycle after sampling	Failure to achieve a clinical pregnancy after transfer of at least four high-quality embryos in a minimum of three fresh or frozen cycles in women [2]
	FSH < 10 mIU/mL, LH < 10 mIU/mL and estradiol (E2) < 50 pg/mL measured on day 2-3 of a menstrual cycle	
	Consent to participate in this study	Consent to participate in this study
Exclusion criteria	Women aged 40 years or older	Women aged 40 years or older
	Chromosomal abnormalities, immunogenetic disorders, uterine anomalies (congenital uterine anomaly, fibroid, polyps, intrauterine adhesions), PCOS, endometriosis, abnormal blood glucose level or thyroid diseases	
	Receive steroid hormone within the preceding 2 months	

RIF recurrent implantation failure, FSH follicle-stimulating hormone, LH luteinizing hormone, PCOS polycystic ovary syndrome

## RNA Isolation and Quality Control

Total RNA was extracted from cells and tissues using TRIzol reagent (Invitrogen, Gibco-BRL, Bethesda, MD, USA) in accordance with the manufacturer's instructions. The concentration and quality of the extracted RNA were then determined on using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity and genomic DNA contamination were further assessed by standard denaturing agarose gel electrophoresis.

## RNA-Seq

RNA samples from the RIF and control groups were sent to Cloud-Seq Biotech (Shanghai, China). Briefly, total RNA was purified following removal of ribosomal RNA (rRNA) with a Gen-Seq® rRNA Removal Kit (GenSeq, Inc.). Each rRNA-depleted sample was then subjected to library construction with a GenSeq® Low Input RNA Library Prep Kit (GenSeq, Inc.) per in accordance with the manufacturer's instructions. Samples were quality-controlled and quantified using the BioAnalyzer 2100 system (Agilent Technologies, Inc., Santa Clara, CA, USA). Library sequencing was performed on an Illumina Novaseq 6000 platform (Illumina, San Diego, CA, USA). Raw data were normalized using edgeR (v3.16.5). Differentially expressed mRNAs and lncRNAs were defined by an FC  $\geq 2$  and  $p$  value  $< 0.05$ .

## Hierarchical Clustering

Differentially expressed mRNAs and lncRNAs (FC  $\geq 2$  and  $p$  value  $< 0.05$ ) were calculated with normalized counts. Cluster analysis was generated using the heatmap2 package in the R software. Different endometrial samples were usually clustered in different branches.

## GO and KEGG Enrichment Analyses

Gene ontology (GO) analysis and pathway analysis were used to determine the functions of these dysregulated mRNAs and lncRNAs. GO terms were grouped into three categories: biological process (BP), cellular component (CC) and molecular function (MF) (<http://www.geneontology.org>). Dysregulated mRNAs and lncRNAs were mapped to GO terms in the DAVID (<https://david.ncifcrf.gov/>). The  $p$  value denotes the significance of GO terms enrichment in the genes ( $p$  value  $< 0.05$  is recommended). The enrichment score equaled  $-\log_{10}(p \text{ value})$ . The pathway analysis was based on the functional analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (<http://www.genome.jp/kegg/>). The  $p$  value denotes the significance of

the pathway correlated to the conditions ( $p$  value  $< 0.05$  is recommended).

## PPI Network Construction

The PPI pairs between differentially expressed mRNAs were evaluated using the String database (version 11.5; <https://cn.string-db.org/>) with a medium confidence score of  $> 0.4$ . Next, PPI network visualization was performed using Cytoscape. The hub nodes with a high network topology property indicator score were considered to have an important role in the network. The hub nodes in the PPI network were analyzed by CytoNCA plug-in of Cytoscape, and degree centrality, betweenness centrality and closeness centrality were calculated. The "Molecular Complex Detection" (MCODE) plugin of Cytoscape was then employed to identify the hub gene clusters exhibiting a high degree of interaction.

## Construction of ceRNA Network

To construct the ceRNA network, differentially expressed mRNAs of interest in the hub nodes of the PPI network were selected. The miRNA-mRNA interaction pairs were obtained from the prediction of TargetScan (<https://www.targetscan.org/>). Target genes with a context score percentile  $< 50$  were excluded from the TargetScan algorithm. The interactions between miRNAs and differentially expressed lncRNAs were predicted using the miRcode (<http://www.mircode.org/>) with R software. Next, lncRNA-miRNA-mRNA relationships were constructed based on their shared miRNAs between the miRNA-mRNA and miRNA-lncRNA interactions. The lncRNA-miRNA-mRNA (ceRNA) network was constructed by using Cytoscape 3.9.1.

## CMap Analysis

CMap (<https://portals.broadinstitute.org/CMap/>) is a database of gene expression profiles from cultured human cells treated with bioactive small molecules. It is designed to help scientists to discover functional connections among diseases, genetic perturbation, and drug action. Up-regulated tags of the 14 hub genes were loaded into the CMap online tool, 1046 bioactive molecules were obtained. A norm\_cs score ranging from -1 to 1 was used to estimate the closeness between query signatures and compounds: a positive score indicates a promotive effect of the compound on the query signatures, whereas a negative score denotes an inverse effect of the compound on the query signatures. Drugs with negative scores are typically interpreted as having potential therapeutic effects on the biological processes represented by the query signatures.

## Cell Culture

10 tissue samples were used for primary cell culture; 4 of them were used for the CCK8 and 6 were used for RT-qPCR. The extraction method of the primary cells was performed as described by Lyu et al. [12].

### A Cell Counting Kit-8 (CCK8)

CCK8 assay was used to determine the viability of human embryonic stem cells (hESCs) and Ishikawa (ISK) cells under the treatment of ML-193. Briefly, cells were seeded onto a 96-well plate (hESCs,  $1 \times 10^4$  cells/well; ISK cells,  $5 \times 10^3$  cells/well). After 1 day, the cells were treated with ML-193 (0, 5 and 10 mM) for 0 or 24 h. Next, 10  $\mu$ L of CCK8 solution (NCM, Suzhou, China) was added to each well, and the cells were incubated at 37°C for 2 h. The absorbance at 450 nm was measured using an enzyme-labelling instrument (Infinite M2000 PRO; Tecan, Männedorf, Switzerland). 5 replicate wells were tested for each group.

### Real-time Polymerase Chain Reaction (RT-qPCR)

Total RNAs isolated from cells and frozen endometrial tissues were reverse-transcribed to complementary DNA using a high-capacity complementary DNA reverse-transcription kit (Wuhan Servicebio Technology Co., Ltd., Wuhan, China). The expression of lncRNAs in endometrial tissues and the expression of mRNAs in hESCs and ISK cells were measured by RT-qPCR using SYBR Premix Ex Taq (Wuhan Servicebio Technology Co., Ltd.) on an ABI 7900HT instrument (Thermo Fisher Scientific).  $\beta$ -actin was served as an internal control. All primer sequences used in this study were listed in Supplementary Table S1. The  $2^{-\Delta\Delta C_t}$  method was used to calculate the relative expression of each gene.

### Statistical Analysis

Lists of differentially expressed mRNAs and lncRNAs were generated by screening with Student's t-test, using the criteria of an FC of  $\geq 2.0$  and a  $p$  value of  $< 0.05$  between the RIF and control groups. In the GO and KEGG pathway analyses, Fisher's exact test was used to evaluate the significance of the GO terms or pathway identifier enrichment in the differentially expressed genes. A  $p$  value of  $< 0.05$  was considered statistically significant. The statistical analysis was performed with GraphPad Prism software 9.0.0 (GraphPad Software, La Jolla, CA, USA).

## Results

### Differentially Expressed mRNAs and lncRNAs

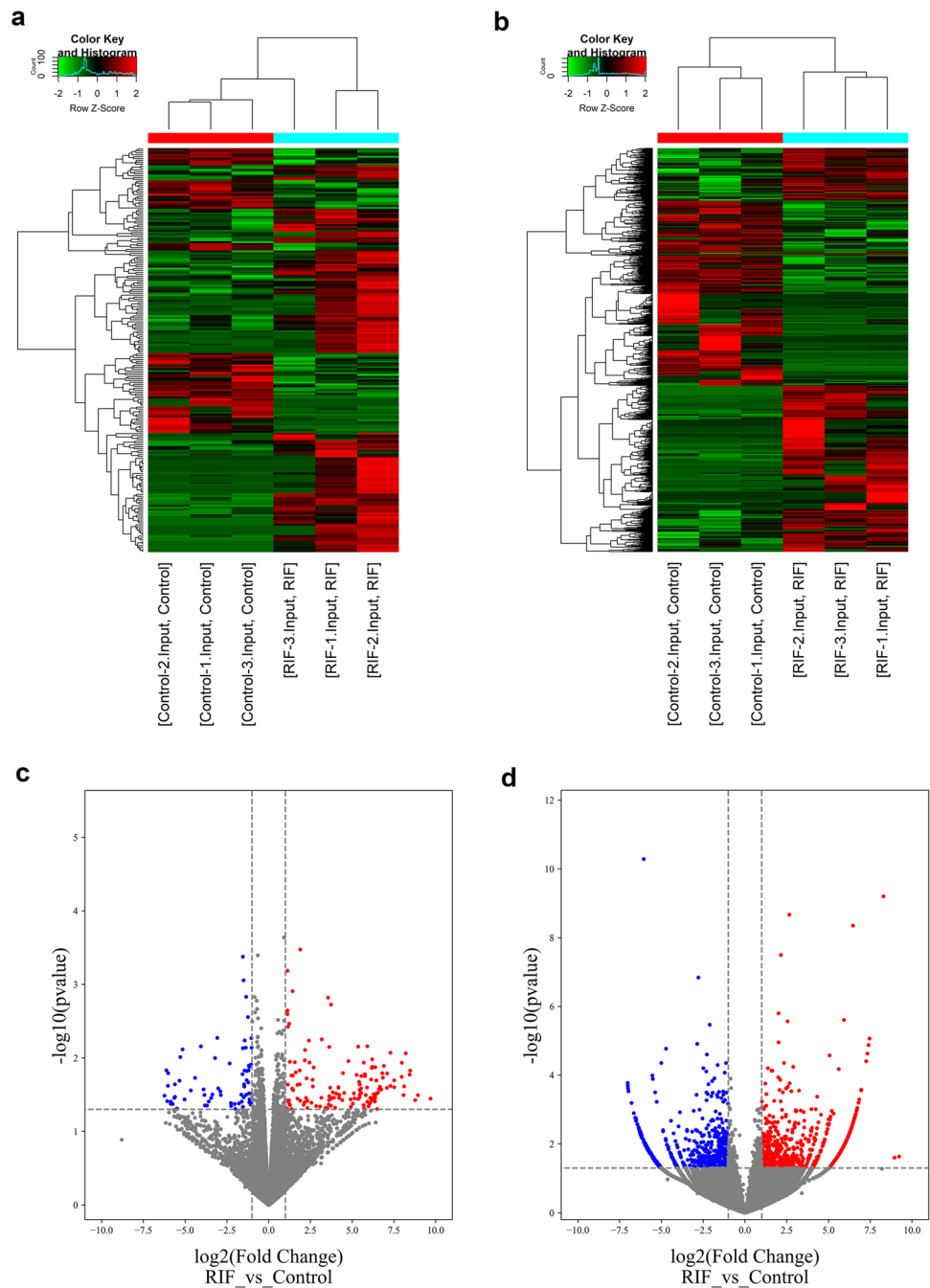
RNA-seq was used to explore the mRNA and lncRNA expression profiles in endometrial tissues from the RIF group ( $n = 3$ ) and control group ( $n = 3$ ) (Table 1). The mean ages of the patients in the RIF and control groups were  $34.00 \pm 3.30$  and  $30.86 \pm 3.39$  years, respectively. Differentially expressed mRNAs (Fig. 1a) and lncRNAs (Fig. 1b) were clustered hierarchically according to their expression levels. Volcano plots of the significant differentially expressed mRNAs and lncRNAs were highlighted between the RIF and control groups are shown in Fig. 1c-d. The expression profiles of 213 mRNAs (Supplementary Table S2) and 1485 lncRNAs (Supplementary Table S3) were identified in the RIF and control groups ( $FC \geq 2$  and  $p$  value  $< 0.05$ ). Among them, 143 mRNAs were up-regulated and 70 mRNAs were down-regulated, while 809 lncRNAs were up-regulated and 676 lncRNAs were down-regulated. Table 2 shows the top 10 up- and down-regulated mRNAs in the RNA-seq study. Table 3 showed the top 10 up- and down-regulated lncRNA transcripts. The classification and chromosome distribution of the dysregulated lncRNAs are shown in Supplementary Fig. 1.

### Gene Ontology and Pathway Analyses

Many lncRNAs act as cis-regulators and that their functions were linked to parental genes with protein-coding ability. GO and KEGG analyses were performed to reveal significant enrichment pathways and to further understand the potential biology of the dysregulated mRNAs and lncRNAs, the GO and KEGG analyses were further performed. GO enrichment analysis was performed for up- and down-regulated mRNAs ( $FC \geq 2$  and  $p$  value  $< 0.05$ ) between the RIF and control groups, as well as for the lncRNA parental genes. In the GO analysis, the top 10 significant GO terms according to the enrichment score ( $-\log_{10}(p$  value)) of the dysregulated genes were considered. For up-regulated mRNAs (Fig. 2a), organic anion transport (GO:0015711) was enriched most in BP, the endoplasmic reticulum lumen (GO:0005788) was enriched most in CC, and transporter activity (GO:0005215) was enriched most in MF. For down-regulated mRNAs (Fig. 2b), cell killing (GO:0001906) was the most enriched term in BP, immunological synapse (GO:0001772) was the most enriched term in CC, and IgG binding (GO:0019864) was the most enriched term in MF.

For up-regulated lncRNAs (Fig. 2c), microtubule cytoskeleton organization (GO:0000226), microtubule-based

**Fig. 1** Expression levels of mRNA and lncRNA in RIF. **a–b** Hierarchical cluster analyses of differentially expressed mRNAs and lncRNAs (fold change  $\geq 2$  and  $p$  value  $< 0.05$ ) between RIF and control groups. Red and green represent up-regulated and down-regulated expression respectively. **c–d** The volcano maps of the differentially expressed mRNAs and lncRNAs. X-axis,  $\log_2$  represents the fold change; Y-axis, expressed as the  $p$  value. The two vertical lines represent 2-fold regulation, and the horizontal line represents a  $p$  value of 0.05. The red and green dots represent the mRNAs and lncRNAs that are significantly up-regulated and down-regulated, respectively



process (GO:0007017) and cellular localization (GO:0051641) were enriched most in BP; intracellular (GO:0005622), organelle (GO:0043226) and cytoplasm (GO:0005737) were enriched most in CC. And iron binding (GO:0043167), IgG binding (GO:0019864) and anion binding (GO:0043168) were enriched most in MF. For down-regulated lncRNAs (Fig. 2d), flavonoid metabolic processes (GO:0009812), xenobiotic glucuronidation (GO:0052697) and cellular glucuronidation (GO:0052695) were the most enriched terms in BP; cytoplasm (GO:0005737), intracellular (GO:0005622) and endomembrane system

(GO:0012505) were the most enriched terms in CC; and glucuronosyltransferase activity (GO:0015020), catalytic activity (GO:0003824) and enzyme binding (GO:0019899) were the most enriched terms in MF.

Further, pathway analyses were performed to test for statistical significance of the differentially expressed mRNA and lncRNA parental genes. The pathway analyses revealed 13 pathways that corresponded to the up-regulated mRNA transcripts, and the most enriched were fat digestion and absorption, *Staphylococcus aureus* infection, and the peroxisome proliferator activated receptor

**Table 2** Top 10 dysregulated mRNAs in RIF endometrium

Up-regulated mRNAs			Down-regulated mRNAs		
Seqname	Gene name	logFC	Seqname	Gene name	logFC
ENSG00000158874	APOA2	9.6920018	ENSG00000095777	MYO3A	-6.2353224
ENSG00000118137	APOA1	8.9349876	ENSG00000131094	C1QL1	-6.1202867
ENSG00000163586	FABP1	8.7784687	ENSG00000197888	UGT2B17	-6.0743592
ENSG00000171557	FGG	8.4700726	ENSG00000185271	KLHL33	-6.0304804
ENSG00000171759	PAH	8.4415567	ENSG00000152578	GRIA4	-6.0171861
ENSG00000118271	TTR	8.2069909	ENSG00000179873	NLRP11	-5.8654646
ENSG00000145321	GC	8.1080001	ENSG00000173585	CCR9	-5.8639400
ENSG00000176566	DCAF4L2	8.0746149	ENSG00000159763	PIP	-5.7515278
ENSG00000129514	FOXA1	7.9754664	ENSG00000167759	KLK13	-5.6486623
ENSG00000148584	A1CF	7.9617238	ENSG00000228570	NUTM2E	-5.6138084

**Table 3** Top 10 dysregulated lncRNAs in RIF endometrium

Up-regulated lncRNAs			Down-regulated lncRNAs		
Seqname	Gene name	logFC	Seqname	Gene name	logFC
ENST00000514279	AFP	9.2245373	TCONS_I2_00021371	XLOC_I2_011327	-7.0227377
ENST00000562665	RP11-1223D19.1	8.9493885	ENST00000523827	EPHX2	-7.0176140
NR_131963	LVCAT5	8.2895469	TCONS_I2_00006747	XLOC_I2_003645	-6.9989903
TCONS_I2_00022232	XLOC_I2_011515	7.4518966	ENST00000480898	GSTT1	-6.9706537
ENST00000513540	RP11-19C24.1	7.3985189	ENST00000450650	CAPN13	-6.7845071
ENST00000432120	RP11-119K6.6	7.3273325	ENST00000562922	RP11-575I8.1	-6.6567424
ENST00000567574	RP11-218F4.1	7.2591380	TCONS_00001528	XLOC_000877	-6.5655982
ENST00000546162	ABCC6P1	6.9595720	TCONS_00003845	XLOC_001650	-6.5491328
ENST00000417524	USP32P1	6.9524983	ENST00000567966	RP11-345M22.3	-6.5189199
ENST00000549369	NAV3	6.8413749	ENST00000419363	FTOP1	-6.5125209

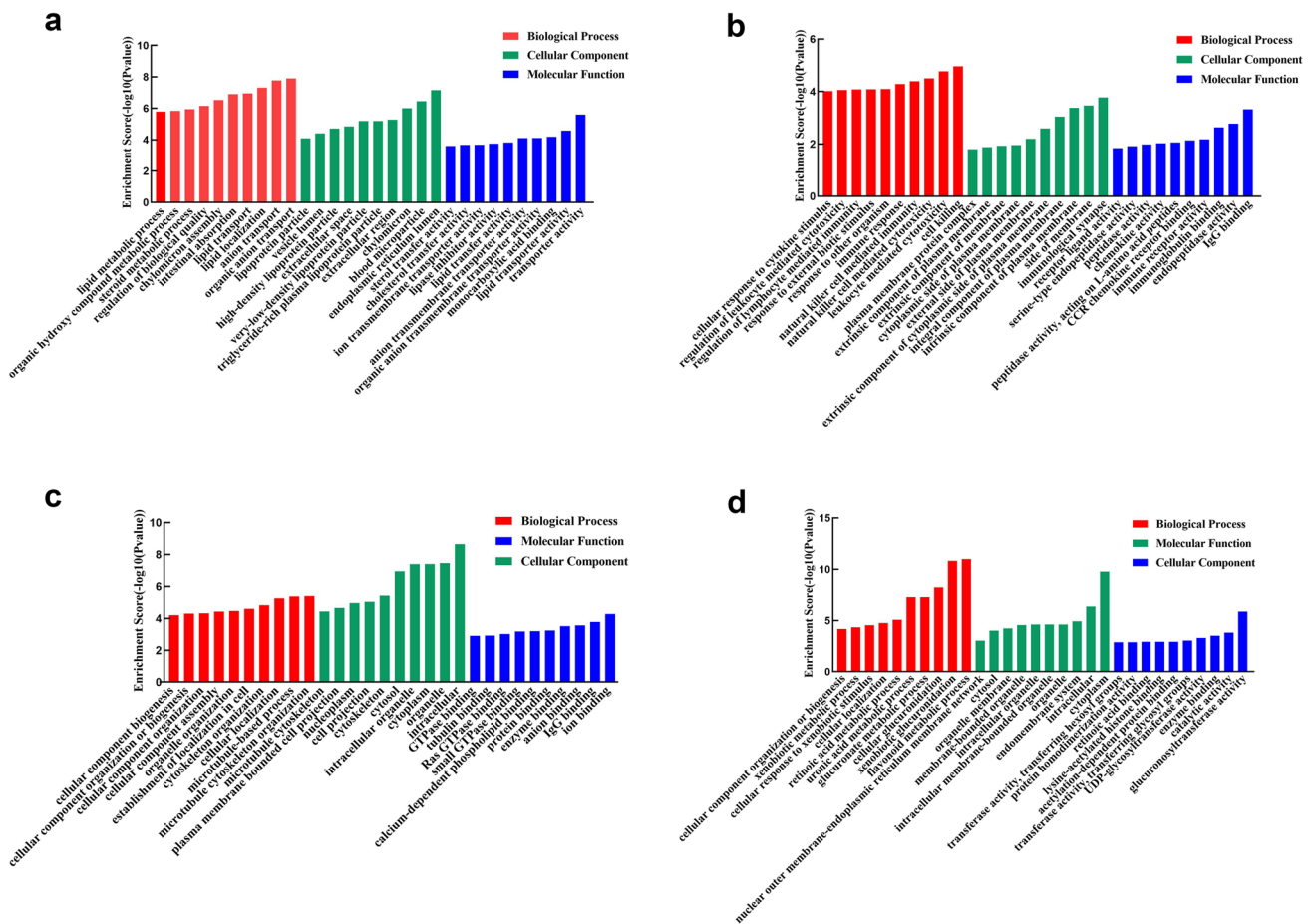
(PPAR) signaling pathway (Fig. 3a). Among the down-regulated pathways, the chemokine signaling pathway was the most enriched (Fig. 3b). In addition, 35 pathways were enriched for up-regulated lncRNAs, and 26 pathways were enriched for down-regulated lncRNA. Targets of up-regulated lncRNA were mainly enriched in the pathways of platelet activation, inflammatory mediator regulation of TRP channel, and the oxytocin signaling pathway (Fig. 3c). Targets of down-regulated lncRNA were mainly enriched in the pathways of bile secretion, ascorbate and aldarate metabolism, and porphyrin and chlorophyll metabolism (Fig. 3d).

### Construction of the PPI Network

To explore the functional connection of key genes, a PPI network was constructed from 213 dysregulated mRNAs. Of these mRNAs, 115 of 213 genes were found to be involved in the interactions based on the predictions from the String database (median confidence score of > 0.4). There were 115 nodes and 358 edges in the PPI network

(Fig. 4a). According to the ranking of network topology property indicators, we defined hubs as the top 10 nodes ranked separately by degree centrality, betweenness centrality, and closeness centrality were defined as hubs (Table 4). Furthermore, the keyword “reproductive or decidualization or endometrial receptivity or recurrent implantation failure” was entered into the GeneCards (<https://www.genecards.org/>), and the first 300 results were screened. Among the hub nodes, *AFP*, *ALB*, *CFTR*, *TF* and *TTR* emerged as key nodes because of their numerous connections with the results obtained from GeneCards.

MCODE, a plugin of Cytoscape, was utilized to screen key modules from the PPI network. Application of the criteria of a k-core is 2 revealed 5 subnetworks. Among them, a subnetwork with the highest MCODE score of 13,231 comprising 14 nodes and 86 edges, was selected (Fig. 4b). There were 14 up-regulated genes in this subnetwork (*AFP*, *ALB*, *APOA1*, *APOA2*, *APOB*, *APOH*, *FABP1*, *FGA*, *FGG*, *GC*, *ITIH2*, *SERPIND1*, *TF* and *TTR*), and these 14 genes were identified as hub genes.



**Fig. 2** GO analyses of the differentially expressed mRNAs and lncRNAs. **a–b** The top 10 GO terms of the three different gene functions for up-regulated and down-regulated mRNAs. **c–d** The top 10 GO

terms of the three different gene functions for up-regulated and down-regulated lncRNAs. Red: biological process; Blue: molecular function; Green: cellular component

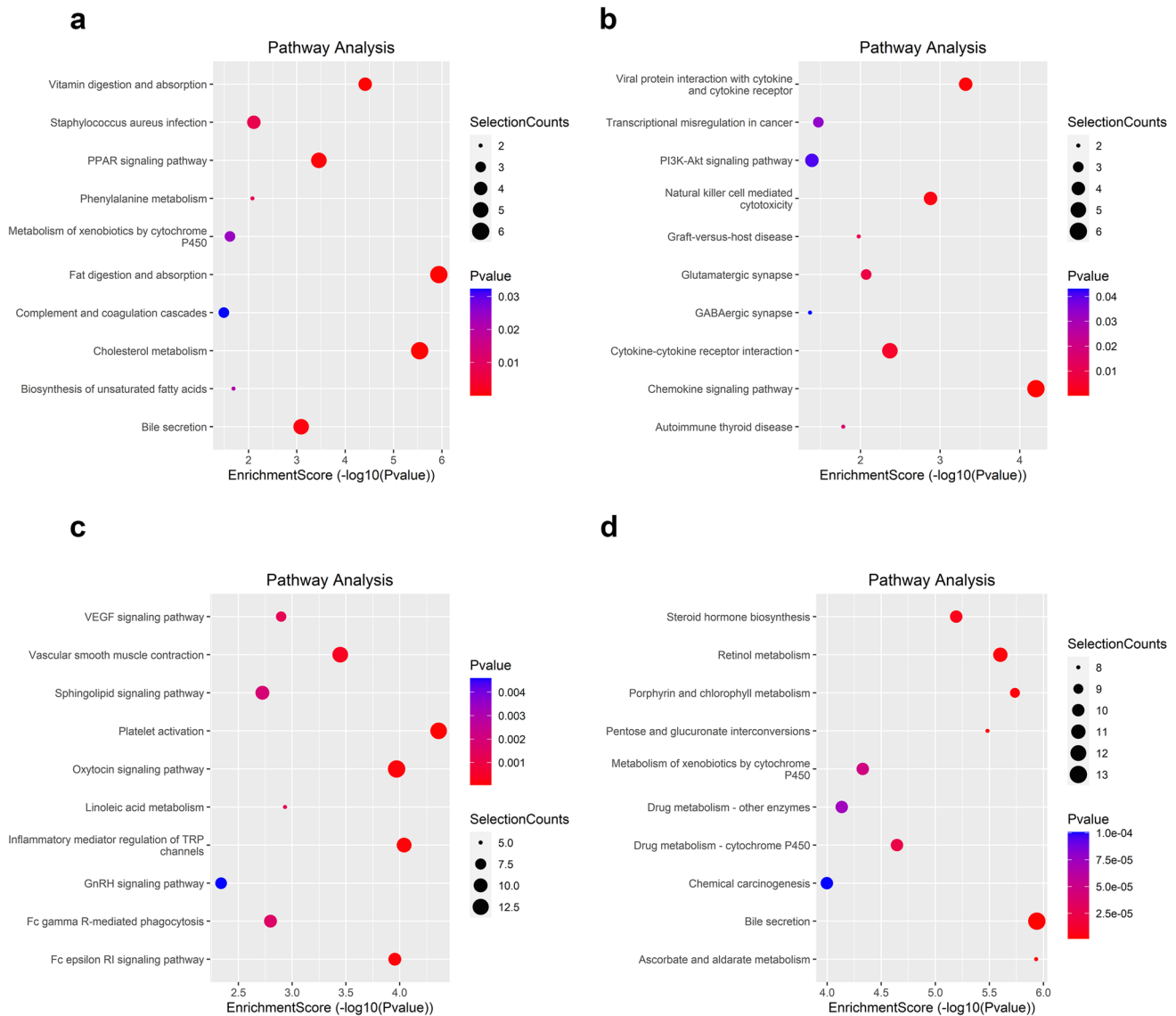
### Construction of the lncRNA-miRNA-mRNA ceRNA Regulatory Network

5 mRNAs of particular interest among the key nodes of the PPI network (*AFP*, *ALB*, *CFTR*, *TF* and *TTR*) were selected to further explore the lncRNA-miRNA-mRNA regulatory network. After separately identifying and integrating the 35 miRNA-mRNA and 177 miRNA-lncRNA interactions (Supplementary Table S4), the ceRNA network was constructed. 3 lncRNAs within the ceRNA network were randomly selected to conduct RT-qPCR and exhibited the same change trends as shown in our RNA-seq results (Supplementary Fig. 2). The ceRNA network contained 57 nodes (5 mRNAs, 28 miRNAs and 24 lncRNAs) and 212 edges (Fig. 5). Among the ceRNA relationships, the top 5 lncRNAs with the highest degree centrality were identified, and included ZNRD1-AS1 (degree = 27), TPTEP1 (degree = 23), NPHP3-AS1 (degree = 14), ANKRD18CP (degree = 12), and LINC00284 (degree = 11). The top lncRNAs were the nodes of which indicating important roles in the ceRNA

network. The top 5 miRNAs were hsa-miR-27a-3p (degree = 14), hsa-miR-24-3p (degree = 13), hsa-miR-129-5p (degree = 11), hsa-miR-4770 (degree = 10), hsa-miR-613 (degree = 10).

### Identification and Validation Effect of ML-193 on Endometrial Cell Proliferation

In the present study, CMap was employed to identify potential compounds that could disturb the gene expression pattern of the 14 hub genes. The up-regulated tag of the 14 hub genes were submitted to the CMap online tool for analysis. The instances for each compound according to the permuted results of the CMap analysis are listed in Supplementary Table S5. 3 drugs (ML-193, bisphenol-a, and capsazepine) were selected from the top 10 drugs, and only ML-193 showed a proliferative effect as indicated by CCK8 analysis (Supplementary Fig. 3). Thus, ML-193, with the most significant negative enrichment score, was identified



**Fig. 3** Pathway analyses of the differentially expressed mRNAs and lncRNAs. **a–b** The top 10 for the up-regulated and the down-regulated mRNA enriched signaling pathways. **c–d** The top 10 pathways for the up-regulated and down-regulated lncRNA target genes. The

X-axis shows the enrichment score ( $-\log_{10}(p \text{ value})$ ). The size of the dot in the bubble map indicates the number of enriched genes, and the red indicates the  $p$  value

as a potential therapeutic compound for RIF. The chemical structure of ML-193 were shown in Fig. 6a.

In addition, the effect of ML-193 on endometrial cells was validated. The results showed that 24 h of treatment with ML-193 promoted proliferation of hESCs and ISK cells (Fig. 6b-c).

### Validation of ML-193 Effect on Expression of Hub Genes and HOXA10

14 hub genes were validated to determine whether ML-193 could modulate their expression. Under treatment with

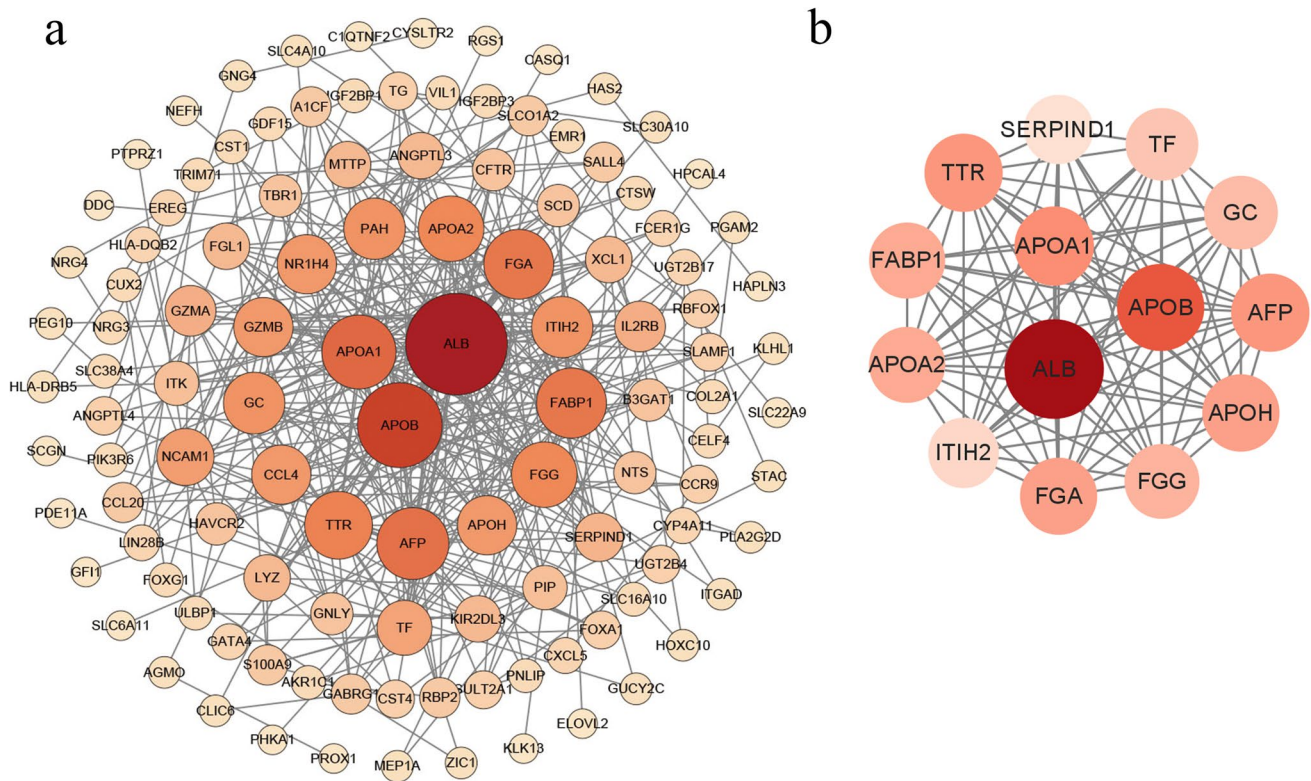
ML-193, the expression of mostly 14 hub genes was decreased in hESCs (Fig. 7a) and ISK cells (Fig. 7b).

*HOXA10*, a biomarker of ER, was also examined. After treatment with ML-193, the expression level of *HOXA10* was increased in both hESCs (Fig. 7c) and ISK cells (Fig. 7d).

### Discussion

RIF is a complicated clinical disorder in human reproductive medicine, which affects the physical and mental health of couples as well as constituting a huge challenge





**Fig. 4** Construction of a PPI network and identification of hubgenes from the PPI network. **a** Protein–protein interaction (PPI) network of 115 differentially expressed mRNAs between RIF and control groups.

The diameter of each circle implies degree centrality. **b** The sub-network with 14 hubgenes that extracted from the PPI network with the “Molecular Complex Detection (MCODE)” algorithm

**Table 4** The hub nodes in the protein–protein interaction network according to the score of network topology property indicators

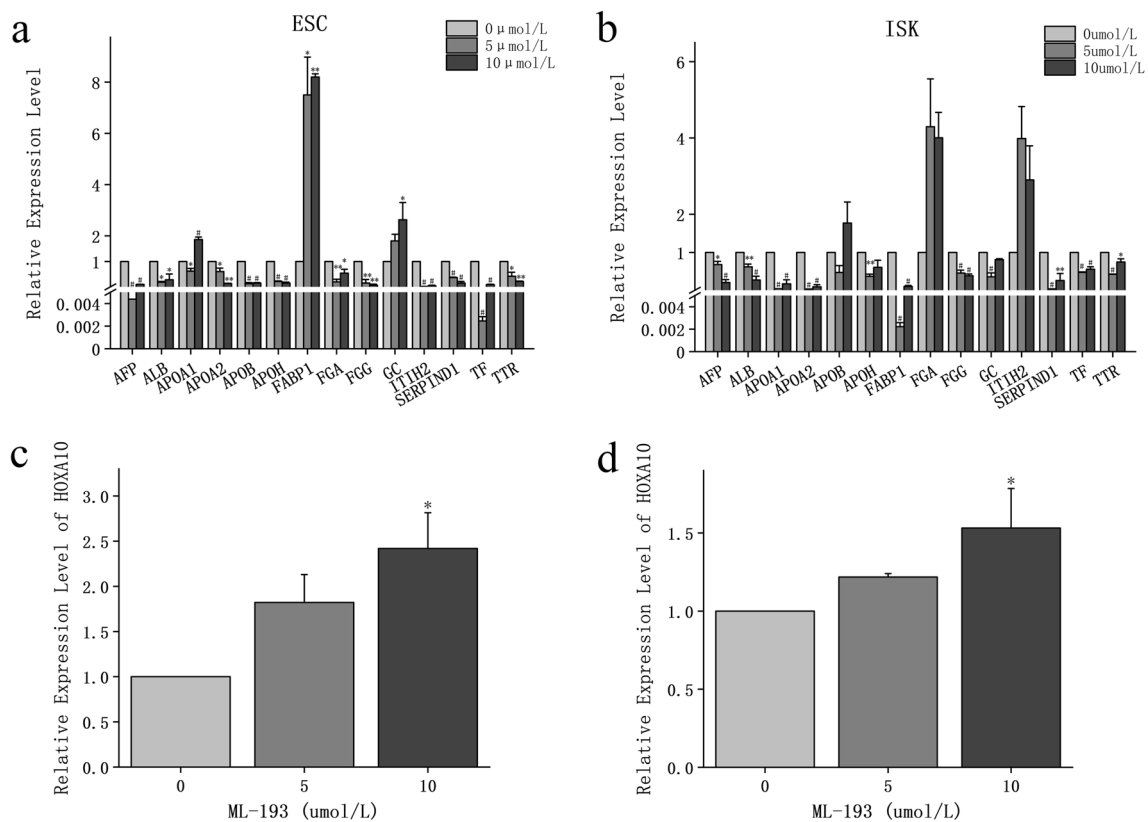
Rank	Node	Degree	Node	Betweenness	Node	Closeness
1	ALB	35	ALB	0.398993	ALB	0.481013
2	APOB	27	NCAM1	0.159758	AFP	0.425373
3	APOA1	22	AFP	0.127715	APOB	0.422222
4	AFP	21	APOB	0.119304	APOA1	0.402827
5	FABP1	20	CCL4	0.092289	NCAM1	0.395833
6	FGA	20	CFTR	0.089918	FGA	0.393103
7	TTR	19	TBR1	0.086203	TTR	0.391753
8	FGG	18	ANGPTL4	0.067633	TF	0.391753
9	APOA2	18	GZMB	0.066833	CCL4	0.390411
10	PAH	16	TTR	0.063071	FABP1	0.390411

for clinicians. The present study reports a comprehensive analysis of mRNAs and lncRNAs expression profiles in RIF and constructs PPI and ceRNA networks. According to the above regulatory relationships, 14 hub genes were identified. ML-193, which may be useful for treatment of RIF were identified based on hub genes by CMap. Under treatment with ML-193, hESCs and the ISK cell proliferation increased, hub genes were mostly down-regulated and ER maker *HOXA10* was up-regulated. This study provided a basis for exploring the pathogenesis and therapeutic

strategies for RIF from the perspective of the regulatory relationship between lncRNAs and mRNAs. Notably, the data indicate that ML-193 could be considered a potential therapeutic agent for RIF by enhancement of ER.

Inflammatory and immune response were essential for ER to embryo implantation and early placental development [13, 14], and their abnormalities were considered the causes of RIF [15]. In the present study, GO analysis revealed that down-regulated mRNAs were mostly associated with immune responses through several molecular mechanisms,





**Fig. 7** The expression of hub genes and *HOXA10* under the treatment of ML-193 in hESC and ISK. **a-b** The expression of hub genes in hESC and ISK. **c-d** The expression of *HOXA10* in hESC and ISK, \* $p < 0.05$ , \*\* $p < 0.01$ , # $p < 0.001$

such as cell killing, immunological synapses, and IgG binding (Fig. 2b). Indeed, in preparation for decidualization, immune cells played key roles in immune tolerance and the promotion of pregnancy [16, 17]. KEGG pathway analysis revealed that these genes were related to two critical inflammatory and immune associated signaling pathways, PPAR signaling pathway and inflammatory mediator regulation of transient receptor potential channels. PPAR signaling pathway ranked third in the KEGG analysis of up-regulated mRNAs (Fig. 3c). Activation of the PPAR signaling pathway has been thought to affect female reproduction by interfering with biological functions such as immune tolerance, inflammatory responses and trophoblast invasion [18, 19]. Inflammatory mediator regulation of transient receptor potential channels was found in the up-regulated lncRNAs (Fig. 3a). Mei et al. [20] found genes related to inflammatory mediator regulation of transient receptor potential (TRP) channels had a lower expression level in the chorion-attached endometrium compared to the non-chorion-attached endometrium, this may be related pregnancy failure by dysregulation of maternal immune tolerance. Altogether, our data suggested dysregulated mRNAs and lncRNA may be through involvement in inflammatory and immune responses, to affect ER and embryo implantation.

PPI and lncRNA-miRNA-mRNA ceRNA networks provided the evidence of the functional mechanism of the dysregulated mRNAs and lncRNAs in RIF. The PPI network construction revealed interactions between the dysregulated mRNAs ( $FC \geq 2$  and  $p < 0.05$ ). The 14 hub genes of particular focus in PPI network were *AFP*, *ALB*, *APOA1*, *APOA2*, *APOB*, *APOH*, *FABP1*, *FGA*, *FGG*, *GC*, *ITIH2*, *SERPIND1*, *TF* and *TTR* (Fig. 4b). Among these genes, *APOA1* had higher expression in the mid-secretory phase endometrium, which might be a cause for RIF [21]. *TTR*, *ALB* and *TF* were related to ER. *TTR*, the carrier for thyroxine and retinol, was a protein marker in the receptive stage of the human endometrium [22, 23], *ALB* and *TF* may be involved in the preparation of ER by regulating iron metabolism and immunological tolerance [24]. *FGG* associated with endometrial inflammation [25], as we all know, inflammatory abnormalities were considered the causes of RIF [15]. *FGA* could promote the migration and invasion of endometrial stromal cells [26], but few studies have examined the relationship between *FGA* and ER. *AFP* was highly expressed in the endometrium of endometrial cancer patients [27], no report to date has indicated that the expression of *AFP* was involved in RIF. Other genes (*APOA2*, *APOB*, *APOH*, *FABP1*, *GC*, *ITIH2* and *SERPIND1*) have not been reported

in the pathophysiology of the endometrium. In summary, abnormal expression patterns of these genes were speculated may contribute to impaired ER and the occurrence of RIF. Accumulating reports were also indicated that lncRNAs, as protein-coding gene modulators, served a vital role in mammals through the ceRNA network [28]. Through back-tracing the 5 initially selected mRNAs (*TTR*, *TF*, *ALB*, *AFP*, and *CFTR*), lncRNA-miRNA-mRNA relationships were connected based on their shared miRNAs. Accordingly, a ceRNA network was constructed in the present study to better understand the pivotal combined roles of lncRNAs with miRNAs and mRNAs.

Various treatments were available depending on the etiology of RIF, but molecular and cellular abnormalities pose great challenges to treatment [29]. ER was a recognized cause of RIF, and identifying new molecular therapeutic agents to improve ER is critical. Therefore, CMap analysis of the 14 hub genes in this study was performed to explore the compounds that could be used to treat RIF. Based on genome-wide expression profiling of gene transcripts, CMap proposed a data-driven and reliable method to identify new drugs or reposition existing drugs [30]. ML-193, the top drug in this analysis, was an antagonist to GPR55, and several studies have shown that ML-193 can exhibit anti-inflammatory activity [31–33]. For example, Kurano et al. [31] found that ML-193 treatment attenuated the elevation of inflammatory cytokines in plasma or tissues in mice, helping to improve insulin resistance and prevent organ damage. Gajghate et al. [32] found that ML-193 reduced inflammatory cell infiltration and increased the number of immune cells during stroke in the brain tissue of mice, thereby attenuating the inflammatory response. Li et al. [33] found that ML-193 exhibited anti-inflammatory activity in LPS-stimulated human dental pulp cells by inhibiting TLR4/Myd88/NF- $\kappa$ B signaling. However, its role against RIF has not been elucidated. The present study showed that ML-193 could promote the proliferation of hESCs and ISK cells (Fig. 6b–c). Successful pregnancy required a receptive endometrium of adequate thickness, insufficient endometrial thickness was closely associated with pregnancy failure [34]. The number of stromal and epithelial cells was reduced in thin endometrium [35]. In addition, proliferation of hESCs was necessary for decidualization, and a decrease in the proliferation rate inhibited decidual formation, leading to pregnancy failure [36]. Thus, ML-193 was speculated whether improves ER by promoting endometrial thickness and decidualization. And ML-193 treatment further down-regulated the mRNA levels of mostly 14 hub genes (Fig. 7a–b), which were up-regulated in RIF. More importantly, among the 14 hub genes, *TTR*, *ALB* and *TF*, which were previously discussed that may involved in ER, were all down-regulated both in hESCs and ISK under the treatment of ML-193. These results suggested that ML-193 may have an effect

on ER, so the effect of ML-193 on ER marker *HOXA10* was further explored.

Our results showed after treatment with ML-193, the expression level of *HOXA10* was increased in both hESCs (Fig. 7c) and ISK cells (Fig. 7d). Study has found that the expression of *HOXA10* was significantly lower in the RIF patients endometrium [37]. As an imperative contributor to embryo im-plantation, *HOXA10* played different roles in stromal and epithelial cells [38, 39]. During embryo implantation, expression of *HOXA10* was increased in endometrial stromal cells to govern stromal cell transformation to decidual cells [40]. Decidualization was necessary to create a receptive endometrium [41]. Abnormalities in the decidualization process were closely related to RIF [42]. The endometrial epithelium was the first part of the embryo to come into contact with the mother at implantation [43]. *HOXA10* was known to play a vital role in regulating changes in epithelial cell function during the establishment of ER [40]. ITGB3 and E-cadherin, which were related to ER, acted as an adhesion medium between the embryo and epithelial cells [44, 45]. Studies showed up-regulation of *HOXA10* increase the expression of ITGB3 and E-cadherin in epithelial cells, thereby improving ER and subsequently increasing the embryo adhesion and implantation [45, 46]. Therefore, ML-193 was speculated to improve ER by affecting *HOXA10*, which regulated stromal cells decidualization as well as adhesion of embryos and epithelial cells. These findings were expected to provide insight into the development of new therapeutic drugs for RIF. The in vitro cellular models used in the study were a limitation of this study, and the specific mechanism of action of ML-193 on ER required further animal experiments.

## Conclusion

RIF is a major limiting factor in the success rates of in-vitro fertilization, it not only seriously threatens women's physical health but also damages mental health. In this study, 14 hub genes were identified through a comprehensive bioinformatic analysis of the relationship between lncRNAs and mRNAs in RIF. Additionally, ML-193 was identified as a potential therapeutic agent for RIF. Furthermore, the 14 hub genes and the ER marker *HOXA10* were down- and up-regulated, respectively, under treatment with ML-193. These results indicated that ML-193 showed promise as a potential therapeutic agent for RIF by enhancing ER.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s43032-024-01630-8>.

**Acknowledgements** The authors would like to thank all the subjects for their generous participation in this study.

**Author Contribution** Lin Liu, Yidan Liu, Yu Tian, Ying Cao and Ting Wang contributed equally to this work. Lin Liu designed and supervised the study. Yidan Liu and Yu Tian conducted the experiments and drafted the manuscript. Ting Wang and Ying Cao collected the samples and analyzed the data. Run Yang, Simin Liu and Shengyan Mi contributed the methodology. Jing Wang and Xiaoling Ma modified the format. All authors read and approved the final manuscript.

**Funding** This work was financially supported by the National Natural Science Foundation of China (82360307), the Talent Innovation and Entrepreneurship Project of Lanzhou City (2022-RC-47), Gansu Provincial Science and Technology Program (20JR10TA715, 21JR7RA391), the Medical Innovation and Development Project of Lanzhou University (lzuyxcx-2022-191), Science and Technology Project of Lanzhou (2023-ZD-93), Fund of the First Hospital of Lanzhou University (ldyyyn2022-60), the First Hospital of Lanzhou University Excellence Program (20220060074, 20220060073).

**Data Availability** The datasets analyzed during the current study are included within the article and its additional files.

**Code Availability** Not applicable.

## Declarations

**Ethics Approval and Consent to Participate** The study was approved by the Research Ethics Committee of the first Hospital of Lanzhou University. Written informed consents were obtained from all patients prior to study commencement.

**Consent for Publication** Not applicable.

**Conflict of Interest** The authors declare no competing interests.

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