Trends in Immunotherapy (2022) Volume 6 Issue 2
doi:10.24294/ti.v6.i2.1457

ORIGINAL RESEARCH ARTICLE

Expressions of miR-199a-5p and miR-125b-5p and their target genes in the endometrium of recurrent implantation failure patients following in uterus infusion of autologous peripheral blood mononuclear cells

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ABSTRACT

Despite numerous advances in fertility techniques, some individuals experience implantation failure. One of the therapeutic approaches is the study of immunological aspects of the implantation process in recurrent implantation failure (RIF) patients. Peripheral blood mononuclear cell (PBMC) therapy and platelet-rich plasma are currently available cell therapies. The aim of this study was to determine the expressions of the *FGFR-2* and *LIF* genes that are regulated by miR-199a-5p and miR-125b-5p. These genes play a fundamental role in implantation in RIF patients treated with PBMCs. 20 patients clinically diagnosed with RIF were randomly assigned to a RIF patient with PBMCs intrauterine infusion group (n = 10) and RIF group (n = 10). Normal, healthy females (n = 10) comprised the control group. In order to examine the efficacy of the PBMCs injection in the treatment group, expressions of miR-199a-5p and miR-125-5p and *FGFR-2* and *LIF* as their target genes, were evaluated in all three groups and were compared the results. We discovered that the RIF group had higher expressions of miR-199a-5p and miR-125-5p along with decreased expressions of their target genes. However, both *FGFR-2* and *LIF* gene had elevated expressions in the RIF patients with PBMCs intrauterine infusion group compared to the RIF group, with significant decrease in miR-199a-5p and miR-125b-5p reciprocally. The treatment with PBMCs can be effective in changing the expression of microRNAs and genes associated with endometrial receptivity and by changes in the expression of them and their role during embryo development improve this process.

Keywords: Endometrium; Peripheral Blood Mononuclear Cells; Repeated Implantation Failure; MicroRNA

1. Introduction

Reproduction is a dynamic process and the greatest show of creation in the world and numerous factors influence the reproductive process. Successful implantation of an embryo is the most important step in the reproductive process[1]. Well-functioning endometrium and high-quality embryos are two main factors for successful implantation. Many factors affect endometrial function and receptivity. Implantation

ARTICLE INFO

Received 10 April 2022
Accepted 2 May 2022
Available online 22 December 2022

failure is one of the major reasons for infertility, because embryo implantation is an important step. Most treatment for infertility, such as Assisted Reproductive Technology (ART), is performed with the intent to increase the success rate of implantation. Despite numerous advances in fertility techniques, the rate of implantation after embryo transfer has not increased. In Vitro Fertilization (IVF) can produce blastocysts, which can implant in the uterus; however, an estimated upper than 40%–60% of embryos produced by IVF techniques don't implant after transfer[2,3]. Some reasons for Repeated Implantation Failure or Recurrent Implantation Failure (RIF) include decreased endometrial acceptance, fetal malformation, immunological factors, genetics, uterine abnormalities and decreases in endometrial thickness[4,5]. Most current treatments such as hysteroscopy, myomectomy, aspirin therapy for endometrial thickness, intravenous immunoglobulin injection as immunoglobulin therapy (two days before transfer, administration of heparin from 14 days before the start of the IVF cycle), prednisolone, and endometriosis treatment are appropriate for stimulation[6]. One of the therapeutic approaches is devoted to the study of the immunological aspects of the implantation process in RIF patients[7]. The maternal immune system regulates the differentiation of endometrial cells from a wide range of cytokines for adequate endometrial receptivity and embryo implantation[8]. Peripheral Blood Mononuclear Cells (PBMCs) include lymphocytes (NK cells, T cells, B cells) and monocytes, which can release a significant amount of biologically active paracrine factors that lead to beneficial effects on the regenerative potential of endometrial cells receptivity. Cytokines, growth factors, polypeptides and proteins have specific cell surface receptors that bind to initiate intracellular signals to regulate cell function and by controlling the expression of proteins involved in Intrauterine adhesion (IUA), increase the ability endometrial receptivity[9,10]. Therefore, it seems that PBMCs can play an important role in increasing endometrial receptivity by inducing the production of several cytokines. For the first time, Fujita *et al.* concluded that some immune cells could affect uterine differentiation[11], and then, some studies have shown that these cells impact the endometrial receptors by producing several important cytokines in implantation such as TNF-α, IL-1α, and IL-1β. Eftekhar *et al.* introduced intrauterine injections of autologous PRP and reported their efficacy in the treatment of thin endometria[12]. Zadehmodarres *et al.*, in a case report, successfully administered PRP therapy to treat a thin endometrium[6]. Hashii *et al.* reported that PBMCs could stimulate progesterone production by luteal cells[13,14]. Salamonsen *et al.* suggested the effect of PBMCs on stimulating several inflammatory factors and secretion of proteases and their inhibitors in the secretory phases to regulate

endometrial function[15]. Yoshioka *et al.* reported that injection of autologous PBMCs could dramatically increase clinical pregnancy rates[16]. In addition, some studies have investigated the effect of cell secretion and its role in the treatment of RIF in infertile patients. These cell secretions contain microRNAs, which have a major role in regulating physiological processes such as differentiation, apoptosis, proliferation, and development[5,17]. The members of the miR-125 family (miR-125a and miR-125b) and also miR-199a family have several functions in human body such as directly or indirectly regulation endometrial receptivity by alter in function of target genes. Liang *et al.*, in their study stated that miR-199a-5p and miR-125b-5p expression reduces endometrial receptivity in embryo implantation process[18]. Also according to the study of Shi *et al.*, miR-199a-5p expression changes have been seen in women with history of recurrent implantation. In their study, miR-199a-5p expression had a 2.5-fold increase in RIF patients[19].

In another study, Chen *et al.* examined the role of the miR-125b and its target gene (Matrix Metallopeptidase 26; MMP26) in endometrial receptivity in women undergoing IVF-ET with elevated progesterone and concluded that in successful subjects, miR-125b expression was lower than in those with implant failure and overexpression of miR-125b significantly reduced the number of implantation sites[20]. Fibroblast growth factor receptor 2 (*FGFR-2*) gene is one of the most important genes in endometrial receptivity which has high expression level during implantation window and performs its biological activity through the MAPK signaling pathway, which is regulated by miR-125b-5p[13,21,22]. Also Leukemia Inhibitory Factor (*LIF*) gene is another important gene in the implantation window that functions through the JAK-STAT signaling pathway and is regulated by miR-199a-5p[23–26]. Although the role of these miRNAs has been mentioned in many studies, but there is no direct study about the function of any of these miR-199a-5p/miR-125b-5p either individually or as a group. Our study aims to determine the *FGFR2* and *LIF* genes and the miR-125b-5p and miR-199a-5p microRNAs expressions that regulate their expression in RIF patients treated with PBMCs. None of the previous studies have examined the effect of PBMCs on the expression of these two genes and their controlling microRNAs, and this is novelty of this study.

2. Methods

In this study we have proposed a new method to identify PBMCs effect on miRNA and their target genes expression rate that interfere in embryo implantation. This way consists of the three steps. First, determination the significant miRNAs and target genes expression in RIF group and RIF patients with PBMCs intrauterine infusion group. Second, evaluation of expression of relative proteins after PBMCs therapy and finally, protein-protein interaction in to main genes.

2.1 Setting and study design

This experimental research study was conducted at the Reproductive Medicine Unit, Payambaran Hospital Tehran, Iran, from 2017 to 2019. A total of 30 women agreed to participate in this study. From these, 20 endometrial specimens were obtained from 20 patients clinically diagnosed with RIF and based on physician diagnosis; they received an IVF cycle (RIF patients with PBMCs intrauterine infusion group which in the future is called in the following text “RIF with PBMCS group”) and (RIF group). In addition, we obtained 10 normal endometrial tissues from healthy women (control group) (**Figure 1**).

2.2 Study groups and treatment

We studied 30 cycles in 30 individuals, from which 20 RIF patients were randomly divided into two groups by tossing coins. 10 patients were allocated per group and also, a control group that consisted of 10 healthy women. Group I (n = 10) patients had at least two episodes of RIF. These patients received injections of autologous PBMCs (RIF with PBMCs group). Group II (n = 10) patients had at least two episodes of RIF without any treatment and didn’t receive PBMCs (RIF group). Group III consisted of 10 normal fertile women who underwent IVF for gender determination

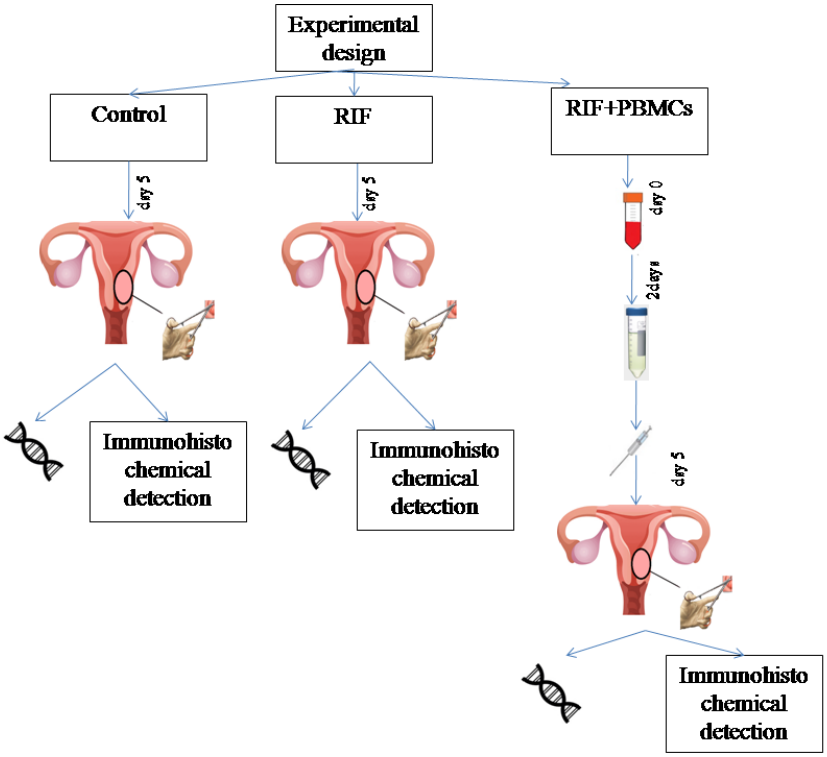

Figure 1. Schematic diagram of study design and work flow.

Table 1. Demographic information of experimental groups

Studied groups	Age	Infertility duration	Number of children	Number of follicles (MII)	Number of embryo (good quality)
Control group (10 patients)	30–35	-	1–3	8–11	6–8
RIF group (10 patients)	30–35	On average 5–9 years	-	7–10	5–7
RIF with PBMCs group (10 patients)	30–35	On average 5–9 years	-	7–10	5–7

(PGD) and they had one child of each gender (control group). Instead of PBMCs, distilled water was injected for the control group and the RIF group. Demographic information of experimental groups has been shown in **Table 1**.

2.3 Inclusion and exclusion criteria

1) Study inclusion criteria consisted of: participants between the ages of 30–35 years and those with a higher number of follicles that were between 5 and 10 mm. For RIF group and RIF with PBMCs group, it was necessary that patients had at least two episodes of RIF and in control group, all 10 women must have normal endometrial tissues.

2) Exclusion criteria consisted of: IVF treatment because of tubal, male or unexplained infertility; recurrent abortions attributed to hormonal, chromosomal, or anatomical causes; current infections; any autoimmune, coagulation, neoplastic, metabolic, liver, or cardiovascular diseases; presence of antiphospholipid antibodies; alcohol or smoking histories and patients with OHSS syndrome, uterine pathologies (such as polyps, myomas, endometriosis, adenomyosis, and congenital anatomical anomalies, as well as chronic endometritis) and status of parents.

2.4 Ethical standards

The Ethics Committee of Shahid Beheshti University of Medical Sciences approved this study (IR.SBMU.RETECH.REC.1396.999). After receiving a sufficient explanation about this study, each participant signed an informed consent for study participation. It was emphasized to them, that they would be randomly divided into study groups.

2.5 *In vitro* fertilization (IVF)

Ovulation induction was performed for women

from all three groups. According to the standard protocol, since the third day of menstruation, Gonadotropin and antagonist injection was started (Aventis Pharma Co., Tokyo, Japan) based on ultrasound-guided. When the diameters of at least one of the follicles reached 20 mm, an injection of hCG (10,000 IU) was administered. Subsequently, 36 h after the hCG injection, follicular evacuation was done followed by cytoplasmic sperm injection. Our approach was to freeze embryos because of taking an endometrial biopsy sample and uterine manipulation; it was not possible to direct embryo transfer in current cycle and after a 48 to 72 h culture, the embryos were frozen for future transfer in all three groups.

2.6 Isolation and intrauterine administration of peripheral blood mononuclear cells (PBMCs)

In the treatment group (RIF with PBMCs group), immediately after oocyte collection in previous stage (IVF cycle), 16–20 mL of peripheral blood samples was taken and the PBMCs were isolated according to Standard protocol for PBMCs isolation. After centrifugation, PBMCs were obtained from the middle layer of the samples by Ficoll-Hypaque gradient (Sigma-Aldrich, USA) and after washed twice in RPMI (RPMI1640, Gibco) medium by PBS (Gibco) plus 10% serum albumin (serum protein substitute, SAGE). The PBMCs were placed in an incubator with 5% CO_2, at 37 °C. After 48 h, 200 µL of the cell suspensions that contained 1×10^7–2×10^7 cells were gently injected into the uterine cavity. The RIF group and control group didn't receive PBMCs and instead distilled water was injected for them to eliminate the effect of injection as an interfering agent.

2.7 Tissue processing

At 72 h after received intrauterine injections of PBMCs and distilled water, endometrial biopsy samples were obtained from all of the study participants to evaluate the miRNA and their target gene expression. The biopsies were taken by pipelle biopsy instrument (Unimar Inc., Wilton, CT, USA). The samples were divided into two sections. One sample was put in a formalin (10%) container for histological studies of *LIF* and *FGFR-2* gene expressions according to immunohistochemical techniques. The second part was stored in an RNA later vial (Qiagen, Germany) and immediately frozen at –80 °C for molecular analysis. Endometrial sampling has been performed for DNA and miRNA extraction and tissue size does not matter and will not affect test results.

2.8 Primer design for miRNA and its target gene

In reviewing previous studies, miR-199a-5p and miR-125b-5p were founded which are potentially over expressed in RIF patients. Next, their target genes were determined by the specific software miRwalk (htpp://miRwalk.umm.uni-heidelberg.de/) and target scan (htpp://www.targetscan.org/). The design of primers used by oligo 7 software (Molecular Biology Insights Inc., Cascade, CO), and gene runner version 5.1 (Informer Technologies Inc., Spain). Finally validated them with NCBI BLAST. The primers information is provided in the table below (**Table 2**).

Table 2. Designed primers for qRT-PCR reaction

Gene name	Primer sequence	Gene bank accession no.
FGFR2	F: CCAACTGCACCAACGAACTG R: GGTCCAAGTATTCCTCATTGGT	NM_000141.4
LIF	F: CTCGCCCATCACCTCATCTC R: GCAGAGCTGTTTCACGCAAA	NM_002309.5
hsa-miR-199a	F: GTATACCCCAGTGTTCAGACT R: GTGCAGGGTCCGAGGT	NR_029586.1
hsa-miR-125b	F: GTATACTCCCTGAGACCCTAA R: GTGCAGGGTCCGAGGT	NR_029671.1

2.9 RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Total RNA and microRNA extraction were performed for all specimens by a kit (Gene All Bio-

technology Co., South Korea) according to the manufacturer's instructions. The RNA extracted and purified was assessed on a Nanodrop (Eppendorf , Germany) with an optical density (OD) of 260/280 nm, from which single-strand cDNA was synthesized using according (YektaTajhiz, Tehran, Iran). Control and confirm of cDNA synthesis were done by GAPDH gene primers using PCR technique. Expressions of miR-125b-5p, miR-199a-5p, and their target genes, *FGFR-2* and *LIF*, were assessed by quantitative real-time PCR (qRT-PCR) or RT-PCR using a Rotor Gene Q instrument (Qiagen, Valencia, CA, USA). The qRT-PCR reactions were performed in a total volume of 13 µL, which included DNA Master SYBR Green I Mix (7 mmol/L; YektaTajhiz, Tehran, Iran), nuclease-free water (4 mmol/L), mixed forward and reverse primers (1 mmol/L) that were specific for each of the genes, and synthesized cDNA (1 mmol/L). The qRT-PCR program consisted of 40 cycles of extension for 2 min at 95 °C, 5 s at 95 °C for denaturation, 30 s at 60 °C, and 10 s at 72 °C for amplification.

2.10 Histological assessment

We performed immunohistochemical assessments of the *FGFR-2* and *LIF* proteins from the endometrial tissues of all three groups. Sections of endometrial tissues were de-paraffinized and dehydrated. Nonspecific binding was blocked by 10% normal goat serum in PBS for 20 min. For immunohistochemical distinction of *LIF*, we used anti-LIF antibody (ab113262, Abcam) at dilutions of 1:100 and for *FGFR-2*, we used recombinant anti-FGFR-2 antibody [SP273]-N-terminal (ab227683, Abcam) at dilutions of 1:100 too. The sections were first incubated overnight with the antibody at 4 ℃. Then, they were washed three times with PBS and incubated for 1 h with the second antibody, goat anti-rabbit IgG H&L (FITC; ab671, Abcam) for 30 min. The sections were counterstained with DAPI according to the manufacturer's protocol. Then, fluorescent intensities were evaluated by Image J software for expression proteins and the results were analyzed.

2.11 Protein-protein interaction (PPI) network analysis and classification of *LIF* and *FGFR-2* protein function

One of the most important ways for understanding of cellular processes and evolutionary processes of protein characteristics are protein-protein interaction networks and also have an important role in predicting the protein function in intracellular signal transduction pathways[27–29], in this study, both of *IGF1* and *LIF* proteins were used to constructing a protein-protein interaction network in the String database[30]. And then, to identifying of each highly connected region, KEGG/BioCarta network pathways were evaluated by ClueGO app in Cytoscape software[31].

2.12 Statistical analysis

All statistical analyses were done on datasets using SPSS version 16 software. The relative levels of RNA were analyzed by REST software. The relative levels of RNA and intensity of proteins signals was analyzed by Student's t test and Analysis of Variance (ANOVA). Normal data were written as mean ± standard deviation (SD). P-values < 0.05 were considered to be statistically significant. GraphPad prism software was used to draw the diagrams.

3. Results

3.1 The results of miRNA and target genes determination

Based on previous studies, our candidate miRNAs were miR-199a-5p and miR-125b-5p, which were predicted by algoritms miRwalk and Target scan. miRNAs's target genes data browsing showed that *LIF* and *FGFR-2* are important target genes which are founded in intersections of the two algorithms, we selected them as main targets genes for miR-199a-5p and miR-125b-5p.

3.2 Quantitative measurement of the expressions of miR199a-5p and miR125b-5p and their target genes, *LIF* and *FGFR-2*, after peripheral blood mononuclear cell (PBMCs) injections

According to **Figure 2A**, qRT-PCR assessment of miR-199a-5p and miR-125b-5p showed miR-199a-5p and miR-125b-5p expression levels

increased significantly in the RIF group in comparison with PBMCs + RIF and control groups. Also, there was significant difference between the RIF group and control group; the RIF group had a highest rate of miR-199a-5p and miR-125b-5p expression while the control group had a lowest rate of the expression.

qRT-PCR analysis showed that the highest rate of *LIF* and *FGFR-2* genes expression was in the control group. The expressions of *LIF* (3.5-fold) and *FGFR-2* (2-fold) genes after treatment with PBMCs significantly increased in the RIF with PBMCs group compared with the RIF group which was similar to the control group (**Figure 2B**).

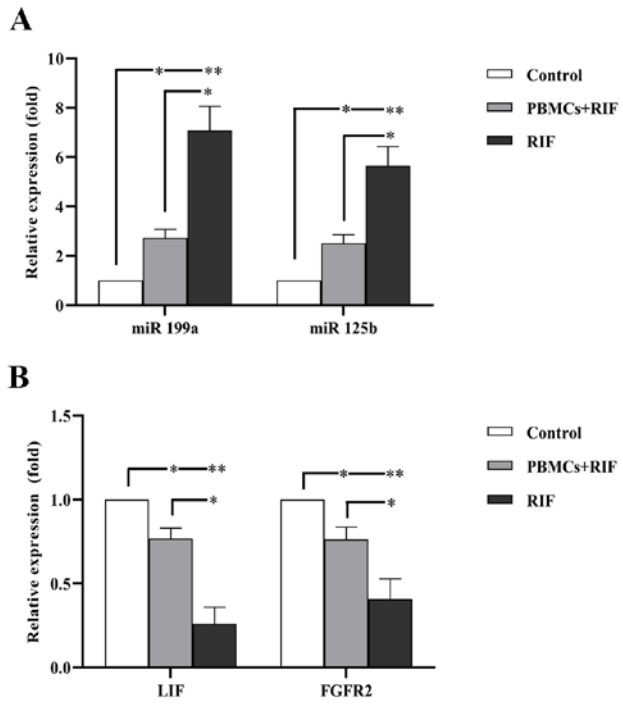

Figure 2. Expression levels of the microRNAs (miRNAs) and genes in endometrial biopsy samples. **(A)** miR199a-5p and miR125b-5 expression in the RIF group compared to the RIF with PBMCs group and control group. miR199a-5p and miR125b-5 expression levels increased significantly in the RIF group in comparison to the RIF with PBMCs group and control group. (**B**) *LIF* and *FGFR-2* genes expression in the RIF group compared to the RIF with PBMCs group and the RIF with PBMCs group compared to the control group. miR199a-5p and miR125b-5 expression levels decreased significantly in the RIF group in comparison to the RIF with PBMCs group and control group.
*: Significant difference at $P < 0.05$.

3.3 Histological assessment of endometrial tissue samples

Immunohistochemical detection of the *FGFR-2* and *LIF* proteins was done throughout the implantation window in endometrial epithelial and glandular cells of the RIF with PBMCs group and compared with the RIF group and control group. The RIF with PBMCs group had higher staining intensity of *FGFR-2* and *LIF* compared with the RIF group (**Figures 3** and **4**).

A comparison of *FGFR-2* protein expression relative to the type of treatment showed that its expression increased in the RIF with PBMCs group, which was similar to the control group. In this regard, there was a significant statistical difference with the RIF group (**Figure 5**).

In confirmation of the results of the histological assessment, a comparison of the *LIF* protein expression level relative to the type of treatment showed that its expression increased in the RIF with PBMCs group, which was similar to the control group. There was a significant statistical difference with the RIF group (**Figure 5**). Based on the results, increased *FGFR-2* and *LIF* proteins expressions were accompanied by decreased expressions of their regulatory microRNAs.

3.4 PPI network and their functional classification

Based on the available database, protein-protein interactions were predicted and validated and according to the **Figure 2A**, results indicated that *LIF* protein has direct interaction with IGF1, CTF1, IL6ST, STAT3, JAK2, LIFR, JAK1, TYK2, IL10 and HBEGF proteins. Also, **Figure 2B** indicated *FGFR-2* protein has interaction with FGF9, FGF3, FGF2, FGF10, FRS2, FGF1, FGF8, PLCG1, FGF7 and FGF4 straightly. **Figure 2** depicted the whole network (**Figure 6**).

The results of KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway in **Table 3** confirmed that the *LIF* protein was enriched in JAK-STAT signaling pathway, signaling pathways regulating pluripotency of stem cells and Th17 cell differentiation, while the *FGFR2* protein was enriched in Rap1 signaling pathway, Ras signaling pathway and Regulation of actin cytoskeleton.

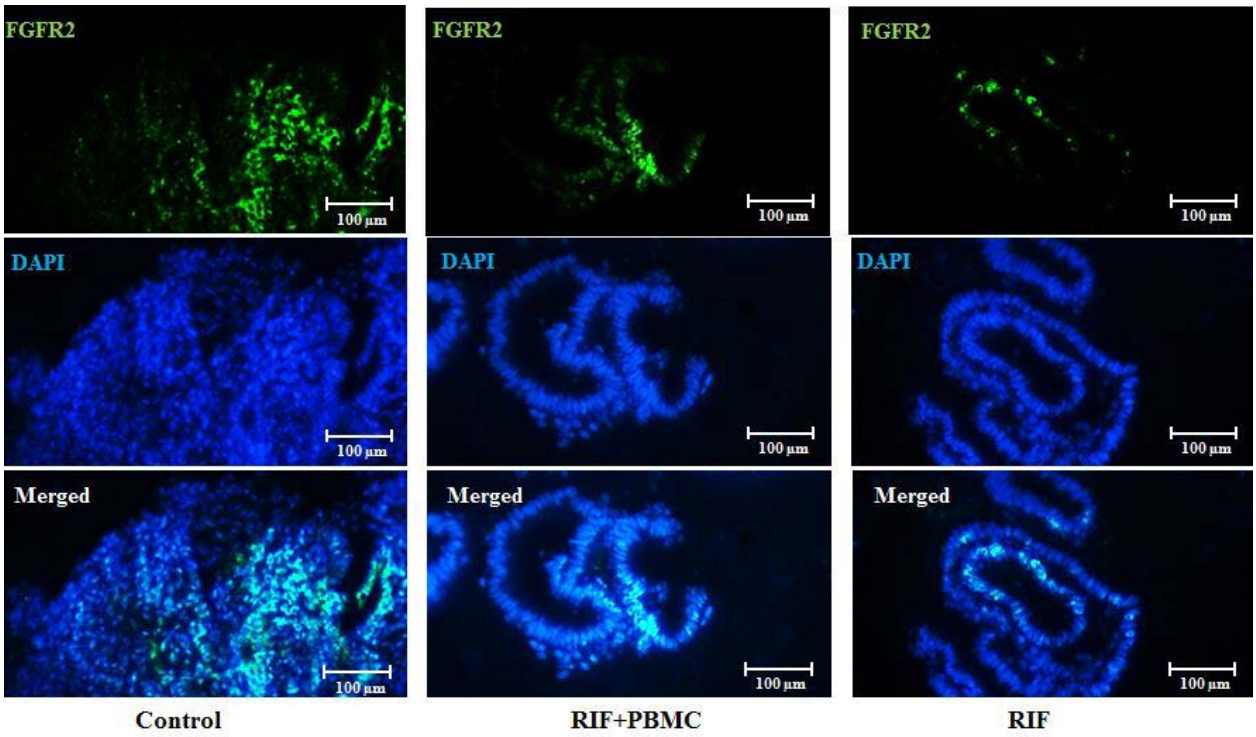

Figure 3. Immunohistochemical staining with DAPI for *FGFR-2* gene and comparing its expression in the three groups studied. (**A**) The control group is identified as DAPI-positive (high expression) in the endometrial tissue. (**B**) The RIF with PBMCs group is identified as (average expression) and (**C**) the RIF group is identified as (low expression). Original magnification ×300.
*: Significant difference at $P < 0.05$.

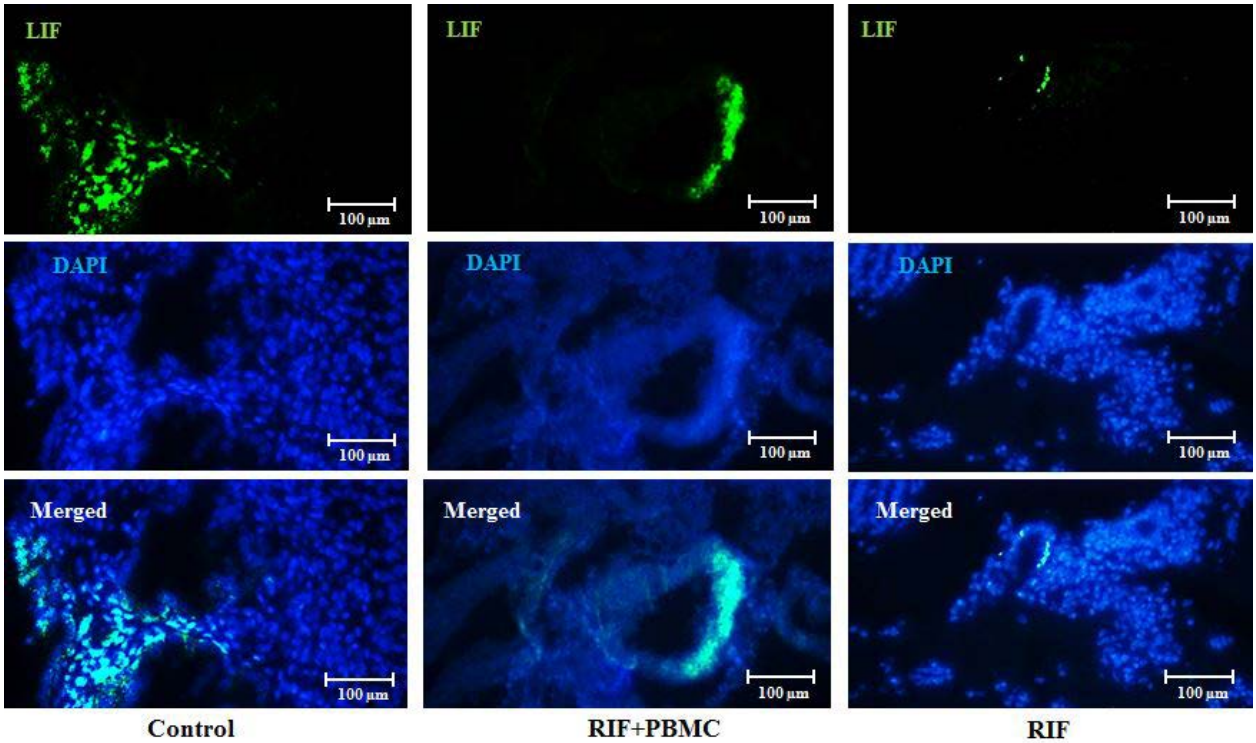

Figure 4. Immunohistochemical staining with DAPI for *LIF* gene and comparing its expression in the three groups studied. (**A**) The control group is identified as DAPI-positive (high expression) in the endometrial tissue. (**B**) The RIF with PBMCs group is identified as (average expression) and (**C**) the RIF group is identified as (low expression). Original magnification ×300.
*: Significant difference at $P < 0.05$.

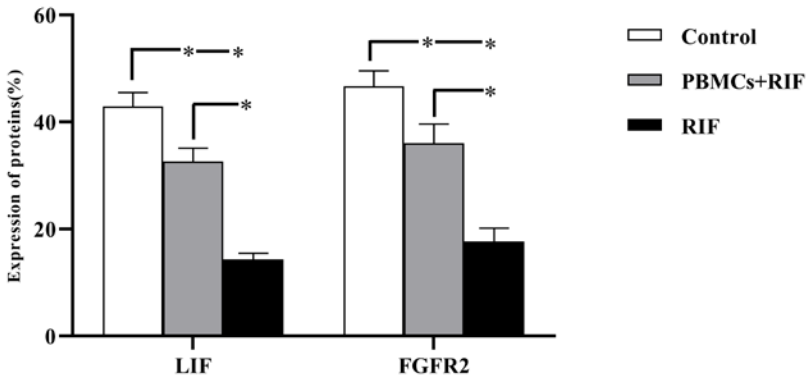

Figure 5. Upregulation of the *LIF* and *FGFR-2* protein in the RIF with PBMCs group in comparison to the RIF group and control group. Quantification of *FGFR-2* and *LIF* proteins expression in the RIF with PBMCs group was significantly increased by PBMCs treatment compared with the RIF group.
*: Significant difference at $P < 0.05$.

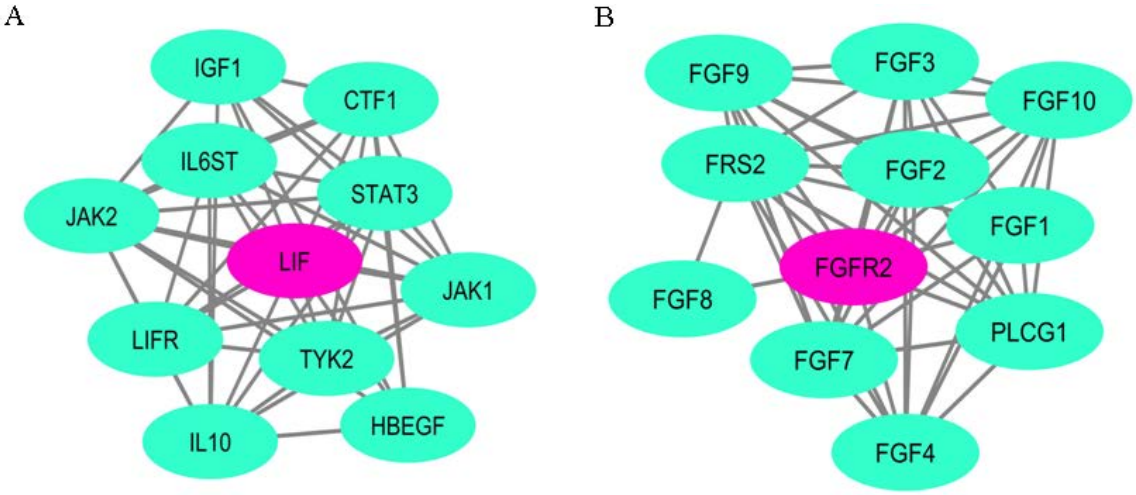

Figure 6. The whole network of *LIF* and *FGFR-2* proteins performed by STRING database and Cytoscape software. (**A**) *LIF* protein and its direct interaction proteins. (**B**) *FGFR-2* protein and its direct interaction proteins.

Table 3. KEGG pathway analysis of *LIF* and *FGFR-2* proteins and directly interaction proteins with them

KEGG ID	KEGG-Term	Nr.Genes	Associated genes	P value
LIF				
KEGG:04630	JAK-STAT signaling pathway	9	CTF1, IL10, IL6ST, JAK1, JAK2, *LIF*, LIFR, STAT3, TYK2	4.28E-14
KEGG:04550	Signaling pathways regulating pluripotency of stem cells	7	IGF1, IL6ST, JAK1, JAK2, *LIF*, LIFR, STAT3	1.98E-10
KEGG:04659	Th17 cell differentiation	5	IL6ST, JAK1, JAK2, STAT3, TYK2	2.30E-07
FGFR-2				
KEGG:04014	Ras signaling pathway	10	FGF1, FGF10, FGF2, FGF3, FGF4, FGF7, FGF8, FGF9, FGFR2, PLCG1	6.98E-15
KEGG:04015	Rap1 signaling pathway	10	FGF1, FGF10, FGF2, FGF3, FGF4, FGF7, FGF8, FGF9, FGFR2, PLCG1	2.08E-15
KEGG:04810	Regulation of actin cytoskeleton	9	FGF1, FGF10, FGF2, FGF3, FGF4, FGF7, FGF8, FGF9, FGFR2	5.48E-13

4. Discussion

The endometrium is a multiplex tissue that consists of several kinds of cells whose functions are mainly regulated by steroid hormones estrogen, progesterone, androgens, and glucocorticoids. Endometrial admission is self-limiting periods that, through mediation by immune cells, cytokines, growth factors, chemokines, and adhesive mole-

cules allow the blastocyst-stage embryos to firmly attach to the endometrial surface. This time period is called Window of Implantation (WOI). The implantation window of human embryos into the endometrium occurs from days 20–24 of the 28-day menstrual cycle. During this process, there is morphological membrane deformation of the endometrial epithelial cells. However, endometrial receptivity may not be faithfully replicated in infertile patients during this period. Any disruption in this process can lead to implantation failure.

As mentioned, one of the dimensions of implantation is its immunological aspect. The maternal immune system regulates endometrial cell differentiation for adequate endometrial receptivity and embryo implantation. Successful implantation largely depends on the immune system's tolerance mechanism[32]. Impairment of the embryo-maternal immune-tolerance pathways can be a cause for RIF. There have been few studies to RIF mechanism. The unique function of the maternal immune system during blastocyst binding to the endometrium and then pregnancy period is a behavior similar to organ transplantation that is tolerated and not rejected by the mother's immune system. Studies have shown that type 2 helper T cell (Th2) cytokines release in women who have normal pregnancy whereas women with recurrent implantation failure induce the production of type 1 helper T cell (Th1) cytokines[33]. Changing the position of immune cells from the blood to stromal tissues will activate them and have a regulatory role by secreting chemokines and cytokines, such as *LIF*[2]. The fundamental, genetic and molecular process of implantation is complicated. Results from several studies have indicated that in addition to microRNAs, numerous genes, molecules, and enzymes play an essential role in embryo implantation. The mechanisms of the effects and interactions between these factors have not been clearly elucidated Leukemia Inhibitory Factor (*LIF)* and Fibroblast Growth Factor Receptor 2 (*FGFR-2)* are two important genes that affect RIF. *FGFR-2* is one of the main genes that influences endometrial receptivity and is controlled by miR-125b-5p[34]. In previous studies, using luciferase reporter assay, it was found that the expression of the FGFR2 gene was directly suppressed by miR-125b-5p activity, and this gene is the direct target of this miR-125b-5p[35]. The *LIF* gene in the JAK-STAT pathway has a critical role in the implantation window, and miR-199a-5p sets it up[13,36]. In 2012, one study was conducted to *LIF* expression evaluation. They confirmed that miR-199a-5p expression, interfered with endogenous *LIF* expression[25]. The important, effective gene panel for endometrial receptors and regulating activity of microRNAs has been identified by researchers. According to the results of this study, both *LIF* and *FGFR-2* genes were more highly expressed in the RIF with treatment group compared with RIF group. There appear to activity of these cytokines can lead to release the protease and created the suitable inflammatory response that can change structure and function in uterine endometrial epithelial cells. These processes along with the factors alter the expression of implicated genes in implantation process. Choi *et al.* assessed the expressions of genes that affected progesterone response to endometrial adhesion. They concluded that mice with defective *LIF* gene expression had no suitable response to embryo implantation. Their study also showed that *LIF* gene expression in RIF patients was inexplicably reduced[37]. In a similar study by Yu *et al.*, *LIF* and *VEGF* gene expressions were examined in mice after injection with PBMCs. The results showed increased expressions of these genes in the intervention group[2]. In 2015, Yu *et al.* evaluated the effect of fetal hCG on PBMC function and its effect on *LIF* and *IL-1β* expressions. They found significantly increased *LIF* and *IL-1β* expressions 24 h after the PBMCs injection. hCG stimulated the secretion of cytokines in PBMCs, which could stimulate trophoblast invasion[38]. Madkour *et al.*, in a study of 27 patients with recurrent abortions, observed a threefold increase in clinical pregnancy rates as a result of the PBMCs injection. They suggested that PBMCs cells with IL8 production played a key role in the interventive approach for protein synthesis in the implantation procedure[8]. Nobijari *et al*. studied 122 couples with RIF who had undergone IVF, treated with PBMCs, and injected PBMCs two days before em-

bryo transfer into RIF women who had undergone IVF. The results confirmed that PBMCs therapy could be an effective treatment for patients with RIF[32]. In this study, in the RIF with PBMCs group, miRNAs were differently expressed than the RIF group. MiR-199a-5p and miR-125b-5p expression is dramatically reduced in the RIF with PBMCs group which was due to the effect of injected PBMCs on microRNA expression and genes associated with endometrial receptivity. The miRNAs expression in the RIF with PBMCs group and control group was similar, indicating the success of treatment with PBMCs, which was able to provide endometrial conditions for a normal immune response.

There are more than 2,000 microRNAs known to regulate multiple genes and their functions[17,18]. Altmäe *et al*. reported that a subset of microRNAs and their target genes could play an essential role in uterine receptivity[13]. The literature supports a major function for microRNAs in human embryonic implantation[17,39]. In 2008, Hu and colleagues showed that miR-21 with its target gene (*Reck*) had a higher expression at the implantation time[40]. Therefore, they suggested that microRNAs could have a key role in embryonic implantation. Similar studies have been done on the role of various microRNAs. On the other hand, Yu *et al.* reported stimulation of cytokine and exosome production by PBMCs. Exosomes contain microRNAs, which has a necessary function in implantation in infertile patients[2]. Rouas *et al.* introduced the important role of miR-31 and its target genes, *FOXP3* and *CXCL12*, in the embryonic implantation window[36]. These genes have an inhibitory role of Th17, which is involved in angiogenesis. Ozcan introduced the miR-30 family for the transformation of epithelial to mesenchymal[38], and Li explained its role in the regulation of the apoptotic process[39]. Song introduced the association of this microRNA in the differentiation of cells during embryo development[41–43]. In 2014, the role of miR-31 in the implantation window was identified by Jesica *et al.* They suggested that it could be used as a suitable biomarker for uterine receptors[44]. Tochigi *et al.* reported that decreased miR-542-3 expression leads to increased expression of *IGFBP-1*, which plays an important role in endometrial decaying[45]. The findings of their study are very similar to our results. Also, the characters of many genes in endometrial receptivity and embryonic implantation have been studied. For example, Labarta *et al.* showed that 140 genes, such as *LIF* and *GPX3*, alter various progesterone levels, which play a role in endometrial adhesion[46]. According to Altmäe *et al.*, 12 genes have been introduced as a panel of important genes in endometrial receptivity. The most important were *LIF*, *FGFR-2*, *CAST*, and *CFTR*. miR-199a-5p, miR-125-5p, miR-30b and miR-30d regulated their functions[13].

In this study, according to the analysis of the PPI network, ten important genes were identified that have direct interaction with each of *LIF* and *FGFR-2* proteins separately and also according to finding of **Table 2**, pathway mining was done and leading to extraction of (KEGG) pathways. This pathway analysis shows that the *LIF* protein was enriched in JAK-STAT signaling pathway, Signaling pathways regulating pluripotency of stem cells and Th17 cell differentiation, while the *FGFR-2* protein was enriched in Rap1 signaling pathway, Ras signaling pathway and Regulation of actin cytoskeleton and confirms the results of other studies.

According to the results of this study, a significant difference existed between the RIF women treated with PBMCs and normal fertile women (control) compared with the RIF negative control group. The expressions of *LIF* and *FGFR-2* genes increased significantly after PBMCs treatment. These results were consistent with other previously mentioned studies. Generally, the results showed that both genes were more highly expressed in the PBMCs treatment group compared with the RIF negative control group. miR-199a-5p and miR-125-5p were also significantly less expressed, which was due to the effect of injected PBMCs on microRNA expression and the genes associated with endometrial receptivity. Therefore, it could be concluded that PMBCs treatment can change the expression of microRNAs and their target genes, and lead to increased embryo implantation.

5. Conclusion

Our study is one of the few studies that have identified the role of PBMCs on *FGFR-2* and *LIF* gene expressions and their regulatory microRNAs. We hypothesized that increases in effective gene expressions in the implantation period and decreases in their regulatory microRNAs would provide a suitable endometrial environment for embryo development and attachment after PBMCs infusion. The results of this study support our hypothesis, as seen by the positive effect of PBMCs on elevating the expressions of embryonic implantation related genes and decreasing their regulatory microRNAs. Finally, we can say there are many other genes that may control the embryo implantation process and have an antagonistic or synergistic effect on the function of other genes. In this study, only the relationship between the two genes and their controlling molecules has been studied, which can be considered as a limitation of the study and further research is needed on other genes and the relationships between genes that control this process and although we achieved good results in this study, our statistical population was small due to cancellation of IVF cycle in many patients, and this was another limitation of this study. Therefore, in order to be able to prove the above results conclusively in future research, larger statistical samples are needed.

Conflict of interest

The authors declare no potential conflict of interest.

Acknowledgements

The authors would like to thank Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, for funding this project.

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