Original Article

Expression Analysis of Genes and MicroRNAs Involved in Recurrent Implantation Failure: New Noninvasive Biomarkers of Implantation

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Abstract

Background: Recurrent implantation failure (RIF) is defined as three or more failed *in vitro* fertilization attempts and is due to several factors such as oocyte and embryo quality. **Methods:** Fifty-one RIF patients and 19 controls were selected based on the inclusion criteria. *EFNB2, CAMK1D, AREG,* and *PTGS2* as well as miR-34, miR-145, miR-204-5p, and miR-26-5p were selected since the microRNAs (miRNAs) targeted the genes based on bioinformatic predictions and literature review. Total RNA was extracted from cumulus cells (CCs) and follicular fluid (FF) of the oocytes. We performed real-time polymerase chain reaction to evaluate the expression of the genes and the miRNAs in CC and FF of pregnant and nonpregnant RIF patients. The expression of *CAMK1D, AREG,* miR-34-5p, and miR-26-5p was higher in CC than FF. **Results:** The expression of *CAMK1D*, *PTGS2,* and miR-26-5p in CC of the pregnant group was higher than FF. The expression of *EFNB2*, *PTGS2*, miR-145, and miR-204-5p was lower in the CC, and the expression of *EFNB2*, *AREG*, miR-34-5p, mR-145, and miR-204-5p was lower in the FF of the pregnant group. The expression of *CAMK1D, AREG, PTGS2*, miR-34-5p, and miR-26-5p was higher in the CC and FF of the high quality (HQ) embryos than non-HQ (NHQ) embryos. The expression of *EFNB2*, miR-145, and miR-204-5p was higher in the CC and FF of the NHQ embryos. The difference was statistically significant for *EFNB2* in CC and FF as well as miR-145 in CC. The level of progesterone and prostaglandin E2 in the FF of the pregnant group was higher than their level in the nonpregnant group. **Conclusion:** *CAMK1D* expression and overexpression of miR-34-5p and miR-26-5p could be considered as markers of successful pregnancy. In addition, the results show that normal FF treatment of RIF patients may result in the production of high-quality embryos.

Keywords: Cumulus oocyte complex, follicular fluid, oocyte quality, repeated implantation failure

Introduction

Assisted reproductive technology (ART) has provided valuable information about human reproduction and has helped the treatment of infertility.[1] It has also improved pregnancy rate after *in vitro* fertilization (IVF).[2] However, reports indicate that posttransfer implantation rate has not increased considerably and is nearly 33% because of recurrent implantation failure (RIF). RIF is defined as three or more failed IVF attempts and is due to several factors such as oocyte and embryo quality.[3] The most widely used approach to assess the quality of embryos is embryo morphology and genetic screening. However, embryo morphology assessment is subjective,[4,5] and genetic screening needs the manipulation

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of embryos and could harm the embryos.[6] Therefore, noninvasive methods are required to evaluate the quality of embryos for IVF. In addition to oocytes, there are somatic cells in follicles including granulosa and cumulus cells (CCs). CCs are in close contact with the oocyte and provide paracrine signals to it.[7] They remain attached to oocytes even after oocyte retrieval for ART[8] and are usually discarded after oocyte retrieval. Since CCs are closely related to oocyte and affect its development, they have been proposed as target cells to evaluate the quality of an oocyte and embryos.[9-11] Granulosa cells and CCs produce follicular fluid (FF), which is essential for oocyte development.[12] Studies show that the constituents of FF affect the maturation of oocytes[13,14] and can be a suitable source for oocyte quality evaluation. Various studies have indicated that CCs and oocyte/embryo quality are affected by gene expression pattern.[9-11,15] Examples of these genes are *AREG, PTGS2, CAMK1D,* and *EFNB1*, which code for amphiregulin (*AREG)*, Cox-2, progesterone synthesizing enzymes, and ephrin-B2, respectively.

AREG has been shown to improve oocyte maturation *in vitro*.[16] *CAMK1D* is a serine/threonine kinase that mediates the expression of enzymes that convert pregnenolone to progesterone.[17] Studies show that *CAMK1D* expression could be used to predict the quality of embryos and implantation.[11,15] *PTGS2* participates in the production of prostaglandins such as prostaglandin E2 (PGE2), which is an important mediator of oocyte maturation and implantation.[18] Finally, *EFNB1* expression has been shown to be related to aneuploidy status of oocytes.[11,19] These genes expression can be affected by several factors including epigenetics factors.

MicroRNAs (miRNAs) are considered as one of the epigenetic factors.[20,21] They are 19–25 nucleotides in length noncoding RNAs and control gene expression posttranscriptionally by suppressing or degrading mRNAs.[22-25] They can also be secreted and transferred to the neighboring cells and control their gene expression.[26,27] These molecules exert their effects via targeting a variety of biological processes such as angiogenesis, apoptosis, metastasis, and autophagy.[28-33] Scores of studies have investigated the role of miRNAs in embryo implantation.[34] For example, some miRNAs including miR-26b has been shown to play a part in atresia and degradation of ovarian follicles as well as the negative regulation of steroidogenic process and granulosa cells apoptosis.[35,36] Others like miR-34-5p, miR-204-5p,[37] and miR-145-5p[38] have been reported to affect embryo quality and implantation.

The quality of oocytes and embryos also depends on hormonal factors such as progesterone and prostaglandins. Studies show that CCs produce progesterone and prostaglandins that influence the quality of embryos and predicts the aneuploidy of embryos.[17,39] FF has also been shown that affects CCs genes and miRNAs expression. Thus, FF and CCs are important factors for oocytes and embryos quality.[40,41] In fact, FF contains various biomolecules,[42,43] and its composition can provide useful information about the quality of oocytes.[44]

Here, we hypothesized that the quality of embryos is affected by the expression of *PTGS2, CAMK1D, EFNB2, AREG,* and their targeting miRNAs including miR-26b, miR-34-5p, miR204, and miR-145-5p expression in CCs of oocytes retrieved from RIF patients and they may be use as biomarkers of successful pregnancy. miRNAs selected that either have not been previously studied or have not been previously evaluated in RIF patients.

Thus, we assessed these genes and the miRNAs expression in the oocytes, CC and FF as well as pregnancy success. We also hypothesized that normal FF may improve oocyte quality and pregnancy outcome. Therefore, we cultured cumulus–oocyte complex in the presence of normal FF to evaluate FF effects on oocyte quality. In addition, progesterone and PGE2 levels were evaluated in the FF of subjects and controls.

Methods

Patients consent form

Informed consent was obtained from all the participants and the study was under the supervision of ethics committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.MSP. REC.1398.364).

Study groups

RIF patients who admitted to the infertility center from February 2019 to February 2020 were selected based on the inclusion/exclusion criteria presented in Table 1. Seventy subjects entered the study based on the criteria, 51 RIF patients and 19 controls. The subjects were divided into 3 groups based on pregnancy/nonpregnancy after implantation: (1) pregnant RIF group, which includes the subjects with a history of RIF who became pregnant after IVF ($n = 19$); (2) nonpregnant RIF, which includes the subjects with a history of RIF who did not become pregnant after IVF ($n = 32$); and (3) healthy oocyte donors had no RIF history. Sperm parameters of the husbands were normal in all subjects.

Stimulation of ovulation

According to the standard protocol in Taleghani Hospital (Tehran, Iran) and under the supervision of gynecologist, 150–300 IU Gonal-F was administered from the 3rd day of cycle and continued until at least two follicles with a diameter of 14–15 mm were observed. Cetrolix (0.25 mg/day) was administered as GnRH antagonist until at least two follicles with a diameter of 18 mm were observed. After observing the follicles, hCG was administered. Oocytes were collected 36 h later under general anesthesia using vaginal sonography.

Cumulus cells and follicular fluid isolation

CCs were mechanically removed from the oocytes. Then, corona cells were briefly treated using 80 IU/ml hyaluronidase at 37°C and were isolated from the oocytes. The CCs were then washed in cold PBS and centrifuged at 200 g for 10 min. The supernatant was discarded, and the cell pellet

Table 1: The inclusion/exclusion criteria used for the selection of subjects and controls

Inclusion criteria	Exclusion criteria
Age <38	Subjects' will
Two histories of RIF after IVF/ICSI	Subjects' whose husbands could not provide semen samples
Regular menstruation (25-35 days)	Ovarian hyper-stimulation syndrome
Not using OCPs and IUD in the past 3 months	
No hormonal disorder	
No uterus anatomical disorder	
No endometriosis	
No thrombophilia	
No active uterine infection and no STD in their husbands	
No history of pre-eclampsia	
No underlying disease such as diabetes and hypertension	
No history of immune-suppressing diseases such as lupus and rheumatoid arthritis	
No genetic disease of the subjects and their partners	
Not using alcohol and cigarettes by the subjects and their partners	

RIF: Repeated implantation failure, IVF: In vitro fertilization, ICSI: Intracytoplasmic sperm injection, OCP: Oral Contraceptive Pills, IUD: Intra Uterine Contraceptive Device, STD: Sexually transmitted diseases

was transferred to the lysis buffer of RNA extraction kit and stored at-80°C until RNA extraction. Oocytes were cultured in human tubal fluid medium (HTF) supplemented with 10% albumin. Finally, oocytes were fertilized via intracytoplasmic sperm injection.

To obtain FF, after removing oocytes from the follicular content, the remaining fluid was centrifuged at 600 g for 10 min. Pelleted granulosa cells and blood cells were discarded and the supernatant was inactivated at 56°C for 30 min. The FF was finally filtered through a 22-µm membrane and stored at −80°C.

Morphological assessment of embryos quality

The quality of the blastomeres was evaluated based on the following criteria: (1) The presence of 4–5 blastomeres on day 2 and at least 7 blastomeres on day 3 without any multinuclear blastomeres. (2) Less than 20% fragmentation on day 2 and day 3.

Genes and microRNAs selection

Several genes were selected based on literature review. These genes have been proved to play a role in the process of ovulation or oocyte and embryo maturation. To select miRNAs that target these genes, we used TargetScan (http://www.targetscan.org/vert_71/) and miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) databases.

RNA extraction, complementary DNA synthesis, and quantitative real-time polymerase chain reaction

Total RNA was extracted using Hybrid-R™ (GeneAll, Korea) according to the manufacturer's instruction.

Complementary DNA (cDNA) synthesis for mRNAs was performed using 500 ng RNA, 1 µLrandom hexamer primer (10 mM), 20 U RiboLock RNase Inhibitor, 200 U RevertAid M-MuLV RT (Cat No. #EP0441; Thermo Fisher Scientific), 4 µl 4X reaction buffer, and 2 µl dNTP (10 mM) in a final volume of 20 µl. The mixture was incubated at 25°C for 5 min, then at 42°C for 60 min, and inactivation at 70 for 5 min.

miRNA cDNA synthesis was performed using 100 ng RNA, 5 µl Stem-loop RT primer (1 µM), 4 µl 5X reaction buffer, 20 U of RiboLock RNAse inhibitor, 2 µl dNTP mix (10 mM), and 200 U of RevertAid M-MuLV RTCat No. #EP0441; Thermo Fisher Scientific in the final volume of 20 µl. The mixture was incubated at 25°C for 5 min, then at 42°C for 60 min, and inactivation at 70°C for 5 min.

Primers were designed for the genes and the miRNAs expression using AlleleID 6. Table 2 present the accession numbers of the genes, miRNAs, and the sequence of the primers used in this study. GAPDH and U6 were used as housekeeping genes.

Quantitative real-time polymerase chain reaction (RT-qPCR) for the genes was performed using 10 µl 2X RealQ Plus MasterMix Green (Amplicon, Denmark), 0.8 µl forward primer (10 pM), 0.8 µl reverse primer (10 pM), and 2 µl cDNA in the final volume of 20 µl. The thermal cycle was 95°C for 15 min for enzyme activation followed by 35 cycles of 95°C for 20 s and 60°C for 1 min.

RT-qPCR for the miRNAs was performed using 10 µl 2X RealQ Plus MasterMix Probe, 0.8 µl forward primer (10 pM), 0.8 µl reverse primer (10 pM), and 2 µl cDNA in the final volume of 20 µl. All RT-qPCR results were analyzed using $2^{-\Delta\Delta Ct}$ method in REST 2009 software.

Oocyte and follicular fluid coculture

The cumulus–oocyte complex of 10 RIF patients were cultured in 10% HTF and 40% normal FF. Afterward, the expression of the genes and the miRNAs were evaluated using RT-qPCR with the protocols previously explained.

Progesterone and prostaglandin E2 enzyme-linked immunosorbent assay

Human prostaglandin and progesterone levels in FFs and

Table 2: The sequence of genes and microRNA primers

Genes		
Gene	**Forward primer**	**Reverse primer**
EFNB2 (NM_004093.4)	CCAAATCCAGGTTCTAGCACAGA	CTCTGAGCCGTTGTTGTTGC
CAMK1D (NM_020397.4)	AAGAGCAAATGGAGACAAGCA	ACTGAGGCTGCTCGAAACAC
AREG (NM_001657.4)	TTCCAACACCCGCTCGTTT	TAATGGCCTGAGCCGAGTATC
PTGS2 (NM_000963)	TCAGCCATACAGCAAATCCTTG	GTCCGGGTACAATCGCACTT
GAPDH (NM_001256799)	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
miRNAs		
miRNA	**Forward primer**	**RT stem-loop**
miR-34-5p (MIMAT0000685)	AGGGTGGCAGTGTCTTAGC	GGTCGTATGCAGAGCAGGGTCCGAGGTATCC ATCGCACGCATCGCACTGCATACGACCACAACC
miR-145-5p (MIMAT0000437)	CGTCCAGTTTTCCCAGGAA	GGTCGTATGCAGAGCAGGGTCCGAGGTATCC ATCGCACGCATCGCACTGCATACGACCAGGGA
miR-204-5p (MIMAT0000265)	ACTTCCCTTTGTCATCCTATG	GGTCGTATGCAGAGCAGGGTCCGAGGTATCC ATCGCACGCATCGCACTGCATACGACCAGGCATAG
miR-26-5p (MIMAT0000083)	CCGCTTCAAGTAATTCAGGAT	GGTCGTATGCAGAGCAGGGTCCGAGGTATCC ATCGCACGCATCGCACTGCATACGACCACCT
U6 (housekeeping)	AAGGATGACACGCAAATTC	GTCGTATGCAGAGCAGGGTCCGAGGTATTCGC ACTGCATACGACAAAAATATGG
Universal reverse primer	GAGCAGGGTCCGAGGT	

AREG: Amphiregulin, miRNAs: Micro RNAs, RT: Reverse transcription

CCs were evaluated using human PGE2 (Cusabio, China) and Human Progesterone (Cusabio, China) enzyme-linked immunosorbent assay kits, respectively, according to the manufacturer's instruction.

Statistical analysis

The comparison between groups was performed using Student's *t*-test or one-way ANOVA and Tukey *post hoc* test. $P < 0.05$ was considered as statistically significant. All tests were performed in triplicate. GraphPad Prism version 7 (San Diego, California, USA) was used for statistical analyses.

Results

Patients, pregnancy, and embryos quality assessment

Embryos quality was assessed based on the criteria mentioned in the materials and methods section. Twenty-two high-quality and nonhigh quality (NHQ) embryos were obtained. After implantation, 19 subjects became pregnant, 11 of whom had high-quality embryos, and 32 subjects did not become pregnant, 11 of whom had high-quality embryos.

Genes and microRNAs selection

Based on the literature review, *EFNB2, CAMK1D, AREG,* and *PTGS2* genes were selected. The results of TargetScan and miRWalk analysis of the selected genes were sorted in MS Excel, and the high score miRNAs were selected. miR-204-5p, miR-145-5p, miR-34-5p, and miR-26-5p were selected as miRNAs that targeted *EFNB2, CAMK1D, AREG,* and *PTGS2,* respectively with the highest score. These miRNAs either have not been studied or have not been evaluated in RIF patients.

The genes expression in cumulus cell and follicular fluid

We evaluated the expression of *EFNB2, CAMK1D, AREG,* and *PTGS2* in CCs and FF in three groups as described in the patients section of materials and methods.

The results indicated that the relative fold change (RFC) of *EFNB2* was higher in the CC (RFC = 6×10^6, $P = 0.04$) and FF (RFC = 3×10^6, $P = 0.023$) of the nonpregnant group than in the pregnant group. On the other hand, the expression of *CAMK1D* was higher in the CC (RFC = 19.9, $P = 0.01$) and FF (RFC = 19.5, $P = 0.025$) of the pregnant group than in the nonpregnant group. In addition, the expression of *AREG* was higher in the CC (RFC = 30 folds, $P = 0.001$) and FF (RFC = 194 folds, $P = 0.006$) of the pregnant group than in the nonpregnant group. Finally, the expression of *PTGS2* was 30 folds ($P = 0.03$) lower in the CC and 3 folds ($P = 0.027$) higher in the FF of the pregnant group than in the nonpregnant group [Figure 1a and b].

MicroRNAs expression in cumulus cell and follicular fluid

miR-145 and miR-204-5p expression in CC was respectively 7.9 ($P = 0.01$) and 1634 ($P = 0.007$) folds lower in the pregnant group than in the nonpregnant group. Similarly, their expression was 9.3 ($P = 0.037$) and 125 ($P = 0.046$) folds lower in the FF of the pregnant group than in the nonpregnant group. On the other hand, the expression of miR-34-5p ($P = 0.03$) and miR-26-5p ($P = 0.0006$) was, respectively, 18 and 19 folds higher in the CC of the pregnant group than that in the nonpregnant group. While miR-34-5p expression was 8.3 ($P = 0.048$) folds higher in the FF of the nonpregnant group, miR-26-5p expression was 3.5-fold higher in the pregnant group ($P = 0.031$) [Figure 2a and b].

Progesterone and prostaglandin E2

The level of progesterone [Figure 3a] and PGE2 [Figure 3b] in the FF of the pregnant group was higher than that in the nonpregnant group. In fact, progesterone concentration was 42.48 ± 2.153 and 30.83 ± 1.128 pg/ml in the FF of the pregnant

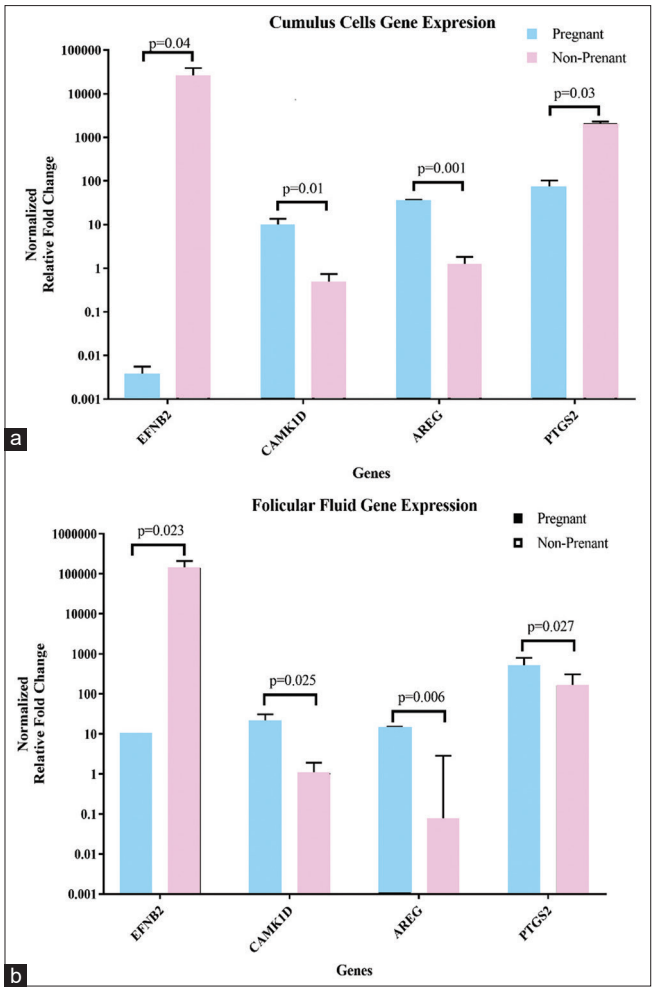

Figure 1: Relative fold change of *EFNB2, CAMK1D, AREG*, and *PTGS2* in the cumulus cell and follicular fluid of pregnant and nonpregnant RIF patients compared to the control group. (a) The relative expression of the genes shows that the expression of *EFNB2*, *AREG*, and *PTGS2* in the cumulus cell of the nonpregnant group was respectively $6 \times 10^6 \pm 12918.08$, 4.6 ± 0.23, and 30 ± 34.17 folds higher than that of the pregnant group. On the other hand, the expression of *CAMK1D* in the cumulus cell of the pregnant group was 19.9 ± 4.87 folds higher than that of the nonpregnant group. The bars indicate the expression in pregnant and nonpregnant groups compared to the control group. The fold change numbers indicate the normalized expression of the either pregnant group to nonpregnant group. (b) In the follicular fluid of the pregnant group, the expression of *EFNB2* and *AREG* was $3 \times 10^6 \pm 70013.56$and 17.7 ± 2.7 folds lower than that of the on-pregnant group. On the other hand, the expression of *CAMK1D* and *PTGS2* was 19.5 ± 10.24 and 3 folds higher in the pregnant group than in the nonpregnant group. The numbers are presented as mean ± standard deviation

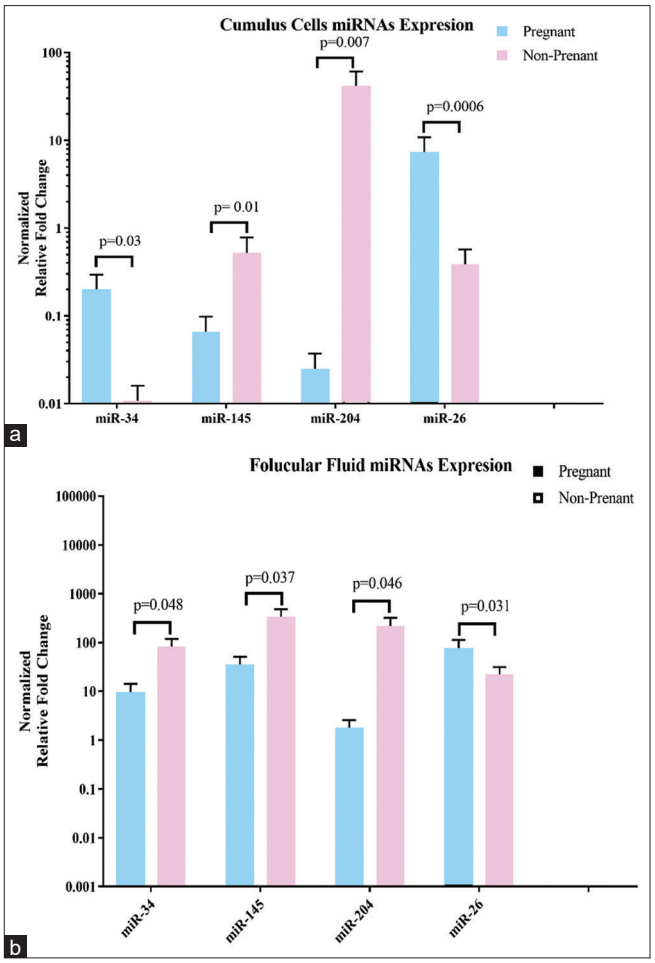

Figure 2: Relative fold change of miR-34-5p, miR-145-5p, miR-204-5p, and miR-26-5p in the CC and FF of pregnant and non-pregnant RIF patients compared to the control group. (a) The relative expression of the miRNAs in CCs shows that the expression of miR-145-5p and miR-204-5p was lower 7.9 ± 0.32 and 1634 ± 20.32 folds, respectively, in the pregnant group than in the non-pregnant group. (b) In the FF also, the expression of miR-145-5p and miR-204-5p was lower 9.3 ± 0.032 and 125 ± 12.3 folds, respectively, in the pregnant group than in the non-pregnant group. The expression of miR-34-5p and miR-26-5p was 18 ± 0.98 and 19 ± 3.6 folds, respectively, higher in the CC of the pregnant group than that in the non-pregnant group. While miR-34-5p expression was 8.3 ± 0.39 folds higher in the FF of the non-pregnant group, miR-26-5p expression was 3.5 ± 0.12 fold higher in the pregnant group

and the nonpregnant group, respectively. PGE2 concentration was 87.31 ± 1.212 and 73.65 ± 2.451 pg/ml in the FF of the pregnant and the nonpregnant group, respectively. The level of progesterone and PGE2 in the control group was 36.6 ± 1.733 and 78.73 ± 2.452, respectively.

The genes and microRNA expression in high quality and nonhigh quality embryos

We also evaluated the expression of the genes and miRNAs in the CC and FF of HQ and NHQ embryos. The expression of *CAMK1D* (CC: 1.8 folds, FF: 3.3 folds), *AREG* (CC: 2.9 folds, FF: 1.5 folds), *PTGS2* (CC: 4.1 folds, FF: 1.3 folds), and miR-26-5p (CC: 1.3 folds, FF: 1.6 folds) was higher in the CC and FF of the HQ embryos than in the NHQ embryos. However, these differences were not statistically significant for *AREG* and *PTGS2* in FF as well as *CAMK1D* in CC [Figure 4]. The expression of *EFNB2*, miR-145, and miR-204-5p was higher in the CC and FF of the NHQ embryos. The difference was statistically significant for *EFNB2* in CC and FF as well as miR-145 in CC [Figure 5]. The expression of miR-34-5p was 3.2 folds higher in the CC of HQ embryos and 2.1 folds lower in the FF of HQ embryos.

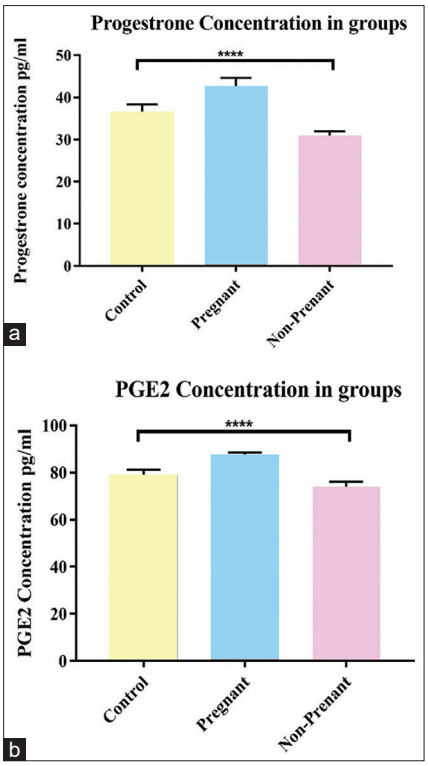

Figure 3: The level of progesterone and prostaglandin E2 in the FF of the pregnant and non-pregnant groups. The level of progesterone and PGE2 in the pregnant group is higher than that in the non-pregnant group. (a) Progesterone concentration was 42.48±2.153 and 30.83± 1.128 pg/ml in the FF of the pregnant and the non-pregnant group, respectively. (b) PGE2 concentration was 87.31± 1.212and 73.65± 2.451 pg/ml in the FF of the pregnant and the non-pregnant group, respectively. The level of progesterone and PGE2 in the control group was 36.6± 1.733 and 78.73± 2.452, respectively.****: *P*-value<0.0001

Cumulus–oocyte complex and follicular fluid coculture

Ten RIF patients CC were treated with normal FF. The treatment of the 10 CCs resulted in six HQ embryos, five of which resulted in successful pregnancy. We assessed the expression level of the genes and the miRNAs in the CC after coculture in the presence of normal FF. The results showed that *EFNB2* was 142 folds down-regulated after the treatment with normal FF ($P = 0$) compared to the CC without FF treatment. However, *AREG* (27 folds), *PTGS2 (*15 folds), and *CAMK1D* (13 folds) were up-regulated. miR-34-5p and miR-26b-5p were up-regulated after the treatment with FF for 3.95 ($P = 0.008$) and 8.7 ($P = 0.001$) folds, respectively. On the other hand, the expression of miR-145-5p and miR-204-5p was down-regulated for 4.4 ($P = 0.004$) and 7.3 ($P = 0$) folds, respectively [Figure 6].

Discussion

In this study, we evaluated the expression of *EFNB2, CAMK1D, AREG,* and *PTGS2* as well as miR-34, miR-145, miR-204-5p, and miR-26-5p in CC and FF of pregnant and nonpregnant RIF subjects. The results showed that the expression of *CAMK1D* and *AREG* as well as miR-34-5p and miR-26-5p was higher in CC of the pregnant group than in the nonpregnant group. In addition, the expression of *CAMK1D* and *PTGS2* as well as miR-26-5p was higher in the FF of the pregnant group than in the nonpregnant group. On the other hand, the expression of *EFNB2* and *PTGS2* as well as miR-145 and miR-204-5p was lower in the CC of the pregnant group than in the nonpregnant group, and the expression of *EFNB2* and *AREG* as well as miR-34-5p, mR-145, and miR-204-5p was lower in the FF of the pregnant group than in the nonpregnant group. The expression of *CAMK1D, AREG, PTGS2*, miR-34-5p, and miR-26-5p was higher in the CC and FF of the HQ embryos than that of the NHQ embryos. However, these differences were not statistically significant for *AREG* and *PTGS2* in FF as well as *CAMK1D* in CC. The expression of *EFNB2*, miR-145, and miR-204-5p was higher in the CC and FF of the NHQ embryos. The difference was statistically significant for *EFNB2* in CC and FF as well as miR-145 in CC.

The expression of *EFNB2* is different in physiological and pathological conditions.[1] *EFNB2* plays an important role in the luteinizing of granulosa cells, and its expression increases in CC of aneuploid oocytes.[2] Studies show that *EFNB2* expression can be used as a predictor of pregnancy.[45-47] Interestingly, we observed that *EFNB2* expression was significantly higher in the nonpregnant group than in the pregnant group. In addition, *EFNB2* expression of HQ embryos was significantly lower than that of NHQ embryos [Figure 5]. Thus, embryos aneuploidy could be the reason for pregnancy failure.

Cox-2 is an enzyme encoded by *PTGS2*. It is an inducible enzyme that converts arachidonic acid to prostaglandins.[48] In addition, it is induced by various stimuli such as mitogens, growth factors, and hormones like progesterone.[49] Studies show that RIF patients express decreased levels of Cox-2.[50] Cox-2 and prostaglandins levels are also reduced in unexplained recurrent miscarriage patients.[51] Gebhardt *et al*. showed that increased expression of *PTGS2* in CC was related to live birth. McKenzie *et al*. also showed that *PTGS2* expression was 6 folds higher in the CC of well-developed embryos. On the other hand, Luz *et al*. found that *PTGS2* expression was down-regulated in CC of infertile women.[52] However, we found that *PTGS2* expression was lower in the CC of the pregnant group than in the nonpregnant group although its expression in the FF[9] of the pregnant group was higher. Regarding these discrepancies, one study has shown that *PTGS2* expression changes rapidly within hours.[53] Therefore, it is possible that we have evaluated the gene expression at a time when its expression was low. Interestingly, miR-26-5p expression was higher in both CC and FF of the pregnant group. Since miR-26-5p targets *PTGS2*, it seems reasonable that *PTGS2* expression is lower in the CC of the pregnant group. However, why the expression of miR-26-5p and FF expression of *PTGS2* is not inversely correlated needs further investigation. One reason could be long noncoding RNAs (lncRNAs). Li *et al*. recently showed that MALAT1 lncRNA directly sponges miR-26 and

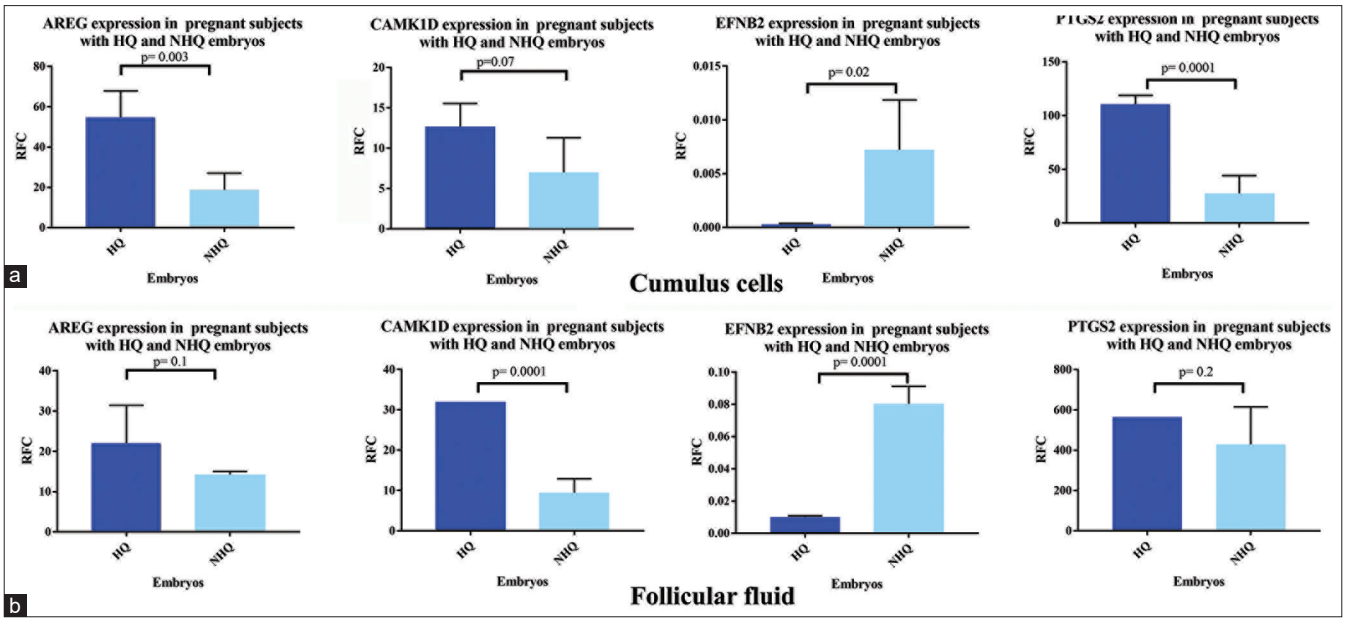

Figure 4: The expression of the genes in cumulus cell and follicular fluid of HQ and nonhigh quality embryos. According to the results, (a) Cumulus cells. The expression of *AREG* ($P = 0.003$), *CAMK1D* ($P = 0.07$), and *PTGS2* ($P = 0.0001$) was higher in the HQ embryos than in the nonhigh quality embryos. However, the di follicular fluid erence of the expression of *CAMK1D* between HQ and nonhigh quality embryos was not statistically significant. In addition, *EFNB2* ($P = 0.02$) expression was lower in HQ embryos than in the nonhigh quality embryos. (b) Follicular fluid. The expression of *AREG* ($P = 0.1$), *CAMK1D* ($P = 0.0001$), and *PTGS2* ($P = 0.2$) was higher in the HQ embryos than in the nonhigh quality embryos, and the expression of *EFNB2* ($P = 0.0001$) was lower in the HQ embryos than in the nonhigh quality embryos. However, the difference of the expression of *AREG* and *PTGS2* between HQ and nonhigh quality embryos was not statistically significant

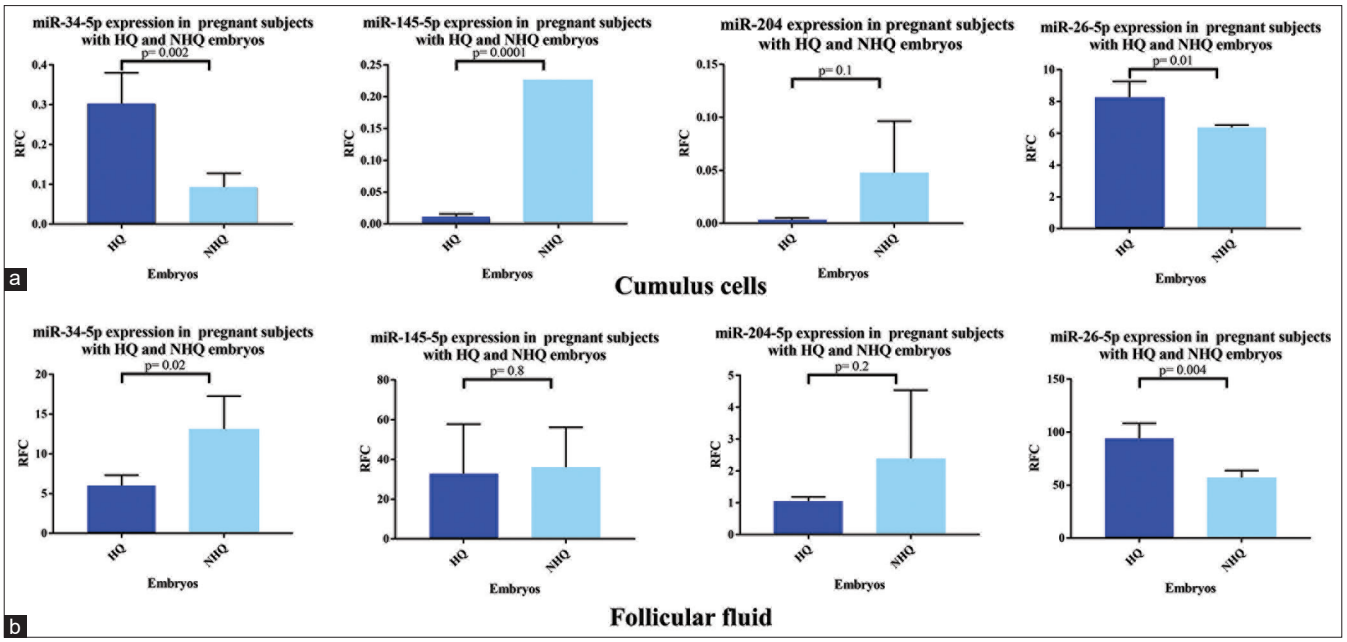

Figure 5: The expression of the miRNAs in cumulus cell and follicular fluid of HQ and nonhigh quality embryos. (a) Cumulus cells. The expression of miR-34-5p ($P = 0.002$) and miR-26-5p ($P = 0.01$) was higher in the HQ embryos than in the nonhigh quality ones. On the other hand, the expression of miR-145-5p ($P = 0.0001$) and miR-204-5p ($P = 0.1$) was lower in the HQ embryos than in the nonhigh quality ones. However, the difference of the expression of miR-204-5p between HQ and nonhigh quality embryos was not statistically significant. (b) Follicular fluid. The expression of miR-34-5p ($P = 0.02$), miR-145-5p ($P = 0.8$), and miR-204-5p ($P = 0.2$) was lower in the HQ embryos than in the nonhigh quality ones although the difference for miR-145-5p and miR-204-5p was not statistically significant. The expression of miR-26-5p ($P = 0.004$) was higher in the HQ embryos than in the nonhigh quality ones

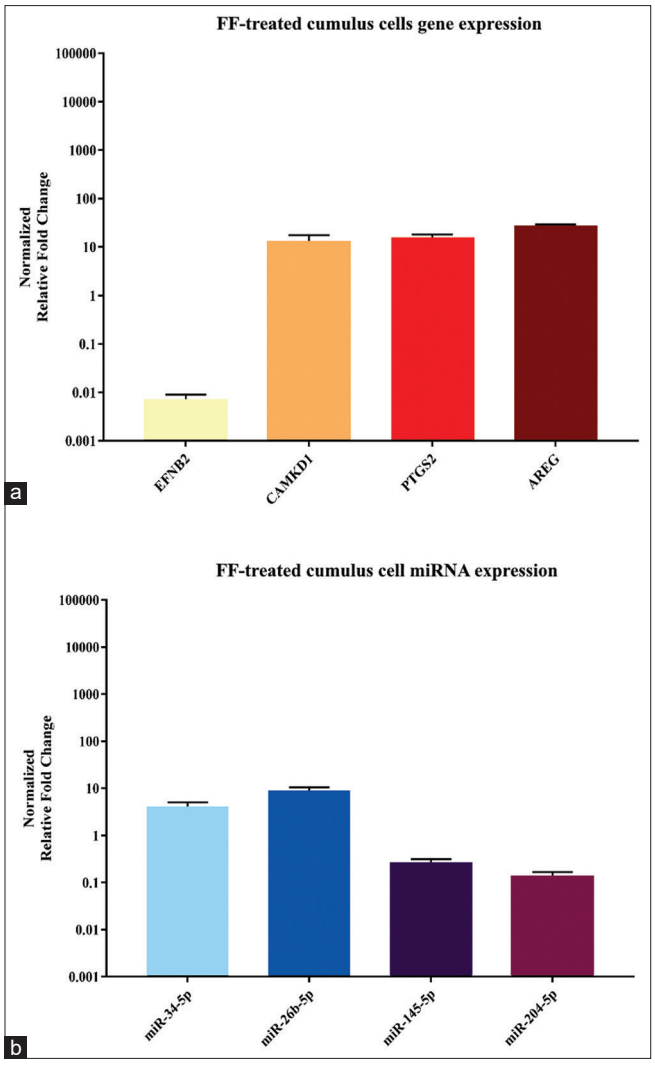

Figure 6: The expression of the genes and the miRNAs in cumulus cell after treatment with normal follicular fluid. (a) After the treatment of cumulus cell with normal follicular fluid, EFNB2 expression decreased 142 folds compared to the nontreated cumulus cell. On the other hand, AREG, PTGS2, and CAMK1D expression increased 27, 15, and 13 folds, respectively, compared to the nontreated cumulus cells. (b) The expression of miR-34-5p and miR-26b-5p increased 3.95 and 8.7 folds after the treatment of cumulus cell with normal follicular fluid, while the expression of miR-145-5p and miR-204-5p decreased 4.4 and 7.3 folds after the treatment of cumulus cell with normal follicular fluid

negates its effects in brain microvascular endothelial cells.[54] The expression of *PTGS2* in the CC of the HQ embryos was significantly higher than that in the NHQ embryos. It was also higher in the FF of the HQ embryos than in the NHQ although statistically insignificant. Note that some studies indicate that too much *PTGS2* expression may lead to pre-eclamptic syndrome.[55] In addition, a study in mice shows that oocytes undergoing germinal vesicle breakdown expressed high levels of *PTGS2* and as the oocytes mature, its expression decreases.[53] Thus, the overexpression of *PTGS2* in the nonpregnant group of our study could be due to the relative immaturity of the oocytes.

AREG is a member of the EGF receptor ligand family.[56] It has been shown that *AREG* in FF was correlated with the number of embryos and pregnancy rate in women undergoing ART.[57] It has also been positively correlated with oocyte competence and maturation.[58] However, Inoue *et al*. showed that *AREG* was inversely correlated with oocyte quality and pregnancy outcome.[56] Our results, however, show that *AREG* expression was higher in the CC and FF of the pregnant group than in the nonpregnant group. In addition, in our study, the expression of *AREG* in the CC and FF of HQ embryos was higher than that in the NHQ embryos. This is in contrast with Inoue's results. These contrasting results could be due to the complex regulation of *AREG*. Studies show that LH, forskolin,[59] FSH,[60] PGE2,[61] and *AREG* itself induced the expression of *AREG*. This means that *AREG* expression is also controlled in an autocrine manner. According to Inoue, since hCG injection to patients decreases *AREG* expression, *AREG* cannot be used as a predictor of oocyte maturation.[56] Fru *et al*. reported that *AREG* expression increases 3 h after hCG administration, and it is not detectable before injection.[62] Therefore, it seems that gene expression in CC, FF, and embryos should be evaluated at various time points since the metabolism and gene expression of these cells are rapidly changing.

CAMK1D is another gene that is implicated in the production of progesterone. Wathlet and colleagues demonstrated that its expression level is strongly related to pregnancy prediction and oocytes' developmental potential.[11] Scarica *et al*. recently showed that its expression in CCs was correlated to blastocyst development.[63] We also observed that *CAMK1D* expression in CC and FF of the pregnant group was higher than that of the nonpregnant group. This is consistent with the level of progesterone in the pregnant group, which was higher than that in the nonpregnant group [Figure 3a]. In addition, our results indicate that miR-145 expression in the FF of the pregnant group was lower than that in the nonpregnant group. Since miR-145 targets *CAMK1D*, this result seems reasonable. However, miR-145 expression in CC of the pregnant group was higher than that in the pregnant group. *CAMK1D* was also higher in the CC and FF of HQ embryos although it was not statistically significant in the CC.

COCs are in close contact with FF and are composed of steroid hormones and metabolites such as polysaccharides and proteins. Any change in FF composition is suggested to affect oocyte maturation and fertilization capacity.[64-66] Therefore, we hypothesized that FF of RIF patients might lack some factors that are important in the development of COCs. When we treated COCs of RIF patients with normal FF, we observed that *EFNB2* expression decreased while *CAMK1D*, *AREG*, and *PTGS2* increased. This is what we observed in pregnant subjects' FF. In fact, six of 10 RIF patients' COCs, which were treated with normal FF, produced high-quality embryos, and 5 of them resulted in successful pregnancies. In line with our results, Liu and colleagues also showed that the level of *AREG* in FF was correlated

with pregnancy rate capacity.[65] Studies show that miRNAs expression changes in RIF patients. For example, Shi *et al*. found that 105 miRNAs were differentially expressed in RIF patients compared to the control group.[37] In our study, we found that miR-34a, miR-145-5p, and miR-204-5p expression was lower, and miR-26-5p was higher in the FF of pregnant subjects compared to the nonpregnant ones. In line with this observation, we also found that miR-145-5p and miR-205-5p decreased in the FF-treated COCs while miR-26-5p increased. These findings may suggest that normal FF treatment of COCs could result in high-quality embryos and increase successful pregnancy rates.

Conclusion

We evaluated the expression of *EFNB2, CAMK1D, AREG,* and *PTGS2* as well as miR-34, miR-145, miR-204-5p, and miR-26-5p in CC and FF of pregnant and nonpregnant RIF subjects. Based on our results, over expression of *CAMK1D* and *AREG* expression and downregulation of *EFNB2* in CC and FF could probably be used as a marker for pregnancy prediction. Furthermore, downlregulation of miR-145 and miR-204 along with the increase in PGE2 and Progestrone level also could be considered as markers of pregnancy. Finally, it seems that the treatment of COC with normal FF can improve embryo quality and pregnancy outcome. Infact, increase expression of *CAMK1D, AREG, PTGS2* along with decline in *EFNB2* expression observed in high-quality and normal FF-treated CC and FF. However, more samples are needed to reliably decide on the predictive values of these markers. The metabolism of oocytes and CCs is fast, and thus, their gene expression is subject to rapid change. Therefore, gene expression of CCs needs to be studied at different time points to determine the best time of sampling and gene expression analysis. We also believe that the role of lncRNAs should be considered when studying miRNAs-genes interactions.

Limitation of study

Therefore, gene expression of CCs needs to be studied at different time points to determine the best time of sampling and gene expression analysis. We also believe that the role of lncRNAs should be considered when studying miRNAs-genes interactions

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical approval

The study was Supervised and approved by ethics committee of School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran (Ethics code:IR.SBMU.MSP.REC.1398.364, Approval date:2019-07-16).

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Conflicts of interest

There are no conflicts of interest.

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