Original Article

Endometrial MicroRNA Signature during the Window of Implantation Changed in Patients with Repeated Implantation Failure

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Abstract

Background: At present, a diagnostic tool with high specificity for impaired endometrial receptivity, which may lead to implantation failure, remains to be developed. We aimed to assess the different endometrial microRNA (miRNA) signatures for impaired endometrial receptivity by microarray analysis.

Methods: A total of 12 repeated implantation failure (RIF) patients and 10 infertile patients, who conceived and delivered after one embryo transfer attempt, were recruited as RIF and control groups, respectively. Endometrial specimens from the window of implantation (WOI) were collected from these two groups. MiRNA microarray was conducted on seven and five samples from the RIF and control groups, respectively. Comparative, functional, and network analyses were performed for the microarray results. Quantitative real-time polymerase chain reaction (PCR) was performed on other samples to validate the expression of specific miRNAs.

Results: Compared with those in the control group, the expression levels of 105 miRNAs in the RIF group were found to be significantly up- or down-regulated (at least 2-fold) by microarray analysis. The most relevant miRNA functional sets of these dysregulated miRNAs were miR-30 family, human embryonic stem cell regulation, epithelial-mesenchymal transition, and miRNA tumor suppressors by tool for annotations of microRNA analysis. Network regulatory analysis found 176 miRNA-mRNA interactions, and the top 3 core miRNAs were has-miR-4668-5p, has-miR-429, and has-miR-5088. Expression levels of the 18 selected miRNAs in new samples by real-time PCR were found to be regulated with the same trend, as the result of microarray analysis.

Conclusions: There is a significant different expression of certain miRNAs in the WOI endometrium for RIF patients. These miRNAs may contribute to impaired endometrial receptivity.

Key words: Embryo Implantation; Endometrial Receptivity; MicroRNA Microarray; Repeated Implantation Failure; Window of Implantation

Introduction

In the past three decades, since the first "test tube baby", Louise Brown, was born in 1978, *in vitro* fertilization-embryo transfer (IVF-ET) has experienced rapid and momentous development. However, the pregnancy rate of IVF-ET remains relatively low up to now.[1] Only approximately 30% of the embryos transferred into the uterus lead to a

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successful pregnancy.[2] Successful implantation depends on the embryo's quality, embryo-endometrium interaction, and endometrial receptivity, of which inadequate endometrial receptivity is responsible for approximately two-thirds of implantation failures.[3-5]

The term, "endometrial receptivity", is introduced to define the state of the endometrium during the window of implantation (WOI), which onsets 4–5 days after the endogenous/exogenous progesterone stimulation and ends 9–10 days afterward.[6] During this period, the endometrium acquires new adhesive properties allowing embryo adhesion and subsequent invasion.[7] Given its key role in successful implantation, predicting and improving endometrial receptivity is critical and may ultimately improve the pregnancy success rate of IVF-ET.[8] Unfortunately, no effective diagnostic tools are yet available to precisely predict endometrial receptivity.[9]

MicroRNAs (miRNAs) are small RNA fragments (18–25 nucleotides) that act as posttranscriptional regulators of various gene targets (either negatively or positively) rather than encoding proteins themselves.[10] miRNAs play a role in some biological processes, such as cellular differentiation, proliferation, and apoptosis, which are involved in implantation.[11-13] Therefore, several studies have been conducted to explore their role in endometrial receptivity. The miRNA expression profiles in human endometrium at different phases have been previously investigated. Kuokkanen *et al*.[14] studied the mRNA and miRNA profiles of fertile women's endometrial epithelial cells in the late proliferative and mid-secretory phases, respectively. They found that miRNA played a role in influencing endometrial receptivity through regulating the relevant genes' expression. Altmäe *et al*.[15] compared the miRNA profile of prereceptive (LH+2) and receptive endometrium (LH+7) from fertile, nonstimulated women and revealed miR-30b, miR-30d, and miR-494's roles in regulating endometrium receptivity. Revel's study[16] showed the different miRNA profiles of the secretory endometrium between patients with repeated implantation failure (RIF) and fertile women. These data have clearly demonstrated that miRNA expression profiles of different populations/stages may differ and therefore should be applied in the diagnosis of endometrial receptivity, but further investigation is required due to study limitations.

Despite its diverse definitions, RIF is generally defined as failure to achieve a clinical pregnancy after transferring at least four good-quality embryos in at least three fresh or frozen cycles.[17] We hypothesized that the endometrial receptivity of RIF patients is low, while that of infertile women, who conceived after only one embryo transfer attempt, is high. The aim of this study was to identify the different miRNA expression profiles between these two populations, which may further provide a good predictor for helping to differentiate the discrepant endometrial receptivity.

Methods

Patients

A total of 22 female infertile patients were enrolled in this study. Twelve patients (numbered RIF1–RIF12), who all had a history of RIF, participated in the study group (RIF group). These participants had previously received *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment and had suffered at least three embryo transfer failures, in which at least four morphologically high-grade embryos were transferred in total. Further, in this group, there were no other obvious explanations for their RIFs, such as polycystic ovary syndrome, ovarian tumors, polyps, fibroids, endometriosis, hydrosalpinx, adenomyosis, and uterine malformation. Ten infertile patients (due to male infertility, tubal factors, or unexplained infertility; numbered C1–C10), who conceived and delivered after the first attempt of embryo transfer, were recruited as the control group.

Inclusion criteria for all participants were age <40 years; regular menstrual cycles; normal uterine cavity confirmed by hysteroscopy, and more specifically, without intrauterine adhesions or inflammation; endometrial thickness in the late follicular phase of ≥7 mm in ultrasonography; normal ovarian reserve (follicle-stimulating hormone <9.6 mU/ml);[18] a normal ovarian response to the stimulation protocol (>8 oocytes retrieved in a controlled ovary hyperstimulation cycle); and no hormone (estradiol/progesterone) applied during the endometrial biopsy cycle.

The study was approved by the Institutional Review Board at Peking University People's Hospital (No. 2011-87) and all participants signed written informed consent.

Endometrial biopsy specimens

Endometrial biopsies were performed by dilation and curettage during hysteroscopy, 5–7 days after ovulation. Ovulation was determined according to ultrasound combined with morning urine LH detection. Endometrial tissue was immediately sent to the laboratory to make sure it was processed within 1 h after the biopsy. Each sample was divided into two portions: one of which was fixed in 10% formalin and processed for histological evaluation (hematoxylin-eosin [H-E]); the second portion was frozen at −80°C for subsequent RNA extraction.

MicroRNA extraction and purifying

Total RNA was isolated from endometrial specimens using Trizol reagent (Invitrogen, USA) following the suppliers' protocol, and miRNA was then purified using the mirVan miRNA Isolation Kit (AM1561, Ambion, USA) according to the manufacturer's instructions. The purity and concentration of RNA was determined by OD260/280 from a spectrophotometer (NanoDrop, ND-1000). The RNA integrity was examined by 1% formaldehyde denaturing gel electrophoresis. RNA with an OD260/280 between 1.8 and 2.0 and no degradation by electrophoresis was considered of good-quality and was included in further experiments.

MicroRNA array and microarray experiments

The transcription analysis of miRNA was performed using an miRNA Array (ID: 046064, Agilent, USA), which contains probes interrogating 2006 human mature miRNAs from miRBase R19.0 and 2164 Agilent control probes.

The miRNA microarray experiments were conducted according to the manufacturer's instructions for the miRNA Complete Labeling and Hyb Kit (Agilent). Then, 200 ng isolated RNA per sample was dephosphorylated and ligated with Cyanine3-pCp, and the labeled RNA was purified and hybridized to miRNA arrays. Images were scanned using the Agilent microarray scanner (G2565CA, Agilent). The arrays were then gridded and analyzed using Agilent Feature Extraction software version 10.10 (Agilent).

Microarray data analysis

The miRNA array data were analyzed for data summarization, normalization, and quality control using GeneSpring software version 13.0 (Agilent). The significance (*P* value) of the normalized value for raw data from each sample of the RIF and control group was calculated by an unpaired *t*-test and then corrected by the Benjamini-Hochberg method. The fold change was also calculated using the normalized value of the raw data. Two criteria were used to select the differentially expressed genes: a fold change ≥2 and a $P < 0.05$. To reduce the false discovery rate of genes, we excluded from our analysis miRNAs whose expression was detected in less than three samples in either the RIF or control groups. Furthermore, we adjusted the threshold to 5- and 10-fold changes to disclose miRNAs whose expression levels were more significantly different between the two groups.

Supervised hierarchical clustering with average linkage clustering analysis was further carried out on these differentially expressed miRNAs using Cluster version 3.0 software and Java Treeview (Stanford University School of Medicine, Stanford, CA, USA) to visually assess the differentially expressed miRNA profiles of the RIF and control groups.

Functional analysis of differentially expressed microRNAs

To discover the patterns and rules of the differentially expressed miRNAs, functional enrichment analysis was performed using tool for annotations of microRNAs (TAM) software (http://www.cuilab.cn/tam).

TAM, the tool for annotations of human miRNAs, is a web-accessible program that integrates miRNAs into different sets according to various rules and provides us with functions of interested miRNAs. Currently, TAM collects 238 miRNA sets, which include 413 distinct miRNAs.[19]

Regulatory network analysis of differentially expressed microRNAs and mRNAs

Based on the idea that miRNAs reduce, at least partially, the expression of targeted mRNAs, we constructed the miRNA-mRNA regulatory network of these differentially expressed miRNAs and those differentially expressed mRNAs we found from mRNA microarray study on the same samples. To improve the quality of prediction, the regulatory relationships were predicted by combining four existing algorithms: TargetScan, miRanda, Pictar, and DIANA, which were implemented with a Bioconductor package (http://bioconductor.org/), miRNAtap, in the R software environment (http://www.r-project.org). The diagram of the network was generated by Cytoscape.

Validation of the microarray data by quantitative real-time polymerase chain reaction

To validate our microarray findings, 10 new samples consisting of 5 from the RIF group (RIF8, RIF9, RIF10, RIF11, and RIF12) and 5 from the control group (C6, C7, C8, C9, and C10) were used to assess the expression of some miRNAs by quantitative real-time polymerase chain reaction (PCR). We selected miRNAs with a high-fold change and/or miRNAs reported in other similar literature before performing the validation. The names of the selected miRNAs and the corresponding primer sequences are listed in Supplementary Table S1.

We applied the poly(A) method to confirm the expression of miRNAs. After being purified with the mirVanaTM miRNA Isolation Kit (Applied Biosystems, USA), total RNA was used for the RT reaction to generate the first strand cDNA using the miRcute miRNA cDNA First-Strand reverse transcription mixture (KR201). Quantitative real-time PCR was then performed according to the miRcute miRNA reverse transcription PCR (RT-PCR) protocol, using U6 as the housekeeping gene. The relative expression was calculated using $2^{-\Delta\Delta Ct}$ method and analyzed with an unpaired *t*-test.

Results

Patients

The clinical characteristics of the two groups are listed in Table 1. There were no significant differences between the two groups in mean age, body mass index, length of menstrual cycle, menstrual duration, or endometrial thickness on the day of LH surge. Participants' additional detailed clinical information is presented in Supplementary Table S2. The histological evaluation results for each sample reported normal mid-secretory endometrium. The micrograph of H-E staining for each sample was similar to that of RIF10 [Supplementary Figure S1].

Results of microarray analysis

The miRNA array identified 105 microarray probes with expression levels in RIF patients that were 2-fold greater compared with those in the control group (93 upregulated and 12 downregulated). With a threshold of 5-fold and 10-fold changes, 70 (67 upregulated and 3 downregulated) and 49 (46 upregulated and 3 downregulated) miRNAs were identified, respectively [Table 2]. However, after the raw signal value correction (>50 for each sample), only 15 miRNAs were found to express in a significantly different way using 2-fold change as the threshold [Table 3]. All the differentially expressed genes are listed in Supplementary Table S3, and the raw data have been uploaded into the Gene Expression Omnibus database (number: GSE71332).

Table 1: Characteristics of the women undergoing endometrial biopsy sampling

Variables	RIF group (n = 12)	Control group (n = 10)	t	P
Age (years)	31.6 ± 4.1	32.1 ± 2.9	−0.33	0.74
BMI (kg/m^2)	22.77 ± 2.63	21.70 ± 2.22	1.01	0.32
Cycle length (days)	30.83 ± 3.10	30.40 ± 4.34	0.27	0.79
Menses duration (days)	5.08 ± 0.90	5.05 ± 0.98	0.08	0.94
Endometrial thickness* (cm)	0.95 ± 0.23	0.97 ± 0.26	−0.19	0.85

Data were presented as mean ± SD. *Endometrial thickness: The thickness of the endometrium on the day when then biopsy was taken. BMI: Body mass index; RIF: Repeated implantation failure; SD: Standard deviation.

Table 2: The number of differentially expressed miRNAs with different FCs*

RIF versus control	Total dysregulated	Upregulated	Downregulated
FCs			
>2	105	93	12
>5	70	67	3
>10	49	46	3

*The criteria for differentially expressed genes were: a greater than 2-FC with a P<0.05 by an unpaired t-test. FCs: Fold changes; RIF: Repeated implantation failure; miRNA: MicroRNA.

Table 3: List of the differentially expressed miRNAs between the RIF and control group with the microarray raw signal value of all samples >50

Systematic name	FC	Mirbase accession number
Upregulated miRNAs		
hsa-miR-374a-5p	7.74524	MIMAT0000727
hsa-miR-145-5p	3.2018807	MIMAT0000437
hsa-miR-30b-5p	2.9336023	MIMAT0000420
hsa-miR-196b-5p	2.5407631	MIMAT0001080
hsa-miR-199a-5p	2.5355365	MIMAT0000231
hsa-miR-199b-5p	2.4879646	MIMAT0000263
hsa-miR-449a	2.3427818	MIMAT0001541
hsa-miR-424-5p	2.190957	MIMAT0001341
hsa-miR-125b-5p	2.1353264	MIMAT0000423
hsa-miR-21-5p	2.0441828	MIMAT0000076
Downregulated miRNAs		
hsa-miR-1207-5p	2.6758146	MIMAT0005871
hsa-miR-4306	2.2878602	MIMAT0016858
hsa-miR-572	2.0804768	MIMAT0003237
hsa-miR-5739	2.1607096	MIMAT0023116
hsa-miR-6088	2.1698172	MIMAT0023713

RIF: Repeated implantation failure; miRNAs: MicroRNAs; FC: Fold change.

In terms of the supervised hierarchical clustering analysis, the dendrograms showed satisfying segregation of the gene expression levels for samples from the two groups, based on the differentially expressed miRNAs [Figure 1]. The first branch in the miRNA heat maps was able to differentiate samples from the RIF group and the control group. This finding suggested a diverse miRNA expression profile for WOI endometrium between RIF patients and those who conceived after their first attempt of IVF/ICSI.

Functional analysis of differentially expressed microRNAs

TAM analysis was used to gain an in-depth understanding of the biological functions of the differentially expressed miRNAs. According to the TAM analysis results, mir-30 family, human embryonic stem cell regulation, epithelial-mesenchymal transition, and miRNA tumor suppressors were the most relevant miRNA functional sets [Figure 2].

Construction of a regulatory network of differentially expressed microRNAs and mRNAs

The relationships between the dysregulated miRNAs and mRNAs were predicted by network regulatory analysis software. A total of 176 interactions between miRNAs and mRNAs were found, of which 122 were for upregulated miRNAs and downregulated mRNAs and 54 were for downregulated miRNAs and upregulated mRNAs. The top core mRNA was ABP1, which was regulated by 13 miRNAs, followed by AQP3, ASS1, and TIMP3 (regulated by 6 miRNAs). The top core miRNA was has-miR-4668-5p, which regulated 14 mRNAs, followed by has-miR-429 and has-miR-5088 (which regulated 9 mRNAs) [Figure 3].

Validation of microRNA expression using quantitative reverse transcription polymerase chain reaction

To validate the differences in transcript levels found in the microarrays, a selected set of miRNAs was chosen for quantitative RT-PCR. New endometrial samples from the RIF group (n = 5; RIF8, RIF9, RIF10, RIF11, and RIF12) and control group (n = 5; C6, C7, C8, C9, and C10) were used for this validation.

Selection for validated miRNAs was done according to the following criteria: (i) miRNAs, the raw signal for each sample in the miRNA microarray analysis was >50 and was differentially up- or down-regulated in samples from the RIF group compared with the control group; and (ii) miRNAs that were in the core mRNA-miRNA network results. The RT-PCR results were in agreement with that of the microarray for all miRNAs: hsa-miR-374a-5p, hsa-miR-145-5p, hsa-miR-30b-5p, hsa-miR-196b-5p, hsa-miR-199a-5p, hsa-miR-199b-5p, hsa-miR-449a, hsa-miR-424-5p, hsa-miR-125b-5p, and hsa-miR-21-5p were elevated and hsa-miR-1207-5p, hsa-miR-4306, hsa-miR-572, hsa-miR-5739, hsa-miR-6088, hsa-miR-4668-5p, hsa-miR-429, and hsa-miR-5088 were

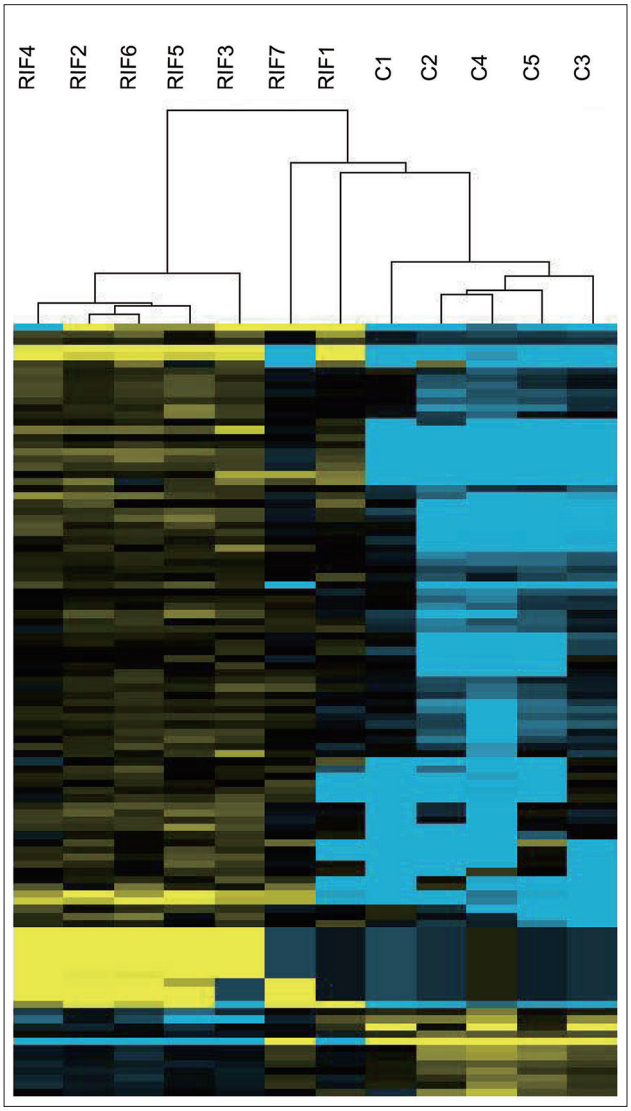

Figure 1: Dendrogram and hierarchical clustering. Expression data from all the differentially expressed miRNAs are analyzed. Each row presents one gene and each column represents an endometrial sample. Column RIF1, RIF2, RIF3, RIF4, RIF5, RIF6, and RIF7 are RIF samples and column C1, C2, C3, C4, and C5 are control samples. Up- and down-regulated miRNAs are, respectively, indicated by yellow and blue, and miRNAs that are lack of significant change are indicated by black. miRNA: MicroRNA; RIF: Repeated implantation failure.

reduced in the RIF group compared with the control group [Figure 4].

Discussion

Until now, an objective diagnosis of endometrial receptivity remained neglected, which limited the improvement of clinical IVF/ICSI success from the endometrial perspective. Therefore, we used a microarray technique to investigate the miRNA profile of women with RIF compared to women who conceived after their first attempt of embryo transfer. We found that 105 differentially expressed miRNAs could result in two distinct groups by hierarchical clustering: RIF endometrium and the control group endometrium.

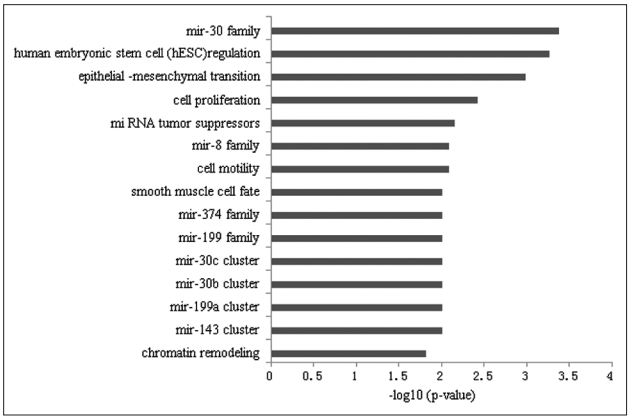

Figure 2: Results of the tool for annotations of microRNA analysis for the deregulated miRNAs between the RIF and control endometrial samples. Mir-30 family, human embryonic stem cell regulation and epithelial-mesenchymal transition were the top 3 relevant miRNA functional sets. miRNA: MicroRNA; RIF: Repeated implantation failure.

Previous research using a miRNA microarray to study endometrial receptivity can be generally grouped into two categories: (i) to compare the dynamic genomic expression profiles of endometrium from the proliferative phase to the WOI in fertile women; and (ii) to investigate the differential genomic expression profiles between fertile and infertile women.

In the first category, 4 studies have been reported. Has-miR-30b, has-miR-30d, and has-miR-494 were considered to play important roles in regulating endometrial receptivity. Compared with the prereceptive endometrium, hsa-miR-30b and hsa-miR-30d were found to be significantly upregulated and hsa-miR-494 was found to be downregulated in receptive endometrium.[15] In our study, hsa-miR-30b was also found to be upregulated in the RIF group. It is indicated that the destroyed endometrial receptivity of RIF patients was related to miRNAs other than hsa-miR-30b.

For the second category, only one study by Revel *et al*.[16] was reported, which found 13 deregulated miRNAs (1 were upregulated and 12 were downregulated). Different microarray platforms contributed mostly to the coincidence of the numbers of dysregulated miRNAs between our results and results of Ariel Revel's study. The miRNA Array card we used contained 2006 mature human miRNAs while the card Revel's group used only contained 381 mature human miRNAs. However, we also obtained two shared deregulated miRNAs: hsa-miR-145 and has-miR-374, which were both upregulated in the RIF patients in our study. ERα, mucin1 and RTKN, which play important roles in the acquisition of endometrial receptivity, have been validated to be the target genes of has-miR-145. In Revel's study, they thought that upregulated hsa-miR-145 might destroy endometrial receptivity in RIF patients by reducing endometrial ERα and mucin1 expression, which was also validated by Western-blot as downregulated.[16] In our study, we also detected the expression of mucin1, ERα, and RTKN by RT-PCR in the WOI endometrium from the two groups.

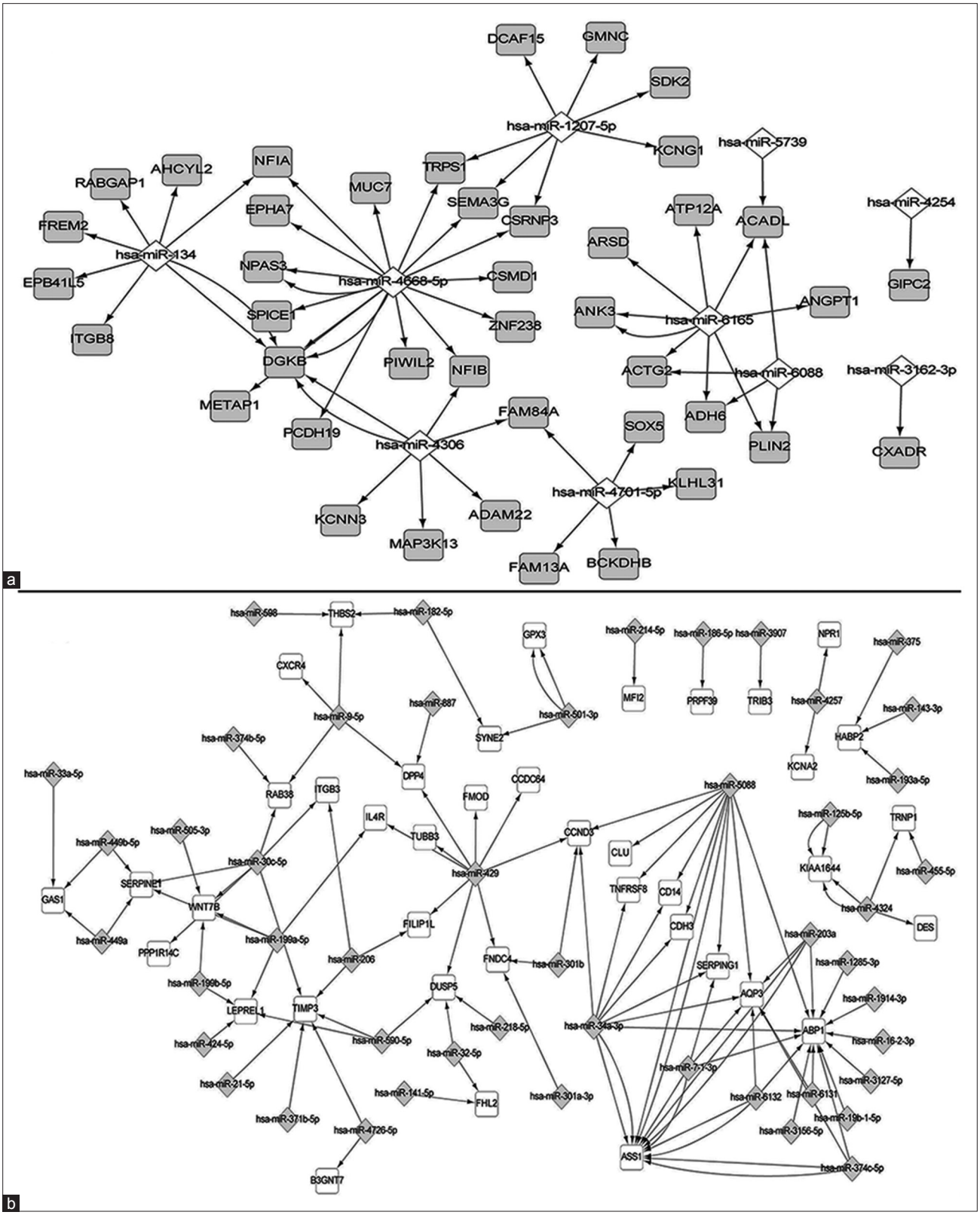

Figure 3: The layout of the miRNA-mRNA regulatory network. The network consists of 54 regulations (a) between downregulated miRNAs to upregulated mRNAs and 122 regulations (b) between upregulated miRNAs to downregulated mRNAs. A diamond marks miRNA and a rectangle marks the mRNA. An edge represents a regulation from miRNA to one of its targets. The miRNAs and mRNAs are colored based on their dysregulation pattern. If the miRNAs (or mRNAs) are upregulated in the RIF group, the nodes are marked by gray, otherwise they are marked by white. miRNA: MicroRNA; RIF: Repeated implantation failure.

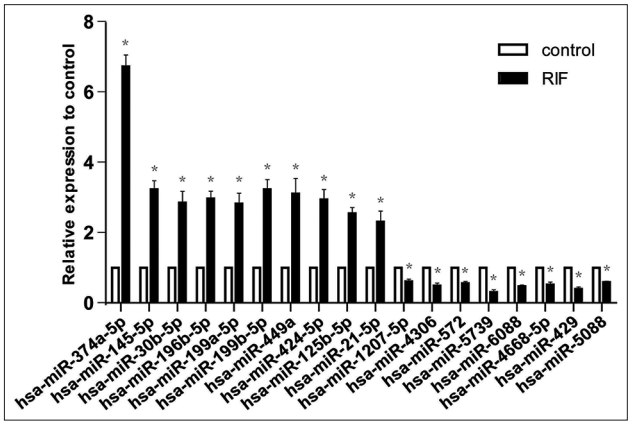

Figure 4: Validation of miRNAs by real-time PCR in new samples (RIF, $n = 5$; control, $n = 5$). Relative levels of the transcripts for the selected 18 miRNAs in the RIF group as compared to the control group are shown. The dysregulation pattern of all the selected miRNAs by real-time PCR is coincident with that by microarray. $*P < 0.05$, vs. control group. miRNA: MicroRNA; RIF: Repeated implantation failure; PCR: Polymerase chain reaction.

Unexpectedly, ERα and RTKN were found to be upregulated in our RIF group, while mucin1 presented with a similar expression levels in both of our groups. Such a result may due to our small sample size (i.e., bias) or by has-miR-145 impairing the endometrial receptivity via regulating the expression of other target genes. Hsa-miR-374, located on chromosome Xq13.2, has been previously shown to constitutively activate Wnt/b-catenin signaling,[20] which has been reported to participate in the implantation process in several studies.[21,22]

Since miRNAs act as the post-transcriptional regulators of mRNA, usually negatively, we created a regulatory network of differentially expressed mRNAs and miRNAs and found 176 regulated pairs. The top 3 core miRNAs were has-miR-4668-5p, has-miR-429, and has-miR-5088, of which has-miR-4668-5p was downregulated, while has-miR-429 and has-miR-5088 were upregulated in the RIF group. The targeted mRNAs of has-miR-429 and has-miR-5088, DPP4, SERPING1, and AQP3 were validated to be downregulated in the new RIF samples in our previous report. These results indicated that the endometrial receptivity of RIF patients may be impacted by the expression of these mRNAs, which were regulated by specific miRNAs. Hence, we should pay more attention to miRNAs in future studies, which may shed some light on potential treatment for RIF.

In conclusion, we performed miRNA microarray on the samples from the RIF and control groups. Differentially expressed miRNAs were found and analyzed for their role in the establishment of endometrial receptivity. We found that has-miR-145, hsa-miR-374, hsa-miR-4668-5p, hsa-miR-429, and hsa-miR-5088 may be relevant to the low endometrial receptivity of RIF patients. We hypothesize that an array including miRNAs may increase the specificity for diagnosing the endometrial receptivity of patients with RIF, and our report provides clues to this diagnostic tool.

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Supplementary information is linked to the online version of the paper on the Chinese Medical Journal website.

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Conflicts of interest

There are no conflicts of interest.

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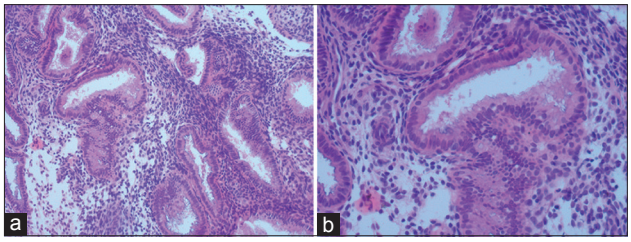

Supplementary Figure S1: The micrograph of H and E dying for the sample of RIF10. The micrographs for the other 21 samples were similar to this, and all the reports were: normal mid-secretory endometrium. (a) Extreme glandular coiling secretory glands set within a spindled edematous stroma. Luminal secretion is most prominent (H and E, original magnification ×100). (b) The coiled spiral arteries are seen within an edematous stroma. Perivascular predecidual reaction has not occurred (H and E, original magnification ×200). RIF: Repeated implantation failure.

Supplementary Table S1: Sequences of miRNAs primers used for real-time PCR amplification

Primers	miRNAs
F: ATAATACAACCTGATAAGTG	hsa-miR-374a-5p
F: GTCCAGTTTTCCCAGGAATCCC	hsa-miR-145-5p
F: GTAAACATCCTACACTCAGC	hsa-miR-30b-5p
F: TAGGTAGTTTCCTGTTGTTGGG	hsa-miR-196b-5p
F: CCCAGTGTTCAGACTACCTGTTC	hsa-miR-199a-5p
F: CCCAGTGTTTAGACTATCTGTTC	hsa-miR-199b-5p
F: TGGCAGTGTATTGTTAGCTGGT	hsa-miR-449a
F: CAGCAGCAATTCATGTTTT	hsa-miR-424-5p
F: TCCCTGAGACCCTTTAACCTGTG	hsa-miR-125b-5p
F: TAGCTTATCAGACTGATGTTG	hsa-miR-21-5p
F: TGGCAGGGAGGCTGGGAG	hsa-miR-1207-5p
F: TGGAGAGAAAGGCAGTAA	hsa-miR-4306
F: CGCTCGGCGGTGGC	hsa-miR-572
F: GCGGAGAGAGAATGGGGAGC	hsa-miR-5739
F: AGAGATGAAGCGGGGGGG	hsa-miR-6088
F: AGGGAAAAAAAAAAGGATTTGTC	hsa-miR-4668-5p
F: TAATACTGTCTGGTAAAACCGT	hsa-miR-429
F: CAGGGCTCAGGGATTGGATG	hsa-miR-5088-5p
F: CTCGCTTCGGCAGCACA	U6
R: AACGCTTCACGAATTTGCGT	

miRNAs: MicroRNAs; PCR: Polymerase chain reaction.

Supplementary Table S2: More clinical information of the women undergoing endometrial biopsy sampling

Case number	Age	Cause of infertility	Number of failed cycles	IVF/ICSI	Number of transferred embryos	Number of high quality embryos	Endometrial thickness on the day of LH surge	Endometrial type on the day of LH surge	The day of sample (post the day of LH surge)
RIF1	34	Tubal	11	ICSI	22	8	1	A	+6
RIF2	33	Tubal	4	IVF	11	7	0.7	A	+7
RIF3	38	Tubal	8	IVF	20	10	0.9	A	+6
RIF4	23	Male	4	ICSI	11	9	0.9	A	+8
RIF5	34	Unexplained	3	IVF	9	6	0.9	A	+6
RIF6	31	Tubal	3	IVF	7	7	1.2	B	+7
RIF7	28	Male	3	ICSI	7	6	1	A	+6
RIF8	35	Male	3	ICSI	6	4	0.8	A	+6
RIF9	32	Tubal	3	IVF	7	6	0.7	A	+7
RIF10	32	Tubal	3	IVF	6	4	1.2	A	+6
RIF11	33	Tubal	4	ICSI	8	4	1.0	A	+7
RIF12	26	Male	4	IVF	9	4	0.7	A	+8
C1	32	Unexplained	0	IVF	2	2	1.1	A	+7
C2	35	Tubal	0	IVF	2	1	0.9	A	+7
C3	29	Male	0	ICSI	2	2	1.1	A	+8
C4	33	Unexplained	0	IVF	2	2	1.1	B	+7
C5	26	Tubal	0	ICSI	2	1	1.2	A	+7
C6	31	Male	0	IVF	2	2	0.9	A	+7
C7	35	Male	0	ICSI	2	2	0.8	A	+8
C8	35	Tubal	0	IVF	3	2	0.7	A	+8
C9	33	Tubal	0	IVF	2	1	1.4	B	+7
C10	32	Male	0	ICSI	2	2	1.2	A	+7

The samples with case number marked by underline were used for real-time PCR and the other samples were used for microarray. IVF: *In vitro* fertilization; ICSI: Intracytoplasmic sperm injection; LH: Luteinizing hormone; PCR: Polymerase chain reaction.

Supplementary Table S3: List of differentially expressed miRNAs

Systematic name	FC	Mirbase accession number
Upregulated miRNAs		
hsa-miR-186-5p	111.32307	MIMAT0000456
hsa-miR-135b-5p	87.14255	MIMAT0000758
hsa-miR-3125	76.56718	MIMAT0014988
hsa-miR-136-5p	73.41167	MIMAT0000448
hsa-miR-204-5p	72.42954	MIMAT0000265
hsa-miR-3907	72.28242	MIMAT0018179
hsa-miR-30d-3p	67.86486	MIMAT0004551
hsa-miR-1288	66.41283	MIMAT0005942
hsa-miR-371b-5p	59.9805	MIMAT0019892
hsa-miR-374c-5p	53.332508	MIMAT0018443
hsa-miR-32-5p	42.89187	MIMAT0000090
hsa-miR-6512-5p	40.44899	MIMAT0025480
hsa-miR-1914-3p	35.808	MIMAT0007890
hsa-miR-205-5p	32.111767	MIMAT0000266
hsa-miR-505-3p	29.47475	MIMAT0002876
hsa-miR-7-1-3p	29.342558	MIMAT0004553
hsa-miR-449b-5p	29.015373	MIMAT0003327
hsa-miR-145-3p	28.210178	MIMAT0004601
hsa-miR-4734	26.772724	MIMAT0019859
hsa-miR-144-5p	25.90218	MIMAT0004600
hsa-miR-9-5p	24.925808	MIMAT0000441
hsa-miR-4690-5p	24.287247	MIMAT0019779
hsa-miR-744-5p	19.679468	MIMAT0004945
hsa-miR-4486	18.95102	MIMAT0019020
hsa-miR-6132	18.29064	MIMAT0024616
hsa-miR-149-5p	17.349735	MIMAT0000450
hsa-miR-1307-5p	16.641792	MIMAT0022727
hsa-miR-424-3p	16.024895	MIMAT0004749
hsa-miR-4324	15.442569	MIMAT0016876
hsa-miR-154-5p	14.907551	MIMAT0000452
hsa-miR-10a-3p	14.901987	MIMAT0004555
hsa-miR-141-5p	14.643423	MIMAT0004598
hsa-miR-501-3p	14.513452	MIMAT0004774
hsa-miR-1290	14.159889	MIMAT0005880
hsa-miR-206	14.120981	MIMAT0000462
hsa-miR-4257	13.665979	MIMAT0016878
hsa-miR-3127-5p	13.506273	MIMAT0014990
hsa-miR-375	13.398406	MIMAT0000728
hsa-miR-3156-5p	13.002842	MIMAT0015030
hsa-miR-598	11.697124	MIMAT0003266
hsa-miR-5088	11.453611	MIMAT0021080
hsa-miR-5096	11.370991	MIMAT0020603
hsa-miR-4746-3p	11.153188	MIMAT0019881
hsa-miR-4726-5p	11.049062	MIMAT0019845
hsa-miR-4656	10.935905	MIMAT0019723
hsa-miR-33a-5p	10.576019	MIMAT0000091
hsa-let-7i-3p	9.9141245	MIMAT0004585
hsa-miR-34c-3p	9.809227	MIMAT0004677
hsa-miR-1285-3p	9.787075	MIMAT0005876
hsa-miR-29c-5p	9.758672	MIMAT0004673
hsa-miR-16-2-3p	9.330457	MIMAT0004518
hsa-miR-142-3p	8.779017	MIMAT0000434
hsa-miR-34a-3p	8.748133	MIMAT0004557
hsa-miR-4695-5p	8.440075	MIMAT0019788
hsa-miR-193a-5p	7.9020715	MIMAT0004614
hsa-miR-374a-5p	7.74524	MIMAT0000727
hsa-miR-182-5p	7.1148996	MIMAT0000259
hsa-miR-203a	6.915573	MIMAT0000264
hsa-miR-301b	6.900287	MIMAT0004958
hsa-miR-450a-5p	6.619105	MIMAT0001545
hsa-miR-6131	6.455001	MIMAT0024615
hsa-miR-887	6.105473	MIMAT0004951
hsa-miR-19b-1-5p	6.0583606	MIMAT0004491
hsa-miR-590-5p	5.9550056	MIMAT0003258
hsa-miR-200c-5p	5.630886	MIMAT0004657
hsa-miR-214-5p	5.396898	MIMAT0004564
hsa-miR-30e-3p	5.378995	MIMAT0000693
hsa-miR-218-5p	4.7786775	MIMAT0000275
hsa-miR-423-3p	4.703984	MIMAT0001340
hsa-miR-455-5p	4.035282	MIMAT0003150
hsa-miR-30c-5p	3.5054796	MIMAT0000244
hsa-miR-1260b	3.3699887	MIMAT0015041
hsa-miR-145-5p	3.2018807	MIMAT0000437
hsa-miR-362-3p	3.0494413	MIMAT0004683
hsa-miR-374b-5p	3.0005975	MIMAT0004955
hsa-miR-30b-5p	2.9336023	MIMAT0000420
hsa-miR-429	2.7092197	MIMAT0001536
hsa-miR-4428	2.6921449	MIMAT0018943
hsa-miR-196b-5p	2.5407631	MIMAT0001080
hsa-miR-199a-5p	2.5355365	MIMAT0000231
hsa-miR-199b-5p	2.4879646	MIMAT0000263
hsa-miR-143-3p	2.4430275	MIMAT0000435
hsa-miR-449a	2.3427818	MIMAT0001541
hsa-miR-6717-5p	2.3069673	MIMAT0025846
hsa-miR-301a-3p	2.30314	MIMAT0000688
hsa-miR-424-5p	2.190957	MIMAT0001341
hsa-miR-3653	2.1542947	MIMAT0018073
hsa-miR-335-5p	2.1516027	MIMAT0000765
hsa-miR-125b-5p	2.1353264	MIMAT0000423
hsa-miR-1305	2.1121273	MIMAT0005893
hsa-miR-365a-3p	2.0961003	MIMAT0000710
hsa-miR-146b-5p	2.0629325	MIMAT0002809
hsa-miR-21-5p	2.0441828	MIMAT0000076
Downregulated miRNAs		
hsa-miR-4668-5p	103.51767	MIMAT0019745
hsa-miR-4254	14.588222	MIMAT0016884
hsa-miR-4701-5p	13.288958	MIMAT0019798
hsa-miR-134	2.7737036	MIMAT0000447
hsa-miR-1207-5p	2.6758146	MIMAT0005871
hsa-miR-4306	2.2878602	MIMAT0016858
hsa-miR-3162-3p	2.2871423	MIMAT0019213
hsa-miR-4788	2.2722929	MIMAT0019958
hsa-miR-6088	2.1698172	MIMAT0023713
hsa-miR-5739	2.1607096	MIMAT0023116
hsa-miR-6165	2.133992	MIMAT0024782
hsa-miR-572	2.0804768	MIMAT0003237

Contd...

Supplementary Table S3: Contd...

miRNAs: MicroRNAs; FC: Fold change.