Original Article

MicroRNA Dysregulation and Steroid Hormone Receptor Expression in Uterine Tissues of Rats with Endometriosis during the Implantation Window

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Abstract

Background: Estrogen receptor (ER) and progesterone receptor (PR) are involved in endometriosis, but the involvement of microRNAs (miRNAs) is unknown. The aim of the study was to explore the correlation between miRNA and ER/PR in uterine tissues of rats with endometriosis during the implantation window.

Methods: Twenty female Sprague-Dawley rats were randomized in three groups: endometriosis (n = 7), fat tissue control (n = 6), and normal (n = 7) groups. The female rats were mated and sacrificed on day 5 (implantation). Uterine tissues were obtained for hematoxylin-eosin staining, immunohistochemistry, and miRNA expression. Reverse transcription polymerase chain reaction (RT-PCR) was used to validate the expression of rno-miR-29c-3p, rno-miR-34c-5p, rno-miR-141-5p, rno-miR-24-1-5p, and rno-miR-490-5p.

Results: The 475 miRNAs were found to differentially express between the endometriosis and normal control groups, with 127 being upregulated and 348 being downregulated. Expression of five miRNAs (rno-miR-29c-3p, rno-miR-34c-5p, rno-miR-141-5p, rno-miR-24-1-5p, and rno-miR-490-5p) were validated by RT-PCR and found to be differentially expressed among the three groups. Expression of ER and PR proteins (immunohistochemistry) in the glandular epithelium and endometrial stroma was significantly different among the three groups (all $P < 0.05$). Five miRNAs were involved in pathways probably taking part in implantation and fertility.

Conclusions: The results suggested that miRNAs, ER, and PR could play important roles in the embryo implantation period of rats with endometriosis. These miRNAs might play a role in endometrial receptivity in endometriosis.

Key words: Endometriosis; Estrogen Receptor; Implantation; MicroRNA; Progesterone Receptor

Introduction

Endometriosis is a common gynecological disease and is characterized by ectopic growth of functional endometrial glands and stroma outside the uterine cavity.[1] Endometriosis is estrogen dependent and is benign hyperplasia of the ectopic endometrium.[2,3] About 25–50% of women with infertility suffer from endometriosis and 30–50% of women with endometriosis show infertility.[4] The incidence of endometriosis has been estimated as 2.37–2.49/1000 person per year.[5] The strong associations between endometriosis, infertility, and impaired daily activities, career, and sexual life have a significant economic burden estimated to US 1.8 billion dollars in Canada[6] and US 18–22 billion dollars in the USA.[7] While it is acknowledged that an association exists between endometriosis and infertility, little is known about how endometriosis causes fertility.

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Endometriosis can be caused by various factors including hormonal status, genetic susceptibility, and environmental factors.[8] The working theory is that endometrial glandular epithelium and mesenchymal cells grow back to the ovaries and pelvic peritoneum along with menstrual blood flow during menstrual period.[1,9] Successful pregnancy is achieved by appropriate implantation, which is dependent on endometrial receptivity and embryo functionality.[10] In a regular menstrual cycle, successful implantation only

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occurs during a short limiting period from day 20 to day 24,[11] i.e., 7–10 days after ovulation, termed as window of implantation (WOI). For rats, the WOI is generally from 4 to 6 days of pregnancy.[12] Disorder of WOI is a major cause for female infertility. Dysregulation of molecular and cellular biomarkers related to endometrial receptivity may contribute to the correlation observed between infertility and endometriosis.[10,13]

MicroRNAs (miRNAs) are noncoding RNAs of 18–24 nt. miRNAs can recognize and combine to the 3' untranslated region (3'UTR) of its target gene, inducing degradation of the mRNA and blocking translation.[14] miRNAs participate in the process of embryo implantation and gene regulation.[15] miRNAs in the endometrium affects the occurrence and development of endometriosis,[13] but most of the existing studies focused on a few specific miRNAs, whereas few studies have interrogated the expression profile changes of miRNA in the endometrium with endometriosis during the implantation window.

The estrogen receptors (ERs) and progesterone receptor (PR) are involved in the pathogenesis of endometriosis.[16] Estrogen binds to the ER present on the endometrial glandular epithelial cells and stromal cells to enhance endometrial growth.[17] Studies have shown that ectopic endometrium contains ER and PR.[16] PR is an important factor in pregnancy decidual formation, and its expression during the implantation window is related to the activity of decidual-specific genes.[18]

We hypothesized that there is a high correlation between the changes in miRNA expression profile and the changes in ER and PR expression in endometriosis versus no endometriosis during the implantation stage. Therefore, the aim of the present study was to compare the expression profiles of some fertility-related proteins (such as ER and PR) and miRNA in the endometrium with and without endometriosis during the implantation window using Sprague-Dawley rats as an experimental animal model. Due to high incidence of endometriosis-related infertility, it is of importance to investigate endometriosis during the implantation window. The results could provide new insights to understand endometriosis-related infertility and offer basis for improving pregnancy for patients with endometriosis.

Methods

Ethical approval

All animal protocols were approved by the Animal Care and Use Committee of the Peking Union Medical College Hospital (Permit Number: XHDW-2016-000).

Animals

Pathogen-free, sexually mature, Sprague-Dawley rats were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (China). The rats included male rats (380 ± 10 g) and nonpregnant female rats (60–70 days old, 200 ± 10 g). The rats were kept at the Laboratory Animal Center of Peking Union Medical College Hospital in cages on bedding (light cycle 14/10 h light/dark) with adequate access to sterilized water and food at 20°C–25°C and relative humidity of 45–60%. The rats were housed five/cage and were adaptively fed for 2–3 days. The estrus cycles of female rats were observed using the vaginal smear method to be about 4–5 days. Twenty female rats were randomized in three groups: endometriosis (n = 7), fat tissue control (n = 6), and normal (n = 7) groups.

Endometriosis model

Rats in the endometriosis group were observed for three consecutive estrus cycles. On the fourth cycle, a piece of uterine tissue was obtained and transplanted to the peritoneal wall using the autotransplantation method described by Vernon and Wilson[19] but with slight modifications. The rats were anesthetized with 5% pentobarbital sodium (50 mg/kg) intraperitoneally and were placed in a supine position. After disinfection, a ventral midline incision of about 5 cm was made on the abdomen to open the abdomen cavity. The uterus was isolated, and a piece of uterine tissue of 1 cm was taken from the left horn. The piece was immediately washed with physiologic saline. The excess fat tissue outside the uterine serosa was removed, and the uterine cavity was longitudinally incised and trimmed into two pieces of 5 mm × 5 mm. Those pieces were stitched to the two sides of the abdominal wall near the area enriched with blood vessels. The abdominal wall was closed layer by layer.

The rats in the fat tissue control group underwent the same surgery as in the endometriosis group, except that the left uterus was ligatured without taking any tissue. Two pieces of adipose tissue taken from the abdomen were stitched at the same location than the uterine tissue in the endometriosis group. For the normal control group, no surgery was performed.

Interventions and specimen collection

On the 28th day after surgery, the abdomen of the rats was open to observe the growth, invasion, and adhesion of the endometriosis tissue, followed by layer-wise suturing. The transplanted intima was evaluated following the scoring method based on a previous study on rat endometriosis model:[20] 0, no epithelium; 1, poorly preserved epithelium; 2, moderately preserved epithelium; and 3, well-preserved epithelium.

Ten male rats were housed in single-spaced cages individually. The female rats of the three groups were caged at the ratio of 1:1 at 18 o' clock every night, and trays were placed at the bottom of the cage. At 8:00–9:00 on the next morning, the trays were checked for vaginal plug. Each female rat after mating was subjected to vaginal smear for the presence of sperm. If both a vaginal plug was found in the tray and sperm was found, the rat was considered pregnant and the day was considered as the 1st day of pregnancy (D1). Pregnant rats were no longer subjected to mating. The rats were sacrificed on day 5 of pregnancy (D5; implantation occurs on day 5 in rats),[12] and uterine tissues were obtained

and divided into two pieces. One piece was placed in 10% formalin at 4°C for hematoxylin-eosin staining and immunohistochemistry. The other piece was rapidly placed into liquid nitrogen at −80°C for gene chip and quantitative polymerase chain reaction (qPCR).

Immunohistochemistry for estrogen receptor and progesterone receptor

Uterine tissues were processed routinely for immunohistochemistry. The samples were incubated with ER (1:2000 Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) and PR (1:10 Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 1–2 h at room temperature. The sample were observed using microscopy.

Immunohistochemistry was evaluated as follows.[21] Based on the positively stained cells, cells were divided as weakly positive (+), score 1; moderately positive (++), score 2; and strongly positive (+++), score 3. According to the number of positive cells, cells were divided as weakly positive (+, the total number of positive cells is <25%), moderately positive (++, the total number of positive cells is 25–49%), and strongly positive (+++, the total number of positive cells is >50%). The degree of positivity was evaluated using the following formula: (+)% × 1+ (++)% × 2+ (+++)% × 3. The result of <1.0 was denoted as +, 1.0–1.5 was denoted as ++, and >1.5 was denoted as +++. The samples were randomly observed at 10×.

RNA extraction

Total RNA was extracted from uterine tissues using the mirVana miRNA Isolation Kit (AM1561, Thermo Fisher Scientific, Waltham, MA, USA), followed by purification and quantification using the Qubit system (Thermo Fisher Scientific, Waltham, MA, USA). An Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA) was used to check the RNA integrity. The purified RNA was subjected to Affymetrix miRNA expression profile chip, and the miRNA expression profile was analyzed by Bo Ao Bio-Tech (Beijing, China).

MicroRNA microarray expression profiling

The Affymetrix miRNA 4.0 chip was applied according to the manufacturer's instructions. The Nanodrop instrument (Affymetrix, Sacramento, CA, USA) was used for quality detection of miRNA extracted from endometrial tissue. Good-quality miRNA from six samples (three from the endometriosis group and three from the normal control group) was subjected to expression profile chip detection.

Quantitative reverse transcription polymerase chain reaction validation of five differentially expressed microRNAs

Fluorescence qPCR was used to validate the expression of miRNAs, with RNA U6 as an internal reference. The conditions of PCR amplification were as follows: 95°C for 15 s; 60°C for 30 s, 40 cycles; 75°C–95°C. All reactions were performed in triplicates. The primers are listed in Table 1. The CT (ΔΔCT) method was used to calculate the fold changes of miRNA expression.

Analysis of microarray results and prediction of the function of microRNAs

The Affymetrix® GeneChip® Command Console® software was used to scan the chips, and the images were saved to DAT file for analysis. The DAT file was converted to CEL file, which were preprocessed using the RMA algorithm for background correction, integration of the probe signal to probeset signal, and interwafer normalization removing intersample variation caused by nonbiological factors. Following this, the preprocessed files were subjected to correlation analysis including cluster analysis and principal component analysis to interrogate the similarity and/or difference among various groups. Three or more biological replicates were provided to analyze differentially expressed genes using the SAM (significance analysis of microarray) R package (Beijing, China). Genes were considered differentially expressed when the *q*-value was ≤5% and the fold change was ≥1.5 or ≤0.5. The target genes of the differentially expressed miRNA were predicted using miRWalk2.0 (PubMed). The function of the predicted target genes was analyzed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG).

Statistical analysis

All data were obtained in triplicate and the results were expressed as mean ± standard deviation (SD). Comparison between two groups was performed using the two-tailed Student's *t*-test. Comparison among three groups was performed using analysis of variance with *post hoc* Tukey's test. Two-sided $P < 0.05$ was considered statistically significant. The statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

Table 1: Primers for the five selected miRNAs

Genes	Primer sequence (5'→3')
U6	Forward: CTCGCTTCGGCAGCACA
	Reverse: AACGCTTCACGAATTTGCGT
Universal miRNA	GTGCAGGGTCCGAGGT
rno-miR-29c-3p	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGACtaaccg
	AS: CCTAGCACCATTTGAAATCGG
rno-miR-34c-5p	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGACgcaatc
	AS: GCAGGCAGTGTAGTTAGCTGATTG
rno-miR-141-5p	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGACcaacac
	AS: GTCCATCTTCCAGTGCAGTGTT
rno-miR-24-1-5p	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGACctgata
	AS: TGCGTGCCTACTGAGCTGATAT
rno-miR-490-5p	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGACacccac
	AS: GCCCATGGATCTCCAGGTG

MiRNA: MicroRNA; RT: Reverse transcription; AS: Antisense strand.

Results

Growth of endometriotic implants

All rats were alive 28 days after surgery. Three rats were randomly selected from each of endometriosis and fat tissue control groups and were subjected to abdominal cavity opening to observe macroscopically the growth of the implanted tissues. No changes were observed in the fat tissues in the fat tissue control group, whereas the ectopic uterine tissues in the endometriosis group were increased in volume, vascularity, and cystic changes, all three features being 2–3 more important than before surgery [Figure 1].

The uterine lining, the myometrium (including the subserosal, vascular, and submucosal layers), and the inner lining of the uterus were observed under light microscopy. Figure 2 shows that the vascular layer of the endometriosis group (averagely 10 mm) was significantly thinner than those of the fat tissue control group (averagely 18 mm) and the normal control group (averagely 18 mm). Atrophy was even observed in the endometriosis group. As shown in Figure 2, in the endometriosis group, the number of endometrial glands was decreased (≤5 in low-magnification fields) and the diameter of the glands (maximum of 5 mm) was reduced. Some glands were hyperplastic (round) with poor body secretion, decidual dysplasia, endometrial epithelial and interstitial disarrangement, and obvious tissue edema. In the fat tissue control group, compared with the endometriosis group, the diameter of endometrial glands (maximum of 10 mm) was larger and the number of endometrial gland was higher (>10 in low-magnification fields). The glandular structure was curved with strong secretion, good decidual growth, and abundant blood vessels. In the normal control group, the diameter of the endometrial gland was the largest (maximum of 10 mm). The glandular structure showed good secretion, good decidua growth, and abundant blood vessels.

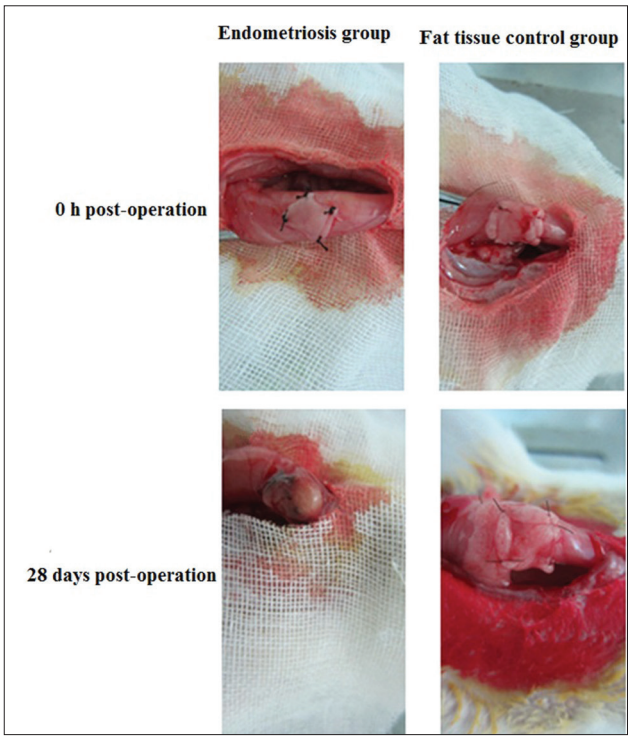

Figure 1: Growth of the implanted tissues in the fat tissue control group and endometriosis group.

Taken together, these results indicated that the implantation of endometrial tissues in the abdomen of rats led to a successful model of endometriosis, as shown by changes in the uterine cavity. The control groups showed normal uterine cavities.

Impact of endometriosis on estrogen receptor and progesterone receptor immunohistochemistry

The expression of ER in glandular epithelium was significantly different between the endometriosis and normal control groups (10.95 ± 0.30 vs. 7.82 ± 0.52, $t = 4.125$, $P < 0.001$) and between the fat tissue control and normal control groups (10.05 ± 0.45 vs. 7.82 ± 0.52, $t = 2.528$, $P = 0.011$), but not between the endometriosis and fat tissue control groups (10.95 ± 0.30 vs. 10.05 ± 0.45, $t = 1.756$, $P = 0.079$; Figure 3a and 3b). The expression of ER in the endometrial stroma was different between the endometriosis and fat tissue control groups (7.58 ± 0.31 vs. 4.33 ± 0.66, $t = 3.627$, $P < 0.001$) and between the endometriosis and normal control groups (7.58 ± 0.31 vs. 3.05 ± 0.33, $t = 4.816$, $P < 0.001$), but no significant difference was observed between the fat tissue control and normal control groups (4.33 ± 0.66 vs. 3.05 ± 0.33, $P = 0.189$; Figure 3a and 3b).

The expression of the PR in the uterine epithelium was different between the endometriosis and fat tissue control groups (2.52±0.52 vs. 5.5±1.28, $t=2.353$, $P=0.019$) and between the endometriosis and normal control groups (2.52 ± 0.52 vs. 7.4 ± 0.93, $t = 3.539$, $P < 0.001$), but not between the fat tissue control and normal control groups (5.5 ± 1.28 vs. 7.4 ± 0.93, $t = 0.970$, $P = 0.332$). The expression of the PR in endometrial

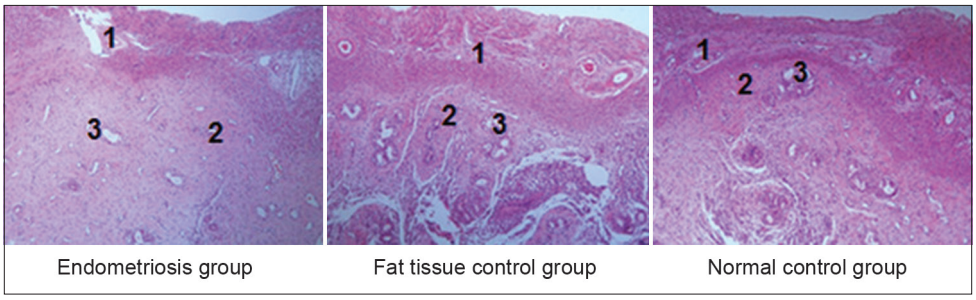

Figure 2: Hematoxylin-eosin staining of the three groups of uterine tissue in rats (original magnification, ×100). 1: Blood vessel; 2:Decidua; 3: Gland.

stroma was different between the endometriosis and fat tissue control groups (0.78 ± 0.22 vs. 2.30 ± 0.74, $t = 2.911$, $P = 0.004$) and between the endometriosis and normal control groups (0.78 ± 0.22 vs. 4.66 ± 0.42, $t = 4.847$, $P < 0.001$), but there was no difference between the fat tissue control and normal control groups (2.30 ± 0.74 vs. 4.66 ± 0.42, $t = 0.937$, $P = 0.349$; Figure 3a and 3c). Taken together, these results indicated that endometriosis affected the expression of ER and PR in uterine tissues.

Differential expression of microRNAs in rat uterine tissues during the window of implantation

The microarray results showed that 475 miRNA transcripts were differentially expressed between the endometriosis and normal control groups, with 127 being upregulated and 348 being downregulated. Table 2 lists the top 20 downregulated miRNAs and top 20 upregulated miRNAs [the heat map is shown in Figure 4a]. In the Circos plot analysis, the

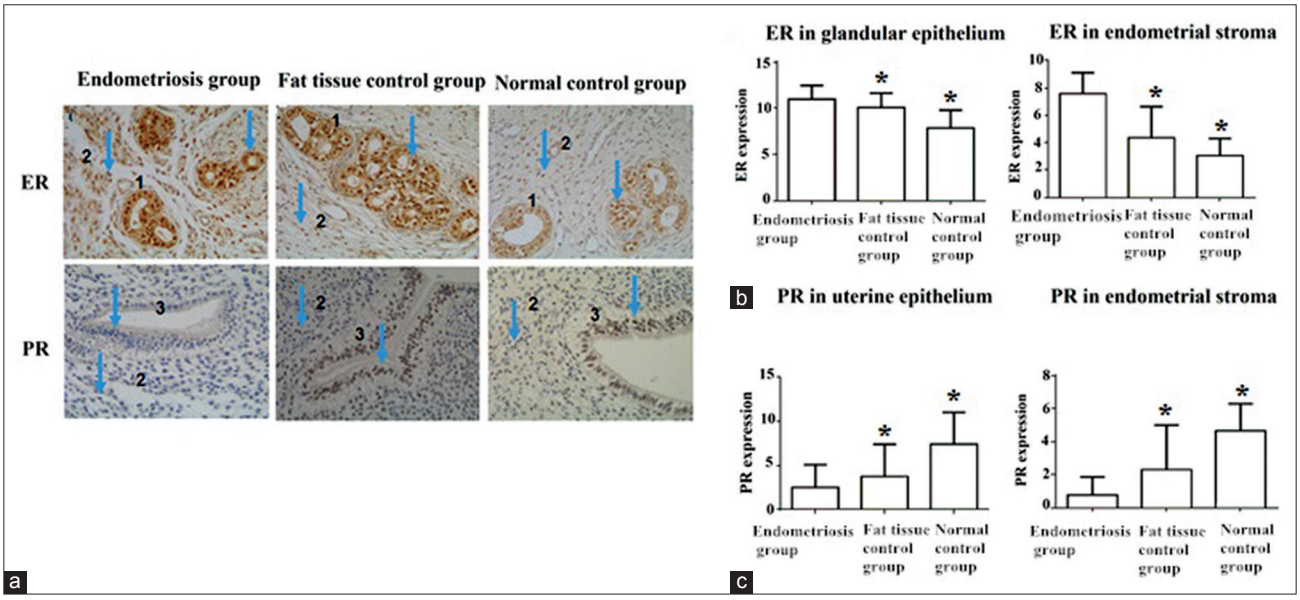

Figure 3: Immunohistochemistry of uterine tissue in rat. (a) Expression of ER and PR in the endometriosis group, fat tissue control group, and the normal control group. 1: Glandular epithelium; 2: Endometrial stroma; 3: Uterine epithelium. The arrows represent positive staining in the nucleus (×400). (b and c) Quantification of the immunochemistry results. *$P < 0.05$, compared with endometriosis group using analysis of variance with Tukey's test for multiple comparisons. ER: Estrogen receptor; PR: Progesterone receptor.

Table 2: Top 20 downregulated and 20 upregulated miRNAs in rat uterine tissues in the endometriosis group compared with paired normal control group

Downregulated miRNAs	FC (abs)	*Q*	Upregulated miRNAs	FC (abs)	*Q*
rno-miR-29c-3p	0.4724	1.05	rno-miR-466b-5p	3.0513	5.51
rno-miR-34c-5p	0.4708	2.69	rno-miR-139-5p	2.0729	4.53
rno-miR-141-5p	0.2688	0	rno-miR-206-3p	3.5532	3.67
rno-miR-24-1-5p	0.4595	0	rno-miR-328a-3p	2.1113	4.02
rno-miR-490-5p	0.2495	0	rno-miR-292-5p	2.1233	3.89
rno-miR-184	0.2167	1.05	rno-miR-6216	1.9126	2.77
rno-miR-181a-1-3p	0.2330	0	rno-miR-188-5p	1.8939	5.01
rno-miR-500-5p	0.2401	0	rno-miR-292-3p	1.8012	3.09
rno-miR-664-1-5p	0.2615	0	rno-miR-139	1.8389	4.68
rno-miR-1949	0.2642	3.13	rno-miR-466d	1.8741	3.78
rno-miR-672-3p	0.2934	1.44	rno-miR-92b-3p	1.8116	4.64
rno-miR-135a-3p	0.2965	0	rno-miR-455-3p	1.7750	5.23
rno-miR-30b-3p	0.3076	0	rno-miR-324-3p	1.6661	2.54
rno-miR-344a-3p	0.3165	0	rno-miR-151-3p	1.6469	1.09
rno-miR-301a-3p	0.3219	0	rno-miR-539-3p	1.6415	3.96
rno-miR-3068-5p	0.3239	0	rno-miR-423-3p	1.6267	4.35
rno-miR-362-3p	0.3353	0	rno-miR-326-3p	1.6113	5.33
rno-miR-455-5p	0.3504	0	rno-miR-423	1.5939	3.87
rno-miR-31a-3p	0.3540	0	rno-miR-320	1.5870	2.98
rno-miR-411-5p	0.3683	4.27	rno-miR-320-3p	1.5663	2.63

MiRNA: MicroRNA; FC: Fold changes.

downregulated miRNAs were more significant than the upregulated ones [Figure 4b], and five downregulated miRNAs with a fold change of ≥2 and $P \leq 0.050$ were selected for PCR validation, based on previous studies and clinical implication. These results indicated that endometriosis affected miRNA expression in the uterine tissues.

Kyoto Encyclopedia of Genes and Genomes and Gene Ontology enrichment analysis

As shown in Figure 5a, the most common pathways involved by the dysregulated miRNAs were cancer, vasopressin-regulated water reabsorption, morphine addiction, and prostate cancer. Interestingly, the estrogen signaling pathway was also one of the common pathways involved by the dysregulated miRNAs. The GO pathway analysis showed that the dysregulated miRNAs were also involved in metabolic process, development process, cell death, rhythmic process, biological regulation, cellular process, and growth [Figure 5b]. These results indicated that the miRNAs differentially regulated in endometriosis were involved in a number of pathways, some of them potentially playing roles in implantation and fertility.

Target genes and function prediction

The target genes of five dysregulated miRNAs (rno-miR-29c-3p, rno-miR-34c-5p, rno-miR-141-5p, rno-miR-24-1-5p, and rno-miR-490-5p) were predicted using miRWalk2.0. The target genes were screened using six types of miRNA target genes prediction procedure: miRWalk, miRanda, miRDB, miRMap, miRNAMap, and RNAhybrid, followed by enrichment analysis using GO and KEGG pathways. Results are summarized in Supplemental Table 1.

Due to the short sequence length of miRNAs, the prediction accuracy was limited, and the identified target genes were

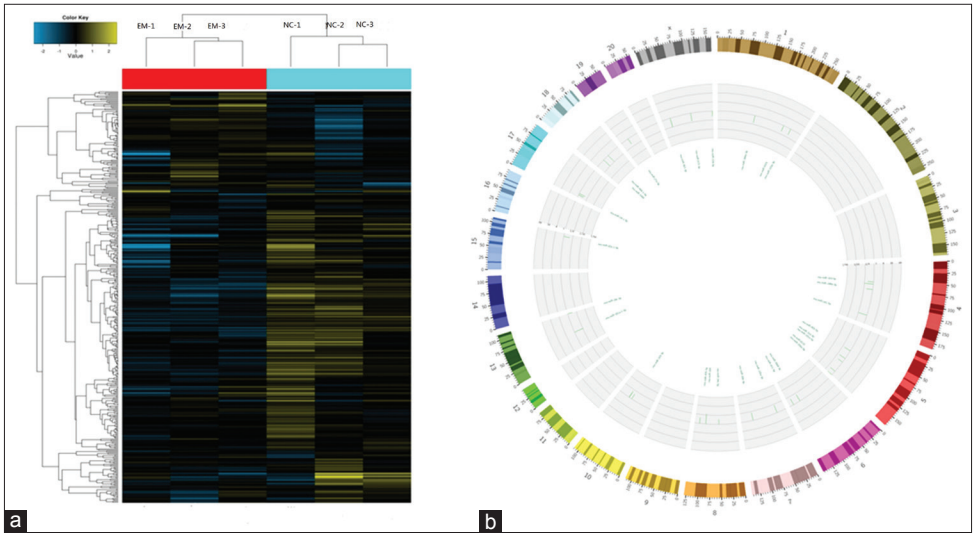

Figure 4: (a) Heat map showing the differentially expressed microRNA between the endometriosis and control groups. Each row represents a microRNA; each column represents a sample. Yellow indicates upregulation; blue indicates downregulation; 2.0 and −2.0 indicate fold change in the corresponding expression profile; 1–3 indicate sample name; EM indicates endometriosis group; and NC indicates normal control group. (b) Circos plot showing the degree of difference in the differential genes based on their location. Green indicates downregulated genes. The length of the bar represents the multiple of the differentially expressed genes. The longer the bar, the greater the difference multiple.

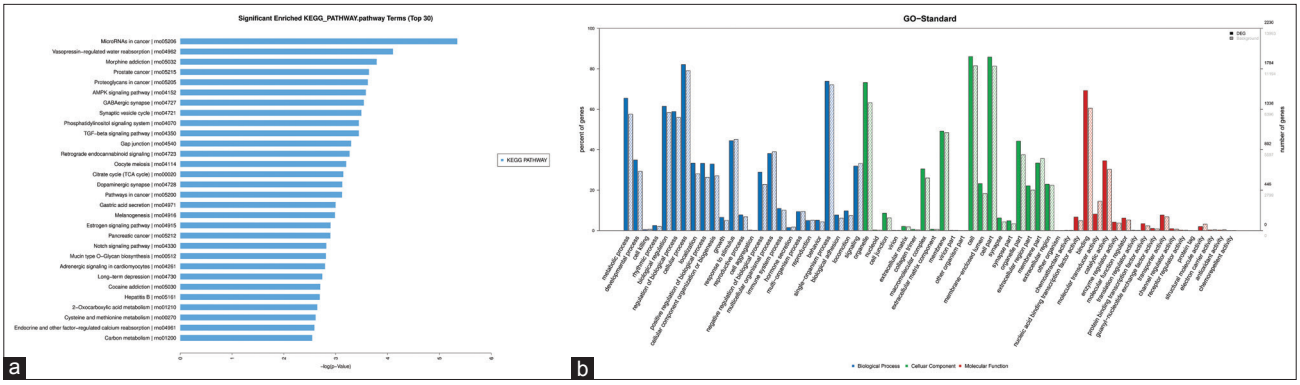

Figure 5: (a) KEGG signaling pathway analysis of target genes for differentially expressed microRNAs. The first 30 KEGG signaling pathways and the most significant *P* value. The X-axis represents the most significant *P* value; the Y-axis represents the KEGG signaling pathway. (b) GO-standard analyzes biological process differentially expressed microRNAs. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

involved in a broad range of pathways including embryo implantation, blastocyst formation, decidualization, luteinizing hormone signaling pathway, sperm-egg recognition, estradiol 17-beta-dehydrogenase, estrogen signaling pathway, ER binding, steroid biosynthesis, steroid hormone receptor binding activity, and steroid dehydrogenase activity [Supplemental Table 2]. Nevertheless, these results showed that these five miRNAs were potentially involved in pathways that were part of the reproductive functions.

Expression of rno-miR-29c-3p, rno-miR-24-1-5p, rno-miR-490-5p, rno-miR-34c-5p, and rno-miR-141-5p among the three groups

Rno-miR-29c-3p expression was different between the endometriosis and fat tissue control groups (0.23430 ± 0.03000 vs. 0.31670 ± 0.02000, P = 0.022) and between the endometriosis and normal control groups (0.23430 ± 0.03000 vs. 0.40590 ± 0.05000, P = 0.001), without significant difference between the fat tissue control and normal control groups (0.31670 ± 0.02000 vs. 0.40590 ± 0.05000, P = 0.085). Rno-miR-24-1-5p expression was different between the endometriosis and fat tissue control groups (0.00030 ± 0.00004 vs. 0.00050 ± 0.00003, P = 0.008) and between the endometriosis and normal control groups (0.00030 ± 0.00004 vs. 0.00060 ± 0.00008, P = 0.001), but not between the fat tissue control and normal control groups (0.00050 ± 0.00003 vs. 0.00060 ± 0.00008, P = 0.101). Rno-miR-490-5p expression was different between the endometriosis and fat tissue control groups (0.00080 ± 0.00011 vs. 0.00130 ± 0.00016, P = 0.028) and between the endometriosis and normal control groups (0.00080 ± 0.00011 vs. 0.00130 ± 0.00004, P = 0.001), but not between the fat tissue control and normal control groups (0.00130 ± 0.00016 vs. 0.00130 ± 0.00004, P = 0.366). The expression of rno-miR-34c-5p was different between the endometriosis and fat tissue control groups (0.00180 ± 0.00020 vs. 0.00280 ± 0.00026, P = 0.015), between the endometriosis and normal control groups (0.00180 ± 0.00020 vs. 0.00360 ± 0.00021, P = 0.001), and between the fat tissue control and normal control groups (0.00280 ± 0.00026 vs. 0.00360 ± 0.00021, P = 0.022). Finally, the expression of rno-miR-141-5p was different between the endometriosis and fat tissue control groups (0.08140 ± 0.01164 vs. 0.12670 ± 0.01542, P = 0.011), between the endometriosis and normal control groups (0.08140 ± 0.01164 vs. 0.19240 ± 0.00422, P = 0.002), and between the fat tissue control and normal control groups (0.12670 ± 0.01542 vs. 0.19240 ± 0.00422, P = 0.032; Figure 6). Taken together, these results suggested that these five miRNAs were involved in endometriosis.

Relationships between microRNAs and estrogen receptor/progesterone receptor

The associations between the expression of the five miRNAs and the expression of ER (ERα and ERβ) and PR were studied using miRWalk 3.0 and TargetScan. Table 3 shows that the five miRNAs had binding sites on the coding DNA sequence and 3'UTR of ERα, ERβ, and PR.

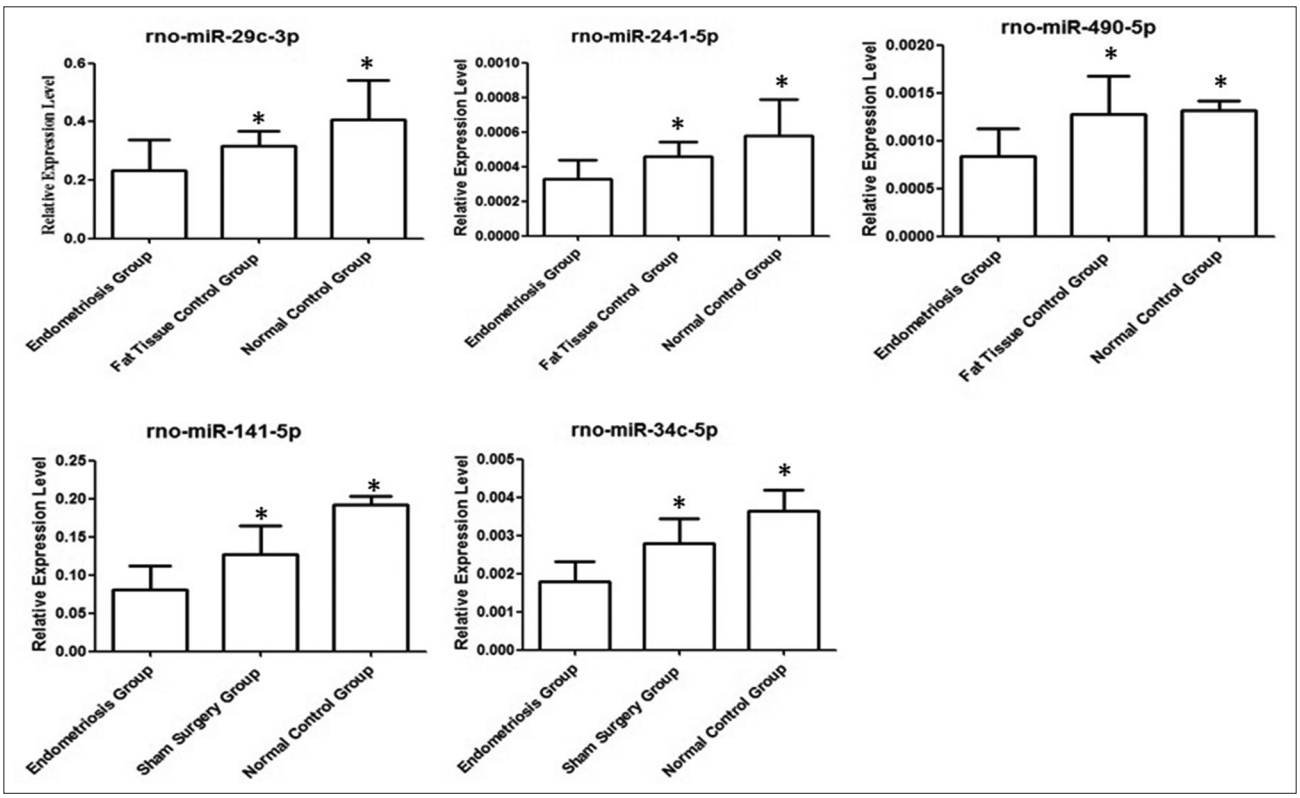

Figure 6: Relative expression of rno-miR-29c-3p, rno-miR-24-1-5p, rno-miR-141-5p, rno-miR-490-5p, and rno-miR-34c-5p among the three groups, according to the ΔΔCT method. *P < 0.05, versus endometriosis group.

Discussion

ER and PR are involved in endometriosis,[2,8,16,18,22] but the involvement of miRNAs is unknown. Therefore, this study aimed to explore the correlation between miRNA and ER/PR in the uterine tissues of rats with endometriosis during the implantation window. The results suggested that miRNAs, ER, and PR could play important roles in the implantation period of rats with endometriosis. These miRNAs might play a role in endometrial receptivity in endometriosis.

The rat model of autologous endometrial implantation is considered to be representative of human endometriosis.[19] The implantation failure observed in endometriosis is possibly related to the upregulation of ER and downregulation of PR, and the high expression of ER might be closely connected with the inflammatory characteristics of endometriosis.[8,18] The present study suggested that multiple differentially expressed miRNAs were detected in the uterine tissue of rats' endometriosis during the implantation window. Changes in the expression of particular miRNAs might play a role in the modulation of endometrial receptivity in endometriosis, ultimately resulting in implantation or failure.[23]

Since Buyalos and Agarwal[24] first proposed their concept of endometriosis-related infertility in 2000, the features of endometriosis infertility gathered great attention from medical workers and researchers. Thus, the pregnancy outcomes of patients with endometriosis obviously improved, mainly with the help of preparative drug regimens and assisted reproduction techniques. Nevertheless, the incidence of endometriosis and endometriosis-related infertility did not really change, mainly because the pathogenesis of endometriosis remains poorly understood and because of the complex process of reproduction, on which endometriosis may affect multiple stages. The present study focused on the embryo implantation and endometrial receptivity, more particularly the miRNAs that could be involved.

In the present study, the Gene Chip technology was used to detect the miRNA array in the uterus of rats with endometriosis during the WOI. We found that 475 miRNA transcripts were differently expressed between the endometriosis and normal control groups, with 127 being upregulated and 348 being downregulated. Among the dysregulated miRNAs, downregulated genes were more significant than the upregulated. We selected five obviously downregulated miRNAs. According to miRNA target gene prediction tools (miRWalk, miRanda, miRDB, miRMap, miRNAMap, and RNAhybrid), we predicted the target genes of rno-miR-29c-3p, rno-miR-34c-5p, rno-miR-141-5p, rno-miR-24-1-5p, and rno-miR-490-5p.

MiR-29c-3p is involved in the regulation of the vascular endothelial growth factor (VEGF)-A mRNA translation in endometrial cells, inhibition of VEGF-A protein expression, and angiogenesis.[25] The present study showed that the expression of miR-29c was increased in eutopic endometrium of baboons and humans with endometriosis, where it might damage the progesterone response by reducing the levels of FKBP4.[26] Removal of the deep infiltrative endometriosis could reverse the progesterone resistance in women with endometriosis.[26] MiR-29c might inhibit endometriosis by inhibiting endometrial cell proliferation and invasion and by promoting apoptosis.

Jeon *et al*.[27] showed that the dysregulated expression of miR-24 might be associated with infertility caused by polycystic ovary syndrome. Indeed, miR-24 inhibits the expression of the *MYC* gene, which is a key factor of multiplication involved in embryo implantation.[27] In the present study, rno-miR-24-1-5p was downregulated in the uterus of rats with endometriosis, supporting this role of miR-24. A study of miR-24 transfection showed that the expression of CDKN1b, which is a target gene of miR-24, was suppressed in the process of embryonic development.[28] MiR-24 might thus regulate the concentration of estradiol and progesterone during follicular fluid formation.[28] The excessive expression of miR-24 might restrain transforming growth factor signaling pathways and inhibit the secretion of estradiol.[28] MiRNA-24 is downregulated in the development of porcine embryos; because miR-24 expression was influenced by the culture conditions, miRNA-24 could be used as a quality index for embryos.[29] Another study showed that the expression of miR-24 was upregulated in the plasma of patients with severe preeclampsia during the gestational period.[30]

MiR-490-3p may increase CDDP sensitivity in ovarian cancer cells by reducing the expression of ABCC2, indicating that the increase of miR-490-3p might be a potential treatment strategy against cisplatin-resistant ovarian cancer.[31] In addition, miR-490-3p mRNA expression was lower in endometrial carcinoma tissue than in normal endometrial tissue.[31] MiR-490-3p expression negatively regulates the infiltration depth and lymph node metastasis in endometrial cancer.[32] MiR-490 expression was significantly lower in ovarian cancer and borderline tumors than that of benign tumor.[33] Its expression in metastatic ovarian cancer (omentum) was lower than that of primary ovarian cancer.[33] It is also negatively associated with FIGO staging and differentiation.[33] MiR-490-3p may inhibit tumor occurrence and development of ovarian epithelial carcinoma by regulating its target gene CDK1.[33] MiR-490-3p might be associated with the occurrence of drug resistance ovarian cancer. MiR-490 is also obviously abnormally expressed in uterine leiomyoma.[34] In the present study, rno-miR-490-3p was downregulated in the uterus of rats with endometriosis.

In maternal plasma, the upregulation of miR-34c expression was directly related to good-quality embryos in D3.[35] There was a strong correlation between miR-34c expression level in sperm and the results of intracytoplasmic sperm injection (ICSI) treatment in cattle.[35] MiR-34c-positive patients are more likely to have good-quality embryo, implantation, pregnancy, and live birth.[35] Paternal miR-34c levels may play a role

Table 3: Relation between the five selected miRNAs (rno-miR-29c-3p, rno-miR-24-1-5p, rno-miR-141-5p, rno-miR-490-5p, and rno-miR-34c-5p) and steroid hormone receptors predicted by miRWalk 3.0

ENSEMBL_ID	mRNA	miRNA	Binding *P*	Position	Binding site		Au	Me	N parings
ENSRNOT00000026350	ERα	rno-miR-24-1-5p	0.850	CDS	944	974	0.4	−9.294	16
		rno-miR-29c-3p	0.850	3'UTR	3590	3607	0.56	−9.86	14
		rno-miR-34c-5p	0.850	CDS	538	566	0.28	−7.912	19
		rno-miR-34c-5p	0.920	5'UTR	170	210	0.4	−4.65	17
		rno-miR-34c-5p	1.000	CDS	628	647	0.34	−10.45	15
		rno-miR-34c-5p	0.920	5'UTR	52	72	0.38	−4.65	17
		rno-miR-141-5p	1.000	CDS	1835	1854	0.43	−6.501	17
		rno-miR-141-5p	0.850	CDS	1538	1555	0.54	−14.161	15
		rno-miR-141-5p	1.000	CDS	1793	1812	0.43	−6.501	17
		rno-miR-490-5p	0.920	CDS	1367	1386	0.47	−7.561	17
		rno-miR-490-5p	0.920	CDS	1836	1858	0.41	−8.871	18
		rno-miR-490-5p	0.900	CDS	1394	1414	0.43	−7.494	16
		rno-miR-490-5p	1.000	CDS	1229	1248	0.47	−7.56	17
		rno-miR-490-5p	0.850	CDS	1794	1816	0.41	−8.87	18
ENSRNOT00000028697	ERα	rno-miR-24-1-5p	0.850	3'UTR	1998	2021	0.43	−8.306	18
		rno-miR-34c-5p	0.920	CDS	1250	1273	0.32	−7.14	18
		rno-miR-34c-5p	0.920	3'UTR	1924	1964	0.46	−5.18	19
		rno-miR-34c-5p	0.920	CDS	934	952	0.35	−6.996	16
		rno-miR-141-5p	0.850	CDS	944	963	0.34	−6.013	16
		rno-miR-490-5p	0.850	CDS	339	367	0.4	−8.55	17
		rno-miR-490-5p	0.850	3'UTR	1900	1918	0.31	−9.585	14
ENSRNOT00000013867	ERβ	rno-miR-490-5p	1.000	CDS	1268	1289	0.44	−9.104	17
ENSRNOT00000043602	ERβ	rno-miR-24-1-5p	1.000	CDS	1243	1268	0.52	−6.464	17
		rno-miR-24-1-5p	0.850	3'UTR	3214	3249	0.46	−3.938	17
		rno-miR-24-1-5p	1.000	CDS	1243	1268	0.52	−6.464	17
		rno-miR-24-1-5p	0.850	3'UTR	3268	3303	0.46	−3.938	17
		rno-miR-24-1-5p	1.000	CDS	1243	1268	0.52	−6.464	17
		rno-miR-24-1-5p	0.850	3'UTR	3385	3420	0.46	−3.938	17
		rno-miR-24-1-5p	1.000	CDS	660	675	0.52	−6.464	13
		rno-miR-34c-5p	0.920	3'UTR	2781	2809	0.54	−5.227	21
		rno-miR-34c-5p	0.920	CDS	1463	1483	0.43	−7.619	15
		rno-miR-34c-5p	0.920	3'UTR	2835	2863	0.54	−5.227	21
		rno-miR-34c-5p	0.920	CDS	1463	1483	0.43	−7.619	15
		rno-miR-34c-5p	0.920	3'UTR	2952	2980	0.54	−5.227	21
		rno-miR-34c-5p	0.920	CDS	1580	1600	0.43	−7.619	15
		rno-miR-34c-5p	0.920	3'UTR	2305	2333	0.54	−5.227	21
		rno-miR-34c-5p	1.000	CDS	987	1007	0.43	−7.619	15
		rno-miR-141-5p	0.880	3'UTR	2445	2486	0.41	−3.793	19
		rno-miR-141-5p	1.000	CDS	2221	2240	0.47	−6.501	17
		rno-miR-141-5p	0.880	3'UTR	2499	2540	0.41	−3.793	19
		rno-miR-141-5p	1.000	CDS	2275	2294	0.47	−6.501	17
		rno-miR-141-5p	0.880	3'UTR	2616	2657	0.41	−3.793	19
		rno-miR-141-5p	1.000	CDS	2392	2411	0.47	−6.501	17
		rno-miR-141-5p	1.000	3'UTR	1969	2010	0.41	−3.793	19
		rno-miR-141-5p	0.920	CDS	1745	1764	0.47	−6.501	17
		rno-miR-490-5p	0.920	3'UTR	2585	2616	0.56	−6.97	19
		rno-miR-490-5p	0.850	CDS	2222	2243	0.44	−8.726	17
		rno-miR-490-5p	0.810	5'UTR	228	251	0.44	−8.527	17
		rno-miR-490-5p	0.920	3'UTR	2402	2423	0.46	−9.547	16
		rno-miR-490-5p	0.920	3'UTR	2639	2670	0.56	−6.97	19
		rno-miR-490-5p	0.850	CDS	2276	2297	0.44	−8.726	17
		rno-miR-490-5p	0.810	5'UTR	228	251	0.44	−8.527	17
		rno-miR-490-5p	0.920	3'UTR	2456	2477	0.46	−9.547	16
		rno-miR-490-5p	0.920	3'UTR	2756	2787	0.56	−6.97	19

Contd...

Table 3: Contd...

ENSEMBL_ID	mRNA	miRNA	Binding *P*	Position	Binding site		Au	Me	N parings
		rno-miR-490-5p	0.850	5'UTR	2393	2414	0.44	−8.726	17
		rno-miR-490-5p	0.810	3'UTR	228	251	0.44	−8.527	17
		rno-miR-490-5p	0.920	3'UTR	2573	2594	0.46	−9.547	16
		rno-miR-490-5p	0.920	3'UTR	2109	2140	0.56	−6.97	19
		rno-miR-490-5p	0.920	3'UTR	1926	1947	0.46	−9.547	16
ENSRNOT00000018796	PR	rno-miR-24-1-5p	0.920	3'UTR	1688	1710	0.44	−11.462	17
		rno-miR-141-5p	0.850	CDS	122	152	0.28	−7.782	19

ER: Estrogen receptor; PR: Progesterone receptor; CDS: Coding DNA sequence; UTR: Untranslated region; miRNA: MicroRNA.

in embryonic development.[35] MiR-34c levels might be used as quality index of ICSI outcome in the human sperm.[35] MiR-34c participates in the regulation of follicular and two-cell stages of embryonic development in bovine.[36] MiR-34c is involved in cervical reconstruction after spontaneous term labor and delivery.[37] MiR-34c is important for the first cellular division in the early embryo development.[38] Upregulation of miR-34c in breast tumor is associated to downregulation of CYP 1A1 and ERα gene expression.[39]

A study in mice found that miRNA-141 downregulated the expression of PTEN (phosphatase and tensin homolog deleted on chromosome ten) and might affect cell proliferation and apoptosis of endometrial cells, playing an important role in embryo implantation.[15] A previous study showed that miR-34c and miR-141 were specific to the epithelial cells and they were highly expressed in abdominal endometriosis tissue.[40] MiR-141 was upregulated in the placenta of patients with preeclampsia, and miR-141 inhibits the trophoblast cells.[41] MiR-141 was expressed in the placenta and was increased in maternal plasma during pregnancy. MiR-141 was downregulated in endometriosis.[23]

Taken together, these results suggested that rno-miR-29c-3p, rno-miR-34c-5p, rno-miR-141-5p, rno-miR-24-1-5p, and rno-miR-490-5p played a number of roles in the fertility and reproduction processes, including the endometrial receptivity. Nevertheless, additional studies are still necessary to determine these roles exactly.

In this study, rno-miR-34c-5p, rno-miR-141-5p, and ER were upregulated in endometriosis. There might be an intimate relationship between miRNA and ER in rat endometriosis-related infertility during the WOI. MiRNA-141 is one of the miRNA-200 family members, and progesterone can reduce miRNA-141 expression to promote stem-like breast cancer cells.[42] MiRNA-141 may directly regulate PR, which is an important transcription factor for the expansion of mammary gland stem cell.[42] The decrease of miR-141 can increase the protein levels of PR and the expression of PR is estrogen dependent on a cellular level.[42] MiR-200a/miR-141 might be related to hormone receptor status in endometrial cancer and may affect prognosis.[43] MiR-34c regulates the reduction of ERα and influences the formation of breast cancer.[39]

According to GO and KEGG-PATHWAY analysis, miRNAs are involved in many relating to fertility activities that molecular functions and biological processes, for example, response to estrogen, response to hormone, luteinizing hormone signaling pathway, ER overload response, ER-nucleus signaling pathway, response to estradiol, response to steroid hormone, protein targeting to ER, ERBB2 signaling, ER-associated ubiquitin-dependent protein catabolic process, regulation of ER to Golgi vesicle-mediated transport, estrogen metabolic process, luteinization, ER to Golgi vesicle-mediated transport, estrogen biosynthetic process, steroid metabolic process, steroid hormone secretion, steroid biosynthetic process, steroid catabolic process, steroid hormone receptor binding, steroid hormone binding, hormone binding, estradiol 17-beta-dehydrogenase activity, steroid dehydrogenase activity, ER binding, steroid hormone receptor activity, steroid binding, and steroid hydroxylase activity. All these pathways may potentially be involved in the regulation of endometrial receptivity, but the exact effects of the miRNAs identified in the present study need to be verified.

The results suggested that dysregulation of miRNAs and abnormal expression of ER, and PR could be observed in rats with endometriosis. These changes might influence the embryo implantation of rats with endometriosis during the implantation window.

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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MicroRNA表达谱与甾体激素受体在内异症大鼠种植窗期的表达研究

摘要

背景：研究发现雌激素受体 (ER)和孕激素受体(PR)表达于子宫内膜异位症，但关于microRNAs (miRNAs)在内异症当中的作用至今未知。本研究的目的是探索miRNA和ER/PR之间的相互调控联系在内异症大鼠种植窗期子宫当中的作用。

方法：20只雌性SD大鼠随机分成三组：内异症组(n=7)、脂肪组织对照组(n=6)和空白对照组(n=7)。交配成功后第5天（种植窗期）取子宫组织，进行H&E染色、免疫组化和miRNA芯片检测。选取rno-miR-29c-3p, rno-miR-34c-5p, rno-miR-141-5p, rno-miR-24-1-5p, and rno-miR-490-5p做实时荧光定量PCR (qRT-PCR)验证。

结果：基因芯片发现内异症组和空白对照组内膜比较有475 miRNAs转录本表达异常，127个miRNAs表达上调，348个miRNAs表达下调。rno-miR-29c-3p, rno-miR-34c-5p, rno-miR-141-5p, rno-miR-24-1-5p, and rno-miR-490-5p的RT-PCR表达量在内异症组、脂肪组织对照组和空白对照组之间有明显表达差异。免疫组化结果示，ER 和PR在子宫内膜腺上皮和腺体当中的表达在三组之间有显著差异($P<0.05$)。推测及5个miRNAs有可能参与生殖和着床。

结论：结果提示miRNAs、ER和 PR在内异症大鼠种植窗期有关键调控作用，miRNA有可能参与内异症内膜容受性的建立过程。

Supplemental Table 1: Target genes prediction

miRNA	Target genes
rno-miR-29c-3p	*Hmgn3, Tpm1, Itgb1, Ccna2, Has3, Sptan1, Sgk1, Mybl2, Col3a1, Rnf19a, Hmgcr, Trib2, Lamtor1, Oxr1, Phc1, Abcb6, Tmem183a, Sms, Morf4l2, Ireb2, Pcdhac1, Hbp1, Col5a3, Commd2, Pcdhac2, Tnfaip1, Smtnl2, Fam167a, Ankrd49, Zhx1, Chfr, Smek2, Vegfa, Tmprss3, Sspn, Gpr37, Kdm2a, Calu, LOC100912146, Slc37a4, Prss35, Ccnt2, Ascc1, Pdik1l, Klhl8, Ccdc47, Ddx3x, Col1a1, Cerk, Ankrd13b, Zbtb5, Gtf3c3, Ybx3, Mgat4b, Insig1, Vav2, Psma3, Kctd5, Rb1cc1, Tmem164, Dnmt3a, Bcl2l2, Pdgfa, Hmgcs1, Gemin2, Serpina5, Ercc6, Taf5, Fert2, Abcg8, Col6a2, Dot1l, Trpv4, Atrn, Crispld1, LOC100910316, Ddx56, Asic1, Snx4, Hn1, Trim63, Cacna1c, Aco1, Pigc, Nup210, Blmh, Zfp282, Grip1, Col4a2, Pmp22, Gjd2, Pdgfra, Agpat4, Slc43a2, Serpina5, Ammecr1l, Fdft1, Adamts7, Dusp2, Ppic, Klhdc3, Tfeb, Mlf1, Tmem135, Glis2, Ythdf1, Camk2b, LOC100912534, Bmf, Zfp384, Dbt, Col4a2, Rnd3, Fermt2, Tubb2b, Ppfia4, Fam83f, Rap1, gds1, Ing2, Lamc1, Dnajb11, Elf2, Lysmd1, Slc6a7, Tbl1xr1, Camk2b, Ing4, Ddx1, Zfp384, Tdg, Cnr1, Col4a1, Stard8, Moap1, Foxj2, Cldn1, Cd276, Plag1, Atp1b1, Dpf1, Bmpr1a, Dennd6a, Elf2, Aacs, Sestd1, Fam213a, Zfp384, Nckipsd, Ppp2ca, Snx24, Impdh1, RGD1309748, Ifi30, LOC685909, Nsun6, Ric8a, Srpx, Osbp, Cwc25, Tmod1, Spry1, Klhl28, Chsy1, Stmn2, Pkmy, Bhmt, Larp4b, Agrn, Adamts9, Zer1, Psme4, Pcolce, Hexa, Cspg4, Atad3a, Slmap, Ppm1d, Cdhr1, Atp5g1, Proser1, Cmpk1, Ccne1, Arf5, Acpl2, Ankrd13b, Fam76b, Tmem169, Slc1a4, Smpd3, Surf6* rno-miR-34c-5p *Ldha, Pdgfra, Acsl1, Dpysl4, Pnoc, Nup210, Gpr85, Akap6, Slc6a1, Pias3, Acsl4, Map2k1, Svop, Pacs1, Gpr64, Ptov1, Rragc, Fbxo30, Pck1, Rarg, Aldoa, Pgm1, Slc4a3, Vamp2, Tat, Tp53, PrkcbScnn1a, Map1a, Efnb1, Vps52, Tyro3, Akt2, Arnt2, Cacnb3, Cntn2, Arrb2, Eno3, Notch1, Prkag1, Alpl, Syt1, Jag1, Mecr, Coro1b, Rgs9, Itsn1, Ralgds, Ndst1, Ptpn5, Scn1b, Scn11a, Ppt2, Phlpp1, Lmna, Kitlg, Bcl2l2, Hr, Pafah1b2, Des, Calu, Ppap2a, Inhbc, Ap2s1, Birc3, Dab2, Frk, Gng5, Alcam, Sec61a1, Inpp4a, Lect1, Smad7, Pi4kb, Suox, Dgkz, Capn6, Taldo1, Dvl1, Flot2, Cntnap1, Dll1, Synj1, Slc25a27, Bmp7, Ncdn, Klf4, Stag3, Pitpnb, Pkia, Kidins220, Calcr, Plod1, E2f5, Dync1i2, Kcnh2, Calb2, Rab8a, Ccnf, Tbp, Baalc, Prkcd, Capn8, Marf1, Igsf6, Trak2, Slc38a5, RGD621098, Scd1, Lzts3, Aip, Rnf34, Mtmr4, Vat1, Hn1, Recql5, St6, galnac1, Polq, F13b, Swt1, Sdhc, Grem2, Xbp1, Zmiz2, Thoc3, Fkbp8, Snx15, B3, gat3, Taf5, Ppp, 1r11, Rai14, Vtcn1, Ppm1j, Tbck, Pkp4, Edf1, Irf5, Mob1a, Ppp4r2, Crbn, Hdac1, Mfsd2a, Ago4, Agtrap, H6pd, Mapre3, Mycn, Brf1, Ei24, Hexa, Ap1s2, Aff4, Kat7, Ccdc64, Rrn3, Gli2, Elk4, Diexf, Irx2, Mex3c, Afg3l2, Uri1, Uhrf2, Tmem79, Pogz, Arhgap1, Zmynd19, Zer1, Tspan9, Rngtt, Zmym4, E2f6, Nol10, Daam1, Myo1f, Rdx, Csk, Tbc1d2b, Slc35g2, Lpin2, Nono, Map3k14, Cops4, Tmem150c, Zic5, Rras, Nrip3, Polr3e, Mta2, Tmem109, Hsd17b8, Sar1a, Ccne2, Tmem55a, Fam83h, Ppp2r3a, Rad54l2, Atg16l1, Ing5, Nanos2, Tbl1xr1, Mob3b, Slc30a3, Gjb2, Scn3a, Trat1, LOC498350, Fam167a, Hsf2bp, RGD1559864, Efcab11, Fgd6, Coro1c, LOC685707, Pigz, Ttc19, LOC100365958*</td></tr><tr><td>rno-miR-141-5p</td><td>*Dcun1d2, Atrn, Ahcyl1, Spag9, Tmem169, Zfp282, Cenpl, LOC681325, Tsr1, Pik3c3, Oprm1, RGD1563222, Ripk1, Trnt1, Gxylt1, Upf3b, Cnksr3, Slc25a24, Arl5b, Bpnt1, H3f3b, Zfp367, Zfp329, Nup35, Gtf3c4, Cd40, Cenpl, Oxsr1, Tsr1, Rgs12, Depdc1b, Cluh, Polh, Drap1, Klf10, Zmat3, Tmem237, Ctbp2, Ets1, Klk9, Cyp4v3, Stc1, Gfra3, Dock8, Anxa5, Sspn, Ssu72, Elk4, Cenpe, Vps13d, Ptcd3, Cluh, Olig3, Triqk, Maml1, Il7r, Atp5sl, Ccp110, Hat1, Klf6, Gpkow, Glg1, Oaz1, Ube2k, Exo1, Acer2, Nudt2, Lsm14a, Atoh8, Foxk2, Hspa12a, Myog, Epor, Dlx3, Slc18a2, Capn6, Slamf8, Plxna2, Htr5b, Rnf215, Rgs4, Aar2, Ttc33, Cthrc1, Ralyl, Ppp1r3c, Hcrtr2, Ndp, Pdzd4, Aff4, Nfyb, Tekt2, Adamts3, Cenpe, Mall, Csgalnact2, Eif4a2, Muc1, Tmprss11g, Cyp2j10, Hnrnpu, Gtf2h1, Caps2, Tmed7, Nr4a3, Slamf1, Kdsr, Col4a3bp, Irf1, Kat6a, Itga8, Rfesd*</td></tr><tr><td>rno-miR-24-1-5p</td><td>*Thnsl2, Tle3, Cald1, Tmprss15, Ndst1, Bcl2l1, Nmnat3, Mier1, Slitrk1, Duox2, Arhgef2, Kcnip2, Dpysl4, Capzb, Slc6a1, Sema5a, Adcy5, Capn3, Mtss1, Ifrd2, Ogn, Srpk2, Pacsin2, Anks3, Cebpz, Ddx24, LOC100911034, Mx1, LOC100910156, Gphn, Fam98a, Rasgrp1, Ube2a, Gata2, Nasp, Aard, Pkd2l1, Nelfb, LOC100910156, Eif4b, Sim2, Fmr1, Tmem178a, Gzf1, Ncoa3, Rhoq, Ppp1r12b, Kcnab1, Acer2, Btbd3, LOC689959, Hivep2, Rnf41, Ncoa3, Enkur, Zfp280d, P2ry6, Calu, Angel2, Kcnip2, Hprt1, Zfp131, Tmpo, Map3k12, Prkcz, Capn1, Rcan2, Bcl2l1, Hspa4, Mybl1, Ung, Synj1, Ube2k, Chrna9, Rfx7, Cd200, Hprt1, Psmd3, Erlin2*</td></tr><tr><td>rno-miR-490-5p</td><td>*Ceacam3, Snap29, Prl8a9, Mnt, Fermt2, Slc25a42, Rgs17, Trat1, Pdcl3, Tgfa, Xlr4a, Inpp5d, Snx5, Pcyox1, Armc5, Tox, Dapp1, Zfx, Ugp2, Psg29, Ythdf3, Nhlrc2, Cdh2, P2ry6, Shisa7, Psip1, Fermt1, Fam84a, Dynlrb2, Tox3, Tmeff1, Angptl3, Cyp4v3, Ifit2, Slirp, Tmod3, Pou2af1, Tollip, Synj1, Sspn, Npap60, Akirin1, Hemgn, Syt4, Fndc3a, Kdsr, Ankrd39, Akr1c1, Nol9, Rbm47, F8, Gpr22, Sox2, Acsm5, Ankrd6, Sds, Prpf18, Psmc6, Foxh1, March11, Abhd15, Pacsin2, Cgnl1, Kcns3, Uba6, Mfsd4, Nol9*</td></tr></table>

Supplemental Table 2: Biological processes of fertility

Term	*P*
Decidualization	2.996553e-01
Luteinizing hormone signaling pathway	2.538776e-02
Embryo implantation	4.861362e-01
Sperm-egg recognition	9.635398e-01
Embryo development	2.559116e-06
Embryo development ending in birth or egg hatching	6.257047e-05
In utero embryonic development	1.053563e-03
Embryonic hematopoiesis	1.309090e-03
Embryonic organ development	2.325896e-03
Embryonic morphogenesis	3.471296e-03
Embryonic organ morphogenesis	1.405830e-02
Embryonic process involved in female pregnancy	1.501105e-02

Contd...

Supplemental Table 2: Contd...

Term	*P*
Embryonic eye morphogenesis	2.770223e-02
Embryonic camera-type eye morphogenesis	2.967756e-02
Embryonic brain development	6.807751e-02
Embryonic skeletal system development	8.099038e-02
Embryonic limb morphogenesis	8.397211e-02
Embryonic appendage morphogenesis	8.397211e-02
Embryonic camera-type eye development	9.260243e-02
Embryonic cranial skeleton morphogenesis	9.329879e-02
Embryonic lung development	1.593654e-01
Embryonic nail plate morphogenesis	1.593654e-01
Embryonic digit morphogenesis	1.466280e-01
Embryonic epithelial tube formation	1.397467e-01
Postembryonic development	1.667276e-01
Negative regulation of embryonic development	1.739488e-01
Embryonic hindlimb morphogenesis	1.975084e-01
Embryonic ectodermal digestive tract development	2.933430e-01
Post-embryonic camera-type eye development	2.456853e-01
Embryonic heart tube left/right pattern formation	2.933430e-01
Regulation of embryonic cell shape	2.933430e-01
Embryonic body morphogenesis	3.096681e-01
Embryonic retina morphogenesis in camera-type eye	3.096681e-01
Embryonic skeletal system morphogenesis	3.028371e-01
Embryonic genitalia morphogenesis	4.059758e-01
Embryonic heart tube anterior/posterior pattern specification	4.059758e-01
Embryonic hindgut morphogenesis	4.059758e-01
Embryonic placenta development	4.177397e-01
Embryonic camera-type eye formation	4.327177e-01
Embryonic axis specification	4.349932e-01
Embryonic forelimb morphogenesis	4.741165e-01
Embryonic neurocranium morphogenesis	5.006630e-01
Blastocyst formation	5.637590e-01
Embryonic placenta morphogenesis	6.030869e-01
Embryonic heart tube morphogenesis	6.018272e-01
Embryonic cleavage	6.471783e-01
Embryonic viscerocranium morphogenesis	7.034300e-01
Embryonic heart tube development	6.921679e-01
Embryonic digestive tract morphogenesis	7.493426e-01
Embryonic skeletal joint morphogenesis	8.519678e-01
Ovarian cumulus expansion	1.593654e-01
Ovarian follicle development	3.107337e-01
Oocyte maturation	3.200550e-01
Oocyte development	4.683096e-01
Oogenesis stage	5.802628e-01
Oocyte differentiation	5.637590e-01
Regulation of oocyte development	7.034300e-01
Oogenesis	7.243492e-01
Ovulation	9.781224e-01
Response to estrogen	8.333247e-03
ER overload response	4.142024e-02
ER-nucleus signaling pathway	5.847705e-02
Response to estradiol	6.759002e-02
Response to steroid hormone	1.974922e-01
Protein targeting to ER	2.500520e-01
ERBB2 signaling pathway	2.933430e-01
ER-associated ubiquitin-dependent protein catabolic process	2.924013e-01

Contd...

Supplemental Table 2: Contd...

Term	*P*
Regulation of ER to Golgi vesicle-mediated transport	2.967336e-01
Estrogen metabolic process	3.200550e-01
ER to Golgi vesicle-mediated transport	4.699971e-01
Cellular response to steroid hormone stimulus	4.688486e-01
Estrogen biosynthetic process	7.904665e-01
Steroid metabolic process	8.191961e-01
Steroid hormone secretion	8.301774e-01
Steroid biosynthetic process	9.017780e-01
Steroid catabolic process	9.631421e-01
Female pregnancy	2.489714e-02
Placenta development	1.025969e-01
Maternal placenta development	5.637590e-01
Maternal process involved in female pregnancy	1.495982e-01
Maternal process involved in parturition	7.034300e-01
Parturition	7.794149e-01
Luteinization	3.906199e-01
Vagina development	7.507166e-01
Mating plug formation	2.933430e-01
Mammary gland development	3.571088e-03
Mammary gland epithelium development	2.612118e-02
Mammary gland duct morphogenesis	6.552407e-02
Mammary gland bud morphogenesis	1.219142e-01
Mammary gland bud formation	2.933430e-01
Mammary gland formation	2.456853e-01
Mammary gland epithelial cell differentiation	3.906199e-01
Mammary gland branching involved in pregnancy	6.471783e-01
Mammary gland involution	7.904665e-01
Lateral sprouting involved in mammary gland duct morphogenesis	2.933430e-01
Branching involved in mammary gland duct morphogenesis	1.739488e-01
Development of secondary male sexual characteristics	6.807751e-02
Male germ-line stem cell asymmetric division	6.807751e-02
Female sex differentiation	8.099038e-02
Male sex determination	1.397506e-01
Male meiosis I	7.158036e-01
Female meiosis II	1.593654e-01
Male genitalia development	6.779372e-05
Male sex differentiation	8.081021e-03
Male gonad development	5.531811e-02
Male somatic sex determination	1.593654e-01
Male germ-line sex determination	2.933430e-01
Male genitalia morphogenesis	5.802628e-01
Male gamete generation	6.893504e-01
Sperm ejaculation	7.034300e-01
Regulation of sperm motility	4.059758e-01
Genitalia development	4.125469e-04
External genitalia morphogenesis	1.593654e-01
Female genitalia development	2.070470e-01
Female gonad development	2.154032e-01
Female meiotic division	2.665900e-01
Female genitalia morphogenesis	4.059758e-01
Regulation of female gonad development	7.034300e-01
Female mating behavior	8.519678e-01
Female gamete generation	9.655210e-01
Chromosome separation	1.062218e-01
Reproduction	6.353662e-01

Contd...

Supplemental Table 2: Contd...

Term	*P*
Sexual reproduction	8.838225e-01
Reproductive behavior	8.134496e-01
Reproductive structure development	5.004314e-04
Reproductive system development	5.954190e-04
Positive regulation of reproductive process	4.929580e-01

ER: Estrogen receptor.