Identification and Characterization of Progesterone- and Estrogen-Regulated MicroRNAs in Mouse Endometrial Epithelial Cells

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Abstract

In endometrial epithelial cells, progesterone (P4) functions in regulating the cell structure and opposing the effects of estrogen. However, the mechanisms of P4 that oppose the effects of estrogen remain unclear. MicroRNAs (miRNAs) are important posttranscriptional regulators that are involved in various physiological and pathological processes. Whether P4 directly induces miRNA expression to antagonize estrogen in endometrial epithelium is unclear. In this study, total RNAs were extracted from endometrial epithelium of ovariectomized mice, which were treated with estrogen alone or a combination of estrogen and P4. MicroRNA high-throughput sequencing with bioinformatics analysis was used to identify P4-induced miRNAs, predict their potential target genes, and analyze their possible biological functions. We observed that 146 mature miRNAs in endometrial epithelial cells were significantly upregulated by P4. These miRNAs were extensively involved in multiple biological processes. The miRNA-145a demonstrated a possible function in the antiproliferative action of P4 on endometrial epithelial cells.

Keywords

progesterone, estrogen, microRNA, endometrial epithelial cells

Introduction

The ovarian hormones estrogen and progesterone (P4) coordinately regulate the proliferation and differentiation of endometrial cells. These hormones also regulate the remodeling of the uterus to provide a suitable environment for preimplantation embryo development, blastocyst implantation, and pregnancy maintenance. In a normal uterus, the synthesis of estradiol (E2) at every estrus or menstrual cycle results in the proliferation of endometrial epithelial cells.¹ By contrast, P4 inhibits this estrogen-induced cell proliferation and stimulates epithelial differentiation in preparation for embryo implantation.² In endometrial epithelial cells, P4 functions in the regulation of cell proliferation by opposing the effects of estrogen in cell proliferation³ and many other aspects, such as differentiation⁴ and material transportation.^{5,6} However, the mechanisms by which P4 antagonizes estrogen remain unclear. The cellular dynamics in the uterus during the estrus cycle and early pregnancy can be precisely mimicked in ovariectomized mice by exogenous sex steroid hormone treatment.⁷⁻⁹ The effects of E2 and P4 in the uterus are mediated by their cognate transcription factors, namely, estrogen receptor (ER) and progesterone receptor (PR), respectively. Estrogen interacting with ER can promote some genes, whereas

P4-induced transcriptors may influence the transcription of these estrogen-induced genes.² However, the currently identified transcription factors that are directly regulated by P4 cannot completely elucidate the mechanisms of the antagonistic actions of P4 to E2. Other mechanisms by which P4 opposes E2 in endometrial epithelial cells possibly exist.

MicroRNAs (miRNAs) are a class of small noncoding regulatory RNAs (18-22 nucleotides [nts]) that work in the posttranscriptional regulation of gene expression.^{10,11} MicroRNAs have important functions in normal biological processes, but their misexpression has been associated with numerous diseases. Recent studies showed that miRNAs have functions in the postnatal development of mouse uterus¹²⁻¹⁴ and mouse embryo implantation.^{12,15} MicroRNA microarray detection in endometrial

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Figure 1. Length distribution and abundance of sequences for endometrial epithelial cells from progesterone (P4) and control group. Sequence length distribution of clean reads was based on the abundance and distinct sequences, the most abundant size class was 22 nt, followed by 23 nt, 24 nt, and 21 nt. nt indicates nucleotide.

biopsies from in vitro fertilization patients with high or normal P4 levels in serum indicated that miRNAs may influence endometrial receptivity.¹⁶ In the human menstrual cycle, the expression levels of miRNAs in the endometrial epithelium differed between the late proliferative and mid-secretory phases.¹⁷ Aberrant miRNA expression levels are associated with endometriosis, uterine leiomyoma, and endometrial carcinoma.¹⁸⁻²¹ However, whether P4 directly induces miRNA expression in the endometrial epithelium and whether these miRNAs facilitate the antagonistic function of P4 to E2 remain unclear.

We speculate the existence of P4-induced miRNAs, which facilitate the physiological effect of P4 on endometrial epithelial cells. In this study, total RNAs were extracted from endometrial epithelial cells of ovariectomized mice, which were treated with E2 alone or a combination of E2 and P4. MicroRNA high-throughput sequencing with bioinformatics analysis was used to identify P4-induced miRNAs, predict their potential target genes, and analyze their possible biological functions in endometrial epithelial cells.

Material and Methods

Ethics Statement

Female Kunming mice were used in this study. All animal procedures were conducted under the protocol approved by the Ethics and Scientific Research Committee in Sichuan University. All efforts were made to minimize suffering.

Animal Treatment and Purification of Endometrial Epithelial Cells

The mice were housed under controlled environmental conditions (20°C, 12-hour light/d) and provided with food and water ad libitum. The 6-week-old mice were ovariectomized via a

dorsal incision under pentobarbital sodium anesthesia. Two weeks after surgery, mice were primed with 100 ng of E2 (Sigma, St Louis, Missouri) in 100 µL of sesame oil by subcutaneous injection for 2 days. All mice were then randomly divided into 2 groups. One group was administered with sesame oil (control group), and the other group was administered with 1 subcutaneous injection of 2 mg of P4 (Sigma) in 100 µL of peanut oil (P4 group). The duration of exposure to E2 and P4 simulated the hormone levels in the murine estrous cycle (preovulatory ovarian estrogen directs epithelial cell proliferation on proestrous and estrous stage, whereas P4 initiates inhibition of epithelial cell proliferation on metestrous stage). The doses of E2 and P4 were in accordance with previously described method.²² All mice were killed, and their uteri were collected 24 hours after the last injection.

Endometrial epithelial cells were enzymatically isolated from the uteri in accordance with a previously described method with some modifications.²³ In brief, mouse uterine horns were cut into small pieces (2-3 mm³) on ice and washed 3 times with Hanks balanced salt solution (HBSS) without Ca²⁺/Mg²⁺ (HBSS + antibiotic). Dispase (6 g L⁻¹; Life Technologies, Inc, New York) and trypsin (25 g L⁻¹; Sigma) were used to digest the tissue at 4°C for 1 hour and for another 1 hour at room temperature. The uterine tissues were passed in and out through a bore pipette, and epithelial plaques were collected in a centrifuge tube. The purity of endometrial epithelial cells was certified by checking cytokeratin 19 by immunohistochemistry.²⁴ The purity was typically ≥95% (Supplementary Figure 5A). The collected cells that were washed 2 times in HBSS were either used for subsequent experiments or stored at -80°C.

Small RNA Library Construction and Solexa Sequencing

Two small RNA (sRNA) libraries pooled from endometrial epithelial cells treated with only E2 (control group, 10 mice) and E2 combined with P4 (P4 group, 10 mice) were constructed and sequenced using an Illumina/Solexa 1G high-throughput sequencer (Illumina, San Diego, California). Total RNA was extracted from endometrial epithelial cells using Trizol reagent (Invitrogen, Carlsbad, California) following the manufacturer's instructions. RNA quality was assessed using an Agilent 2100 bioanalyzer (Agilent, Palo Alto, California), and only samples with an RNA integrity number greater than 9 were used. The sRNA libraries were constructed using the total RNAs of each group. The overall flow of sRNA library construction and solexa sequencing and bioinformatics analysis is shown schematically in Supplementary Figures 1 and 2, respectively. From each group, 20 mg of total RNA was used for library construction using an sRNA sample prep kit (Illumina) following the manufacturer's instructions with minor modifications. In brief, the 18 nt to 30 nt fraction of total RNA was excised and purified by 15% Tris-borate-EDTA denaturing polyacrylamide gel electrophoresis. Subsequently, 3' and 5' RNA adaptors were ligated using T4 RNA ligase. The adaptor-ligated sRNAs were subjected to real-time polymerase chain reaction (RT-PCR) amplification, and the complementary DNA was further

Figure 2. Distribution of small RNAs among different categories for endometrial epithelial cells from progesterone (P4) and control group. The clean reads were annotated and classified as microRNA, ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA), in GenBank and Rfam databases.

amplified with 15 PCR cycles. The PCR products (90 bp, sRNA + adaptors) were purified on 4% agarose gels and used for sequencing analysis on an Illumina 1G Genome Analyzer (Illumina) at the Beijing Genomics Institute (BGI, Shenzhen, China). After masking the adaptor sequences and removing the contaminants, the clean reads were processed for computational analysis.

Bioinformatics Analysis for sRNAs

The basic figure from sequencing was converted into sequence data (raw reads) using the base-calling step. The raw reads were processed to obtain clean reads through the elimination of the following aspects: (1) low-quality reads; (2) reads without 3' primer; (3) reads with 5' primer contaminants; (4) reads without the inserted tag; (5) reads with poly A; and (6) reads shorter than 18 nt. The clean reads were mapped to the Mouse Genome Database (MGD)⁵⁶ using SOAP v1.11 software²⁵ to analyze their expression and distribution. Sequences with perfect matches were retained for further analysis. The sequences were aligned against the known miRNA precursors and mature miRNAs deposited in the miRBase 20.0 to identify conserved miRNAs. The clean reads were compared against the sRNAs (ribosomal RNAs, transfer RNAs, small nuclear RNAs, small nucleolar RNA, and miRNA) deposited in the GenBank and Rfam⁵⁷ databases to annotate the sRNA sequences using tag 2 annotation software (developed by BGI). Given that some sRNA tags could be mapped to more than 1 category, we used the following priority rules to ensure that every unique sRNA was mapped to only 1 annotation: Genbank > Rfam > known miRNA > repeat > exon > intron.

Differential Expression Analysis

To identify P4-induced miRNAs in mouse endometrial epithelial cells, the expression abundances of miRNAs in the P4 and control groups were normalized to obtain the expression in transcripts per million. If the normalized expression value of a given miRNA was 0, the expression value was modified to 0.01. If the normalized expression of a given miRNA was less

than 1 in both libraries, it was removed in differential expression analysis. The fold changes and *P* values were calculated from the normalized expression using the following formulas: the normalization was carried out as calculation of transcripts parts per million. Normalized expression = (Actual miRNA sequencing reads count/Total clean reads count) × 1 000 000.²⁶

Fold change = $\log_2(\text{P4/control})$.

P value:

$$p(x|y) = \binom{N_2}{N_1}^y \frac{(x+y)!}{x!y! \left(1 + \frac{N_2}{N_1}\right)^{(x+y+1)}} \quad C(y \leq y_{\min}|x) = \sum_{y=0}^{y \leq y_{\min}} p(y|x)$$

$$D(y \leq y_{\max}|x) = \sum_{y \geq y_{\max}}^{\infty} p(y|x)$$

N_1 and x represent the total count of clean reads and normalized expression, respectively, for a given miRNA in the control group sRNA library. N_2 and y represent the total count of clean reads and normalized expression, respectively, for a given miRNA in the P4 group sRNA library. The *P* values were corrected by false discovery rate (FDR) according to Benjamini-Hochberg correction, and *P* value (FDR) < .01 was considered significant.²⁷

Target Prediction and Pathway Analysis

We predicted the target genes of miRNAs in mice at a genome level. TargetScanMouse 6.2⁵⁸, miRDB⁵⁹ and miRanda⁶⁰ were used for target gene prediction. We selected the intersection of the 3 algorithms for each miRNA as its potential targets. Furthermore, GeneTrail online service was utilized for pathway analysis according to all differentially expressed potential targets of miRNAs.²⁸ The minimum number of genes of the potential pathway affected by differentially expressed miRNAs was 15, and the *P* values were corrected by FDR according to Benjamini-Hochberg correction, and *P* values (FDR) < .05 was considered significant.

Quantitative Real-time PCR

Differentially expressed miRNAs were validated by relative quantitative real-time PCR (qRT-PCR) according to the

manufacturer's protocol. The bulge-loop miRNA qRT-PCR primer sets (1 reverse transcription primer and a pair of quantitative PCR primers for each set) specific for each miRNA were designed by RiboBio (Guangzhou, China). Real-time PCR amplification was performed in a mixture of 16 mmol L⁻¹ real-time PCR Master Mix (Bio-Rad, California) and 500 nmol L⁻¹ of each primer in a final volume of 20 µL. Thermocycling was conducted using an Opticon DNA Engine (Bio-Rad, California). The values for relative quantification were calculated using the 2^{-ΔΔCt} method after the threshold cycle. Data were normalized to mouse Rnu6 (gene ID: 19862, NR_003027.2) levels to compensate for unequal amounts of miRNA in the samples. Data were then exported into an Excel workbook for analysis.

Antibody Array

The array used was the Master Antibody Microarray (AA0008, consisting of 656 antibodies in duplicates; Abnova, Taiwan). A total of 518 antibodies can be used for mice. An Antibody Array Detection Kit (AA0011; Abnova) was used following the manufacturer's protocol. Lysis beads were used for protein extraction, and a spin column was used for protein purification. The purified proteins with an ultraviolet absorbance greater than 40 optical density were used for subsequent experiments. After biotinylation, blocking, and coupling, proteins were incubated with Cy3-streptavidin for 20 minutes at room temperature in the dark. After washing the proteins, the wash solution was discarded. The fluorescence signals were scanned by a GenePix 4000B laser scanner (Axon Instruments, Sunnyvale, California) and analyzed with the Genepix software package (Axon Instruments, Inc).

Primary Culture and Treatment of Endometrial Epithelial Cells and Stromal Cells

The ovariectomized mice were primed with 100 ng of E2 for 2 days. Endometrial epithelial cells were then enzymatically isolated according to the aforementioned methods. After epithelial plaques were collected, the residual cells were incubated in fresh HBSS containing 0.5 mg L⁻¹ collagenase (Sigma) at 37°C for 30 minutes and then passed through a 70-µm filter to obtain the stromal cells.²⁹ The purity of endometrial stromal cells was certified by checking desmin. The purity was typically ≥ 95% (Supplementary Figure 5A). Both epithelial and stromal cells were grown in 12-well plates with complete media, consisting of phenol red-free Dulbecco's modified Eagle medium (DMEM)/F12 (Life Technologies, Inc) supplemented with charcoal-stripped 10% fetal bovine serum (FBS). The cells were allowed to grow for 2 days in fresh complete media and depleted in phenol red-free DMEM/F12 supplemented with charcoal-stripped 1% FBS for 1 day. Both epithelial and stromal cells were then treated with E2 (10⁻⁸ mol L⁻¹) as the control or various combinations of E2 (10⁻⁸ mol L⁻¹), P4 (10⁻³, 10⁻⁶, and 10⁻⁹ mol L⁻¹), and RU486

(10⁻⁶ mol L⁻¹; Sigma) for 24 hours. The cells were harvested for RT-PCR to detect the expression of miRNA.

Transient miRNA Antagomir Transfection

Ovariectomized mice were primed with 100 ng of E2 for 2 days. Transfection was performed according to the manufacturer's instructions (RiboBio). The antagomirs were dissolved in sterile PBS. The antagomirs for mmu-mir-145a-5p were directly injected into one side of the uterine horn for each mouse, and the control antagomirs were injected into the contralateral horn. After transfection, the mice were treated with a single subcutaneous injection of 2 mg of P4 in 100 µL of peanut oil. After 24 hours, mice were killed and their uteri were collected. Endometrial epithelial cells were enzymatically isolated from each uterine horn according to the aforementioned methods.

Cell Cycle Analysis by Flow Cytometry

The cell cycle progression of purified endometrial epithelial cells was assessed using flow cytometry. The cells were suspended in cold 70% ethanol for 10 hours at 4°C. The cells were then centrifuged at 1000 rpm for 5 minutes. The pellets were washed 2 times with cold PBS and incubated with 5 µL of RNase (20 µg mL⁻¹ final concentration) at 37°C for 30 minutes. The cells were chilled over ice for 10 minutes, stained with propidium iodide (50 µg mL⁻¹ final concentration) for 5 minutes and analyzed by flow cytometry. Flow cytometry was performed using an Epics Elite ESP instrument (Beckman Coulter, California). DNA histograms were further used for cell distribution analysis at different stages of the cell cycle.

Statistical Analysis

Statistical analyses for solexa sequencing and pathway analysis have been described previously. The data of qRT-PCR were analyzed using Student *t* test, and the results of cell cycle analysis were analyzed with the Wilcoxon signed rank test according to the paired design. A *P* value <.05 was considered significant.

Results

Small RNA Library Construction and Solexa Sequencing

A total of 13 203 894 and 12 037 813 raw reads were provided for endometrial epithelial cells from the P4 and control groups, respectively. After removing the low-quality reads, adaptors, and insufficient tags, 12 715 086 and 11 836 869 clean reads of 18 nt to 30 nt were ultimately obtained (Table 1). A total of 9 331 471 P4 sequences and 8 977 258 control sequences accounted for 73.39% and 75.84% of the total reads, respectively (Table 1). These sequences were perfectly mapped to the MGD. All identical sequence reads were grouped together to simplify the sequencing data. A total of 847 578 and 829 791 unique sequences for the P4 and control groups remained, respectively, for further analysis

Table 1. Summary of Solexa Sequencing Data for Small RNAs in P4 and Control Group.^a

Categories	P4 Group		Control Group	
	Unique sRNA	Total sRNA	Unique sRNA	Total sRNA
Raw reads		13 203 894		12 037 813
Clean reads	847 578	12 715 086	829 791	11 836 869
Perfect match to mouse genome	595 270	9 331 471	617 994	8 977 258
Specific sequences	728 031	897 406	710 244	852 475
Common sequences	Unique sRNAs:	119 547	Total sRNAs:	22 802 074
Total	Unique sRNAs:	1 557 822	Total sRNAs:	24 551 955

Abbreviations: P4, progesterone; sRNA, small RNA.

^a Unique sRNA means the types of small RNA; total sRNA means the numbers of small RNA; specific sequence means specific small RNAs in each group; common sequence means the same small RNAs in both groups.

Table 2. The Most Abundance MicroRNAs in P4 and Control Group.^a

MicroRNA	Normalized Expression		Fold Change	P Value (FDR)	Sig-lable
	P4	Control			
mmu-let-7c-5p	152197.2403	172467.8207	-0.18038501	0	
mmu-let-7f-5p	124539.7003	114495.2267	0.12131826	0	
mmu-let-7b-5p	76728.9344	108742.523	-0.50307358	0	
mmu-let-7a-5p	60675.091	59153.9029	0.03663101	9.10e-53	
mmu-miR-29a-3p	9441.3046	7031.4202	0.42517011	0	
mmu-miR-1a-3p	8157.7112	565.6901	3.85008061	0	b
mmu-miR-21a-5p	7794.8352	8831.9808	-0.18021851	7.50e-174	
mmu-let-7d-5p	7537.0312	7380.1611	0.03034406	1.38e-05	
mmu-let-7e-5p	7165.8973	7223.1939	-0.01148953	.11857838	
mmu-let-7g-5p	6319.5011	6289.5855	0.00684572	.396369754	
mmu-miR-192-5p	4330.9971	11214.1986	-1.37255541	0	b
mmu-miR-320-3p	4856.1213	7954.1304	-0.71189976	0	

Abbreviation: FDR, false discovery rate; P4, progesterone.

^a Normalized expression = (Actual microRNA sequencing reads count/Total clean reads count) × 1 000 000; fold changes = $\log_2(\text{P4/control})$; P values manifest the significance of microRNAs differential expression between 2 groups.

^b Significant difference.

(Table 1). The size distribution of the sRNAs of the total small sequences in the 2 libraries was similar (Figure 1). The lengths of the majority of sRNAs ranged from 21 nt to 24 nt, and the most abundant class size in the sRNA sequence distribution was 22 nt, which accounted for 43.25% and 49.00% in the P4 and control groups, respectively.

To assess the efficiency of high-throughput sequencing for sRNA detection, all clean sequence reads were also annotated and classified through alignment with GenBank and Rfam databases. Classification annotation revealed that 9 358 569 and 9 275 701 reads were annotated and classified as miRNA in the P4 and control groups, respectively (Figure 2).

Differential Expression of Known Conserved miRNAs

A total of 6 836 012 and 6 742 592 miRNA precursor sequences (representing 5122 and 4013 unique miRNA precursors and 551 and 509 unique mature miRNAs, respectively) in the P4 and control groups, respectively, had perfect matches to known mouse miRNAs deposited in miRBase 20.0. Generally, only mature miRNA can act on their target gene mRNA

3-untranslated region. Thus, we analyzed the differential expression of miRNA with mature miRNA sequences. After the removal of normalized miRNAs with expression of less than 1 in both groups, 358 known mature miRNAs remained for differential expression analysis (Supplementary Table 1).

The top 10 highly expressed miRNAs in the P4 group were mmu-let-7c-5p, mmu-let-7f-5p, mmu-let-7b-5p, mmu-let-7a-5p, mmu-miR-29a-3p, mmu-miR-1a-3p, mmu-miR-21a-5p, mmu-let-7d-5p, mmu-let-7e-5p, and mmu-let-7g-5p. The top 10 highly expressed miRNAs in the control group were mmu-let-7c-5p, mmu-let-7f-5p, mmu-let-7b-5p, mmu-let-7a-5p, mmu-miR-192-5p, mmu-miR-21a-5p, mmu-miR-320-3p, mmu-let-7d-5p, mmu-let-7e-5p, and mmu-miR-29a-3p. Their expression levels exceeded 7000 reads per 1 000 000 reads (Table 2). Compared with miRNA expression in the control group, 146 mature miRNAs in the P4 group were significantly upregulated with fold change ($\log_2 \text{P4/control}$) > 1 and P value < .01, and 17 mature miRNAs were significantly downregulated with fold change ($\log_2 \text{P4/control}$) < -1 and P value < .01 (Figure 3 and Supplementary Table 1). The differentially expressed miRNAs had fold changes ranging from 1-fold to

Figure 3. Comparison of expression levels of microRNAs in endometrial epithelial cells from P4 and control group. The horizontal and vertical axes show the expression levels of microRNAs in the 2 groups. The red points represent microRNAs with fold changes >2 and P value $<.01$; the blue points represent microRNAs with $1/2 < \text{fold changes} < 2$; the green points represent microRNAs with fold changes $<1/2$ and P value $<.01$. Fold changes = microRNA expression levels of P4 group/microRNAs expression levels of control group. P4 indicates progesterone. (The color version of this article is available at <http://rs.sagepub.com>.)

15-fold. Among the upregulated miRNAs, mmu-miR-3473e expressed the highest fold change. Among the downregulated miRNAs, mmu-miR-148b-5p expressed the highest fold change. The members of the mmu-let-7 family exhibited high expression levels in both groups, but no differential expression was observed between the 2 groups.

Validation of miRNA Expression

Quantitative RT-PCR was applied to validate the reliability of the sequencing data. Nine highly expressed miRNAs in the P4 group were randomly selected for further analysis to compare the expression levels between the P4 and control group. The results show that the expression levels of miR-23a-3p, mmu-miR-26a-5p, mmu-miR-1a-3p, mmu-miR-133a-3p, mmu-miR-195a-5p, mmu-miR-3473b, mmu-miR-204-5p, mmu-miR-145a-5p, and mmu-miR-143-3p in the P4 group were higher than those in the control group (Figure 4). The expression patterns were consistent with the solexa sequencing results.

Target Gene Prediction and Pathway Analysis

The main function of miRNA is posttranscriptional regulation of its target gene expression. Thus, target prediction is critical for the investigation of miRNA function. A total of 1280

potential target genes (1155 genes for upregulated miRNA and 125 genes for downregulated miRNA in the P4 group compared with the control group) were predicted for 163 differentially expressed miRNAs (Supplementary Table 2).

Pathway analysis using GeneTrail showed that the upregulated miRNAs in the P4 group participated in 22 possible Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways ($FDR < .05$). These pathways were involved in Wnt/ β -catenin signaling, focal adhesion, tight junctions, regulation of actin cytoskeleton, pathways in cancer, cell cycle, and so on (Table 3). The GO enrichment analysis from cellular components showed that 978 (84.68%) genes were clustered into intracellular regions, and 350 (33.30%) genes were clustered into the nucleus. The analysis of molecular function showed that 1081 genes were assigned different functions, and most of the functions were related to binding activity in 799 (69.18%) genes. The results from GO enrichment analysis are shown in Supplementary Material (Supplementary Table 3 and Supplementary Figure 3).

The KEGG pathway analysis showed that the downregulated miRNAs in the P4 group were not enriched in any pathway ($FDR > .05$). However, GO enrichment analysis from cellular components showed that 102 (81.6%) genes were clustered into intracellular regions. The analysis of molecular function showed that 123 genes were assigned different functions, and most of the functions were also related to binding activity in 87 (69.60%) genes. The results from GO enrichment analysis are shown in Supplementary Material (Supplementary Table 4 and Supplementary Figure 4).

Validation of Target Protein Expression

We selected an antibody array to validate miRNA target gene expression. In the results, we found 82 downregulated proteins in the P4 group ($P4/\text{control expression} < 0.77$) and 85 upregulated proteins in the P4 group ($P4/\text{control expression} > 1.3$; Supplementary Table 5). Among the 82 downregulated proteins, 20 proteins were consistent with the potential targets for P4 upregulating miRNA (Ywhag, Vegfa, Tgfb2, Tfdp2, Slc2a3, Slc2a1, Pdgfra, Mmp15, Mc11, Lamc1, Ikbkb, Gria1, Gabra1, Cul2, Ctnna1, Cldn2, Ccne2, Ccne1, Bcl6, and APC).

Western blot was applied to validate the reliability of the antibody array results. Five proteins were selected for further analysis to compare the expression levels between the P4 and control groups. The results show that the expression levels of Cdkn2a, Ccnd1, Ccne1, and Ccne2 were consistent with the antibody array results. But the expression of Pcnal was lower in the P4 group than in control group (Supplementary Figure 5B).

mmu-mir-145a-5p is Involved in the Antiproliferative Action of P4 on Endometrial Epithelial Cells

To investigate whether the P4 upregulating miRNAs affect the proliferation of endometrial epithelial cells, we chose miR-

Figure 4. Relative expression of 9 differentially expressed microRNAs validated by quantitative real-time polymerase chain reaction (PCR). miR-23a-3p, mmu-miR-26a-5p, mmu-miR-1a-3p, mmu-miR-133a-3p, mmu-miR-195a-5p, mmu-miR-3473b, mmu-miR-204-5p, mmu-miR-145a-5p, and mmu-miR-143-3p were upregulated in progesterone (P4) group compared with control group. The relative quantification of expression was calculated using the $2^{-\Delta\Delta C_t}$ method after the threshold cycle (C_t) and was normalized with the C_t of Rnu6. The relative expression levels were presented as the $2^{-\Delta\Delta C_t}$ means $\pm$ standard errors (SEs). The error bars indicate the standard error of the $2^{-\Delta\Delta C_t}$ mean values. Statistical significance was determined by Student t test, * represents $P < .05$ was considered significant. $n = 5$.

145a, a tumor-suppressing miRNA, to examine its function in the cell cycle. Target gene prediction showed that miR-145a could act on cyclin D2 and cyclin-dependent kinase 6 (CDK6), which were directly involved in cell cycle regulation. The results show that mmu-mir-145a-5p was expressed in a dose-dependent manner to P4 in cultured mouse endometrial epithelial cells. The expression of mmu-mir-145a-5p increased with the increase in P4 (Figure 5A). And the expression of mmu-mir-145a-5p was reduced to control levels following P4 + RU486 treatment (Figure 5B). In normal endometrial epithelial cells, P4 can induce cell cycle arrest in the G_1 -S checkpoint with low cell population in S phase and high population in G_1 phase.³⁰ To ascertain whether mmu-mir-145a-5p regulates the proliferation of endometrial

epithelial cells, antagomirs were used in specifically blocking the function of mmu-mir-145a-5p in endometrial epithelial cells in vivo. The results show that antagomirs for mmu-mir-145a-5p slightly decreased the population of endometrial epithelial cells at G_1 phase and increased the cell population at S phase compared with the control antagomirs (Figure 5C and D). These results indicate that mmu-mir-145a-5p was involved in the anti-proliferative action of P4 on endometrial epithelial cells.

The expression of mir-145a was also examined in cultured endometrial stromal cells. The results show that mir-145a was also expressed in a dose-dependent manner to P4 in vitro (Figure 5A). And the expression of mmu-mir-145a-5p was reduced to control levels following P4 + RU486 treatment (Figure 5B).

Table 3. KEGG Pathway Analysis for Upregulated MicroRNA in the P4 Group.

Category	Subcategory	Expected Gene Number	Observed Gene Number	P Value (FDR)
KEGG	Axon guidance	8.08089	34	6.10034e-12
KEGG	Focal adhesion	12.0341	32	2.37017e-06
KEGG	Prostate cancer	5.46478	20	2.37017e-06
KEGG	Pathways in cancer	19.9988	42	1.60741e-05
KEGG	Glioma	4.30206	16	1.78204e-05
KEGG	Renal cell carcinoma	4.41833	16	2.14785e-05
KEGG	Pancreatic cancer	4.36019	15	6.97965e-05
KEGG	Neurotrophin signaling pathway	8.37157	22	7.48029e-05
KEGG	Oocyte meiosis	7.32512	20	8.85589e-05
KEGG	Wnt signaling pathway	9.30175	23	.000106373
KEGG	MAPK signaling pathway	16.2781	33	.000149678
KEGG	Small-cell lung cancer	5.46478	16	.000173494
KEGG	TGF- β signaling pathway	5.29037	15	.000384718
KEGG	Tight junction	8.37157	20	.000384735
KEGG	Regulation of actin cytoskeleton	13.3131	26	.00116469
KEGG	Ubiquitin-mediated proteolysis	8.83666	19	.00180909
KEGG	Chagas disease	6.68563	15	.00359197
KEGG	Cell cycle	8.08089	17	.00363103
KEGG	Chemokine signaling pathway	11.8016	22	.0041787
KEGG	Insulin signaling pathway	8.48784	17	.00520242
KEGG	Leukocyte transendothelial migration	7.09258	15	.00520242
KEGG	Endocytosis	13.8945	22	.022916

Abbreviations: FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Discussion

In endometrial epithelial cells, P4 antagonizes the effects of estrogen in many aspects, such as gene transcription,² cell proliferation,³ differentiation,⁴ substance transportation.^{5,6} The effects of E2 and P4 in the uterus are generally facilitated by their nuclear receptors, ER and PR, respectively. P4, through binding with PR, can directly promote the expression of numerous transcription factors to antagonize estrogen regulation of its downstream gene transcription.² Our results indicate that upregulated miRNAs by P4 could be involved in the regulation of some transcription factors. Thus, miRNA-mediated posttranscriptional inhibition of transcription factors may be a potential mechanism of P4 opposing estrogen.

Substance transportation, which is an important function of endometrial epithelial cells for preimplantation embryo development and blastocyst implantation, is also regulated by estrogen and P4. By adjusting the transportation of various substances, estrogen and P4 regulate the uterine luminal fluid volume, pH, metabolites, ionic compositions, and nutrients.³¹ The concentrations of glucose in uterine fluid are lower in the proestrus stage than those in the metestrous stage. Some studies showed that most glucose transporters (SLC2A family) are highly expressed in luminal and glandular epithelial cells of the estrous stage but poorly expressed in endometrial epithelial cells of the metestrous and diestrous stages.³² Our results suggest that P4-induced miRNAs were clustered into transmembrane transporter activity and possibly involved in the regulation of glucose transporter function in endometrial epithelial cells.

Wnt/ β -catenin signaling is recognized as an important regulatory pathway of endometrial proliferation and differentiation.³³

A central feature in Wnt signaling is the destruction complex, which is a multiprotein complex consisting of the scaffold proteins AXIN1, AXIN2, β -catenin, APC, casein kinase 1 (CK1), and glycogen synthase kinase 3 β (GSK-3 β).³⁴ During the menstrual cycle, E2 enhances Wnt/ β -catenin signaling in the proliferative phase, whereas P4 inhibits Wnt/ β -catenin signaling.³⁵ However, the putative mechanisms underlying E2- and P4-mediated Wnt/ β -catenin activation in the uterus are poorly understood. Our results showed several key proteins of the destruction complex, such as GSK-3 β , APC, β -catenin, and CK1, were potential target genes of P4-induced miRNAs (mmu-miR-26a, mmu-miR-150, mmu-miR-214, and mmu-miR-30). These results suggest that miRNAs could be an important medium for P4 to regulate Wnt/ β -catenin signaling.

In a normal uterus, P4 antagonizes the effect of estrogen on cell proliferation to inhibit the proliferation of endometrial epithelial cells.³ The cell cycle is regulated by the actions of the cyclin family members with their CDK partners.³⁶ Tong and Pollard demonstrated cyclin D1 localization as a key point of regulation in endometrial epithelial cells.¹ E2 causes its nuclear accumulation, and P4 retains it in the cytoplasm by inhibiting GSK-3 β activity.³⁷ In our study, both KEGG pathway and GO enrichment analysis showed that upregulated miRNAs in the P4 group were involved in the cell cycle. Cyclin D1, cyclin D2, CDK6, cyclin E1, and cyclin E2 were potential target genes of upregulated miRNAs (mmu-miR-23a, mmu-miR-145a, mmu-miR-494, mmu-miR-1a, and mmu-miR-143) in the P4 group. Antibody array and Western blot validated that the expression levels of cyclin E1 and cyclin E2 were downregulated in the P4 group. Surprisingly, PCNA, as a proliferation

Figure 5. A, Expression of mmu-miR-145a-5p detected by quantitative real-time polymerase chain reaction (PCR). Both cultured epithelial cells and stromal cells were treated with only E2 (10^{-8} mol L⁻¹) as control or various combinations of E2 (10^{-8} mol L⁻¹) and P4 (10^{-3} mol L⁻¹, 10^{-6} mol L⁻¹, and 10^{-9} mol L⁻¹) for 24 hours. The vertical axis represented the mmu-miR-145a-5p relative expression ratios of E2 combining P4-treated group to E2 alone treated group. This result was repeated 5 times. B, Both cultured epithelial cells and stromal cells were treated with only E2 (10^{-8} mol L⁻¹) as control or various combinations of E2 (10^{-8} mol L⁻¹) + P4 (10^{-6} mol L⁻¹) and E2 (10^{-8} mol L⁻¹) + P4 (10^{-6} mol L⁻¹) + RU486 (10^{-6} mol L⁻¹) for 24 hours. C and D, Flow cytometric analysis cell cycle of endometrial epithelial cells transfected with the antagonist specifically blocking the function of mmu-miR-145a-5p or control antagonist in vivo. The cell population in G₁ phase treated with antagonists for mmu-miR-145a-5p is lower than that in control. And the cell population in S phase treated with antagonists for mmu-miR-145a-5p is higher than that in control. The cell population percentages of cell cycle were analyzed with the Wilcoxon signed rank test according to the paired design, and this result was statistically significant ($P < .05$, $n = 5$). E2 indicates estradiol; P4, progesterone.

marker, increased in the P4 group in antibody array result. It was contrary to other results. Western blot revealed that this is an obvious experimental error of antibody array. This discrepancy prompts that the antibody array needs to be further verified by other methods.

We selected an upregulating miRNA to investigate whether these miRNAs regulate the proliferation of endometrial epithelial cells. MiR-145a (homologous with human mir-145), a major tumor-suppressing miRNA, has a crucial function in regulating smooth muscle cell differentiation³⁸ and inducing apoptosis.³⁹ MiR-145a is downregulated in many cancers, including endometrial,⁴⁰ prostate,⁴¹ bladder,^{39,42} and colon.⁴³ Studies on colon cancer and oral squamous cell carcinoma suggest that miR-145a may be directly involved in cell cycle regulation.^{44,45} Target gene prediction showed that miR-145a could act on cyclin D2 and CDK6, which were directly involved in cell cycle regulation. Our results indicate that mmu-mir-145a may participate in the effect of P4 on the antiproliferation of endometrial epithelial cells. However, this conjecture still requires further studies.

Estradiol and P4 have a direct function in endometrial epithelial cells. Estrogen can directly regulate some genes expression, such as PR, in isolated endometrial epithelial cells.⁴⁶ A recent study on ablation of PR in the uterine epithelium of mice demonstrated that PR in the uterine epithelium is critical for the functions of P4, such as antiproliferation regulation of epithelial target gene expression and uterine function and development.⁴⁷ Without endometrial stromal cells, E2 cannot promote isolated mouse endometrial epithelial cell proliferation in vitro.⁴⁸ The importance of stromal PR in P4 action in the endometrium has been established by epithelial and stromal tissue recombination studies from neonatal wild-type and PR knock out mice.⁴⁹ Progesterone induces paracrine growth inhibitors in the stromal cells, such as fibroblast growth factors,⁵⁰ to act on epithelial cells in a paracrine manner.⁵¹ Thus, E2 and P4 possibly have both direct and indirect functions to specifically regulate the expression of miRNA in epithelial cells. The overall role of estrogen and P4 regulation of miRNA expression has been reviewed recently.⁵²⁻⁵⁴ However, studies about miRNA in normal endometrium are relatively rare. No systematic and elaborate study has profiled miRNA through the entire menstrual cycle using both endometrial epithelial and stromal cells, as has been done at the mRNA level. Using in vitro cultures of primary endometrial stromal or epithelial cells from normal women, Chegini group showed that E2 decreased stromal cell expression of miR-20a, miR-21, miR-23a, and miR-23b but increased expression of miR-26a, miR-17-5p, and miR-542-3p. While medroxyprogesterone acetate decreased stromal cell expression of miR-20a, miR-21, miR-26a, and miR-23a but increased expression of miR-17-5p and miR-542-3p. In glandular epithelial cells, E2 decreased expression of miR-20a, miR-21, miR-542-3p, and miR-23a but increased expression of miR-26a, miR-17-5p, and miR-23b. While medroxyprogesterone acetate increased miR-20a, miR-26a, miR-23a, miR-17-5p, and miR-23b but decreased miR-21 and miR-542-3p expression.^{20,55} These studies suggest a complicated mechanism for steroidal regulation of miRNAs in endometrial stromal and glandular epithelial cells. MicroRNA

may play a role in cross-talk between the uterine epithelium and stroma, but so far, there is no direct study on the relationship of miRNA and this cross-talk. In this study, we found that mir-145a expression was P4 dependent in cultured mouse endometrial epithelial cells and stromal cells in vitro. These results indicate that mir-145a could be directly regulated by P4, which influences its target gene posttranscription. Further studies are necessary to determine its function in endometrial stromal cells.

In conclusion, P4 could specifically regulate the expression of miRNAs in endometrial epithelial cells, which possibly facilitated the effect of P4 on the structure and function of the endometrial epithelium by regulating their target genes.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

The online supplementary figures and tables are available at <http://rs.sagepub.com/supplemental>

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