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# Genomic Profiling of MicroRNAs and Messenger RNAs Reveals Hormonal Regulation in MicroRNA expression in Human Endometrium

**Abbreviated title:** microRNA regulation in the human endometrium
**Summary sentence:** Global microRNA and mRNA expression arrays demonstrate distinct gene expression signatures in the human late proliferative phase and mid-secretory phase endometrial epithelium.
**Key words**: cell cycle, endometrium, microarray, microRNA

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## ABSTRACT

MicroRNAs (miRNAs), a class of small non-coding RNAs that regulate gene expression play fundamental roles in biological processes, including cell differentiation and proliferation. These small molecules mainly direct either target mRNA degradation or translational repression, thereby functioning as gene silencers. Epithelial cells of the uterine lumen and glands undergo cyclic changes under the influence of sex steroid hormones, E2 and P4. Because the expression of miRNAs in the human endometrium has been established, it is important to understand whether miRNAs have a physiological role in modulating the expression of hormonally induced genes. The studies herein establish concomitant differential miRNA and mRNA expression profiles of uterine epithelial cells purified from endometrial biopsies in the late proliferative and mid-secretory phase. Bioinformatics analysis of the differentially expressed mRNAs revealed the cell cycle as the most significantly enriched pathway in the late proliferative phase endometrial epithelium ($P = 5.7 \times 10^{-15}$). In addition, the WNT signaling pathway was enriched in proliferative phase. The twelve miRNAs (MIR29B, MIR29C, MIR30B, MIR30D, MIR31, MIR193A-3B, MIR203, MIR204, MIR200C, MIR210, MIR582-5p, and MIR345) whose expression was significantly upregulated in the mid-secretory phase samples were predicted to target many cell cycle genes. Consistent with the role of miRNAs in suppressing their target mRNA expression, the transcript abundance of the predicted targets, including cyclins and cyclin-dependent kinases as well as *E2F3* (a known target of MIR210), was decreased. Thus, our findings suggest a role for miRNAs in down regulating the expression of some cell cycle genes in the secretory phase endometrial epithelium, thereby suppressing cell proliferation.

## INTRODUCTION

The human endometrium undergoes cyclic changes regulated by the female sex steroid hormones, estradiol-17β (E2) and progesterone (P4). E2 elicits a wave of uterine epithelial cell proliferation whereas progesterone inhibits this E2 induced epithelial cell proliferation, promotes differentiation and has decidualizing effects on endometrial stroma later in the secretory phase. E2 and P4 act through their cognate nuclear receptor transcription factors, estrogen receptor 1 (ERS1) and progesterone receptor (PGR). Experiments using tissue recombinants of uterine epithelium and stroma whose estrogen status differ because of inactivating mutation suggest that E2 stimulates uterine epithelial cell proliferation through epithelial ESR1 [1]. This contrast to the situation in mice where E2 acts on epithelial cell proliferation via paracrine influences downstream of the binding of E2 to stromal ESR1 and E2 induced uterine epithelial cell proliferation is inhibited by P4 actions mediated via PGR in the stroma [1, 2]. Coordinated and synchronized action of E2 and P4 is essential for controlled proliferation of the endometrium and for uterine receptivity at the time of implantation. Identification of the molecular mechanisms involved in normal hormonal regulation of human endometrium is therefore an important step towards understanding molecular deregulation occurring in pathological situations and also may expose novel therapeutic opportunities.

E2 elicits rapid responses in the human and mouse uterus by activating the so called canonical cell cycle pathway [2-4]. In the mouse uterus, paracrine signaling through the epithelially expressed insulin-like growth factor 1 (IGF1) receptor leads to the activation of PI3-kinase pathway followed by AKT activation that results in an inhibitory phosphorylation of glycogen synthase kinase 3 beta (GSK3B) thereby allowing accumulation of cyclin D1 in the nucleus, retinoblastoma protein phosphorylation and the progress of the cells through the restriction point into S-phase [2, 4, 5]. In addition, parallel activation of DNA replication licensing involving the minichromosome maintenance proteins (MCMs) is necessary for cell cycle progression into S-phase in the mouse endometrial endometrium [6]. However, in the more complex regenerative human endometrium the molecular mechanisms underlying its cyclic changes are not as clearly elucidated. Our previous studies on the human endometrial biopsies demonstrated increased epithelial cell expression of markers of proliferation (MKI67, PCNA and MCM2) and cell cycle regulatory proteins (cyclin A1, CCNA and cyclin E1, CCNE1) during the proliferative phase of the menstrual cycle compared with low expression in the secretory phase epithelium [7]. Further inhibition of GSK3B by lithium chloride in reconstructed human endometrial biopsies xenotransplanted into immunocompromised mice resulted in epithelial cells entry into S-phase [8]. These results provide evidence that similar cell cycle regulators are activated by E2 and inhibited by P4 in the human endometrium as in the mouse [7].

MicroRNAs (miRNA), a class of small non-coding RNAs, function as post-transcriptional regulators of gene expression. MicroRNAs base-pair with the 3' untranslated regions (UTRs) of their target mRNAs, leading to mRNA degradation or translational repression [9]. The 5' sequences of the miRNAs, particularly those at nucleotide positions 2-7 relative to the 5' end of the miRNA, are important for binding to the target. Individual targets of miRNAs responsible for the phenotypes have been proposed in experimental settings, although it is likely that many miRNAs function through co-operative regulation of multiple mRNAs [10].

While it is established that miRNAs are expressed in the human endometrium [11, 12], the hormonal regulation of miRNAs in the function of human endometrial epithelium and their role in differentiation of endometrium into its receptive state still remain unknown. MicroRNAs have been reported to regulate many cellular processes that are known to occur during the cyclic changes in the endometrium such as cell proliferation and differentiation [13]. Interestingly, aberrant miRNA expression has been associated with human endometrial disorders such as endometriosis, endometrial hyperplasia and carcinoma [11, 12, 14, 15]. In addition, miRNAs were recently described to play a role in the postnatal development of

mouse uterus and oviducts [16-18] and in mouse embryo implantation [16, 19, 20]. Taken together, the previous human and mouse studies strongly suggest that in human endometrium, miRNAs are likely to have a regulatory function during the physiologic cycle phases.

In the present study, high density gene expression arrays were utilized to identify specific miRNAs involved in the hormonal regulation of the normal human endometrium by E2 and P4. Well characterized endometrial biopsy samples were taken from eight healthy mid-reproductive aged women, and simultaneous mRNA and miRNA profiles were established for endometrial epithelial cell preparations either during late proliferative or mid-secretory phase of the menstrual cycle. The rationale for examining the endometrial epithelium during the time of maximum proliferation (late proliferative phase) and maximum progesterone action (mid-secretory phase) was to elucidate the action of progesterone opposing estrogen effects. The expression profiling of mRNAs and microRNA in the same samples allowed us to identify specific mRNAs that may be post-transcriptionally repressed by the miRNAs in the human endometrial epithelium and to determine some cellular functions and molecular pathways targeted by these differentially expressed miRNAs.

## MATERIALS AND METHODS

### General Participants

This study was approved by The Clinical Investigations/Institutional Review Board at Albert Einstein College of Medicine (AECOM) and was conducted in accordance with the Declaration of Helsinki for Medical Research involving Human Subjects at the General Clinical Research Center (GCRC). Written, informed consent was obtained from all participants of this study.

Participants were recruited from the community and were healthy volunteer women aged between 18 to 36 years, with no history of infertility, who met the following criteria: 1. regular 25-35 day menstrual cycles; 2. No use of hormonal contraception within 3 months; 3. At least 90% normal weight for height [21]. Screening done solely for study purposes included history and physical exam, a negative cervical cytology, a negative urine pregnancy test and normal saline hysterosonogram to rule out any intrauterine pathology. Sixteen women attended the GCRC for endometrial biopsy and 14 biopsies were performed. Endometrial biopsies from total of eight women, 4 late proliferative phase and 4 mid-secretory phase, met the criteria and were analyzed during the course of the study. The study participant characteristics are detailed in Supplemental Table S1 (all Supplemental Data are available online at www.biolreprod.org).

### Endometrial Biopsy Protocol

Endometrial biopsy samplings from the fundal area, as outlined previously [7], were performed using a Pipelle catheter (Unimar, Inc, Wilton, CT). Portions of the tissues were saved in formalin, 10% vol/vol solution for hematoxylin and eosin (H&E) staining, and for histological evaluation. The main portion of the tissues was further processed for endometrial epithelial cell isolation.

Late proliferative phase biopsies were performed on cycle day (CD) 12 ± 1 to target the time of maximal endometrial response to $E_2$ [22]. Serum $E_2$ and $P_4$ were obtained on the day of the biopsy; the sample was excluded if $P_4 \geq 3.5$ ng/ml, indicative of ovulation [23]. To target the endometrial window of receptivity and maximum progesterone action, secretory biopsies were taken during the mid luteal phase on CD 19-23. Mid-secretory phase was confirmed post-biopsy by serum $P_4$ level $\geq 3$ ng/ml and by histology [23, 24]. The histological evaluation of the H&E stained tissue specimens was performed blindly by two persons, and cycle phases were assigned according to the published criteria [24]. Histological dating was correlated with CD and blood E2 and P4 levels on the day of the endometrial biopsy. Dating of all biopsies was within a 2-day window from the cycle day as calculated from the last menstrual period (LMP).

**Serum Hormone Assays**

Fluoro-immunoassays were used to measure serum $E_2$ and $P_4$ levels (Perkin-Elmer Life and Analytical Sciences; Wallac; Turku, Finland) as previously described [7, 25]

**Epithelial Cell Isolation**

After sampling, the endometrial biopsies were transported to the laboratory in McCoy modified 5A medium on ice. Isolation of the epithelial cells from stromal cells was performed according to the modified protocol by Satyaswaroop et al. [26]. Briefly, after removal of blood and mucus, the endometrial biopsy was minced into 1 mm pieces. Minced pieces were digested with type I collagenase (Worthington, Lakewood, NJ; 230u/mg) in modified McCoy modified 5A medium containing 20,000 IU/ml DNase I (Sigma, saint Louis, MI) at 37 ºC for 1 hour. Collagenase treated cells were centrifuged and suspended into RNase free PBS. The suspension was strained through a 80 um nylon mesh where the epithelial cell microaggregates retain in the mesh, whereas stromal cells and red blood cells filter through. Isolated epithelial cells were then washed with RNase free PBS, centrifuged and stored in TRIzol Reagent (Invitrogen, Carlsbad, CA) at -80 ºC. The purity of the epithelial cell preparation was determined by intracellular immunohistochemistry (IHC) using anti-Pan cytokeratin antibody (clone PCK-26, Sigma, Saint Louis, MI) on isolated epithelial cell preparation. Epithelial cells were attached and fixed with ethanol onto Superfrost plus slides. The fixative was rinsed off with PBS and slides were incubated with Triton X-100, 0.3% vol/vol solution in PBS for 10 minutes to permeabilize the membranes followed by IHC. The stained cells were observed under light microscopy. The number of cells showing cytoplasmic cytokeratin staining (only epithelial cells and not stromal cells stain positive for cytokeratin) indicated that the isolation procedure yielded >90% pure endometrial epithelial cell preparation (data not shown).

**RNA Extraction**

Total RNA and miRNA-enriched RNA were extracted from isolated uterine epithelial cells using MirVana miRNA Isolation Kit (Ambion, Austin, TX) following the manufacturer's protocol. RNA yield and quality was assessed by electrophoresis using the Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA) and spectrophotometric analysis.

**Sample and Micro Array Processing, HG U133 Plus 2.0 Array**

Eight endometrial epithelial cell samples were used for microarray analysis. Forty ng of total RNA from each sample was used to generate cDNA which was amplified using NuGEN Ovation RNA Amplification version 2 (NuGEN Inc. product# 3100) The resulting cDNA was purified by Zymo Research DNA Clean Concentration 25 (Zymo Research Inc.) and the cDNA quality was assessed by Bioanalyzer. Following cDNA amplification and purification, the product was fragmented (50-100bp) and labeled with biotin using NuGEN FL-Ovation cDNA Biotin Module V2 (product #4200) in preparation for hybridization to Affymetrix GeneChips. We used 3.75 ug of amplified and labeled cDNA in the hybridization cocktail for GeneChip analysis. Detailed protocols for sample preparation using the NuGen Ovation amplification and labeling protocols can be found at http://www.nugeninc.com. All samples were subjected to gene expression analysis via the Affymetrix Human Genome U133 Plus 2.0 arrays. Hybridization, staining and washing of all arrays were performed in the Affymetrix fluidics module. Streptavidin phycoerythrin stain (SAPE, Molecular Probes) is the fluorescent conjugate used to detect hybridized target sequences. The detection and quantification of target hybridization was performed with a Affymetrix GeneChip scanner. Data were assessed for array performance prior to analysis. The probe level intensities were processed using robust multi-array average (RMA) method for background correction, normalization, and log2 transformation of perfect match values [27].

**Microarray Data Analysis**

mRNA microarray data were analyzed using microarray data analysis software ArrayAssist 5.0.1 (Stratagene). Data were log2 transformed. Students' two tail *t*-tests were conducted between the

proliferative phase and mid-secretory phase groups for each transcript and fold-change was determined. Raw *P* values were adjusted by the Benjamini-Hochberg false discovery rate to yield adjusted *P* values. The criteria for significance of differentially regulated genes were established as greater than a two-fold change with an adjusted *P* value < 0.05. We performed an unsupervised two-way hierarchical clustering analysis (Distant metrics: Pearson Centered, Linkage Rule: Centroid) on the 8 samples in each dataset with all probe sets included.

**Sample and Micro Array Processing, miRCHIP V1 array**

A custom-manufactured Affymetrix GeneChip from Ambion was designed to miRNA probes derived from Sanger mirBase and published reports by Asuragen [28-31]. Antigenomic probe sequences were provided by Affymetrix and derived from a larger set of controls used on the Affymetrix human exon array for estimating background signal, as described below. Other non-miRNA control probes on the array were designed to lack sequence to the human genome and can be used for spike-in external reference controls.

Samples for miRNA profiling studies were processed by Asuragen Services (Austin, TX), according to the company's standard operation procedures. The miRNA-enriched RNA samples were obtained using miRVana miRNA Isolation Kit (Ambion Inc., Austin, TX) and were provided to Asuragen Services. The 3'ends of the RNA molecules were tailed and biotin-labeled using mirVana miRNA Labeling Kit (Ambion Inc., Austin, TX). The kit's dNTP mixture in the tailing reaction was replaced with a proprietary mixture containing biotin-modified nucleotides (PerkinElmer, Waltham, MA). Hybridization, washing, staining, imaging, and signal extraction were performed according to Affymetrix-recommended procedures, except that the 20X GeneChip Eukaryotic Hybridization Control cocktail was omitted from the hybridization. The signal processing implemented for the Ambion miRCHIP V1 array was a multi-step process and involved probe specific signal detection calls, background estimates and correction. For each probe, an estimated background value was subtracted that was derived from the median signal of a set of G-C matched anti-genomic controls. Arrays within a specific analysis experiment were normalized together according to the variance stabilization method (VSM) described by Huber et al. [32]. Detection calls were based on a Wilcoxon rank-sum test of the miRNA probe signal compared with the distribution of signals from GC-content matched anti-genomic probes. For statistical hypothesis testing, a two-sample *t*-test, with the assumption of equal variance, was applied and this test defined which probes were considered to be significantly differentially expressed based on cutoff values of *P* value < 0.05 and greater than 2-fold or less than -2-fold difference in expression. To reduce the false discovery rate of miRNAs, we excluded miRNAs whose expression was detected in less than three out of four specimens in either the late proliferative phase or mid-secretory phase group. We performed an unsupervised two-way hierarchical clustering analysis (Distant metrics: Pearson Centered, Linkage Rule: Centroid) on the 8 samples in each dataset with differentially expressed miRNAs (greater than 1.5-fold or less than -1.5-fold difference in expression with *P* value <0.05). A heatmap was generated and the dendrogram illustrates relationships between the specimens.

**Real Time Quantitative RT-PCR (qrtPCR)**

One μg of total RNA from each sample was used for reverse transcription (RT) reaction to generate cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers. Quantitative rtPCR were performed in triplicate using SYBR Green PCR master mix (Applied Biosystems) following the manufacture's instructions and ABI Prism 7900HT (Amersham-Pharmacia). PCR primers were designed to be intron spanning and to amplify 68-300 bp fragments (please see Supplemental Table S2). Messenger RNA expression of *TGF3B* was analyzed using Taqman Assay (Applied Biosystems). The data were normalized to expression levels of the housekeeping gene GAPDH and the relative expression was calculated using the $2^{-\Delta\Delta CT}$ method [33].

TaqMan MiRNA assays (Applied Biosystems) were used to quantify the expression of miRNAs according to the manufacturer's instructions. The mean Ct value was determined from four PCR replicates. The data were normalized to expression levels of SNORD48 (RNU48). Relative gene expression of the late proliferative vs. mid-secretory phase samples was assessed using the $2^{-\Delta\Delta CT}$ method [33].

**Bioinformatics**

The Database for Annotation, Visualization and Integrated Discovery (DAVID, 2008 6th version) and Ingenuity Pathway Analysis software (IPA 7.6) were used to identify enriched cellular and molecular functions among differentially expressed gene transcripts. The DAVID and the Gene Ontology Project (http:www.geneontology.org) were employed to classify differentially expressed genes into functionally related groups of genes. Target genes of the miRNAs were predicted by using the following algorithms: miRanda (http://microrna.sanger.ac.uk/cga-bin/targets/v5), PicTAr (http://pictar.bio.nyu.edu), and TargetScan (http://www.targetscan.org).

**RESULTS**

Endometrial biopsies were obtained from eight mid-reproductive aged women. The ages of these women ranged from 19-36 years with a mean age of 29.0 in late proliferative phase group and from 22-30 years with a mean age of 24.3 in the mid-secretory phase group. The mean serum estradiol levels (pg/ml) were 170.43 ± 36.17 in the late proliferative phase group and 121.55 ± 43.56 in the mid-secretory phase group. Their mean serum progesterone levels (ng/ml) during the late proliferative and mid-late secretory phase were 0.39 ± 0.19 and 12.20 ± 1.2, respectively. General data on the women in the study are presented in Supplemental Table S1.

**Late proliferative phase and mid-secretory phase endometrial epithelia exhibit distinct miRNA and mRNA expression profiles**

To establish the miRNA and mRNA profiles of endometrial epithelium, microarray analyses were performed on a set of four late-proliferative and four mid-secretory phase epithelial samples of the endometrium. To identify the most informative set of differentially expressed genes between late proliferative and mid-secretory phase groups, we ranked each gene by the probability that the mean of its expression values are statistically distinct between the two groups using the Student's *t*-test. We focused our attention on genes meeting our designated criteria: $P < 0.05$ and fold change greater than 2 or less than -2. In this fashion we identified 3244 differentially expressed mRNAs between the late proliferative vs. mid-secretory phase epithelium of the endometrium; 2206 genes were upregulated and 1038 downregulated (Supplemental Fig. S1). Of the human miRNAs on the miRCHIP V1, 49 miRNAs were differentially expressed between the two groups (24 microarray probes are published miRNAs and 25 probes represent novel predicted miRNA sequences). The transcript abundance of 12 published miRNAs was increased and that of 12 published miRNAs was decreased in the late proliferative phase vs. the mid-secretory phase epithelial samples (Table 1).

To visually assess the differentially expressed gene profiles we performed an unsupervised hierarchical clustering analysis separately for mRNAs and miRNAs using the eight well-characterized endometrial epithelial samples. The dendrograms show a complete segregation of the late proliferative and mid-secretory phase samples into two groups based on their mRNA (Fig. 1A) and miRNA (Fig. 1B) expression patterns. Notably, all four late proliferative phase samples cluster robustly together as do the mid-secretory phase samples even when the expression data from all > 48,000 probes of the HG U133 Plus 2.0 array is utilized for the analysis. These data suggests that the late proliferative phase endometrial epithelium exhibit unique mRNA and miRNA expression signatures compared with the mid-secretory phase endometrial epithelium.

**Late proliferative phase endometrial epithelium expresses increased transcript abundance of cell cycle regulators**

Next, we analyzed the transcriptome of the late proliferative phase endometrial epithelium using the Database for Annotation, Visualization and Integrated Discovery (DAVID, 2008 6th version) bioinformatics program. The genes that showed significantly increased transcript abundance in the late proliferative phase samples were enriched for the following functional gene ontology groups: cell cycle (GeneOntology enrichment score 25.32, *P* value = $7.5 \times 10^{-29}$), cell cycle phase, M phase, mitosis, cell division, regulation of cell cycle and DNA replication. Also, the bioinformatics analysis revealed cell cycle regulation as the most significantly enriched functional pathway in the late proliferative phase endometrial epithelium (Fig. 2, KEGG pathway cell cycle, *P* value = $5.7 \times 10^{-15}$). These results are in agreement with the previous global gene expression study of proliferative phase versus secretory phase total endometrium [34]. The differentially expressed transcripts were uploaded to the IPA database to explore for enriched biological functions and pathways. As previously reported in the studies using the whole endometrial specimens [34], the cell cycle, DNA replication, recombination and repair as well as cellular growth and proliferation were biological functions differentially regulated between late-proliferative and mid-secretory endometrial epithelium (Fig. 3). The enriched canonical pathways were Mitotic Roles of Polo-Like Kinases, WNT/β-catenin and sonic hedgehog signaling, cell cycle regulation by the B cell translocation gene (BTG) proteins, and checkpoint regulation of the cell cycle (G1/S and G2/M) (Fig. 3.)

**miRNA gene signatures of late proliferative and mid-secretory phase endometrial epithelia**

We then explored the specific miRNAs that showed differential expression between the late proliferative and mid-secretory phase epithelial cell samples. As shown in Table 1, the expression of several miRNAs was altered in epithelial cells of the endometrium between the two physiological phases of the menstrual cycle. Among the miRNAs with lower transcript level in the late proliferative phase samples than in the secretory phase samples, MIR210 and MIR193A were the most highly decreased by the microarray analysis. The other notable miRNAs in this list are MIR29B and MIR29C as well as MIR30B and MIR30D that belong to MIR29 and MIR30 miRNA clusters, respectively. Among the significantly upregulated late proliferative phase miRNAs were MIR503 and MIR450 that are members of the same miRNA cluster together with MIR542-3P.

Using qrtPCR we validated the expression of select miRNAs. As shown in Figure 4, the level of expression of MIR210, MIR29B, MIR29c, MIR30B, MIR30D, MIR193A, MIR200C and MIR31 was significantly decreased in late proliferative phase compared with mid-secretory phase endometrial epithelium, thus validating our miRCHIP V1 data. Of the upregulated miRNAs, the expression of MIR503, showed significantly increased expression in the late proliferative phase samples than in mid-secretory phase samples. The expression levels of MIR405, MIR542-3P, MIR214 and MIR134, did not reach statistical significance although the trend in increased expression level in late proliferative phase vs. mid-secretory phase endometrial epithelium was consistent with the microarray data. Surprisingly, MIR214 demonstrated the highest level of fold change in expression between the two groups by the microarray analysis but the qrtPCR did not validate this result. The MIR222 showed a 2.4-fold and 1.7-fold increase in transcript abundance by miRNA microarray and by qrtPCR, respectively.

**Cell cycle genes are targeted by miRNAs exhibiting increased transcript abundance in the mid-secretory phase endometrial epithelium**

For further study, we investigated the miRNAs whose transcript abundance was increased in the mid-secretory phase samples compared with the late proliferative phase samples because these miRNAs are likely to negatively modulate the expression of cell cycle regulators. Using the bioinformatics online sites (miRanda, Targetscan, PicVert), we identified the predicted target mRNAs of these specific miRNAs. The predicted targets were then further characterized by employing GeneOntology analysis (DAVID

http://david.abcc.ncifcrf.gov) to identify targets that are functionally involved in the cell cycle pathway (KEGG pathway). Table 2 illustrates the predicted cell cycle targets of each miRNA. With the mRNA expression results, it was striking to note that the transcript abundance of nineteen predicted cell cycle genes was decreased in the mid-secretory samples by the mRNA array data as would be expected if their expression was modulated by the respective miRNAs. Quantitative rtPCR of these nineteen cell cycle genes validated robustly the results from the array (Fig. 5). Additionally, we have previously reported increased transcript abundance of cyclin A, cyclin E and *MCM2* in the late proliferative phase compared to the mid-secretory phase laser capture microdissected glandular epithelial samples, thus validating the expression of these transcripts in independent biological repeats [7].

**DISCUSSION**

In the current study, we demonstrate that the mRNA transcript profile of the late proliferative phase endometrial epithelium is enriched for the genes involved in regulating the cell cycle. Moreover, we show that miRNAs are differentially expressed during the physiological phases of the menstrual cycle, suggesting that they are hormonally regulated in the human endometrial epithelium. The miRNAs that show increased transcript abundance during the mid-secretory phase are predicted to target several cell cycle regulators. Consistent with the miRNA data, the transcript levels of several of these cell cycle regulators are lower during the mid-secretory phase than during the late proliferative phase. These results suggest that miRNAs may post-transcriptionally downregulate the expression of the cell cycle genes, thereby suppressing cell cycle progression and cell proliferation in the human secretory phase endometrial epithelium.

Our global mRNA and miRNA microarray analysis yielded specific gene expression signatures for the late proliferative and mid-secretory phase epithelial samples. To our knowledge this is the first report demonstrating the differential expression profiles of miRNAs in the human endometrial epithelium according to the menstrual cycle phases in mid-reproductive aged women who had been extensively screened for hormonal status and absence of intrauterine pathologies. Grouping of endometrial samples based solely on their mRNA expression profiles and histological menstrual cycle stage has been previously described for the entire endometrial samples including the epithelium and stroma [34]. Consistent with the previous expression profiles of the whole endometrium, our current data defines the proliferative phase transcriptome as one that is characterized by cell cycle regulatory functions reflecting the active mitogenic state of the cells [34, 35]. Furthermore, the two separate pathway analysis programs identified the cell cycle as the most significantly enriched pathway. Also, it is noticeable that our findings of increased transcript levels of the members of DNA replication licensing, such as *MCM2-7* and origin recognition complex proteins, in the late proliferative phase samples is in agreement with the prior expression studies [36]. These data suggest that in addition to the cell cycle the DNA replication licensing is likely another key regulatory point in hormonal regulation of epithelial cell proliferation in the human endometrium, as previously demonstrated in the mouse endometrium [6]. Additionally, bioinformatics analysis of the mRNA array data indicate that WNT/β-catenin signaling previously implicated in the hormonal regulation of proliferative and secretory phase endometrium [34, 35], is a significantly regulated "canonical pathway" in endometrial epithelium across the menstrual cycle. Specifically, we observed upregulation of WNT ligands (*WNT5A and WNT7A*) and lymphoid enhancer binding factor 1 (*LEF1*) in the late proliferative phase compared to mid-secretory phase endometrial epithelium, whereas the transcript abundance of WNT-inhibitors, dikkopf homolog (*DKK1*) and forkhead box O1 (*FOXO1*) was increased in the mid-secretory epithelial cells. Interestingly, Tulac et al. has shown that *WNT7A* mRNA expression is restricted exclusively to luminal epithelium of the human endometrium using in situ hybridization [37]. Furthermore, using WNT-activated Ishikawa cells Wang et al. recently demonstrated that progesterone induction of DKK1 and FOXO1 resulted in inhibition of WNT signaling [38]. While WNT signaling is a well known pathway in the hormonal regulation of human endometrium based on gene expression data [34, 35] and from the studies in the mouse uterus [1, 39], we recognize that

the molecular mechanisms of sex steroid regulation still remains obscure. Since these are secreted molecules they may mediate epithelial and stromal interactions mediating the hormonal effects on endometrial cells.

Importantly, specific miRNAs with increased transcript abundance in the mid-secretory phase samples were predicted to target several genes involved in DNA replication licensing and in the cell cycle (Table 2). For example, the key regulators of the cell cycle, cyclins and their partners cyclin-dependent kinases (CDKs), were predicted to be targeted by MIR31, MIR29B/29C, and MIR30B/30D; whereas the expression of the *MCM2* was predicted to be regulated by MIR31, MIR30B and MIR30D and that of *MCM4* by MIR210 (Table 2). MIR210 has been reported to downregulate the expression of E2F transcription factor 3 (*E2F3*) [40] which is an important transcription factor that induces the expression of cell cycle regulated genes and promotes cell cycle progression. The transcript levels of several putative cell cycle targets were decreased in the mid-secretory phase samples as would be expected if their expression was downregulated by miRNAs, whereas the levels of others were unchanged. For instance, cell division cycle 7 (*CDC7*) was predicted to be targeted by at least two miRNAs; yet its mRNA level showed no significant change between the cycle phases. miRNA regulation of this target, however, may occur through translational repression in which case only protein level would be altered. Of note is that the same miRNAs that are putative regulators of the genes involved in the DNA replication licensing may also regulate cyclins and CDKs. Recently, tumor suppressive miRNAs, MIR16 and MIR34A family, have been reported to modulate the expression of multiple cell cycle regulators [41-43]. To this end, our observations, although only based on the gene expression data, perfectly fit to a present view of miRNA mediated regulation of gene expression: a single miRNAs can regulate multiple genes that have related functions.

Two miRNAs, MIR221 and MNIR222 are known to negatively regulate the expression of cyclin-dependent kinase inhibitor 1B (CDKN1B; also known as p27/KIP1), by suppressing the translation of mRNA into protein [44]. CDKN1B is an important inhibitor of the cell cycle and it executes this function by binding and inhibiting CCNE/CDK2 and CCNA/CDK2 complexes in the early G1 phase [45]. The recent immunohistochemistry studies by Niklaus et al. demonstrated that CDKN1B protein expression is higher in epithelial cells of the human endometrium in the late proliferative phase than in the mid-secretory phase [7]. Here, we observed increased transcript abundance of MIR222 in the late proliferative phase samples, whereas no significant change was found in the CDKN1B mRNA level, as expected. Qian et al. [46] recently reported downregulation of MIR222 expression in endometrial stromal cells, permitting cyclin-dependent kinase inhibitor 1C (CDKN1C; also known as p57/KIP2) expression, and thereby suppressing stromal cell proliferation in vitro. Increased expression of MIR221 and MIR222 has been observed in several human cancers, supporting their function as oncogenes [47-49]. Interestingly, aberrant MIR221 expression was recently reported in atypical endometrial hyperplasia and endometrial cancer [12]. Although further validation studies are still needed, our results suggest that MIR222 may play cell cycle regulatory role in the human endometrial epithelium.

One prior study has explored the differential expression of miRNAs in the human endometrium according to the menstrual cycle phases but failed to show any significant differences in the miRNA profiles [11]. Of note is that this study limited the research to the entire endometrium including the glandular and luminal epithelium as well as the stroma as this study was primarily designed to explore the role of miRNAs in endometriosis. There is evidence that the uterus is a complex tissue comprising different resident cell types that response differently to hormonal exposure [7]. Two markers of proliferation, MKI67 and MCM2, are expressed at the peak level in luminal and glandular epithelial cells during the proliferative phase, whereas the expression of these proliferative markers in stromal cells shows much less change across the two cycle phases [7, 50]. Collectively, these findings indicate that the responses to sex steroid hormones are cell type specific and thus to understand the mechanism of action of these

hormones it is essential to study responses in particular cell types. Separating the two cellular compartments for molecular studies, however, has its limitations in that this approach precludes any opportunities to investigate mechanistic basis of epithelial and stromal cell interactions, thus paracrine influences in mediating hormonal effects on these two cell types.

The identification of protein-coding genes that are regulated by a specific miRNA has proved difficult despite the development of computational approaches to predict miRNA targets. The ability to identify target mRNA is further hampered by the fact that only partial sequence complementary between miRNA and the 3' untranslated regions of target mRNA is needed and that target selectivity of miRNAs may depend on the cellular environment. To circumvent some of these difficulties we studied the expression profiles of miRNAs and mRNA in the same epithelial cell samples of the endometrium to identify differentially expressed miRNAs and to investigate the influence of these miRNAs on putative target gene transcript levels. This approach can be successful if the miRNAs of interest affect transcript abundances of target mRNAs, but fails if the target genes are regulated only by translational suppression. Most miRNAs are believed to negatively regulate their target gene expression by causing degradation of mRNA transcripts or translational repression which would lead to inverse relationship in expression between miRNA and its target. This relationship, however, may be more complex than previously thought and more recently miRNAs were described to be capable to activate translation of their targets [51].

The array data presented here has revealed several miRNAs whose transcripts are significantly increased in abundance in the endometrial epithelium during the mid-secretory phase compared with the late proliferative phase. Target prediction analysis identified many cell cycle regulators as putative target mRNAs of these upregulated miRNAs. These results suggest a new level of regulation of gene expression that may be involved in the hormonal regulation of the epithelial cell proliferation in the human endometrium by E2 and P4. Ongoing studies are focusing to validate the true cell cycle targets of the differentially expressed miRNAs and to define the functions of these miRNAs in the human endometrial epithelium.

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**Figure legends**

**Figure 1**. Dendogram and unsupervised hierarchical clustering. Expression data from all mRNA probes (Fig. 1A) and expression data from differentially expressed miRNAs showing greater than 1.5 or less than -1.5 fold change in expression with $P$ value < 0.05 (Fig. 1B) in four late proliferative (pp) and four mid-secretory phase (sp) samples. The dendrogram on the top displays the relationship between the samples based on the gene expression patterns. Clustering analyses robustly separate the late-proliferative phase samples from the mid-secretory phase samples and assign each sample to the correct menstrual cycle phase. The expression intensity of each gene in each sample varies from high (red) to low (green).

**Figure 2.** Transcripts regulating the mammalian cell cycle are upregulated in the proliferative phase endometrial epithelium. Genes with greater than 2-fold increase in transcript abundance with $P$ <0.05 in the late proliferative phase samples compared with the mid-secretory phase samples from the HG U133 Plus 2.0 arrays are indicated in purple boxes and represent total of 38 upregulated genes. Green and white boxes indicate cell cycle genes that showed no differential expression between the late proliferative compared with the mid-secretory phase samples. Diagram is adapted from KEGG PATHWAY database (www.genome.jp/kegg/pathway.htlm).

**Figure 3.** Transcripts mediating cell cycle regulation are differentially regulated between the late proliferative compared to mid secretory endometrial epithelium. Differentially expressed transcripts were subject to bioinformatics analysis by IPA. **A.** Most significantly enriched groups relating to molecular and cellular functions. **B.** Most significantly enriched canonical signaling pathways. Total

number of genes in pathway listed in bold. For each pathway, percentage of genes that are of decreased (green) or increased (red) transcript abundance is indicated at top of chart. Portion of pathway genes that are not significantly changed are also depicted (white). Yellow square for each pathway indicates –log(*P*-value). The yellow arrow marks $P = 0.05$.

**Figure 4.** Expression of selected upregulated and downregulated miRNAs in the late proliferative phase endometrial samples relative to the mid-secretory endometrial phase samples by qrtPCR. All samples were normalized to *RNU48*. Relative gene expression of the late proliferative vs. mid-secretory phase samples was assessed using the $2^{-\Delta\Delta CT}$ method [33]. Data shown indicate relative expression of the late proliferative phase samples (gray bar) with respect to the mid-secretory phase samples (black bars), set to one. The error bars show standard error of the mean (SEM). $*P < 0.05$; $** P < 0.01$; $*** P < 0.001$

**Figure 5.** Quantitative rtPCR validates gene expression array data from the cell cycle ontological group. Nineteen genes identified as differentially regulated cell cycle members from the HG U133 Plus 2.0 array validated using qrtPCR. All samples were normalized to the housekeeping gene, GAPDH. Data shown indicate relative expression of the late proliferative phase samples (gray bar) with respect to the mid-secretory phase samples (black bars), set to one. The error bars show standard error of the mean (SEM). $*** P < 0.001$; $** P < 0.01$; $*P < 0.05$

Table 1. MicroRNAs differentially expressed in the late proliferative phase compared to the mid-secretory phase endometrial epithelium.

| **Down-regulated in late proliferative endometrium** | | | | **Up-regulated in mid-secretory endometrium** | | | |
|---|---|---|---|---|---|---|---|
| miRNA gene | Accession No. | Fold change | *P* | miRNA gene | Accession No. | Fold change | *P* |
| *MIR210* | MIMAT0000267 | 7.1 | 0.0003 | *MIR214* | MIMAT0000271 | 4 | 0.02 |
| *MIR193A-3P* | MIMAT0000459 | 5.2 | 0.0002 | *MIR503* | MIMAT0002874 | 3.6 | 0.007 |
| *MIR345* | MIMAT0000772 | 3.3 | 0.002 | *MIR134* | MIMAT0000447 | 3.1 | 0.03 |
| *MIR29B* | MIMAT0000100 | 2.8 | 0.0007 | *MIR450* | MIMAT0001545 | 3 | 0.003 |
| *MIR29C* | MIMAT0000681 | 2.6 | 0.005 | *MIR382* | MIMAT0000737 | 2.6 | 0.03 |
| *MIR30B* | MIMAT0000420 | 2.6 | 0.01 | *MIR376A* | MIMAT0003386 | 2.6 | 0.04 |
| *MIR204* | MIMAT0000265 | 2.6 | 0.04 | *MIR369-5P* | MIMAT0001621 | 2.4 | 0.006 |
| *MIR203* | MIMAT0000264 | 2.5 | 0.000086 | *MIR222* | MIMAT0000279 | 2.4 | 0.04 |
| *MIR582-5P* | MIMAT0003247 | 2.3 | 0.01 | *MIR370* | MIMAT0000722 | 2.3 | 0.01 |
| *MIR30D* | MIMAT0000245 | 2.2 | 0.005 | *MIR542-3P* | MIMAT0003389 | 2.2 | 0.04 |
| *MIR200C* | MIMAT0000617 | 2.1 | 0.004 | *MIR105* | MIMAT0000102 | 2.1 | 0.01 |
| *MIR31* | MIMAT0000089 | 2.1 | 0.02 | *MIR127* | MIMAT0000446 | 2.1 | 0.01 |

miRNAs significantly ($P < 0.05$) differentially regulated (fold change ± > 2.0) as determined by Student's *t*-test from microarray analysis of the late proliferative phase epithelial samples as compared to the mid-secretory epithelial samples are listed in order of the fold change. The accession number is for the mature miRNA sequence.

**Table 2.** Predicted cell cycle targets of the twelve miRNAs showing increased transcript abundance in the mid-secretory phase endometriual epithelium.
The predicted targets of the microRNAs were identified using the following three online algorithms: miRanda http://microrna.sanger.ac.uk/cga-bin/targets/v5 (gray background), Target scan http://www.targetscan.org (blue background), PicTar http://pictar.bio.nyu.edu (white background). Genes with transcript abundance enriched in the mid-secretory phase epithelial samples by the microarray and qrtPCR (fold change >2, $P$ <0.05). *E2F3* is a known target of miR-210 [40].

| MicroRNA | *MIR210* | *MIR31* | *MIR203* | *MIR204* | *MIR200C* | *MIR29B* | *MIR29C* | *MIR30B* | *MIR30D* | *MIR193A-3P* | *MIR345* | *MIR582-5P* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Predicted mRNA targets** | *E2F3** | *MCM2** | *BUB1B** | *CDC7* | *HDAC2* | *CCNA2** | *CCNA2** | *CCNB1** | *CCNB1** | *HDAC3* | *ABL1* | *TGF3B** |
| | *MCM4** | *ORC1L** | *CDKN2C** | *GADD45* | *HDAC6* | *CCNE1* | *CCNE1* | *MCM2** | *MCM2** | *CCND1* | *TFDP1* | *BUB1B** |
| | *PLK1** | *ORC4L* | *RB1* | *CCND2** | *YWHAG* | *SKP2** | *CDK2** | *CCNA1* | *CCNA1* | *E2F6* | *E2F6* | *MAD2L1** |
| | *CHK2* | *CDK1** | *PRKDC* | *CDC7* | *EP300* | *CDKN2A** | *CDKN2A** | *ORC2L* | *ORCL5* | | *HDAC4* | *HDAC2* |
| | *CDKN1C* | *CDK2** | *E2F3** | *SMAD4* | *CDKN3** | *CDC7* | *CDC7* | *CDC7* | *E2F6* | | *HDAC8* | *HDAC6* |
| | *E2F3** | *CDK4** | *SMAD3* | *CDC25B* | *E2F3** | *TGF3B** | *HDAC8* | *HDAC8* | *HDAC8* | | *CDKN1A* | *BUB3* |
| | | *E2F4* | *ATM* | *CDC14B* | *CDKN1B* | *HDAC4* | *TP53* | *CCNE2** | *CCNE2** | | *CDC25B* | *CDKN1C* |
| | | *E2F6* | *ABL1* | *WEE1* | *CDK2** | *CCND2** | *HDAC4* | *ORC2L* | *ORC2L* | | *CDKN1A* | *CDK6** |
| | | *MAD2L2* | *ABL1* | *CDC25B* | *CCNE2** | *CDC42* | *CCND2** | *TFDP1* | *TFDP1* | | *E2F3** | *CDKN2B* |
| | | *TFDP3* | | *YWHAG* | *CDC14B* | *E2F7* | *CDC42* | *ABL1* | *ABL1* | | | |
| | | *SMC1B* | | | *HDAC4* | *CDC7* | *E2F7* | *DBF4** | *DBF4** | | | |
| | | *E2F2* | | | *YWHAG* | *CDK6** | *CDC7* | *E2F3** | *E2F3** | | | |
| | | | | | *EP300* | *CDK6** | *CDK6** | *HDAC5* | *HDAC5* | | | |
| | | | | | *E2F3** | *CCND2** | *CDK6** | *CDC7* | *CDC7* | | | |
| | | | | | *YWHAG* | *TGF3B** | *CCND2** | *ABL1* | *CCNE2** | | | |
| | | | | | | *ABL1* | *TGF3B** | *CCNE2** | | | | |
| | | | | | | *HDAC4* | *ABL1* | | | | | |
| | | | | | | | *HDAC4* | | | | | |

Abbreviations: *ABL1* (C-ABL oncogene 1); *ATM* (ataxia telangiectasia mutated homolog); *BUB1B* (budding inhibited by benzimidazoles 1 homolog beta); *BUB3* (budding inhibited by benzimidazoles 3); *CCNA1* (cyclin A1); *CCNA2* (cyclin A2); *CCNB1* (cyclin B1); *CCND2* (cyclin D2); *CCNE1* (cyclin E1); *CCNE2* (cyclin E2); *CDC7* (cell division cycle 7); *CDC14B* (cell division cycle 14 homolog B); *CDC25A* (cell division cycle 25A); *CDC25B* (cell division cycle 25B); *CDC42* (cell division cycle 42); *CDK1* (cyclin-dependent kinase 1); *CDK2* (cyclin-dependent kinase 2); *CDK4* (cyclin-dependent kinase 4); *CDK6* (cyclin-dependent kinase 6); *CDKN3* (cyclin-dependent kinase inhibitor 3); *CDKN1A* (cyclin-dependent kinase inhibitor 1A); *CDKN1B* (cyclin-dependent kinase inhibitor 1B); *CDKN1C* (cyclin-dependent kinase inhibitor 1C); *CDKN2A* (cyclin-dependent kinase inhibitor 2A); *CDKN2B* (cyclin-dependent kinase inhibitor 2B); *CDKN2C* (cyclin-dependent kinase inhibitor 2C); *CHECK2* (CHK2 checkpoint homolog); *DBF4* (DBF4 homolog); *E2F3* (E2F transcription factor 3); *E2F4* (E2F transcription factor 4); *E2F6* (E2F transcription factor 6); *E2F7* (E2F transcription factor 7); *EP300* (E1A binding protein p300); *GADD45* (growth arrest and DNA-damage-inducible, alpha); *HDAC2-6* (histone deacetylase 2-6); *HDAC8* (histone deacetylase 8); *MAD2L1* (MAD2 mitotic arrest deficient-like 1); *MAD2L2* (MAD2 mitotic arrest deficient-like 2); *MCM2* (minichromosome maintenance complex component 2); *MCM4* (minichromosome maintenance complex component 4); *ORC1L- ORC6L* (origin recognition complex, subunit 1-6 homolog-like); *PLK1* (polo-like kinase 1); *PRKDC* (protein kinase, DNA-activated, catalytic polypeptide); *RB1* (Retinoblastoma 1); *SMAD3* (SMAD family member 3); *SMAD4* (SMAD family member 4); *SMC1B* (structural maintenance of chromosomes 1B); *SKP2* (S-phase kinase-associated protein 2 [p45]); *TFDP1* (transcription factor Dp-1); *TFDP3* (transcription factor Dp-3); *TGF3B* (transforming growth factor, beta 3); *WEE1* (WEE1 homolog); *YWHAG* (tyrosine 3-monooxygenease/tryptophan 5-monooxygenase activation protein, gamma polypeptide).

**Figure 1.**

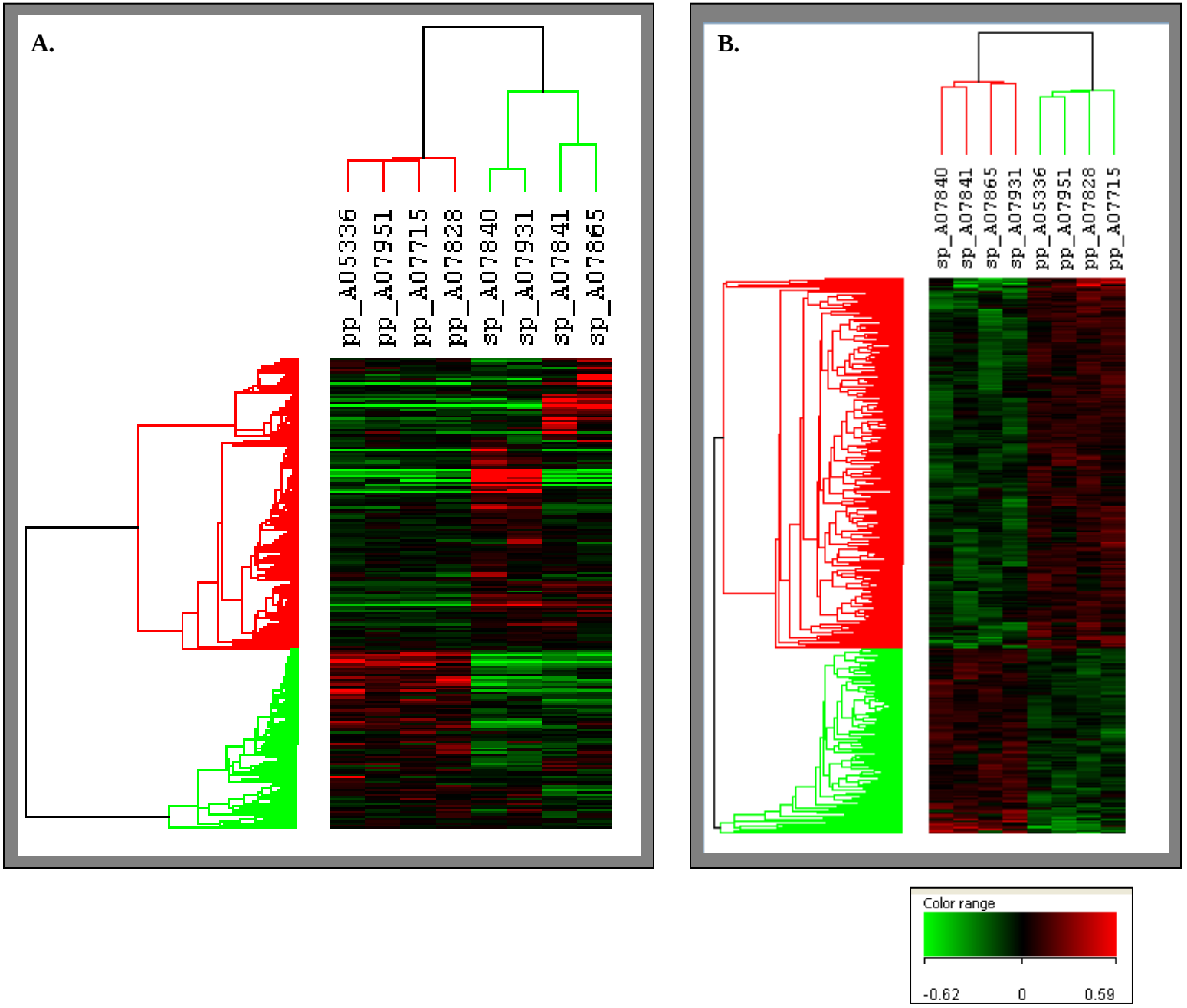
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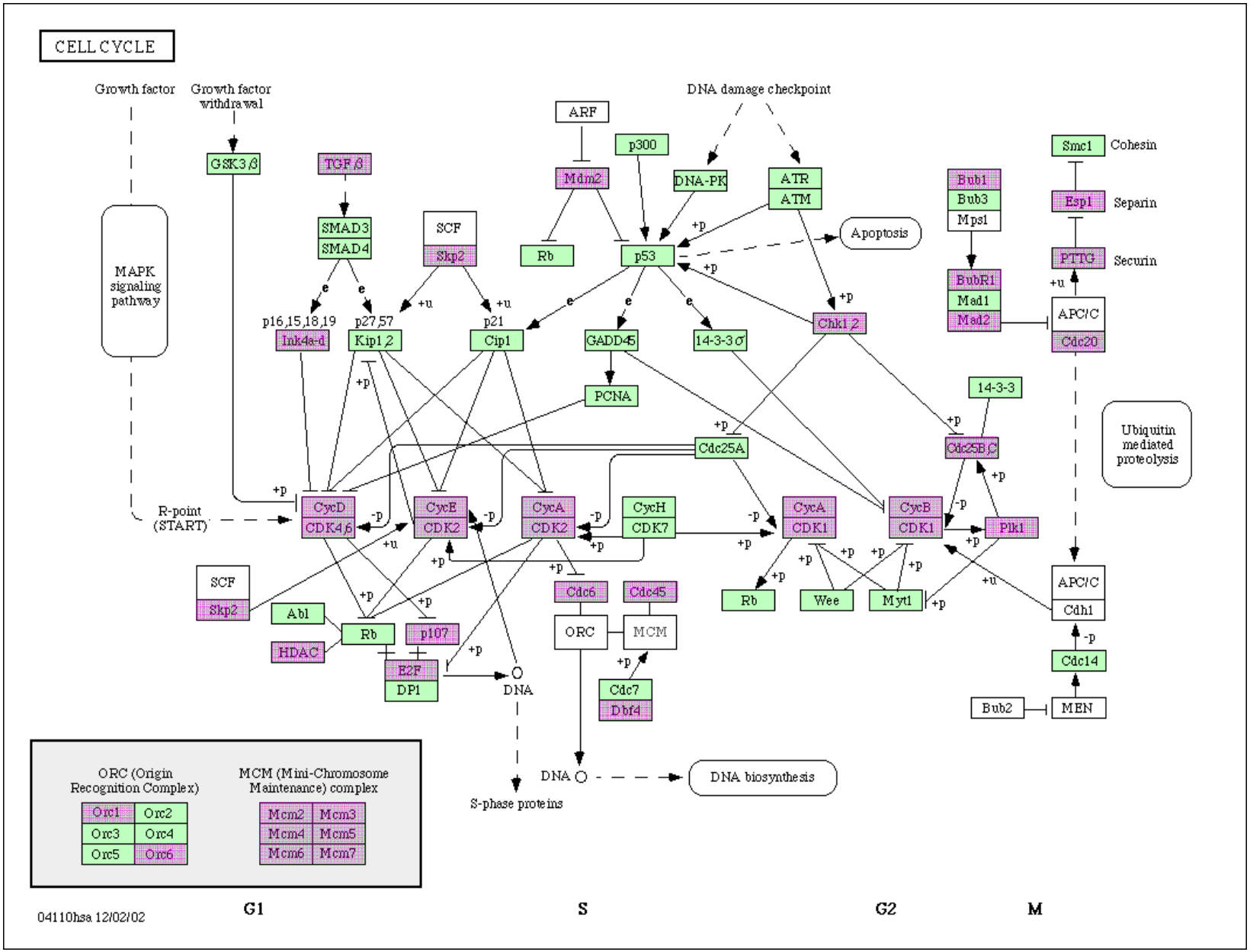
**Figure 2.**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TGF β
SMAD3
SMAD4
MAPK signaling pathway
R-point (START)
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p16,15,18,19
Ink4a-d
p27,57
Kip1,2
p21
Cip1
ARF
Mdm2
p300
DNA damage checkpoint
DNA-PK
ATR
ATM
Rb
p53
Apoptosis
GADD45
14-3-3 σ
Chk1,2
PCNA
Cdc25A
CycD
CDK4,6
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CycB
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Bub3
Mps1
BubR1
Mad1
Mad2
Smc1
Cohesin
Esp1
Separin
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HDAC
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Cdc45
ORC
MCM
Cdc7
Dbf4
Rb
Wee
Myt1
APC/C
Cdh1
Cdc14
Bub2
MEN
S-phase proteins
DNA
DNA biosynthesis
ORC (Origin Recognition Complex)
Orc1
Orc2
Orc3
Orc4
Orc5
Orc6
MCM (Mini-Chromosome Maintenance) complex
Mcm2
Mcm3
Mcm4
Mcm5
Mcm6
Mcm7
04110hsa 12/02/02
G1
S
G2
M

**Figure 3.**

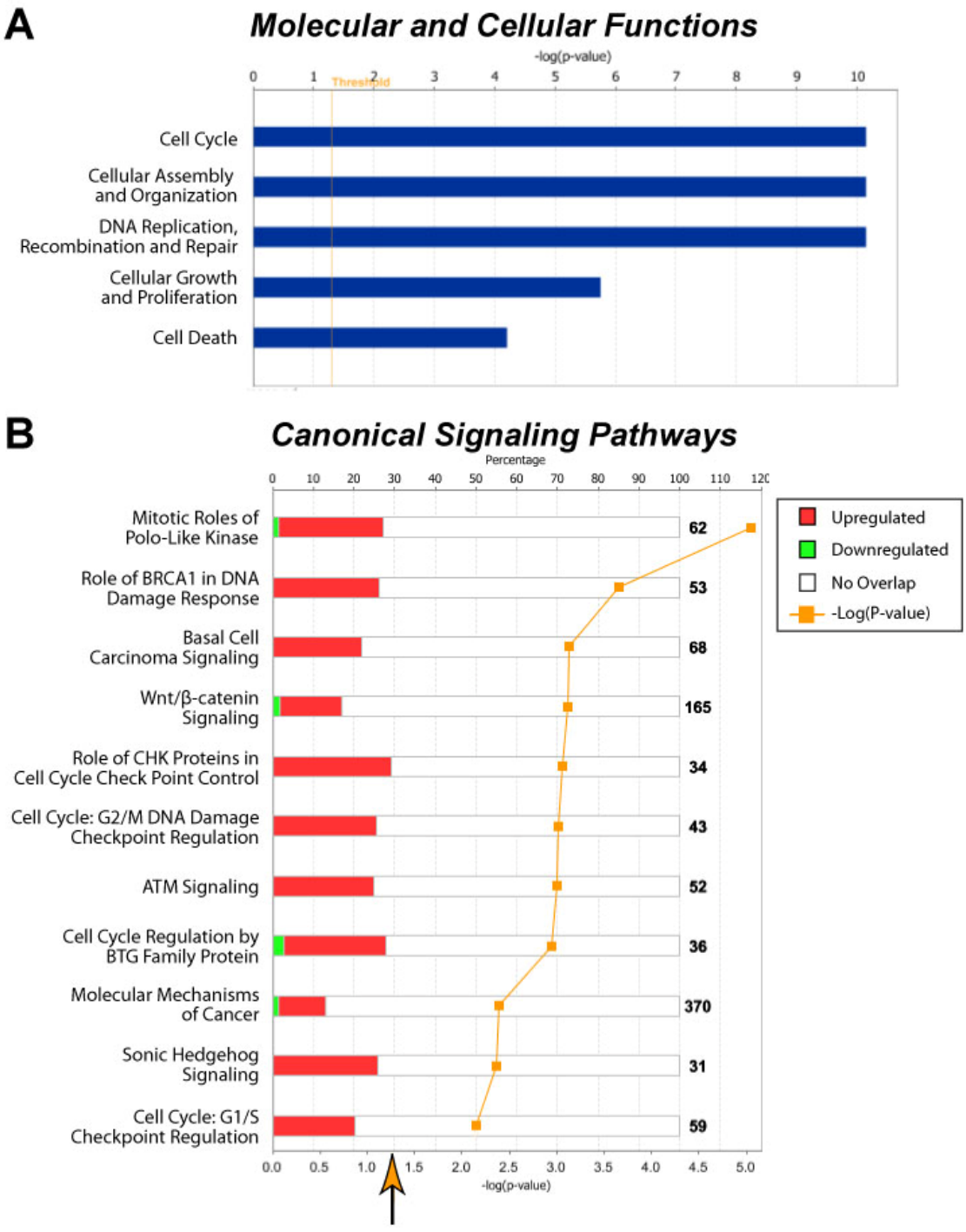

A
Molecular and Cellular Functions
-log(p-value)
0 1 2 3 4 5 6 7 8 9 10
Threshold
Cell Cycle
Cellular Assembly and Organization
DNA Replication, Recombination and Repair
Cellular Growth and Proliferation
Cell Death
B
Canonical Signaling Pathways
Percentage
0 10 20 30 40 50 60 70 80 90 100 110 120
Mitotic Roles of Polo-Like Kinase
Role of BRCA1 in DNA Damage Response
Basal Cell Carcinoma Signaling
Wnt/β-catenin Signaling
Role of CHK Proteins in Cell Cycle Check Point Control
Cell Cycle: G2/M DNA Damage Checkpoint Regulation
ATM Signaling
Cell Cycle Regulation by BTG Family Protein
Molecular Mechanisms of Cancer
Sonic Hedgehog Signaling
Cell Cycle: G1/S Checkpoint Regulation
62
53
68
165
34
43
52
36
370
31
59
Upregulated
Downregulated
No Overlap
-Log(P-value)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0
-log(p-value)

**Figure 4.**

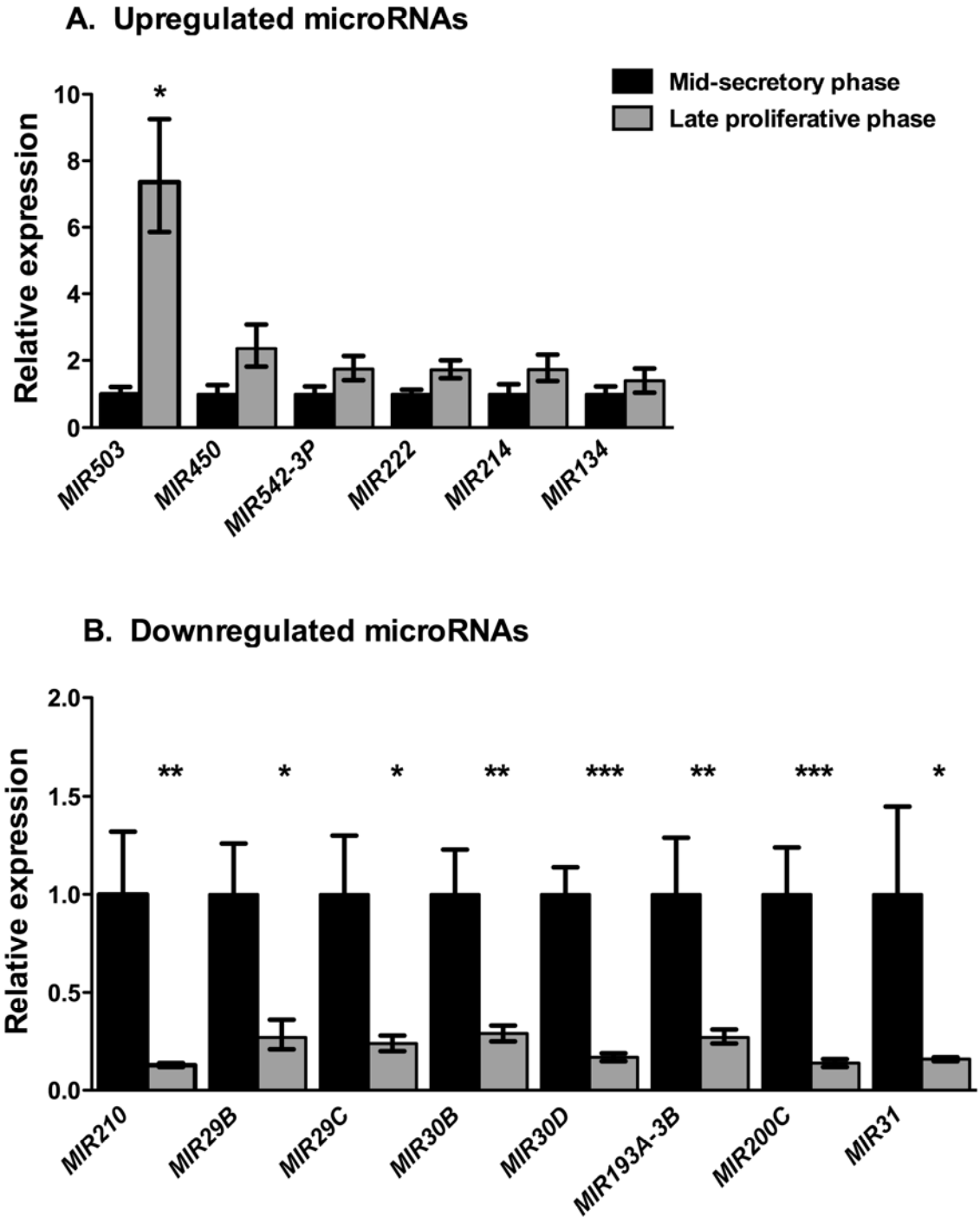
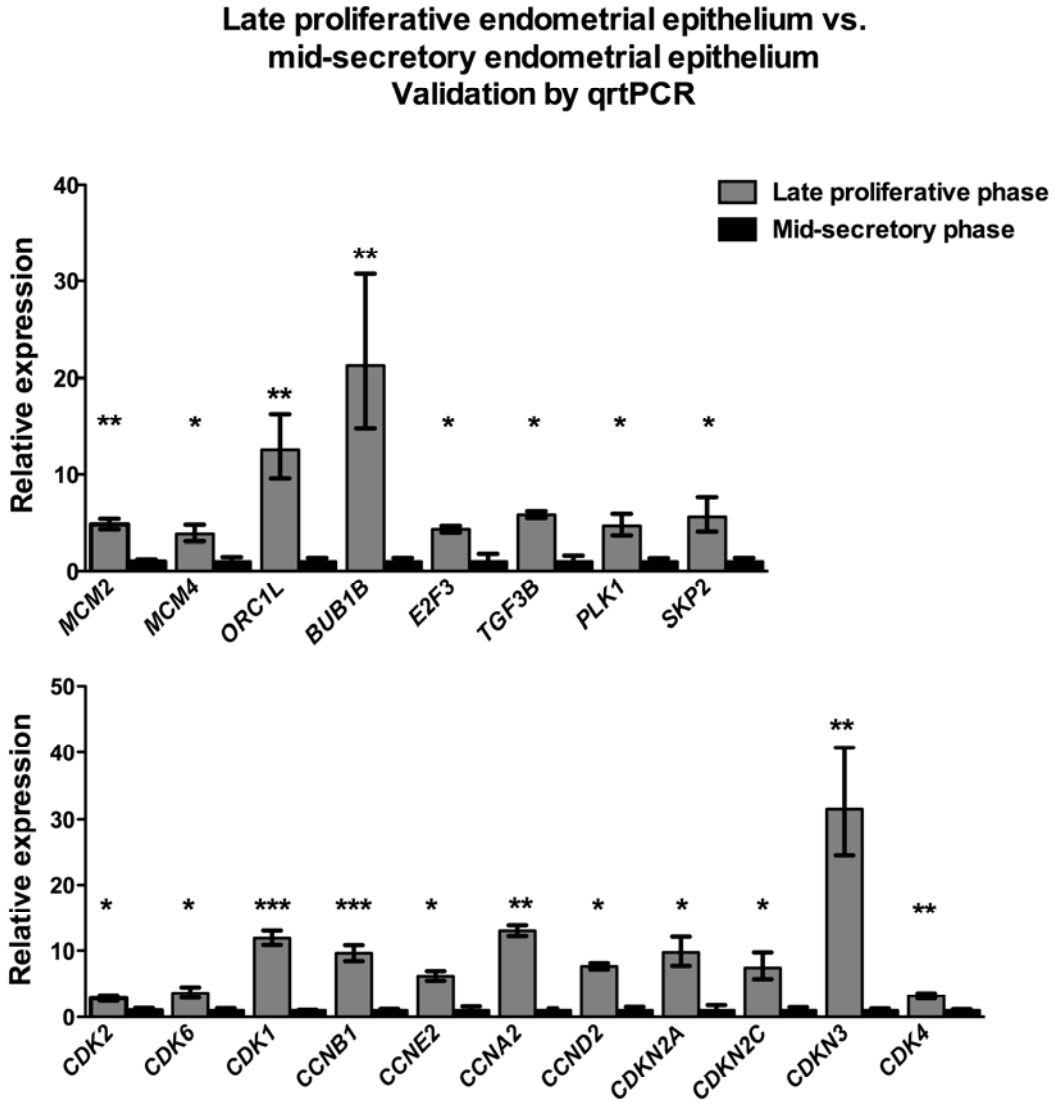

A. Upregulated microRNAs
Mid-secretory phase
Late proliferative phase
Relative expression
10
8
6
4
2
0
*
MIR503
MIR450
MIR542-3P
MIR222
MIR214
MIR134
B. Downregulated microRNAs
Relative expression
2.0
1.5
1.0
0.5
0.0
**
*
*
**
***
**
***
*
MIR210
MIR29B
MIR29C
MIR30B
MIR30D
MIR193A-3B
MIR200C
MIR31

**Figure 5.**


Late proliferative endometrial epithelium vs.
mid-secretory endometrial epithelium
Validation by qrtPCR
Late proliferative phase
Mid-secretory phase
Relative expression
MCM2
MCM4
ORC1L
BUB1B
E2F3
TGF3B
PLK1
SKP2
CDK2
CDK6
CDK1
CCNB1
CCNE2
CCNA2
CCND2
CDKN2A
CDKN2C
CDKN3
CDK4