Transcriptome sequencing of endometrium revealed alterations in mRNAs and lncRNAs after ovarian stimulation

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Abstract

Research question Using RNA-sequencing analysis, we investigated the relationship between ovarian stimulation and endometrial transcriptome profiles during the window of implantation (WOI) to identify candidate predictive factors for the WOI and to optimize timing for embryo transfer.

Methods Twelve women with normal basal hormone levels and regular ovulation were randomly assigned into three groups based on sampling time: late-proliferate phase (P group), and receptive phase in natural cycles (LH+7, N group) and stimulated cycles (hCG+7, S group). Transcriptome profiles of mRNAs and long non-coding RNAs (lncRNAs) were then compared among the three groups. Validation was performed using real-time qPCR.

Results Comparison of transcriptome profiles between the natural and stimulated endometrium revealed 173 differentially expressed genes (DEGs), with a > 2-fold change (FC) and $p < 0.05$, under the influence of supraphysiological estradiol (E_2) induced by ovarian stimulation. By clustering and KEGG pathway analysis, molecules and pathways associated with endometrial receptivity were identified. Of the 39 DEGs common to the three groups, eight genes were validated using real-time PCR. ESR1, MMP10, and HPSE were previously reported to be associated with endometrial receptivity. In addition, three novel genes (IL13RA2, ZCCHC12, SRARP) and two lncRNAs (LINC01060, LINC01104) were new potential endometrial receptivity-related markers.

Conclusion Using mRNA and lncRNA sequencing, we found that supraphysiological E_2 levels from ovarian stimulation had a marked impact upon endometrial transcriptome profiles and may result in a shift of the WOI. The precise mechanisms underlying the supraphysiological hormone-induced shift of the WOI require further research.

Registration number ChiCTR180001453

Keywords Ovarian stimulation · Window of implantation · Endometrial receptivity · mRNAs · lncRNAs

Introduction

A viable embryo, receptive endometrium, and the synchronization of these two factors provide three essential elements for

a successful pregnancy [1]. The endometrium is a dynamic tissue which grows and sheds monthly under the control of estradiol (E_2) and progesterone. The window of implantation (WOI) is a period of time usually 5–10 days after ovulation

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that lasts 12 to 48 h, when the endometrium has transformed for the implantation of embryos [2]. During this time-specified and hormone-regulated phase, the endometrium gains the optimal ability to accept embryos, termed receptivity [3]. The acquisition of endometrial receptivity is the result of the sequential effect of E_2 and progesterone [4]. Implantation failure makes up 30% of all pregnancy losses when excluding embryonic factors [5].

Controlled ovarian stimulation (COH) allows the simultaneous development of multiple follicles, but it induces supraphysiologic levels of E_2 , which increases the risk of ovarian hyperstimulation syndrome (OHSS) and defective endometrial receptivity [6]. In the 1990s, it was first noticed that high responders to COH had lower implantation rates compared with normal and low responders [7]. However, this phenomenon was not found in frozen-thawed embryo transfer (FET) cycles and donor cycles, indicating that endometrial receptivity was likely impaired by COH treatment [8]. Progesterone and estrogen were both found to be essential for the preparation of receptive endometrium, while the degree of endometrial estrogen exposure was related to impairment of endometrial receptivity [9]. However, the mechanism remains unclear.

Differences in endometrial morphology between natural and stimulated cycles have been extensively reported. The endometrium of stimulated endometrium was advanced, with delayed gland and advanced stroma development, sometimes accompanying stromal edema [10]. The asynchronization of endometrial development may lead to the failure of implantation. It was also found that greater than 3 days difference between the endometrial maturity and menstrual period resulted no pregnancy [11]. During stimulated cycles, pinopodes developed in advance and exhibited morphology different from those in natural cycles [12]. Although the morphological assessment of endometrium has benefits, this kind of evaluation is observer-dependent and thus biased. Moreover, there is no consensus regarding the morphological evaluation of endometrium.

The altered expression of molecular markers related to endometrial receptivity has been reported. Estrogen and progesterone receptors [13], leukemia inhibitory factors, cell adhesion molecules, such as integrins, abnormal expression of growth factors, and some immune cells, [14–18] were shown to be related to the process of embryo implantation or the establishment of endometrial receptivity. However, whether a certain molecular change will influence the outcome of pregnancy remains unknown [11].

One of the major challenges in this field is to find new potential markers of endometrial receptivity. Early and intensive endeavors explored gene profiles during the peri-implantation phase in a natural cycle [19–27]. In 2004, Mirkin et al. [28] reported for the first time that COH leads to differential gene expression. Subsequent work using DNA

microarrays found more transcriptome changes induced by supraphysiologic estrogens [29–33]. However, due to various factors, including inter-patient variability, limited sample sizes, and different timing of biopsy, the results of these studies were highly heterogeneous and few genetic overlaps were found [34]. It has to be pointed out that gene array techniques have limitations. The recent and rapid emergence of next-generation sequencing (NGS) technology is overcoming some of the array analysis shortcomings [35]. Unlike traditional Sanger sequencing and microarray, RNA-seq is a deep and high-throughput sequencing method. Instead of specific probe hybridization, NGS can detect and quantify the entire transcriptome, including encoding RNA (mRNA) and long non-coding RNA (lncRNA), providing more comprehensive transcriptomic information. NGS is very suitable for detecting rare transcripts in complex and unknown tissues, such as the endometrium, and for revealing important pathways for further analysis. To our knowledge, this is the first report to compare the differences in gene expression between natural and stimulated cycles using NGS.

In this study, we used NGS to investigate the effects of ovarian stimulation on functional genomic profiles of the endometrium, and to discover potential regulatory genes and involved pathways to provide a better understanding of embryo implantation and the WOI.

Material and methods

Human subjects

Twelve women with normal ovulation and basal hormonal levels undergoing IVF/ICSI-ET at the Reproductive Department of Beijing Chaoyang Hospital were recruited. Inclusion criteria were as follows: (1) age 20–35 years; (2) body mass index (BMI) 18–23 kg/m²; (3) regular menstrual cycle with confirmed ovulation, basal follicle stimulating hormone (FSH) or LH < 8 IU/L; (4) gynecological ultrasound showed uterus, uterine cavity, and bilateral attachment had no abnormalities, endometrial thickness on ovulation day $\geq$ 6 mm; (5) COH protocol: GnRH long-agonist or GnRH antagonist protocols. Exclusion criteria were as follows: (1) uterine malformations; (2) endometrial lesions: endometritis, polyps, submucosal fibroids, uterine adhesion; (3) endometriosis; (4) uterine surgery that may damage the endometrium: curettage, polyp removal, submucosal myomectomy; (5) hydrosalpinx. Subjects were evenly and randomly allocated to three groups according to the sampling time.

This study was registered in the Chinese Clinical Trials Registration Center with registration number: ChiCTR180001453. The study was approved by the ethics committee of Chaoyang Hospital and informed consent was

signed before the biopsy. Patients were required to use condoms for contraception during the study.

Controlled ovarian stimulation and tissue collection

In natural cycles, we assigned the day of the urinary LH peak (hemtrue®, Shanghai, China) as LH+0. Samples were collected in late-proliferative phase (P group) and on the day of LH+7 (N group). Patients in P group were biopsied on the cycle day 11–14 in their regular 28–30 periods. In the meantime, ultrasound was used to monitor ovulation. Histological analysis was performed to reconfirm the biopsy time.

Biopsies were performed at the fundal and upper part of the uterus under sterile condition using Pipelle (Yajie®, Jiangxi, China). Biopsies were performed by the same surgeon. After washing to remove mucus and blood, the endometrium samples were divided into two parts. One was placed in 4% paraformaldehyde (Cat no. KGIHC016CS, KGI Bio, China) for histological assessment, and the other was snap-frozen and stored in liquid nitrogen for further processing.

In stimulation cycles, ovarian stimulation was performed using standard long GnRH-agonist or GnRH-antagonist protocol. Long GnRH-agonist protocol: 7 days after ovulation, a subcutaneous injection of 0.05 mg triptorelin acetate (Decapeptyl®, Ferring Pharmaceuticals, Switzerland) was administered daily for 14 days until criteria of downregulation was met ($E_2 < 50$ pg/ml, Luteinizing hormone (LH) and FSH < 5 IU/ml, endometrial thickness < 5 mm, and the maximal follicle diameter was less than 10 mm). Then individualized injections of 150–225 IU rFSH (Gonal-f®; Serono, Geneva, Switzerland) were administered daily for ovarian stimulation. Triptorelin acetate was continued until trigger day. For the GnRH-antagonist protocol, rFSH was started on the 2nd day of menstruation, and 0.25 mg cetrorelix acetate (Cetrotide®; Serono, Geneva, Switzerland) was added daily until trigger day and serum E_2 had reached 400 pg/ml or when a follicle > 14 -mm diameter was confirmed by ultrasound. When the leading follicle was 18–20 mm in diameter and there were more than three follicles at 16 mm, 5000–10000 IU HCG (Livzon Pharmaceutical, Guangdong, China) was injected to trigger final oocyte maturation. Oocyte retrieval was performed 34–36 h after hCG injection. Serum E_2 levels were measured on the same day with an electro-chemiluminescence immunoassay kit (Roche Diagnostics GmbH, Mannheim, Germany). Endometrial biopsies were done 7 days after hCG administration in four patients with a high risk of developing OHSS.

RNA extraction and RNA-Seq analysis

RNA extraction and RNA-sequencing

Total RNA was extracted using the TRIZOL method according to the protocol recommended by the manufacturer (Life

Technologies, Inc., Gaithersburg, MD, USA). The total RNA quantity was measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc. DE, USA) and RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). RNA samples with an RNA integrity number (RIN) ≥ 7 and 28S:18S $\geq 1.5:1$ in electrophoresis were chosen for mRNA Library Construction. Samples were sequenced according to the product protocol using the mRNA-Seq Sample Preparation Kit (Cat no. RS-930-1001 Illumina containing 1 box, part no. 1004814 and 1 bag, part no. 1004815). The libraries were sequenced on the Illumina HiSeq X Ten generating 150 bp paired end reads.

Detection of differentially expressed genes

Raw reads were mapped to the human genome (hg19) using hist2 software. Gene expression quantity was performed using feature-count with the ensemble gene Annotation file by the t Strand-Specific params. The differentially expressed genes (DEGs) were identified by edgeR R package. RPKM (Reads Per Kilobase per Million mapped reads) was used to calculate gene expression. Qualified gene expression was defined as gene count-per-million (CPM) > 2 in more than 2 samples within one group. Reads with low quality were discarded. The DEGs were identified with the p value < 0.05 and fold change (FC) > 2 . P -values were corrected using false discovery rate (FDR) (5%).

Gene annotation: gene ontology and pathway analysis

DEGs were used for gene ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes database) pathway analysis. GO and KEGG pathway enrichment analysis was performed using the ClusterProfiler package from BioConductor (<http://www.bioconductor.org/>). GO was classified as cellular component (CC), biological process (BP), and molecular function (MF). Hypergeometric exact test was used to classify enrichment GO categories and KEGG pathway, and only the terms with $p < 0.05$ were considered enriched.

Quantitative RT-PCR

The remaining RNA of each sample after sequencing was used for quantitative RT-PCR. Genomic DNA elimination reaction was first performed. 5× gDNA Eraser Buffer (2 µl, TAKARA) and gDNA Eraser (1 µl, TAKARA) were added to 2 µg of total RNA. RNase Free dH₂O was then added to make the total reaction volume to 10 µl. The mixture was heated at 42 °C for 2 min and chilled on ice. Reverse-transcription reaction was then performed. cDNA was synthesized in a total volume of 20 µl containing 10 µl reaction solution from step 1 and 10 µl Master mix (4 µl 5× PrimeScript Buffer2 for Real Time, 1 µl PrimeScript RT Enzyme

Mix1, 1 µl RT Primer Mix, 4 µl RNase Free dH₂O, all from TAKARA). The subsequent incubation process was 15 min at 37 °C, then 5 sec at 85 °C. The cDNA was stored at 4 °C for further use.

RT-PCR was performed using cDNA in a total of 20 µl PCR mix. The total mix contained 1 µl cDNA, 300 nM forward primer, 300 nM reverse primer, and 1× SYBRgreen PCR Master Mix (Applied Biosystems). The Q-PCR was performed in a MicroAmp® Optical 96-well Reaction Plate (Applied Biosystems) with an ABI Prism Optical Adhesive Cover (Applied Biosystems) in the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The selected protocol consisted of 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by a dissociation curve step of 15 s at 95 °C, 1 min at 60 °C, 30 s at 95 °C, and 15 s at 60 °C. Standard curve material consisted of pooled endometrial RNA from all three groups. All reactions were performed in triplicate. Supplemental Table S1 shows the primer sequences, forward (F) and reverse (R), for each gene in RT-PCR.

Relative gene expression in the P and S groups compared with the N group was calculated by delta delta Ct. Validation data and presented as mean ± standard error of mean (SEM).

Results

Demographic data

There were no significant differences in age, duration of infertility, and basal hormone levels among the three groups.

The serum E₂ levels in the stimulation cycle group were significantly higher than that in the natural cycle, and the histology dating was consistent with the time of sampling. The results are shown in Table 1.

Different gene expression profiles between natural and stimulated cycles

Twelve samples were analyzed using RNA-seq. Four triplicate libraries were generated and sequenced to a depth of 55 million to 82 million reads (Supplemental Table S2). The reads were aligned to a human reference genome (hg19) using hisat2 software with the –rna-strandness RF. On average, 91.70% of sequences were aligned to the genome, with an average of 78.13% of the reads being uniquely aligned; 93.39% of the uniquely aligned reads were aligned to mRNA, with 58.18% of reads aligned to exons.

The reads of each gene were counted using the feature count. Reads aligned to multiple genes were discarded, and the reads of genes were normalized, and an average of 19702 genes were detected (Supplemental Table S3). The edger was used to identify differentially expressed genes. Genes with a > 2-fold change with a *p* value < 0.05 were identified as differentially expressed genes (DEGs).

Among the 18487 genes, there were 1063 DEGs in P group compared with the N group, of which 513 (48.3%) were upregulated and 550 (51.7%) were downregulated. Compared with the S group, there were 173 DEGs in the natural cycle group, of which 130 (75.1%) were upregulated and 43 (24.9%) were downregulated. Thirty-nine

Table 1 Demographic data of the 12 subjects

	Stimulated cycle (hCG+7) <i>n</i> = 4	Natural cycle		<i>p</i> value
		Proliferative phase <i>n</i> = 4	LH+7 <i>n</i> = 4	
Age (years)	32.5 ± 2.5	31.0 ± 3.7	34.3 ± 2.1	NS
Cause of infertility	Tubal	Tubal	Tubal	NS
Duration (years)	3.0 ± 1.8	2.8 ± 1.0	2.3 ± 1.3	NS
BMI (kg/m ²)	21.2 ± 1.7	19.8 ± 0.90	20.6 ± 2.4	NS
Period (days)	31.75 ± 3.3	27.5 ± 1.0	29.0 ± 1.2	NS
Basal FSH (mIU/ml)	5.2 ± 1.7	7.6 ± 1.6	6.5 ± 1.5	NS
AFC	14 ± 5.2	16 ± 2.9	13.2 ± 5.4	NS
Histology	Secretory phase	Proliferative phase	Secretory phase	—
rFSH dosage (IU)	2362.5 ± 256.17	—	—	—
rFSH duration (days)	10.75 ± 1.89	—	—	—
E ₂ on the hCG day (pg/ml)	3121.5 ± 1445.63	—	—	—
No. of retrieved oocytes	15.5 ± 6.76	—	—	—

All the data are presented as mean ± standard deviation (SD), NS indicates no significant difference between groups (*p* > 0.05)

BMI, body mass index; AFC, antral follicle count; COH, controlled ovarian stimulation; rFSH, recombinant follicle stimulating hormone; hCG, human chorionic gonadotropin; E₂, estradiol

Fig. 1 **a** Principal component analysis (PCA) of DEGs. **b** Unsupervised hierarchical clustering of gene list of 12 samples. *x*-axis represents samples and *y*-axis represents the expression abundance of different genes. The color from green to red represents the gene expression abundance from low to high. Volcano plot of differentially expressed genes. **c** N vs P.

d N vs S. The *x*-axis represented the $\log_2$ of fold change (FC) and *y*-axis represented $\log_{10}$ of *p* values. Genes with $\text{FC} > 2$ and $p < 0.05$ were painted as red dots. Red dots on the left were downregulated genes and on the right were upregulated genes. The leftover blue dots were the genes without significant difference

overlapping genes were in common between the two comparisons.

Clustering and principal component analyses (PCA) of the gene expression patterns for the three groups are shown in Fig. 1a. The samples were labeled with three groups in the PCA analysis. The 4 samples in P group were randomly distributed, while samples in N and S group were well clustered. Plus, the two groups can also be separated, indicating the difference between N and S group was originating from inter-group

variability rather individual heterogeneity. The heat map showed significantly different expression patterns between the N, S, and P groups (Fig. 1b), and genes representing the secretory phase were more similar in groups N and S compared with group P. These DEGs could be almost clustered together according to the abundance of gene expression. Volcano plots representing the distribution of the different genomic profiles from the compared groups are shown in Fig. 1c, d.

Fig. 2 The top 10 enrichment GO (gene ontology) and KEGG pathway terms in (1) N vs P and (2) N vs S. x-axis represents negative $\log_2 p$ values. A GO analysis for biological process (BP); B GO analysis for molecular function (MF); C GO analysis for cellular components; (CC) D 10 most significant KEGG pathways

Gene ontologies clustering and pathway analysis

Gene ontology and pathway analysis of genes in the natural cycle and stimulation cycle were performed to find possible biological processes related to endometrial receptivity (Fig.

S1). The involved DEGs belong to almost all the signaling pathways and biological functions. Interestingly, of the many involved pathways, immune system and cancer-specific cell types, proliferation, and immune responses exhibited the most significant differences.

Notably, analysis of KEGG pathways of the DEGs showed that genes associated with the estrogen signaling pathway exhibited significant differential expression in the stimulation cycle (Fig. 3). Under the influence of excessive estrogen,

Potential biomarkers of endometrial receptivity in stimulated cycles and validation of data by RT-PCR

Known genes and their biological functions that were differentially expressed in both the natural and stimulated cycles are

Fig. 4 Validation data of the selected genes using quantitative RT-PCR. x-axis showed the gene names, and the fold changes on a log10 scale comparing P vs N (dark grey) and S vs N (light grey) were displayed on the y-axis. The data are presented as the mean $\pm$ standard error of mean (SEM)

shown in Supplemental Table S4. Based on biological function and relevance to reproduction, and by comparisons with DEGs in related studies, we identified three DEGs, ESR1, MMP10, and HPSE that were related to endometrial receptivity. In addition, ZCCHC12, IL13RA2, SRARP, and two lncRNAs, LINC01060 and LINC01104, were selected as potential novel markers or regulators of endometrial receptivity.

Expression of the eight selected transcripts was examined in the 3 groups by real-time PCR, and the results were consistent with the sequencing data (Fig. 4).

Analysis of lncRNAs

The two differentially expressed lncRNAs, LINC01060 and LINC01104, are intergenic non-coding RNAs, and their functions remain unclear. The website (<http://rtools.cbrc.jp/cgi-bin/RNARNA/index.pl>) was used to predict their target genes for LINC01060 (http://rtools.cbrc.jp/cgi-bin/RNARNA/createRankedHTML_bin.pl?gene=ENST00000513313&fm=1&to=100&ngenes=100&rmeth=min) and LINC01104 (http://rtools.cbrc.jp/cgi-bin/RNARNA/_createRankedHTML_bin.pl?gene=ENST00000452354&to=100&rmeth=min&fm=1&submit=submit). The top 100 target genes were selected for function analysis using topgene (<https://toppgene.cchmc.org/>). We found that the potential target genes of LINC01060, such as MAPK7 and MAP3K2, were enriched in the Erk5 MAPK signaling pathway (https://rgd.mcw.edu/rgdweb/Ontology/view.html?acc_id=PW:0000603, $p=0.0001279$, $fdr=0.04273$), as shown in Fig. S2. However, the target genes of this pathway were not differentially expressed among the N, P, and S groups. Enriched GO and pathways of LINC01104

were not found (https://toppgene.cchmc.org/output.jsp?userdata_id=1b13f39b-d23c-4fac-9ad9-679b0a9b6466).

Discussion

To our knowledge, this is the first study using NGS to compare the differential expression of genomic profiles between stimulated and natural endometrium. Compared with traditional commercial array methods, the NGS provides more comprehensive transcriptome information, including coding and non-coding mRNA, underlying the transition of endometrium [43]. We selected the late-proliferative phase as the non-receptive control group. As shown in Table 2, previous studies generally used LH+2 (i.e., the early secretory phase) as the pre-receptive endometrium model. However, the progesterone-induced transition from the proliferative to secretory phase is more essential for endometrial receptivity. It can be deduced that genes playing a key role in the transition process also have a role in endometrial receptivity or embryo implantation. To date, we have found only one study exploring changes in genomic expression of endometrium between the proliferative and mid-secretory phases in natural cycles [44].

Our results indicated that hyperphysiological E_2 levels resulted in the alteration of endometrial transcriptomic profiles. The hundreds of identified DEGs, whether new or familiar, were related to different aspects of endometrial receptivity. Some DEGs were shown in many studies to be associated with WOI or endometrial receptivity. For example, ESR1 is mainly expressed on endometrial cells and mediates the proliferation and differentiation of endometrial

Table 2 Overview of studies on the differentially expressed genes of endometrium under ovarian stimulation

Study	Comparator Stimulation protocol	Type of GnRH-a/ant	Sample size	Type of array	Fold change	Gene ontology (GO) KEGG pathways	Differentially expressed genes (DEGs)
Mirkin et al. (2004)	LH+8 VS hCG+9 GnRH agonist VS GnRH antagonist	Leuprolide acetate Ganirelix or Cetorelix	15	Affymetrix HG-U95Av2	≥ 1.2		Up: agonist—5 antagonist—6 Down: agonist—1 antagonist—6 Up: 13 Down: 0
Simon et al. (2005)	LH+2/4+7 VS hCG+2/4+7 GnRH agonist VS GnRH antagonist	Buserelin Ganirelix	12	Affymetrix HG-U133A	≥ 2		No relevant alteration was Observed.
Horcajadas et al. (2005)	LH+2/LH+7 VS hCG+7 GnRH agonist	Leuprolide acetate	24	Affymetrix HG-U133A	≥ 3		Up: 281 Down: 277
Macklon et al. (2008)	5 days after ovulation VS 5 days after ovum pick up GnRH antagonist	Ganirelix	8	Affymetrix HG-U133plus2	≥ 1.5	Cell adhesion, T-cell receptor signaling, regulation of signal transduction, cell growth, proliferation, and programmed cell death	Up: 142 Down: 98
Horcajadas et al. (2008)	LH+1/4+3/5/7/4+9 VS hCG+1/4+3/5/7/4+9 GnRH agonist	Leuprolide acetate	49	Affymetrix HG-U133A	—	Biological processes including cell proliferation, angiogenesis, cellular physiological process, cell death, and apoptosis were listed.	Up: 203 Down :97(2-day delay of genomic profile in COH)
Liu et al. (2008)	LH+7 VS hCG+7 Moderate responders VS High responders GnRH agonist	Buserelin (nasal spray)	13	Affymetrix HG-U133A	≥ 2	enzyme, signal transducer, ion-binding protein, transporter, receptor, transcription factor, cell adhesion molecule, reg- ulatory protein Wnt-signaling pathway, MAPK signaling pathway, nicotinamide metabolism	Moderate responders: Up: 5 Down: 2 High responders: Up: 244 Down: 159
Hauzi et al. (2009)	LH+2/LH+7 VS hCG+ 2/hCG+7 Not mention	Not mention	84	Affymetrix HG-U133plus2	> 2	cell division cycle (CDC) members, cyclin-dependent kinases (CDK), and members of the E ₂ F family of transcription factors TGFβ signaling pathway, the complement and coagulation cascades and leukocyte transendothelial migration	Up: 777 Down: 221
Hauzi et al. (2009)	LH+2/LH+7 VS hCG+ 2/hCG+5 GnRH agonist VS GnRH antagonist	Not mention	84	Affymetrix HG-U133plus2	> 2	Chemokines in inflammatory processes, growth factors related to endometrium G1/S checkpoint regulation	GnRH agonist: Up: 731 Down: 451 GnRH antagonist: Up: 634 Down: 210
Senapati et al. (2018)	LH+12 VS hCG+13 GnRH antagonist (7) and GnRH agonist (4)	Leuprolide acetate Type of GnRH-anta not mention	23	Affymetrix Human Gene 2.0 ST array+ Infinium DNA methylation 450 K BeadChip	> 1.5 > 2	Gene enrichment in endometrial remodeling Proteolytic pathway involved in extracellular matrix degradation	FC > 2: 165 DEGs, FC > 1.5: 641 DEGs 3322 genes with differential methylation, HPSE2, SERPINA5, MMP2, and MMP26 were similar to our common DEGs.

cells by E_2 [45]. Polymorphisms of ESR1 were associated with infertility, endometriosis, recurrent spontaneous abortion, and the transportation of embryos in the oviduct [46–49]. Types of ESR1 polymorphism were found to provide a predictive value on the ovarian response to exogenous FSH and the outcomes of assisted reproduction [50, 51]. MMP10, a family member of lytic enzymes that cleave protein components of the extracellular matrix, plays a role in uterine matrix degradation and embryo implantation [52]. Mutations in MMP10 were detected by whole exon sequencing in patients with recurrent spontaneous abortion, suggesting this mutation affected MMP10 function and may lead to miscarriage [53]. A significant decrease in MMP10 expression was found in the placentas of women with pre-eclampsia, demonstrating a role for MMP10 in maternal-fetal communication and maintenance of pregnancy [54]. Similarly, changes in HPSE expression have been found in the placentas of women with pre-eclampsia [55].

We also revealed some new potential markers. For instance, ZCCHC12, encoding a downstream effector of bone morphogenetic protein (BMP) signaling, was previously found by RNA-Seq to be an important DEG in the regulation of endometrial receptivity in natural cycles [35]. Despite this consistent finding, the mechanism has not been elucidated. There is limited available data on SRARP and IL13RA2. SRARP is involved in steroid hormone synthesis, and IL13RA2 belongs to the family, in which many members were found to be closely associated with endometrial receptivity [56].

Two long non-coding RNAs, LINC01060 and LINC011104, were found in our study, and to date, no studies have been reported relating either lncRNA to reproduction. LINC011104 is a non-coding RNA mostly found in fat tissue, lymph nodes, and testes. Enriched GO and pathways of LINC011104 were not found by enrichment analysis, and its function and interaction with other genes require further research. LINC01060 was found to be significantly down-regulated in pancreatic cancer tissues and was closely related to tumor invasion and metastasis [57]. Reported functional studies of its target genes were mainly focused on its role in regulating neurodevelopment. It is noteworthy that LINC01060 was found to have a link with the ERK5 MAPK pathway, which was previously found to participate in embryonic angiogenesis by regulating the expression of vascular endothelial growth factor, essential for embryo implantation [58]. Nevertheless, the target genes of LINC01060 in this signaling pathway were not differentially expressed between the current study groups. However, it is possible that genes with low expression levels may be indispensable for some essential pathways. The current results could also reflect limited sample size or the analytical methods used in our genetic analysis. Information about these lncRNAs and related genes was rather limited, and

further in vitro or in vivo experiments are needed to investigate their functions.

Mirkin et al. [28] first reported transcriptome differences in stimulated endometrium using microarrays, but identified less than 20 DEGs. Simon [33] and Horcajads [32] took a step forward by finding a delay in changes of genomic expression for about 2 days, a feature more significant in the GnRH agonist protocol compared with GnRH antagonist protocol [29]. Liu grouped patients as high and moderate responders based on the level of E_2 and found more DEGs in the high responders [31]. Interestingly, there were few overlapping DEGs among these studies. The most recent study found DEGs that were critical in the remodeling of endometrium and placenta formation, in which the identified MMP2 and HPSE2 were similar to our findings [43]. The variation of enrolled patients, techniques of data processing, and analysis may account for the heterogeneity of DEGs.

Successful embryo implantation depends on the balance of endometrial receptivity and selectivity under the precise control of functional and regulatory genes [59]. Despite many investigations of embryo implantation in vivo or in vitro, little is known of the underlying molecular mechanisms. There are hundreds of candidate genes or pathways with ambiguous function, which require further analysis. To improve our knowledge of the underlying mechanisms of embryo implantation, the signaling pathways of WOI or endometrial receptivity may provide valuable opportunities in the near future.

The current study has shed light on the special role of E_2 during the establishment of endometrial receptivity. For the first time, we identified two lncRNAs that may participate in the regulation of WOI under excessive E_2 levels, providing a new avenue for future research. However, there were some limitations of this study. For example, the sample size of each group was limited. Also, samples in natural and stimulated cycles were not paired due to the difficulties of multiple biopsies on the same patients in one period, possibly causing intra-individual bias. Fortunately, by the time the manuscript was completed, all 12 patients enrolled in the current study were getting a live birth or ongoing pregnancy after standard FET protocol, suggesting that the endometrium was healthy or receptive.

Conclusion

The phenomenon of lower implantation rates in high responders has puzzled reproductive endocrinologists for years. Side effects of excessive E_2 produced by COH have drawn much attention. Supraphysiologic hormonal levels lead to a high risk of OHSS and impaired endometrium function. Our study suggested that high E_2 levels in COH alter the expression of endometrial

receptivity-related mRNA and lncRNA. Further research studying on their pathways in vitro or functional identification in endometrial cell lines is needed to validate the biological function of these identified genes. Our findings may provide new genetic markers or pathways that will contribute to a better understanding of the WOI. Current methods for endometrial sampling and testing require further improvement, and a less invasive and more efficient method for endometrial biopsy is needed for clinical practice.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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