Ovarian Estrogen Synthesis Reduced by Peritoneal Endometriosis in Rat Autologous Transplantation Model

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Research Article

Keywords: peritoneal endometriosis, steroid hormone synthetases, FSHR, ovarian function, rat autologous transplantation model

Posted Date: December 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1160943/v1>

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Abstract

OBJECTIVE: To investigate the effects of peritoneal endometriosis on rat ovaries.

METHODS: A rat model of peritoneal endometriosis was established by autologous transplantation. qPCR was performed to measure mRNA levels of steroid hormone and steroid synthesis-related genes in the ovaries of endometriosis rats. Immunohistochemistry was performed to characterize the distribution of FSHR in the ovaries of endometriosis rats. RNAseq was performed to find pathological changes in the ovaries of endometriosis rats.

RESULTS: By qPCR, it was revealed that mRNA levels of steroid hormone synthesis-related genes were decreased in the ovaries of rats with endometriosis; With IHC, observed that FSHR expression was significantly decreased in the antral follicles of rats with endometriosis. RNAseq revealed that endometriosis affected transcription of the genes related to the microtubule structure and tight junctions of rat ovarian cells.

CONCLUSION: Peritoneal endometriosis decreased the genic expression of ovarian steroid hormone synthetases and FSHR protein level in granulosa cells of antral follicles, and reduced the mRNA levels of the microtubule structure and tight junctions in rat ovarian cells, which contribute to the impairment of ovarian function.

Introduction

Endometriosis is a disease caused by growth of endometrial tissue or cells outside the uterine cavity. Its prevalence rate is about 5% and mainly affects women of reproductive age between 25 and 35 years^[1]. Infertility is one of the main clinical symptoms of endometriosis. Studies have shown that the prevalence of infertility in endometriosis patients is as high as 30-50%^[2,3]. At present, the pathogenesis of endometriosis is not clear, but the hypothesis of menstrual blood reflux proposed by Sampson in 1927 is the most recognized^[2]. Sampson believes that the endometrial cells or tissues that fall off during menstruation enter the abdominal cavity with menstrual blood, and grow in the pelvic peritoneum or ovaries, causing endometriosis. Studies have shown that tissue adhesions caused by the ectopic endometrium with menstrual blood reflux can interfere with pregnancy from various links such as follicular development, ovulation, fertilization, and embryo transportation, then leading to infertility^[1,2,4,5].

The main functions of the ovary are oogenesis and steroid hormone synthesis. Studies have shown that endometriosis can cause cumulus granulosa cell apoptosis^[6], abnormal hormone metabolism^[7], increased inflammation^[8], abnormal energy metabolism^[9], and oxidative stress disorders^[10], which can cause Decreased Ovarian Reserve (DOR), thereby affecting the fertility of patients. In addition, in vitro Fertilization-Embryo Transfer (IVF-ET) technology is one of the main methods for the treatment of infertility caused by endometriosis, and DOR will directly affect the outcome of superovulation during IVF-

ET. However, the clinical situation is very complicated, often accompanied by ovarian ectopic lesions, it is difficult to say whether ovarian dysfunction was resulted from the direct damage or caused by indirect overall regulation.

In order to explore the impact of endometriosis on ovarian function, we established a rat model of endometriosis through autologous transplantation. The genic expression levels of steroid synthesis-related enzymes, and FSHR levels in antral follicular granulosa were used to evaluate ovarian function. Transcriptome sequencing of the ovary was applied to find the related pathways mediating the damages from peritoneal ectopic lesions.

Methods

1 Animals

In this study, 24 female non-pregnant SD rats (body weight of $180 \text{ g} \pm 20 \text{ g}$) aged 6-8 weeks were used, including 12 in the model group and 12 in the sham operation group, all of which were purchased from Shanghai SIPPR-BK Experimental Animal Co., Ltd. (China). The experiment was carried out after 1 week of environmental adaptation in the SPF rodent room of Shanghai Institute for Biomedical and Pharmaceutical Technologies. All procedures were approved by the Animal Ethics Review Committee of Shanghai Institute for Biomedical and Pharmaceutical Technologies.

2 Rat model of endometriosis

The rat model of endometriosis was established using the autologous transplantation method previously used by our team^[11, 12]. Simply, after the rats were anesthetized by inhalation of isoflurane, they were fixed on a standard rat operating table, routinely sterilized, and cut along the midline of the abdomen to fully expose the uterus. After ligation, a segment, about 1.5 cm in length, was cut from the left uterine horn, and the endometrium was separated from the smooth muscle with forceps in normal saline, and was trimmed it into 2 pieces with a size of approximately $5 \text{ mm} \times 5 \text{ mm}$. Suture the small endometrial pieces to the right side of the ventral wall where there are abundant blood vessels, leaving the surface epithelium facing the abdominal wall. Rats in control group only performed to open and close the abdomen, without uterine operation and endometrial transplantation.

On the 21st day since modeling operation, the laparotomy was performed again to check the growth of the ectopic endometrium. The volume of the graft (length $\times$ width $\times$ height) was measured with an electronic vernier caliper, and the rats with an endometrial graft larger than 20 mm^3 were sampled to collect ovaries for follow-up study.

3 RNA isolation, cDNA synthesis and qPCR

Total RNA extracted with TRIZOL reagent (Invitrogen, USA) was reverse transcribed into cDNA using the ReverTra Ace qPCR RT Master Mix (Toyobo, Japan) according to the manufacturer's instructions. Quantitative Real-time PCR was taken with THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan) and gene-specific primers (see Table 1 for primer sequences) on the LightCycler 480 system (Roche, Switzerland). Specificity of each PCR amplification was verified by melting curve analysis. Data were normalized to the expression of the housekeeping gene *Gapdh*.

4 Immunohistochemistry

Ovaries were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin, 4- μ m thick slices were sectioned. After conventional dewaxing and rehydrating procedures, the slices were heated at 98°C in a microwave oven for 20 minutes in citrate buffer (pH 6.0), and then incubated with 3% H₂O₂ for 10 min to remove endogenous peroxidase, and in 10% goat serum at 37°C for 1 h to block the non-specific immune binding. The primary antibodies against FSHR (Invitrogen, PA5-99424, USA) were diluted to 1:200, and sections were incubated with the primary antibody overnight at 4 °C. Following three washes with PBS, the slides were incubated with biotin-conjugated secondary antibody (1:200, Proteintech, SA00004-2, USA) for 1 h. The slides were then marked with an avidin-biotinylated horseradish peroxidase complex solution (1:200, Proteintech, SA00001-0, USA) at 37 °C for 1 h. The marked FSHR proteins were visualized with 3,3-diaminobenzidine chromogen. Sections were counterstained with hematoxylin. The primary antibody replaced with 10% goat serum was served as a negative control. For quantitative analysis of immunohistochemical staining, all antral follicles in each ovarian section were observed and recorded under a microscope (Nikon NI-u, Japan) equipped with Nikon Plan Flour $\times$ 4 (N.A. 0.13, W.D. 17.2 mm, Japan) or $\times$ 10 (N.A. 0.30, W.D. 16.0 mm, Japan) objective lens and a color camera (Nikon DS-Ri2, Japan). The Integrated Optical Density (IOD) / Area of each antral follicle was calculated by Image Pro-Plus 6.0 (Media Cybernetics, USA) to compare the expression of FSHR between two groups of antral follicles.

5 Transcriptome sequencing and bioinformatics analysis

Shanghai Biotechnology Co., Ltd. (China) conducted RNA library construction and transcriptome sequencing on our ovarian RNA samples, including 2 samples in the model group and 2 samples in the control group. The total RNA was first examined by the NanoDrop ND-2000 spectrophotometer (Thermo, USA) / Qubit2.0 and Agilent Bioanalyzer 2100 (Thermo, USA), and then high throughput sequencing was performed using the Illumina NovaSeq6000 sequencing platform (USA). Based on the technology of sequencing by synthesis, the raw data of sequencing were submitted to FastQC for quality validation. Use Seqtk tool to remove unqualified reads, and the removed reads mainly included reads containing adapter, reads with quality of 3'-terminal base less than 20, reads with a length less than 25, and ribosomal RNA

reads. Genome mapping was done using HISAT2^[13], and a genomic version of Rnor 6.0. Used FPKM^[14] to calculate gene expression, and then conducted Pearson correlation coefficient between the samples. The EdgeR package^[15] was used to analyze the different expression of mRNAs (DEMs) between the two groups. Used ggplot2 package to generate a volcano map and a heat map of the DEMs. The genes for $\log_2(\text{Fold Change}) > 1$ were labeled red (corrected $p < 0.05$), and those for $\log_2(\text{Fold Change}) < -1$ were labeled blue (corrected $p < 0.05$). We used the clusterProfiler package^[16] for the enrichment analysis of the Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG). Based on KEGG and GO databases, we also conducted a gene set enrichment analysis (GSEA) to explore the mRNA changes related to endometriosis-induced ovarian injury.

6 Statistics

Data were analysed using Prism 9.0 software. The experimental data are presented as the mean $\pm$ standard deviation. Statistical comparison between two groups was carried out using the unpaired Student's t-test. The experimental data are presented as the mean $\pm$ standard deviation. Data were considered statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

1 Effects of endometriosis on ovarian steroid and estrogen synthesis in rats

Studies have reported that the levels of estrogen and aromatase activity in the follicular fluid of endometriosis-related infertile patients are significantly lower than those of infertile patients with non-endometriosis factors^[17, 18]. For this reason, we used qPCR to detect the mRNA expression levels of steroid hormones (*Cyp11a1*, *Cyp17a1*, and *Cyp19a1*) and steroid (*Cyp51*, *Dhcr24*, and *Sqle*) synthesis-related genes in the ovaries of endometriosis rats. We found that the relative expression levels of aromatase genes *Cyp11a1* (EMS vs. Control: 2.35 ± 0.87 vs. 4.01 ± 1.66 , $p < 0.01$, Fig.1A), *Cyp17a1* (EMS vs. Control: 0.076 ± 0.120 vs. 0.209 ± 0.086 , $p < 0.01$, Fig.1B) and *Cyp19a1* (EMS vs. Control: 0.0048 ± 0.0039 vs. 0.0122 ± 0.0046 , $p < 0.001$, Fig.1C) in the EMS group were significantly lower than those in the control group. Although there was no significant difference ($p > 0.05$) between the two groups, the expression levels of steroid synthesis-related genes (EMS vs. Control: *Cyp51*: 0.186 ± 0.169 vs. 0.232 ± 0.127 , Fig.1D, *Dhcr24*: 0.092 ± 0.063 vs. 0.139 ± 0.049 , Fig.1E, *Sqle*: 0.232 ± 0.151 vs. 0.300 ± 0.134 , Fig.1F) in the EMS group also tended to be lower than the control group.

2 Endometriosis affects the expression of granulosa cell FSHR in rat antral follicles

Considering that the synthesis of estrogen is mainly in ovarian granulosa cells and is regulated by FSH, we used qPCR to detect changes in the mRNA level of *Fshr* in the ovaries of endometriotic rats, and then used immunohistochemistry to detect the expression of FSHR in ovarian granulosa cells of endometriotic rats to evaluate the follicular sensitivity to FSH signals. The mRNA level of *Fshr* in the ovaries of EMS group were significantly lower than those in the control group (EMS vs. Control: 0.00353 ± 0.00161 vs. 0.00626 ± 0.00242 , $p < 0.01$, Fig.2A). IHC showed abnormal expression of FSHR in granulosa cells of antral follicles in rats with endometriosis (Fig.2B). And then, we quantitatively analyzed the expression levels of FSHR in all antral follicles and found that the expression level of FSHR in EMS group was significantly lower than that in the control group (IOD/Area: 0.0114 ± 0.0132 vs. 0.0493 ± 0.0326 , $p < 0.0001$, Fig.2C). It shows that endometriosis affects the sensitivity of antral follicular granulosa cells in response to FSH signals by affecting the expression of FSHR.

3 Endometriosis affects the gene transcription of the microtubule structure and tight junctions in rat ovaries

In order to further explore the pathological changes of rat ovaries in endometriosis, we performed RNA sequencing on 2 ovary samples from control groups and 2 ovary samples from model groups. Person correlation coefficient analysis of the samples (Fig.3A) and heat map analysis of difference expression of mRNAs (DEMs, Fig.3B) demonstrated good reproducibility of samples within each group. We found that 14 genes were up-regulated and 875 genes were down-regulated in the ovaries of the endometriosis group compared with the control group (foldchange > 2, corrected $P < 0.05$, Fig.3C).

The enrichment analysis of DEMs between the two groups found that the most top 30 GO terms were mainly related to the microtubule structure, such as “outer dynein arm”, “inner dynein arm assembly”, “outer dynein arm assembly”, “ATP-dependent microtubule motor activity”, “dynein light chain binding”, “dynein heavy chain binding”, “microtubule bundle formation”, “dynein complex”, “dynein intermediate chain binding”, and so on (Fig.3D). Only one pathway (“tight junction”, no display) was found to be enriched in the KEGG database (counts = 21, enrich factor = 4.00, q value < 0.0001). Further gene set enrichment analysis of the mRNA datasets between the two groups revealed that genes associated with cell junctions (Fig.3E, especially tight junctions, Fig.3F-G), extracellular transport (Fig.3H) and estrogen signaling pathways (Fig.3J) were significantly enriched in the control group. Proteasome-related genes (Fig.3I) were significantly enriched in the endometriosis group.

Discussion

Among the animal models of endometriosis used in existing studies, the rat autologous transplantation model is the most commonly used. This is due to the high success rate of the model, accurate positioning, weak immune rejection, and easy observation. The autologous transplantation model is a peritoneal endometriosis model, in which two endometrial tissue sheets were sutured to the abdominal wall to form ectopic endometrial lesions. This model is difficult to simulate the natural adhesion process

of endometrial cells, and endometrial cells will not attach to other pelvic organs such as ovaries and fallopian tubes. Therefore, the influence of this model on the ovaries is bound to be in an indirect way. Some scholars believe that peritoneal endometriosis may affect the ovarian reserve function by affecting the inflammatory microenvironment in the peritoneal fluid^[5]. In view of the abnormalities of hormones, cytokines, inflammatory factors and oxidative stress markers in the blood of patients with endometriosis^[19], we speculate that peritoneal endometriosis may also affect the ovaries through blood or even nerves (pituitary-gonadal axis).

Endometriosis is an estrogen-dependent, chronic inflammatory disease. Local estrogen synthesis is often increased in ectopic endometrium for the growth of endometriotic lesions depends on estrogen^[20]. However, studies have shown that estrogen levels in serum and follicular fluid are significantly lower in patients with endometriosis than in normal subjects^[7, 17, 18] even though endometriotic lesions contain major enzymes of the estrogen synthesis pathway that can locally complete cholesterol to estrogen synthesis^[21]. Thus, changes in estrogen levels may contribute to the pathogenesis of endometriosis and endometriosis-related infertility. In female mammal ovaries, estrogen synthesis is mainly dependent on theca cells and granulosa cells. In theca cells, cholesterol is transferred from the outer mitochondrial membrane to the inner membrane via Steroidogenic Acute Regulatory protein (StAR), catalyzed by P450cholesterol Side-chain Cleavage Enzymes (P450scc, the coding gene is *Cyp11a1*) to form pregnenolone, and then catalyzed by 3 β -Hydroxysteroid Dehydrogenase (the coding gene is *Hsd3b1* or *Hsd3b2*) and 17 α Hydroxylase (the coding gene is *Cyp17a1*) to form androstenedione. Androstenedione enters granulosa cells and is catalyzed by Cytochrome P450 aromatase (the coding gene is *Cyp19a1*) to form estrogen. Notably, changes in estrogen production usually depend on the amount of pregnenolone synthesized, therefore, the transfer of cholesterol to P450scc belongs to the rate-limiting step of estrogen synthesis. In our study, we found that mRNA levels of *Cyp11a1*, *Cyp17a1* and *Cyp19a1* were significantly lower in endometriosis rats than in controls, which would affect estrogen synthesis in endometriosis rats in the long run, these results were confirmed in human blood and follicular fluid^[7, 17, 18]. Furthermore, we hypothesize that reduced estrogen synthesis in the ovary may be a way for the body to defend itself against the damage caused by estrogen-rich endometriosis lesions.

Granulosa cells are not only involved in the synthesis of estrogen, but also in oocyte growth, differentiation, follicular cavity formation and follicular development, which are essential for maintaining ovarian function. Follicle-stimulating hormone (FSH) is necessary for the maintenance of normal granulosa cell function. FSH can not only promote the growth of granulosa cells and inhibit the apoptosis of granulosa cells, but also activate the cytochrome P450 aromatase of granulosa cells to promote the synthesis and secretion of estradiol^[22]. In addition, for activating follicles, whether FSH can meet the needs of granulosa cells will determine whether the follicle is atresia or develops into a dominant follicle^[23]. Our study found that the expression of FSHR in the antral follicles in the ovaries of endometriosis rats was significantly lower than that of the control group, which would affect the sensitivity of granulosa cells in response to FSH signals, resulting in decreased estrogen synthesis in the

ovaries of endometriosis rats. From a more far-reaching perspective, the reduction of FSHR expression will also affect the development of follicles in endometriosis rats. Although it has not been verified in our research, we can obtain these results from other studies^[24, 25].

The cytoskeleton is the fiber network structure of proteins in cells, in which microtubules are involved in various biological processes such as cell morphology maintenance, material transportation, information transmission and cell division. Through the GO enrichment analysis of DEMs, we found that the cytoskeleton, especially microtubule-related genes, including *Dnah2*, *Dnah1*, *Dynlrb2*, *Krt18*, *Aurkb*, *Drc1*, *Kif19*, *Agbl2*, *Dnah6*, *Wdr63*, *Dnah9*, *Drc7*, *Kif27*, *Kif6*, *Kif5c*, and *Kif9*, were significantly downregulated in endometriosis rats, suggesting that endometriosis may affect ovarian structure and function by affecting the cytoskeleton of ovarian cells. In support of our findings, it has been found that the dynein light chain roadblock-type (DYNLRB) family affects zebrafish follicle development by influencing TGF β signaling^[26, 27]. High expression of AGBL2 can lead to poor prognosis in patients with ovarian carcinoma^[28]. Keratin type I cytoskeletal 18 (KRT18) inhibits the apoptosis of ovarian granular tumor cells by inhibiting Fas signaling^[29]. Aurora Kinase B (AURKB) and Progesterone Receptor Membrane Component 1 (PGRMC1) are jointly involved in late events of bovine granulosa cell mitosis and oocyte meiosis^[30, 31]. Although it is not clear which type of cell has an abnormal cytoskeleton, existing studies have shown that endometriosis affects the cytoskeleton of the oocyte and thus affects its meiosis^[32, 33]. Abnormal microtubules can also cause changes in the morphology of granulosa cells and theca cells, thereby affecting the synthesis of steroid hormones^[34-37]. Microtubules may affect the synthesis of steroid hormones by affecting the transfer of cholesterol to mitochondria^[38]. The above results suggest that endometriosis may affect steroid hormone synthesis and oocyte maturation by affecting the cytoskeleton structure of ovarian cells. In addition, the significant down-regulation of dynein (including *Dnah2*, *Dnah1*, *Dnah6*, *Dnah9*, etc.), kinesin (including *Kif19*, *Kif27*, *Kif6*, *Kif5c*, *Kif9*, etc.) and extracellular transport-related genes, up-regulation of proteasome-related genes and down-regulation of estrogen signaling-related genes in endometriosis rat ovaries suggest that endometriosis affects the microtubule structure and microtubule-related motor protein activity of ovarian cells, which affects extracellular transport, resulting in abnormal protein aggregation and increased proteasome activity, and thus damages the structure and function of ovarian cells.

The types of cell junction mainly include tight junction, adhesive junction and communicating junction. Among them, gap junctions in communicating junction have been confirmed to play a central role in the maturation and fertilization of oocytes^[39]. However, some studies have also found that tight junctions play an important role in the formation of the follicle cavity and the development of the follicle^[40]. Although there are differences between species, tight junction related proteins, mainly including cingulin (CGN), claudin (CLDN), occluding (OCLN) and tight junction protein (TJP), are widely present in granulosa, theca externa and thecal vascular endothelial cells, and the expression of tight junction proteins is regulated by estrogen and androgens^[41-43]. During the development of marmoset follicles, the expression level of tight junction related protein CLDN5 increases in the theca cells^[43]. The *Cldn11* mRNA level of

large antral follicles was also found significantly higher than that of small antral follicles in cattle^[44]. The expression of *Ocln* was more prevalent in bovine theca cells than in granulosa cells and gradually decreased with follicular development^[42]. These studies suggest that tight junctions are involved in follicular development. In addition, overexpression of *Cldn3*^[45], *Cldn4*^[46] and *Cldn7*^[47] leads to the development of ovarian cancer and malignant metastasis, thus homeostasis in tight junctions are essential to maintain normal ovarian structure and function. Through the KEGG enrichment analysis of DEMs and the GSEA analysis of the transcriptome expression profile, we found that the expression of tight junction-related genes, including *Cldn3*, *Cldn4*, *Cldn7*, *Cldn8*, *Cldn10*, *Cldn22*, *Ocln*, *Tjp3*, was significantly down-regulated in the ovaries of endometriosis rats, as compared to the control group. So, we speculate that the decreased estrogen synthesis in the ovary of endometriosis rats will affect the tight junctions between ovarian cells, resulting in abnormal follicular development and damage to ovarian function. In addition, tight junctions are also involved in the formation of the blood-follicle barrier (BFB). Structural abnormalities of BFB caused by down-regulation of tight junction related proteins expression may lead to a large number of factors in the blood to enter the follicular cavity and damage oocytes or granulosa cells.

The ectopic endometrium forms lesions by adhering to specific tissues. Through RNAseq, we did not find that the ovarian samples of rats with peritoneal endometriosis had enriched cell adhesion-related pathways, and there was no discovery to support that the endometrial cells had invaded in the ovaries in our model. In this way, we believe that endometriosis lesions can affect ovarian steroid hormone secretion through indirect pathways, such as neuroendocrine regulation, which gives an explanation for mild infertility owing to peritoneal endometriosis.

Conclusion

This study found that endometriosis caused the granulosa cells of rat antral follicles to reduce their sensitivity to FSH, thus inhibiting the synthesis of ovarian steroid hormones and down-regulating the gene transcription of the microtubule structure and tight junctions in ovarian cells, through indirect pathways such as the neuroendocrine system. Our results clarify part of the pathogenesis of infertility caused by peritoneal endometriosis, and provide a reference for clinical research and development of new technologies for the treatment of infertility associated with endometriosis.

Abbreviations

AURKB	Aurora Kinase B
BFB	blood-follicle barrier
CGN	cingulin
CLDN	claudin
DEMs	difference expression of mRNAs (DEMs)
DOR	Decreased Ovarian Reserve
DYNLRB	dynein light chain roadblock-type family
EMS	endometriosis
GO	Gene Ontology
GSEA	gene set enrichment analysis
IOD	Integrated Optical Density
IVF-ET	in vitro Fertilization-Embryo Transfer
KEGG	Kyoto Encyclopedia of Genes and Genomes
KRT18	Keratin type I cytoskeletal 18
OCLN	occluding
P450scc	P450cholesterol Side-chain Cleavage Enzymes
PGRMC1	Progesterone Receptor Membrane Component 1
StAR	Steroidogenic Acute Regulatory protein
TJP	tight junction protein

Declarations

Acknowledgments

Not applicable.

Author contribution

ZJ worked on the experiment and manuscript writing, ZY directed and participated in the detection of ovarian function indicators, LJ participated in ovarian function evaluation, YL, LP and WX participated in rat model preparation and tissue sampling, ZT participated in the design of the study, SZ conceived and designed this study, responsible for the result analysis and manuscript modification.

Funding

This study was supported by National Natural Science Foundation of China (No. 82071598, 82074475).

Availability of data and materials

The datasets used in this study are available from the corresponding authors upon reasonable request.

Ethics approval and consent to participate

All animal experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were supported by the Animal Ethics Review Committee of Shanghai Institute for Biomedical and Pharmaceutical Technologies.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Reduction of steroid hormone and steroid synthesis-related mRNA levels in the ovary of endometriosis rats. (A) The level of Cyp11a1 mRNA (2.35 ± 0.87 vs. 4.01 ± 1.66 , $p < 0.01$) in the ovary of endometriosis rats ($n=11$) was significantly lower than that in the control group ($n=12$). (B) The level of Cyp17 mRNA (0.076 ± 0.120 vs. 0.209 ± 0.086 , $p < 0.01$) in the ovary of endometriosis rats ($n=12$) was significantly lower than that in the control group ($n=12$). (C) The level of Cyp19 mRNA (0.0048 ± 0.0039 vs. 0.0122 ± 0.0046 , $p < 0.001$) in the ovary of endometriosis rats ($n=11$) was lower than that in the control group ($n=12$). (D) The level of Cyp51 mRNA (0.186 ± 0.169 vs. 0.232 ± 0.127 , $p > 0.05$) in the ovary of endometriosis rats ($n=12$) was lower than that in the control group ($n=12$). (E) The level of Dhcr24 mRNA (0.092 ± 0.063 vs. 0.139 ± 0.049 , $p > 0.05$) in the ovary of endometriosis rats ($n=12$) was lower than that in the control group ($n=12$). (F) The level of Sqle mRNA (0.232 ± 0.151 vs. 0.300 ± 0.134 , $p > 0.05$) in the ovary of endometriosis

rats (n=8) was lower than that in the control group (n=8). Data represent as mean $\pm$ sd of biological duplicate experiments.

Figure 2

Expression of FSHR in ovary of endometriosis rats. (A) The level of *Fshr* mRNA (0.00353 ± 0.00161 vs. 0.00626 ± 0.00242 , $p < 0.01$) in the ovary of endometriosis rats (n=12) was significantly lower than that in

the control group (n=12). (B) Immunohistochemistry showed abnormal expression of FSHR in granulosa cells of the ovary in endometriosis rats (n=8) compared with control group (n=8), NC: Negative Control. (C) Immunohistochemical quantitative analysis showed that the expression of FSHR (IOD/Area: 0.0114±0.0132 vs. 0.0493±0.0326, $p < 0.0001$) in ovarian antral follicles of endometriosis rats (n=35) was significantly lower than that of the control group (n=31).

Figure 3

RNAseq revealed mRNA changes of microtubule structure and tight junctions of ovarian cells in endometriosis. (A) Analysis of pearson correlation coefficient of mRNA datasets of ovary in 2 endometriosis ovaries and 2 control ovaries. (B) Volcano map of differentially expressed mRNAs (DEMs) using ggplot2 packages. The red and blue points indicate the up-regulated and down-regulated DMEs, respectively (fold change > 2 and corrected p value < 0.05). (C) Heat map of the 14 up-regulated DMEs and 875 down-regulated DMEs. (D) The top 30 GO enrichment terms for DEMs between the ovaries of endometriosis and control group. Items with corrected p value (q-value) < 0.05 were considered to be significantly enriched. (E-J) ClusterProfiler package was used to conduct GSEA. Gene sets with $P < 0.05$ and false discovery rate (FDR) < 0.25 were considered to be significantly enriched. The genes of "GO: cell-cell junction" (E), "GO: tight junction" (F), "KEGG: tight junction" (G), "GO: extracellular transport" (H), "KEGG: estrogen signaling pathway" (J) were significantly enriched in control group, and the genes of "KEGG: proteasome" (I) were significantly enriched in endometriosis group. NES: Normalized enrichment score.