The Role of FDX1 in Granulosa Cell of Polycystic Ovary Syndrome (PCOS)

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Abstract

Background: To explore the development mechanism of PCOS and Transcriptomics was applied to seek the key gene.

Methods: Transcriptomics marked by UID (unique identifier) technique of granulosa cell in PCOS and control women was measured. RT-PCR was applied to verify the key gene. Two kinds of PCOS models modeling with Letrozole and Testosterone Propionate were applied to verify the key gene of granulosa cell in PCOS.

Results: The outcome of GO-enrich of transcriptomics concentrated in steroid metabolism and mitochondria. The different genes were sought from coexisting in both steroid and mitochondria. Finally, five different genes including CYP11A1、CYB5R1、STAR、FDX1 and AMACR were obtained. RT-PCR was administrated to furtherly verify the downregulating FDX1 in PCOS, which showed the consistent outcome with the transcriptomics. FDX1 in granulosa cell of antral follicle in both PCOS model rats was measured and was lower than control rats, which was consistent in two kinds of PCOS models.

Conclusion: FDX1 was related with steroid metabolism and mitochondrial and may participate in the development of PCOS.

Background

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women during their reproductive ages, associated with a plethora of cardiometabolic consequences, with obesity, insulin resistance and hyperandrogenemia playing a major role in the degree of such manifestations [1]. As far, the pathogenesis of PCOS was still uncertain. Many mechanisms was mentioned including disturbed HPO axis [2]、hyperinsulinemia[3]、heredity[4] and so on. Granulosa cell was closely neighboring to follicle and played a very import role in the development of follicle, which was paid more attention to the pathogenesis in PCOS. Factors secreted from granulosa cell such as estradiol and insulin-like growth factor were revealed to be essential for follicular growth/development [5]. The granulosa layer is aligned along the follicular basal lamina, no apoptotic cells are observed in growing healthy follicles [5]. Some studies showed that granulosa cell apoptosis increased in PCOS to induce follicular premature atresia [6–9]. DHEA-induced PCOS rat model indicated granulosa cell apoptosis increased [10]. Confusingly, significant differences in the rate of cell death and proliferation in granulosa cell populations in PCOS patients were found, which was associated with decreased expression of apoptotic effectors and increased expression of a cell survival factor [11]. So the status of granulosa cell in PCOS were unclear and granulosa cells of PCOS patients and control women were collected and measured by transcriptomics in this study, exploring the abnormal mechanism of granulosa cell in PCOS.

Ferredoxins are low molecular mass proteins (6–25 kDa) that are negatively charged at neutral pH and contain iron-sulfur clusters as a redox active group[12]. Humans possess two mitochondrial ferredoxins, Fdx1 and Fdx2[13, 14]. Mammalian adrenodoxin (ferredoxin 1; Fdx1) is essential for the synthesis of

various steroid hormones[10]. As a member of the [2Fe-2S] cluster-containing ferredoxin family, Fdx1 reduces mitochondrial cytochrome P450 enzymes (such as CYP11A1), which then catalyze the conversion of cholesterol to pregnenolone, aldosterone, and cortisol [10]. From here we see that FDX1 play a very important role in steroid hormones.

Granulosa cells are essential in normal follicular maturation process since they produce steroidal hormones and growth factors, and they play a crucial role in follicular atresia [15]. FDX1 was found a significant difference between PCOS women and control women after transcriptomics in this study. The relation of FDX1 in granulosa and PCOS was not mentioned. We initially indicated that FDX1 may play a very important role in PCOS.

Methods

Participants and Granulosa cell collect

All PCOS women and control women were recruited from Reproductive Medical Center of Tongji Hospital Affiliated to Tongji Medical College, Huazhong University of science and technology. The inclusive criteria of PCOS women are as follow: 1, diagnosed with PCOS; 2, the ages were not more than 34 years old; 3, Except IVF treatment, no other treatments were done in a month. The exclusive criteria of PCOS women; 1, diagnosed with hyperprolactinemia; 2, abnormal androgen secretion due to adrenal or ovarian tumors; 3, patients with uncorrected thyroid disease; 4, suspected Cushing's syndrome; 5, using estrogen or oral contraceptives and other hormone drugs in recent one month; 6, using other drugs that affect reproductive function or metabolism in the past 2 months (such as anti-obesity drugs, anti-diabetes drugs and traditional Chinese medicine, etc.). Control women receiving IVF treatment were recruited owning to fallopian tube jam□intrauterine adhesions and male factors. The ages of control women were not more than 34 years old. Granulosa cell was collected by Percoll density-gradient centrifugation method[16]. The follicle aspirate was collected and centrifuged at 200g for 10mins. The supernatant was discarded and the sediment was collected and resuspended with PBS. The resuspended solution was pipetted into the 50% Percoll gradient (lot number: P4937, sigma) of equal volume and centrifuged at 400g for 20mins. Intermediate cell layer was collected and resuspended with PBS, which were centrifuged at 200g for 10mins. Finally, the sediment was collected and deposited at -80℃ refrigerator.

Total RNA extraction

Total RNA was extracted by Trizol method. 1ml Trizol reagent (Invitrogen, cat. NO 15596026) was pipetted into each tube of sediment (granulosa cell) at room temperature for 5mins. After centrifuging at 12000g for 15mins, the supernatant were collected into another tube, mixed with 200ul chloroform. After remaining for 15min at room temperature and centrifuging at 12000g 4℃ for 15mins, the water layer of supernatant was removed into a tube mixed with 0.5ml isopropanol, keeping for 10mins at room temperature. After centrifuging at 12000g 4℃ for 10mins, the supernatant was discarded and sediment of RNA was collected resuspended with 1 ml 75% ethanol. After centrifuging at 8000g 4℃ for 5mins, the supernatant was discarded and sediment of RNA was obtained. The RNA was dissolved with TE buffer

and concentration of RNA was measured by Nanodrop™ OneCspectrophotometer (Thermo Fisher Scientific Inc). 1% agarose gel electrophoresis was administrated to observe the integrity of the strip.

UID-mRNA-seq

After the total RNA samples were up to standard, 5ug of total RNA was taken for subsequent experiments. KCTM Stranded mRNA Library Prep Kit (Catalog NO. DR08402, Wuhan Seqhealth Co., Ltd. China) was applied in RNA library preparation according to the manufacturer's instruction. The mRNA was enriched by magnetic beads with oligo (dT). Then, fragment buffer was added to break the resulting mRNA into short fragments. A six base random primer was used to synthesize a single strand of cDNA using the mRNA fragment as a template, and then two strand cDNA was synthesized by adding buffer, dNTPs and DNA polymerase I. After elution and purification, the terminus of double stranded cDNA was repaired with base and added with sequencing adaptor. The 5 'end of cDNA was connected to UID connector. The fragment was caught by magnetic beads and PCR amplification was performed by T100™ Thermal Cycler (BIO-RAD, USA). Agarose electrophoresis was used to detect the quality of the cDNA library. Qubit 3.0 with Qubit™ RNA Broad Range Assay kit (Life Technologies, Q10210) is used to quantify the cDNA library and determine whether the cDNA library concentration is suitable for the computer. After the cDNA library passed the quality inspection, the different cDNA library was sequenced on Illumina sequencer (Illumina NovaSeq 6000) according to the requirements of effective concentration and target offline data volume.

UID-mRNA-seq data analysis

RPKM (Reads per Kilobase per Million Reads) was calculated to estimate gene expression. Differentially expressed genes was were identified using the edgeR package (version 3.12.1) by R4.0.2. Gene expression differences were judged by p-value < 0.05 and fold-change > 2. KEGG enrichment analysis and Go enrichment analysis for differentially expressed genes were administrated by KOBAS software (version 2.1.1)

Quantitative Real-time PCR

Total RNA was extracted by Trizol method. Reverse transcription PCR was implemented by strand cDNA Synthesis kit (11141ES60, Yeasen, China) according to instruction. Quantitative Real-time PCR was implemented by qPCR kit (11201ES08, Yeasen, China) according to instruction. FDX1, forward primer: CTTTGGTGC ATGTGAGGGAA, reverse primer: GCATCAGCCACTGTTTCAGG. The $2^{-\Delta\Delta CT}$ method was used for data analysis.

PCOS modeling

Two PCOS rat model were implemented. Letrozole and Testosterone Propionate were applied into modeling PCOS. All rats included Wistar weighting 250□300g, purchased from the Hubei Provincial Center for Disease Control and Prevention, Wuhan, China and housed in SPF room of experimental animal center, Tongji Medical College, Huazhong University of science and technology. This animal experiment and all operation of rats were approved by the Institutional Animal Care and Use Committee at Tongji

Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Wistar rats modeling with Letrozole were perfused with Letrozole 1mg/kg via stomach, continuing for 21days. Control Wistar rats and model Wistar rats were sacrificed at the end of perfusing. Wistar rats modeling with Testosterone Propionate were injected with 10 mg/kg Testosterone Propionate, continuing for 6 weeks. Control rats and model rats injected with Testosterone Propionate were sacrificed at the end of injection. Ovary was picked up and fixed by paraformaldehyde to Paraffin embedding. All rats(n = 6 for each group) were euthanized by intraperitoneal injection with pentobarbital sodium.

Immunohistochemistry

After paraffin section of ovary dewaxing with xylene and antigen repair with Sodium citrate repair solution, the sections were put into 3% hydrogen peroxide solution and incubated for 25 min at room temperature. After sections washing for 3 times (each time for 5mins), sections were covered with 10% goat serum for 30mins. Then, goat serum was discarded and anti-FDX1 antibody (ab108257, Abcam, UK) was added to incubate for the whole night at 4℃. After washing for 3 times (each time for 5mins), sections were incubated with secondary antibody (ab6721, Abcam, UK) for 1 hour at room temperature. DAB was added into the section to render color for 1mins. After DAB stain stopping, cell nucleus was stained with Hematoxylin for 1 min. After Hematoxylin stain stopping, sections were added with 1% alcohol hydrochloride for several seconds and added with Ammonia to turns blue. Finally, the sections were dehydrated with Gradient concentration alcohol and mounted.

Statistics analysis

Student's t test analysis was applied if data was meeting with normal distribution and homogeneity of variances. Otherwise, Mann-Whitney U was applied. P value < 0.05 was considered significant. IBM SPSS 23 was used to analyze data and GraphPad prism 7 was applied to plot.

Results

Characteristics of clinical baseline

21 control women and 22 PCOS women were included in the experiment. Characteristics of clinical baseline were shown in Table1. Plasma LH and LH/FSH in PCOS women were higher than control women. Plasma PRL in PCOS women was lower than control women. Plasma AMH in PCOS women were higher than control women.

Table 1
Characteristics of clinical baseline

Characteristics	Control(n = 21)	PCOS(n = 22)	P value
Age	28.52 ± 3.63	28.05 ± 3.11	> 0.05
BMI	20.54 ± 1.77	23.51 ± 4.15	> 0.05
FSH (mIU/ml)	7.62 ± 1.99	6.65 ± 1.53	> 0.05
LH (mIU/ml)	4.14(3.22,5.38)	10.18 ± 6.08	**< 0.05**
LH/FSH	0.62 ± 0.25	1.14(0.86,2.12)	**< 0.05**
E_2 (pg/ml)	43.12 ± 15.87	44.49(38.68,59.29)	> 0.05
PRL (ng/ml)	16.41 ± 7.04	10.58(8.17,12.70)	**< 0.05**
P(ng/ml)	0.65(0.44,0.80)	0.64 ± 0.41	> 0.05
T(ng/dl)	38.40 ± 16.22	46.36 ± 23.24	> 0.05
AMH (ng/ml)	4.18 ± 1.54	10.51 ± 4.84	**< 0.05**

UID-mRNA-seq

Granulosa cell of 3 PCOS women and 3 control women were measured via UID-mRNA-sEq. Different expression genes were shown in volcano plot (Fig. 1A) and heatmap plot (Fig. 1B). Totally, 183 up-gene and 400 down-gene were obtained (Fig. 1C).

Go enrichment and KEGG enrichment

Go enrichment and KEGG enrichment according to up-gene and down-gene were implemented. Up-gene Go enrichment showed that up-genes were mainly located in domain steroid and domain mitochondria (Fig. 2A). Down-gene Go enrichment was mainly located in term SH3/SH2 adaptor activity□response to copper ion□regulation of osteoblast differentiation□regulation of ossification and so on (Fig. 2B). Down-gene KEGG enrichment was mainly located in term Terpenoid backbone biosynthesis□Systemic lupus erythematosus□RNA degradation□Primary bile acid biosynthesis□Ovarian steroidogenesis and so on (Fig. 2C). Up-gene KEGG enrichment was mainly located in term Prion disease□MicroRNAs cancer and Hedgehog signaling pathway (Fig. 2D). Combining Go enrichment and KEGG enrichment, domain steroid and domain mitochondrial of Go enrichment were focused and Ovarian steroidogenesis of KEGG enrichment was focused. Finally, Five genes including CYP11A1□CYB5R1□STAR□FDX1 and AMACR were associated with domain steroid and domain mitochondria. FDX1 were focused in the experiment, not mentioned in PCOS so far.

FDX1 mRNA expression

To verify the relation between FDX1 and PCOS, RT-PCR was implemented. Granulosa cell of 21 control women and 22 PCOS women were measured by RT-PCR. Expression

of FDX1 in PCOS were lower than control. (Fig. 3)

FDX1 in PCOS model rat

Two kinds of PCOS model including Letrozole and Testosterone Propionate were applied to verify the role of FDX1 in PCOS furtherly. Protein of FDX1 in PCOS model rats modeling with Letrozole or Testosterone Propionate was lower than control rats. (Fig. 4)

Discussion

Profile of transcriptomics of granulosa cell indicated that abnormality of domain steroid and domain mitochondria may be related closely with the development of PCOS. Five down genes including CYP11A1、CYB5R1、STAR、FDX1 and AMACR were associated with domain steroid and domain mitochondria. FDX1 were rarely to be mentioned in PCOS so far. Relative mRNA expression of FDX1 were measured by RT-PCR to verify the transcriptomics, which showed that mRNA of FDX1 in PCOS was lower than control. Moreover, two kinds of PCOS model rat modeling with Testosterone Propionate and Letrozole respectively showed that FDX1 protein of granulosa cell of antral follicle in control rats were higher than PCOS. FDX1 may participate in the development of PCOS and play a very important role in PCOS.

Down-regulating FDX1 had been verified in PCOS, but the mechanism was unclear. A study indicated that transcription of FDX1 is regulated by the NR5A family and cAMP signaling, and participating in steroid hormone production in ovarian granulosa cells [17]. Another study indicated three Cytochrome P450 family proteins-Cyp1a1, Cyp11a1 and Cyp2u1-interact with Fdx1 to form an interactive network that in the ovarian steroidogenesis pathway [18]. FDX1 was associated with mitochondria and steroid synthesis. A study showed that mitochondrial dysfunction of human granulosa cells may contribute to the decline of steroidogenesis, decreased fertilization rate, oocyte maturation rate, and oocyte quality, and it can ultimately jeopardize fertility [19].

Mitochondrial biogenesis genes were downregulated in granulosa cells of PCOS mice when compared to the non-PCOS granulosa cells [20]. So FDX1 probably participated in the development of PCOS. More work needed to be done in the future, exploring the concrete mechanism of FDX1 in PCOS.

Conclusion

FDX1 was related with steroid metabolism and mitochondrial and may participate in the development of PCOS.

Abbreviations

PCOS
polycystic ovary syndrome;

Declarations

Ethics approval and consent to participate

The trial was approved by Chinese Ethics Committee of Registering Clinical Trials (ChiECRCT-2012030) recognized by Medical ethics committee of Tongji Hospital Affiliated to Tongji Medical College of Huazhong University of science and technology and written informed consent of all people in the trial was obtained.

Consent for publication

Not applicable

Availability of data and materials

All clinical data supporting the conclusions of this article are included within the article. Raw data of transcriptomics could be obtained from SRA (SRR13083683, SRR 13083684, SRR13083685, SRR13083686, SRR13083687, SRR13083688),which was uploaded in NCBI.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

ZW, HD, QW and LY contributed to data collection. PY and DMH contributed to data analysis and plot. ZW and DMH contributed to paper writing and revision. All authors confirmed with the final version of the manuscript

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Figures

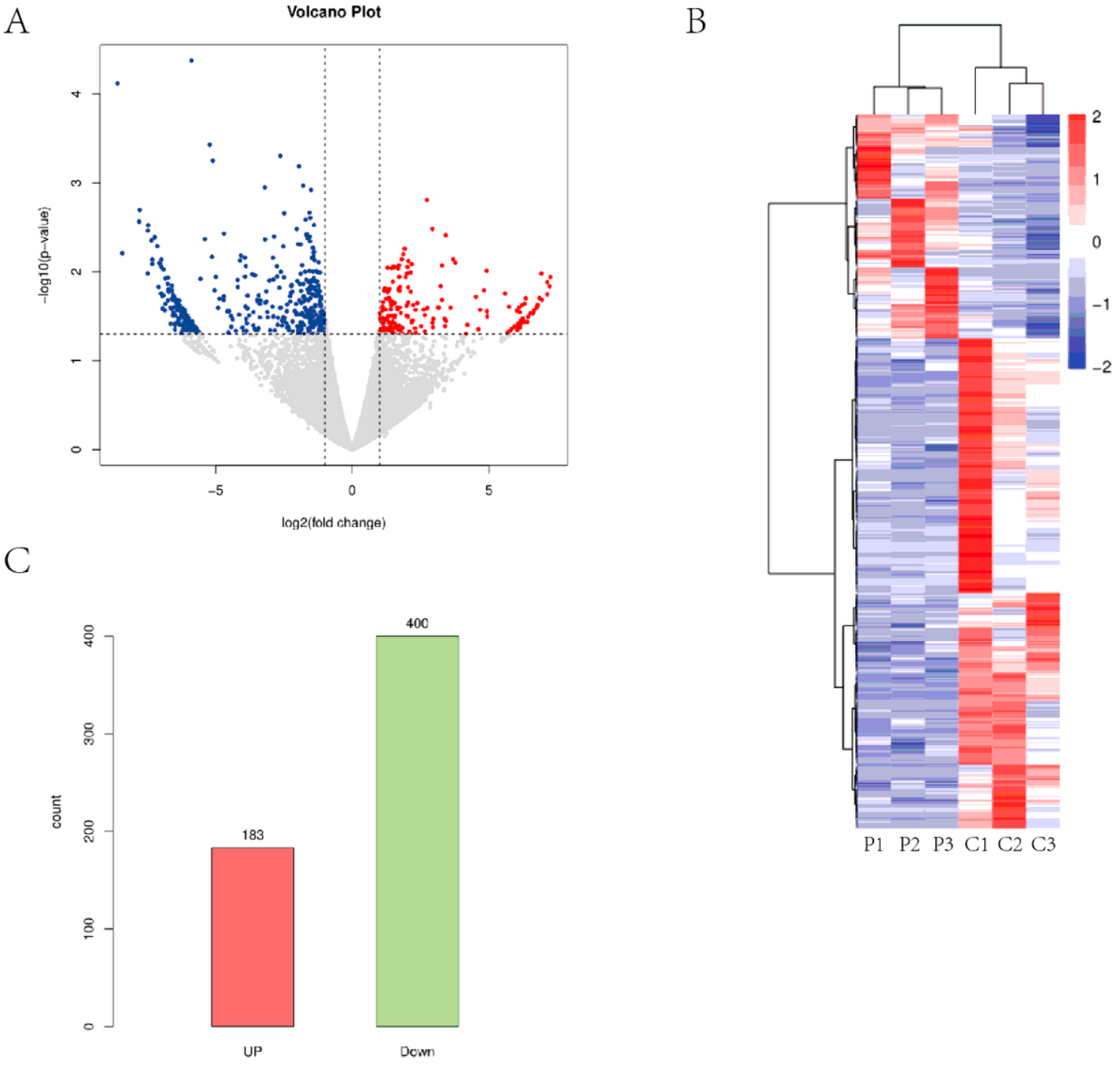

Figure 1

Volcano plot(A)、heatmap plot(B) and Bar plot(C).

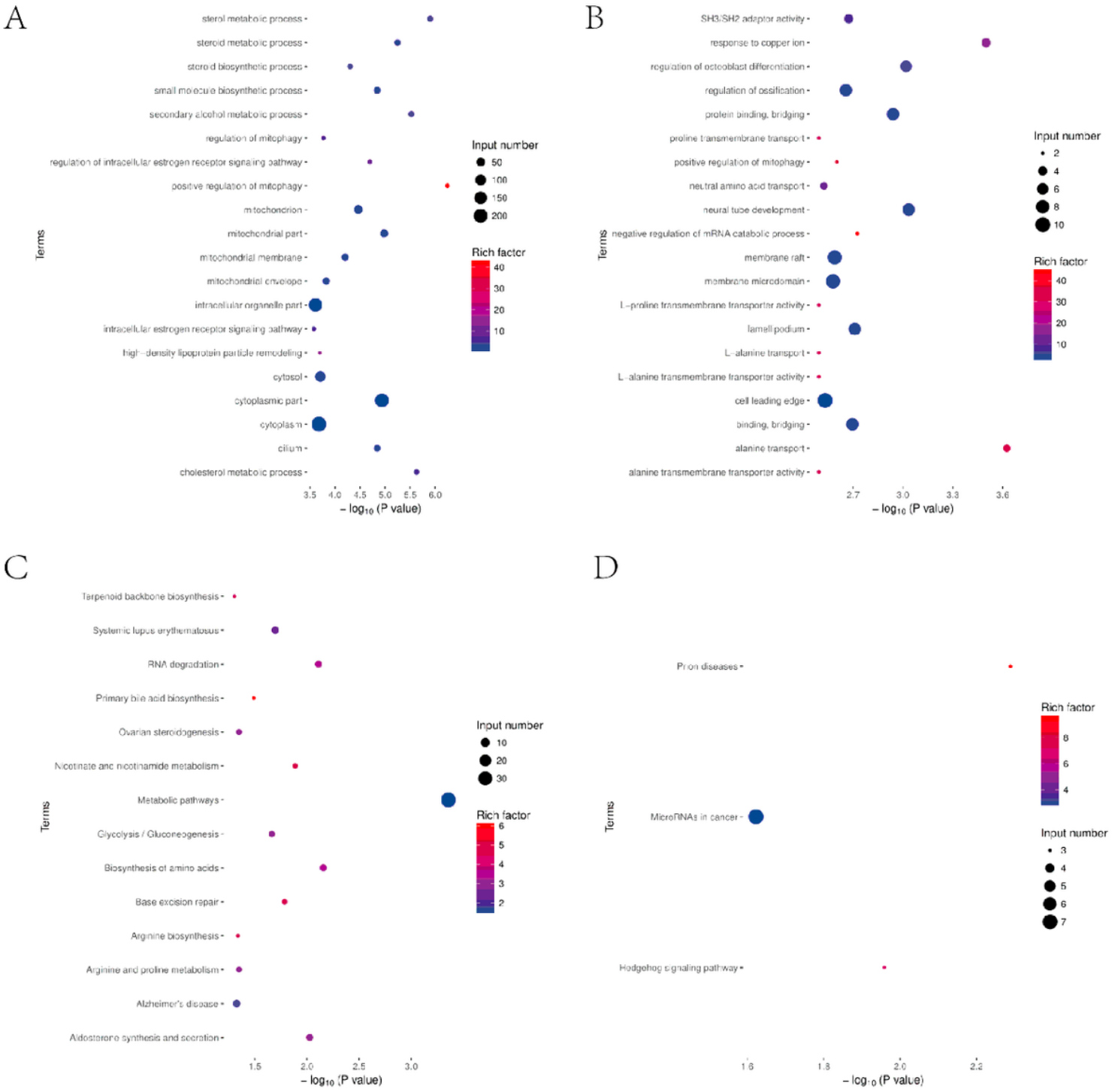

Figure 2

Go enrichment (A, B) and KEGG enrichment (C, D)

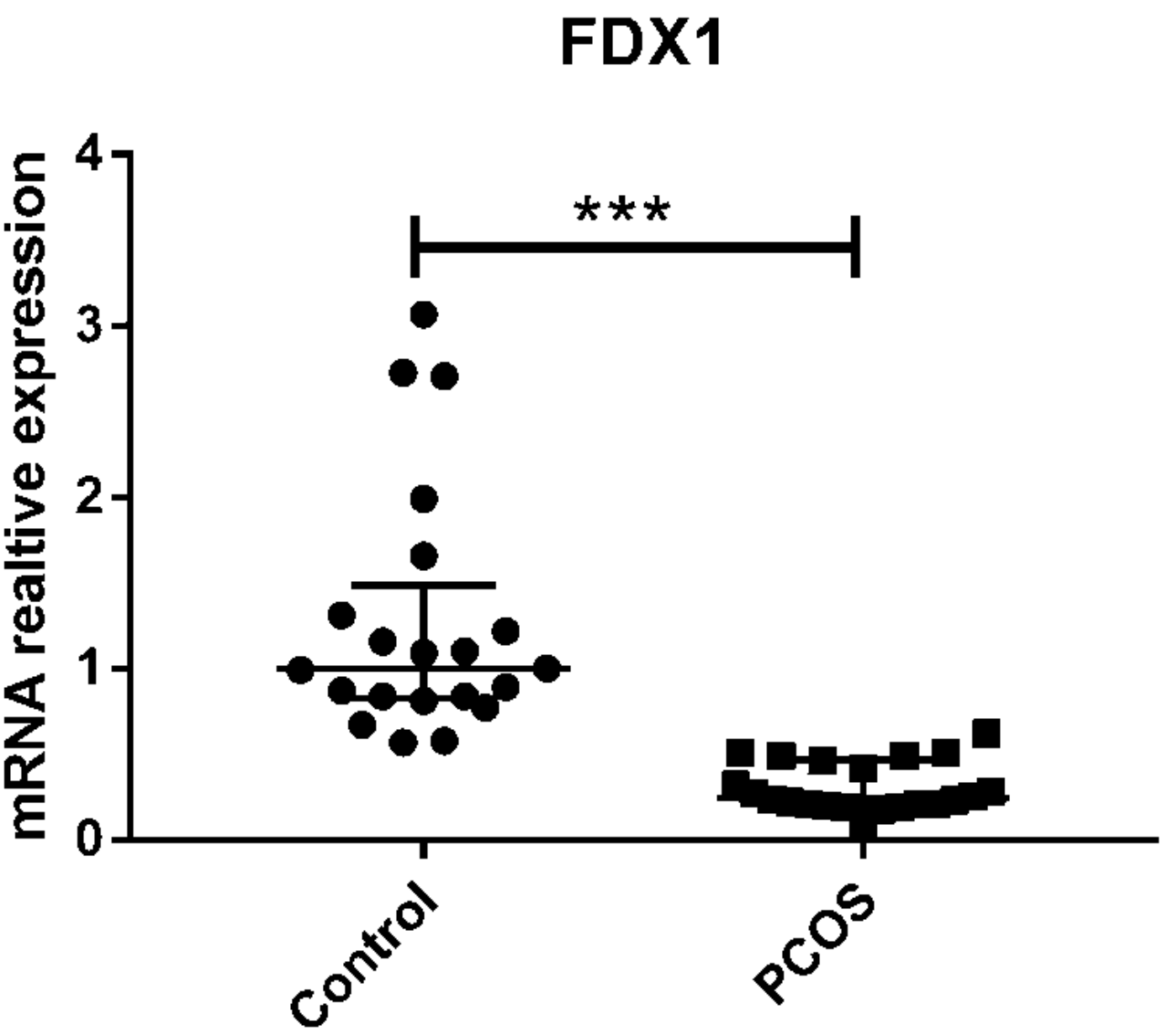

Figure 3

Relative mRNA expression of FDX1.

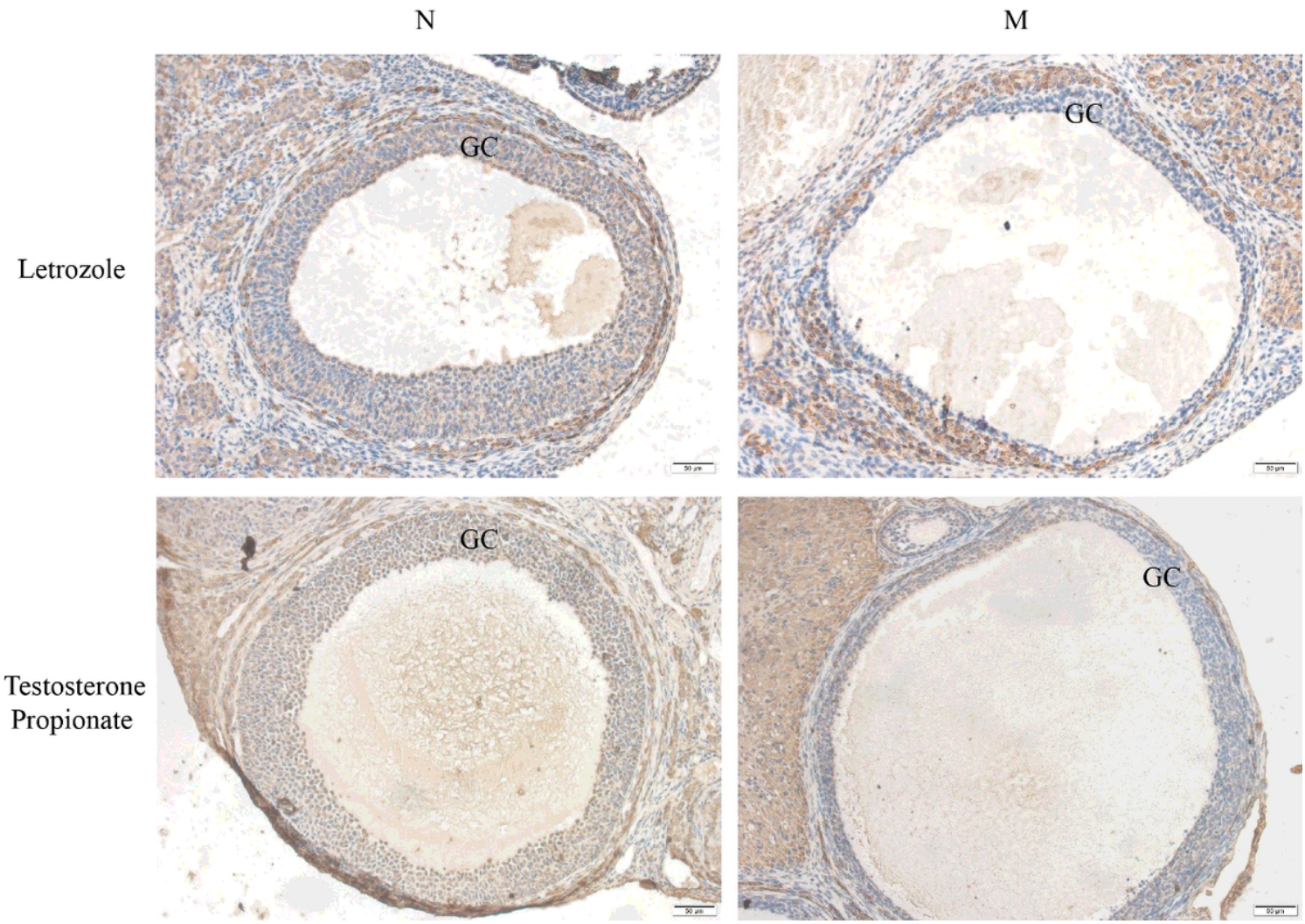

Figure 4

FDX1 in granulosa cell of antral follicle. Scale bar:50um. GC: granulosa cell. (n=3 for each group).

Supplementary Files

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- ARRIVEchecklist.pdf