Differential Gene Expression Analysis of Oocytes in a PCOS Mouse Model Revealed the Relation of Ribosomal Pathway

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Abstract

Polycystic ovary syndrome (PCOS), a common endocrinal disorder, is associated with impaired oocyte development, which leads to infertility. However, the pathogenesis of PCOS has not been completely elucidated. This study aimed to analyze the differentially expressed genes (DEGs) and epigenetic changes in the oocytes of the PCOS mouse model to identify the etiological factors. In this study, RNA-sequencing analysis revealed that 90 DEGs were upregulated and 27 DEGs were downregulated in the PCOS mouse model. DNA methylation analysis revealed 30 hypomethylated and 10 hypermethylated regions in the PCOS group. However, the DNA methylation status was not correlated with differential gene expression. The pathway enrichment analysis revealed that five DEGs (*Rps21*, *Rpl36*, *Rpl36a*, *Rpl37a*, and *Rpl22l1*) were enriched in ribosome-related pathways in the oocytes of the PCOS mouse model, and the immunohistochemical analysis revealed significantly upregulated expression levels of *Rps21* and *Rpl36*. These results suggest that differential gene expression in the oocytes of the PCOS mouse model is related to impaired folliculogenesis. These findings improved our understanding of the pathogenesis of PCOS.

Introduction

Polycystic ovary syndrome (PCOS), a common endocrinal disorder, is characterized by polycystic ovary (PCO), irregular menstrual cycles, and hyperandrogenism. PCOS is a systemic disease, the features include cardiovascular disease, type 2 diabetes and infertility[1]. Previous studies have performed the genetic analysis of the blood[2, 3], skeletal muscles[4], adipocytes[5], whole ovaries[6], granulosa cells[7], and cumulus cells[8] of patients with PCOS. Several genes were reported to be associated with endocrinal disorders, insulin resistance, hyperandrogenism, and ovulation disorders.

The prevalence of PCOS, which affects females of reproductive age and is associated with infertility, is 8–13%[9, 10]. The endocrinal changes and hyperandrogenism affect the intrafollicular environment, alter the interaction between granulosa cells and oocytes, and consequently impair oocyte maturation[11, 12]. In addition, granulosa cells are highly responsive to follicle-stimulating hormone[13], which decreases oocyte quality and increases the risk of developing ovarian hyperstimulation syndrome during in vitro fertilization (IVF)[14]. Patients with PCOS undergoing IVF exhibit increased intrafollicular testosterone and circulating insulin concentrations[15], which suggested that implantation failure and decreased oocyte quality can be attributed to alterations in the follicular microenvironment[15, 16]. However, limited studies have performed the genetic analysis of oocytes in PCOS.

PCOS is a complex and heterogeneous disease with both genetic and environmental factors[17–19]. The changes in the nutritional environment factors during the intrauterine period may affect specific genes and epigenetic modifications[20], and lead to the development of PCOS[21]. The high androgen intrauterine environment affects fetuses and could contribute to PCOS at reproductive age[22, 23]. Thus, various PCOS mouse models have been established using androgen[24]. Prenatal treatment with 5 α -dihydrotestosterone (DHT) leads to the development of PCO morphology in mice.

Previous studies have suggested that PCOS adversely affects oocyte maturation and quality. However, the mechanisms underlying PCOS-induced impaired oocyte maturation and quality and the genetic profiles of oocytes in PCOS have not been completely elucidated. In this study, the global gene expression levels and DNA methylation status in the oocytes of the DHT-induced PCOS mouse model were analyzed to examine the gene expression alterations in oocytes under PCOS conditions.

Results

Bodyweight, estrous cycle, and ovarian morphology

The schedule of drug administration and the evaluation of phenotypes in the PCOS group are shown in Fig. 1a. The bodyweight of the PCOS group was similar to that of the control group (Fig. 1b). All mice of the control group exhibited the physiological estrous cycles, which last 3–4 days. In contrast, the estrous cycle of mice belonging to the PCOS group was irregular and characterized by persistent diestrus phases (Fig. 1c).

The ovarian morphology was not affected in the control group. In contrast, a trend of decreased corpus luteum size and increased small antral follicle number was observed in the PCOS group. Large antral follicles and preovulatory follicles were similar in both groups (Fig. 1d). These findings are consistent with the previously reported characteristics[24].

DEGs in oocytes of PCOS mouse model

In total, 118 DEGs were identified between the PCOS and control groups. Of the 118 DEGs, 90 were upregulated and 28 were downregulated in the oocytes of the PCOS group. The volcano plot of DEGs is shown in Fig. 2a.

GO and KEGG pathway enrichment analyses of DEGs

The GO enrichment analysis of DEGs between the control and PCOS groups revealed that 10 ontologies were significantly enriched (Fig. 2b,c). Among the upregulated genes, five (*Rps21*, *Rpl36*, *Rpl36a*, *Rpl37a*, and *Rpl22l1*) were enriched in the GO term negative regulation of B cell proliferation. Additionally, *Btla* and *Ctla4* were enriched in the following two terms: osteoblast differentiation and translation. Among the downregulated genes, *Hspa1a* was enriched in the GO term regulation of neuron differentiation, which plays an important role in fertilization and embryonic development[32]. Additionally, *Pax6* and *Timp2* were enriched in oocyte embryo implantation, whereas *Pax6* and *Rap1gap* were enriched in the negative regulation of cell proliferation.

KEGG pathway enrichment analysis revealed that the DEGs were significantly enriched in the ribosomal and tight junction pathways (Table 1). The tight junction pathway in the PCOS group was inhibited contrary to that in the control group. In contrast, the ribosomal pathway, in which *Rps21*, *Rpl36*, *Rpl36a*, *Rpl37a*, and *Rpl22l1* were enriched, was upregulated in the PCOS group.

Table 1
Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially expressed genes.

KEGG term name	Genes	P-value
Pathways in which upregulated genes in PCOS group are enriched		
mmu03010: Ribosome	<i>Rps21</i> , <i>Rpl36</i> , <i>Rpl36a</i> , <i>Rpl37a</i> , and <i>Rpl22l1</i>	0.0049
Pathways in which downregulated genes in PCOS group are enriched		
mmu04530: Tight junction	<i>Myh15</i> , <i>4930544G11Rik</i> , <i>Myh7</i> , and <i>Cldn10</i>	0.0284

Immunohistochemical analysis of Rps21 and Rpl36 in ovaries

Immunostaining was performed to validate and quantify the protein levels of Rps21 and Rpl36 in the ovaries of the control and PCOS groups.

Oocytes in the primordial follicles exhibited strong (3+) Rps21 and Rpl36 immunostaining intensities in both control and PCOS groups. Oocyte in the small antral follicles almost exhibited moderate (2+) intensity, and in the large antral follicles exhibited weak (1+) (Fig. 3a,b). To quantify the staining intensities of oocytes in all follicles, the histo scores were calculated (Fig. 3c,d). The staining intensities of Rps21 and Rpl36 were significantly upregulated in the PCOS group ($P < 0.05$, both proteins).

Next, the Rps21 and Rpl36 staining intensities in each type of follicles were evaluated. The oocytes in the small antral follicles of the PCOS group exhibited strong (3+) and moderate (2+) Rps21 and Rpl36 staining intensities. The percentage of cells exhibiting strong (3+) Rps21 and Rpl36 staining intensities in the PCOS group were significantly higher than those in the control group ($P < 0.01$, both proteins). The Rps21 staining intensity in the oocytes in the large antral and preovulatory follicles of both groups was weak (1+). The Rpl36 staining intensity was weak (1+) in the oocytes in the large antral and preovulatory follicles of the control group but moderate (2+) in some oocytes in these follicles of the PCOS group (Table 2).

Moreover, the staining intensities of Rps21 and Rpl36 in the cumulus cells of the PCOS group were stronger than those in the cumulus cells of the control group.

Table 2
Rps21 and Rpl36 staining intensities in preantral, small antral, large antral, and preovulatory follicles.

Rps21		Staining intensity		
Follicle type		(1+)	(2+)	(3+)
Preantral follicle	Control			159 (61.2%)
	PCOS			145 (60.7%)
Small antral follicle *	Control		42 (16.2%)	21 (8.1%)
	PCOS		24 (10.0%)	36 (15.1%)
Large antral follicle	Control	33 (12.7%)		
	PCOS	32 (13.4%)		
Preovulatory follicle	Control	5 (1.8%)		
	PCOS	2 (0.8%)		
Rpl36		Staining intensity		
Follicle type		(1+)	(2+)	(3+) %
Preantral follicle	Control			87 (62.6%)
	PCOS			70 (59.3%)
Small antral follicle *	Control		14 (10.1%)	20 (14.4%)
	PCOS		3 (2.5%)	29 (24.6%)
Large antral follicle	Control	17 (12.2%)		
	PCOS	10 (8.5%)	3 (2.5%)	
Preovulatory follicle	Control	1 (0.7%)		
	PCOS	2 (1.7%)	1 (0.8%)	
Note: Values represent oocyte number (%) at each follicle stage.				
* $P < 0.01$. The Mann-Whitney U-test				

Correlation between DNA methylation and gene expression in PCOS

The oocytes of the control and PCOS groups were subjected to genome-wide DNA methylation analysis. In total, 40 significant DMRs were identified between the control and PCOS groups ($p < 0.01$). In the PCOS group, 30 hypomethylated regions and 10 hypermethylated regions were identified. No significant

difference in DMRs residing in chromosomal locations was observed (Fig. 4a,b). In total, 19 hypomethylated regions and 7 hypermethylated regions were located within the intragenic regions (Fig. 4c, d). The genomic location of DMRs in the intragenic region except for the intron are shown in Table 3. There are 3 first exons, 7 other exons, and no promoter region. The DMRs in the first exon, involving *Kcnv2*, *Clip3*, and *Gm32269*, were not significantly correlated with differential gene expression in the oocytes.

Table 3

Differentially methylated regions of genomic locations in the intragenic region without an intron.

Chr	Position		Average methylation levels		Genomic locations	Gene locus
	start	end	control	PCOS		
Hypomethylated regions						
4	128539059	128539140	77%	21%	Other exon	Csmd2
8	84702764	84702834	87%	32%	Other exon	Lyl1
8	48235749	48235815	62%	10%	Other exon	Tenm3
13	56810384	56810444	70%	13%	Other exon	Trpc7
15	91159052	91159119	56%	9%	Other exon	Abcs2
15	76306488	76306554	48%	7%	Other exon	Oplah
17	27113869	27113931	88%	22%	Other exon	Itpr3
19	27323896	27323957	72%	11%	First exon	Kcnv2
Hypermethylated regions						
7	30295287	30295404	30%	88%	First exon	Clip3
14	17660304	17660361	15%	79%	First exon	Gm32269
Chr, chromosome.						

Discussion

Various studies have examined the quality of oocytes and embryos in PCOS. One study reported that patients with PCOS were associated with decreased fertilization rates[33]. The intra-cytoplasmic sperm injection of oocytes from females with PCOS resulted in delayed embryonic development and hyperandrogenism[34].

Additionally, granulosa cells and cumulus cells are reported to affect oocyte quality and oocyte maturation in patients with PCOS[8, 35]. Meanwhile, the interaction between granulosa, cumulus cells, and oocytes is critical for oocyte maturation[36, 37]. This indicated that oocytes are also important for follicle development. However, limited studies have performed the genetic analysis of oocytes in PCOS. This may be due to the difficulty associated with the accessibility of human oocytes.

Several types of androgens, including DHT and dehydroepiandrosterone, have been used to model PCOS in mice[24]. Prenatal treatment with DHT resulted in irregular estrous cycles, PCO-like ovarian morphology, and increased levels of serum luteinizing hormone (LH) without marked effects on bodyweight. This model mimics the PCOS phenotype, which is characterized by physiological bodyweight and enhanced LH secretion. In this study, the oocytes of the PCOS model exhibited a PCO-like ovarian morphology and ovulatory dysfunction but not high testosterone levels at the time of oocyte retrieval.

RNA-Seq revealed that compared with those in the oocytes of the control group, the *Igfbp5* expression levels were upregulated and the *Hspa1a* and *Zscan4b* expression levels were downregulated in the oocytes of the PCOS mouse model. The expression levels of IGFBP5 in the cumulus cells of immature follicles were significantly higher than those in the cumulus cells of mature follicles in patients with PCOS. The upregulated levels of IGFBP5 are reported to inhibit oocyte maturation in patients with PCOS[38]. A previous study reported that HSPA1A is involved in embryonic maturation and that the expression of HSPA1A was downregulated in the granulosa cells of patients with PCOS[39]. The downregulation of *Hspa1a* in the oocytes of the PCOS mouse model may impair its function in embryonic maturation. ZSCAN4B, a *Zscan4* paralog, is reported to be involved in DNA-binding transcription factor activity and embryo implantation[40]. These findings demonstrated that the DEGs of oocytes in the PCOS mouse model are associated with the disorders of oocyte or embryo maturation and implantation in PCOS.

GO and KEGG pathway enrichment analyses revealed that the ribosomal pathway was upregulated in the oocytes of the PCOS mouse model. Five upregulated DEGs (*Rps21*, *Rpl36*, *Rpl36a*, *Rpl37a*, and *Rpl22l1*) were enriched in the ribosomal pathways. In particular, immunohistochemical analysis revealed that the expression levels of *Rps21* and *Rpl36* in the oocytes of the PCOS group were higher than those in the oocytes of the control group. The expression levels of *Rps21* and *Rpl36* in the oocytes based on the follicle stage were examined using immunohistochemical analysis. The *Rps21* and *Rpl36* levels were upregulated in the preantral follicles of both control and PCOS groups but downregulated in the oocytes of the small and large antral follicles (later stages of follicle development). Additionally, the *Rps21* and *Rpl36* levels in the oocytes in the small antral follicles of the PCOS group were higher than those in the oocytes in the small antral follicles of the control group. The main role of ribosomes is to translate mRNAs into proteins in the nucleolus[41]. The number of ribosomes increases with age, which may affect the accuracy and efficiency of ribosome translation and consequently result in the production of poor-quality proteins and oocytes[42]. The deletion of *Rps26*, a ribosomal protein component, in oocytes inhibited oocyte growth and impaired follicle development by downregulating the protein levels of oocyte-

derived Gdf9, Bmp15, and Gj4 (an oocyte-granulosa cell gap junction protein)[43]. Additionally, in the fertilization process, the ribosomal pathway is associated with poly-pronuclear (PN) and poly-PN arrest zygotes, which exhibit defects in the meiosis process and oocyte maturation, and is repressed in the early stage of oocytes in patients with PCOS[44]. These findings suggest that the upregulation of the ribosomal pathway in PCOS is involved in oocyte development and maturation during follicle development. The upregulation of the ribosomal pathways promotes translation and oocyte growth. However, the aberrant overexpression of the ribosomal pathway could inhibit the oocyte maturation process and impair embryonic development. In this study, small antral follicles in the PCOS group exhibited upregulated protein levels of Rps21 and Rpl36, which suggested that the altered expression of these ribosomal proteins in follicles at this stage is associated with the disorders of subsequent follicle maturation.

This study has some limitations. The findings of this animal study have not been validated in human samples. Additionally, the mechanism underlying the androgen-induced alteration in gene expression during fetal development was not elucidated in this study. DNA methylation, an epigenetic modification, regulates the identity of a cell without changing the DNA sequence[45]. During the gestation period, the environment promotes changes in DNA methylation[46–48]. Therefore, DMRs were analyzed in this study. However, the methylation alterations determined in this study were not associated with changes in gene expression. Finally, the small number of oocytes used for DNA methylation analysis may skew the results.

In summary, this study performed a genome-wide analysis of oocytes from the PCOS mouse models. The findings of this study indicate that the upregulated ribosomal pathway may impair oocyte maturation in PCOS.

These findings are expected to improve our understanding of the mechanism of follicle development disorders and contribute to favorable fertility outcomes in patients with PCOS. Future studies must focus on the altered gene expression of oocytes in patients with PCOS and determine the functions of these genes in PCOS phenotype using animal models and human samples.

Methods

Animal models

Adult female and male C57BL/6J mice purchased from CLEA Japan Inc. (Tokyo, Japan) were paired. The copulatory plugs of female mice were examined. The day of copulatory plug appearance was determined as the first day of gestation. Pregnant mice were subcutaneously injected with 100 μ L sesame oil containing 250 μ g DHT (PCOS group) or 100 μ L sesame oil (control group) on days 16–18 of gestation. Female offspring (aged 7–9 weeks) of the PCOS and control groups were analyzed as described previously[24–26]. The animal experiments were approved by the Tokyo University of Agriculture and Nagoya University Graduate School of Medicine.

Assessment of mouse phenotype

The bodyweight of mice was measured every week from 3 weeks of age until they were euthanized. The estrus cycle was determined based on the presence of epithelial cells in the vaginal smears. The smears of mice were examined daily from the day of the vaginal opening (postnatal day 21–56). The estrus cycle stages, which were characterized by the presence of nucleated cells, epithelial cells, and leukocytes, were determined as diestrus, proestrus, and estrus, respectively. The morphology of the ovary was determined using paraffin-embedded ovarian tissue sections. The sections were sliced to a thickness of 6 μm , mounted on micro slide glass (CRE-01; Matsunami Glass Industries, Kishiwada, Osaka, Japan), and stained with hematoxylin and eosin.

Oocyte collection

Adult female C57BL/6J mice (aged 7–9 weeks) of the control and PCOS groups were injected with equine chorionic gonadotropin. At 44–48 h post-treatment, germinal vesicle stage oocytes were collected from the ovarian follicles. The samples were treated with hyaluronidase to remove the cumulus cells and stored at -70°C .

RNA-Seq analysis

The oocytes ($n=22-23$) collected from the control or PCOS group were randomly divided into six groups according to storage time. Individual libraries were generated for each sample ($n=3$ for the control group; $n=3$ for the PCOS group).

Total RNA was extracted from the oocytes using the RNeasy Plus Micro kit (Qiagen, Germany), following the manufacturer's instructions. The libraries were prepared using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (#634888, Takara), and the Nextera XT DNA Library Preparation Kit (Illumina, USA) and sequenced using the PE100 strategy (HiSeq2500, Illumina, USA).

The sequencing reads from each sample were trimmed and filtered. The raw data obtained after quality control were aligned to the mouse reference genome (mm10; Genome Reference Consortium Mouse Build 38) using HISAT2 (version 2.1.0; <http://daehwankimlab.github.io/hisat2/>). The expression levels of the reads were counted with featureCounts (version 1.6.2)[27]. The gene expression patterns in different oocytes were analyzed using principal component analysis. Differentially expressed genes (DEGs) among the different groups were evaluated using multiple t-tests. DEG analysis was performed using edgeR (version 3.22.5; R-packages; <https://bioconductor.org/packages/release/bioc/html/edgeR.html>) under the following criteria: $P < 0.05$; $|\text{fold-change}| > 2$. Gene ontology (GO) and pathway enrichment analyses were performed using DAVID Bioinformatics Resources 6.8 and Kyoto Encyclopedia of Genes and Genomes (KEGG) for the functional annotation of the DEGs.

Bisulfite treatment

The target-enriched DNAs from six samples (one sample set of approximately 330 oocytes; $n=3$ for the control group; $n=3$ for PCOS group) were subjected to bisulfite treatment using the EZ DNA Methylation-

Gold kit (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions. The enriched DNA solution (20 µL) was incubated with 130 µL of freshly prepared CT conversion reagent at 64°C for 2.5 h. The 10 min incubation step at 98°C was omitted because the target-enriched DNA was already denatured. After purification and desulfonation, bisulfite-treated DNA was eluted with 20 µL of M-elution buffer.

Post-bisulfite adaptor tagging (PBAT) library construction and Illumina sequencing

Bisulfite-treated DNA was used for library preparation according to the PBAT protocol[28]. The constructed SureSelect Methyl-Seq (SSM)-PBAT libraries were sequenced using the Illumina HiSeq2500 system (Illumina, San Diego, CA, USA). The SSM-PBAT reads were aligned to the mouse genome (mm10; Genome Reference Consortium Mouse Build 38) using the Bismark tool (<http://www.bioinformatics.babraham.ac.uk/projects/bismark/>). The significance of DNA methylation at each CpG site and CpG islands was evaluated using the Fisher's exact and the Mann-Whitney U tests. Differential methylated regions (DMRs) between the control and PCOS groups were defined as those with $P < 0.01$ using DSS-single software[29].

Immunohistochemical analysis

The ovarian sections (6-µm thick) from each mouse were mounted on micro slide glass (CRE-01; Matsunami Glass Industries), pre-treated with 0.3% H₂O₂, and blocked with 10% bovine serum albumin for 30 min. Next, the sections were incubated with anti-Rps21 (Proteintech, USA, 1:200 dilution) and anti-Rpl36 (Sigma-Aldrich, USA, 1:400) antibodies at 4°C overnight, followed by incubation with secondary antibodies (Beyotime, Shanghai, China, 1:2000 dilution) for 2 h at 25°C. The sections were then incubated with 3,3'-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark) and counterstained with hematoxylin. Negative control sections were incubated with phosphate-buffered saline instead of primary antibodies. The immunoreactive oocytes were counted using Zeiss ZEN 2 (blue edition) microscope and Zeiss software version 1.0en.3.1 (Carl ZeissMicroscopy GmbH, Jena, Germany). The follicles were classified as follows: preantral (oocyte with 2–5 layers of cuboidal granulosa cells), small antral (oocyte surrounded with more than five layers of granulosa cells and/or one or two small areas of follicular fluid), large antral (comprised a single large antral cavity), preovulatory (comprised a single large antrum and an oocyte surrounded by cumulus cells at the end of a stalk of mural granulosa cells), and atretic cyst-like follicles (large fluid-filled cyst with an attenuated granulosa cell layer and dispersed theca cell layer)[25, 30].

The histo scores were calculated as follows: 0, negative; 1⁺, weak; 2⁺, moderate; and 3⁺, strong. The percentage of cells at each intensity level was calculated as follows: $[1 \times (\% \text{ cells at } 1+ \text{ intensity level}) + 2 \times (\% \text{ cells at } 2+ \text{ intensity level}) + 3 \times (\% \text{ cells at } 3+ \text{ intensity level})]$ [31].

The significance between groups was determined using Mann-Whitney U-test. The Mann-Whitney U-test was used for the non-normally distributed variables. The histo scores were analyzed using GraphPad

Prism software version 7.01 (GraphPad Software, San Diego, CA). Differences were considered significant at $P < 0.05$.

Declarations

Authors' contributions

A.I., and S.O. conceptualized and designed the study; N.N., S.O., and T.K. developed the methodology; N.N., and T.K. helped with the animal experiments. N.N., and S.O. prepared the manuscript, analyzed and interpreted the data, and performed statistical analysis; N.N., S.O., B.B., S.Y., S.I., H.Kobayashi, and T.K. provided technical and material support; analyzed the data with assistance from T.S., A.Y., H.T., R.S., M.M., N.M., A.M., and Y.K.; T.M., T.N., M.G., and H.Kajiyama supervised the entire project. All authors have read and approved the final version of the manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed in this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All applicable international, national, and/or the ARRIVE guidelines for the care and use of animals were followed. The study was approved by the ethics committee of Nagoya University Graduate School of Medicine (approval number 31254), and Tokyo University of Agriculture (approval number 170174).

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Figures

Fig. 1

Figure 1

Assessment of bodyweight, estrous cycle, and ovarian morphology in polycystic ovary syndrome (PCOS) mouse model.

(a) Schematic illustration of oocyte retrieval. s.c., subcutaneously. **(b)** Evaluation of bodyweight. Data are represented as mean $\pm$ standard error of mean. **(c)** Representative estrous cycles. E, estrus; P, proestrus; D, diestrus. The PCOS mouse model mostly exhibited irregular estrus cycles. **(d)** Morphology of the ovaries. Bars, 200 μ m. CL, corpora lutea.

Fig. 2

Figure 2

Differentially expressed genes in oocytes of polycystic ovary syndrome (PCOS) mouse model.

(a) Volcano plot with default log (fold-change) thresholds of -1 and 1 and an adjusted p-value threshold of 0.05 . The expression of 90 genes was upregulated ($> \log(\text{fold-change})$ thresholds of 1), whereas that of 28 genes was downregulated ($< \log(\text{fold-change})$ thresholds of -1). **(b,c)** Results of gene ontology (GO) analysis performed using DAVID Bioinformatics Resources 6.8. GO terms in which the upregulated **(b)** and downregulated **(c)** genes are enriched.

Fig. 3

Figure 3

Immunostaining of Rps21 and Rpl36 in ovary.

(a) Rps21 and (b) Rpl36 immunohistochemical staining intensities in the oocytes of the preantral, small antral, and large follicles of the control and 5 α -dihydrotestosterone-treated groups. Bars, 200 μ m. Histo scores of (c) Rps21 (n = 260 oocytes for the control group; n = 239 oocytes for the PCOS group) and (d)

Rpl36 (n = 137 oocytes for the control group; n = 118 oocytes for the PCOS group) immunohistochemical staining intensities in different follicles of the ovary. Data are represented as the mean $\pm$ standard error of mean. * $P < 0.05$ using the Mann-Whitney U-test.

Fig. 4

Figure 4

Differentially methylated regions (DMRs) in chromosomes and genomic locations.

The DMRs in chromosomes. **(a)** Thirty hypomethylated and **(b)** ten hypermethylated regions in the mouse model for polycystic ovary syndrome. The DMRs in genomic locations. **(c)** Thirty hypomethylated and **(d)** ten hypermethylated regions. The DMRs were classified into 5 genomic locations; promoter (within 500-bp upstream from the first exon) or first exon, last exon, other exon, intron, and intergenic region. There was no DMRs in the promoter and last exon. chr, chromosome.