

# Aberrant activation of the Hedgehog signaling pathway in granulosa cells from patients with polycystic ovary syndrome

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## Research

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# Abstract

The molecular mechanism that triggers polycystic ovary syndrome (PCOS) is mysterious. Abnormal development of ovarian granulosa cells (GCs) is one of the causes of PCOS. Herein, we carried out RNA-seq to detect the different gene expression levels in ovarian GCs between 3 patients with PCOS and 4 normal controls, and found that Hedgehog signaling pathway (Hh) members, *Ihh* and *Ptch2* were abnormally highly expressed in the PCOS group. To further verify the above results, GCs from 22 patients with PCOS and 21 controls with normal ovulation were collected to perform the RT-PCR analysis. The qPCR results also indicated that the expression levels of other Hh signaling pathway downstream members, *Ptch1*, *Gli1*, and *Gli2* in the PCOS group were significantly higher than those in the control group. These results suggest that abnormally activated Hh signaling pathway, especially *Ihh* signal, may have a profound influence on PCOS.

## Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women, which affects 10% reproductive age women(1). The Rotterdam Criteria requires women to conform to two of three following symptom: oligoovulation or anovulation, clinical or biochemical hyperandrogenism and polycystic ovaries (PCO)(2). In the late development stage of PCOS, the complication associated with metabolic diseases such as diabetes, abdominal adiposity, high cholesterol and hypertension most occur(3–5). Therefore, the PCOS patients need a long term therapy to avoid the consequence. But the etiology, especial molecular mechanism and pathogenesis are still unclear.

Folliculogenesis, a highly coordinated event in the development and release of oocytes, is disrupted in PCOS. In PCOS women, excessively primordial follicles were recruited and subsequent development is arrested at the early preantral stage, resulting in the formation of multiple cysts(6). Increased GnRH pulses favor promoted LH production, which along with excess insulin stimulates ovarian theca cells to produce more androgen resulting in cessation of follicular growth and dominant follicle selection, thus affecting ovulation. Therefore, one of the most important symptoms of PCOS is the disordered follicular development. However, the precise molecular defects of follicular development in PCOS still remain unknown. The process of follicular development was accurately regulated by the communication among the oocyte, granulosa cells (GCs) and theca cells(7–9), which involving several signaling pathways(10–14).

The hedgehog (Hh) gene was first cloned in *Drosophila*(15). It plays a very important role in the embryonic development and remodeling processes of adult tissues(16). Hh signaling regulates cell fate determination, proliferation and differentiation(17). Moreover, over-activation of Hh signaling is associated with the tumorigenesis(18–21). In mammals, the Hh pathway consists of three Hh ligands, indian (*Ihh*), desert (*Dhh*), and sonic (*Shh*). The membrane receptors are patched (*Ptch1,2*) and transmembrane signal transducer protein smoothed (*SMO*)(22). In the absence of ligand binding, *PTCH* maintains *SMO* in an inactive state. Binding of Hh ligand to *PTCH* relieves inhibition of *SMO*, and

allows the activation of Hh-induced intracellular transcriptional effectors Glioma-associated oncogene homolog (Gli-1, 2, 3), leading to the induction of target gene expression(23).

Recently, expression of components in the Hh pathway were observed in both GCs and residual ovarian tissue, and the expression were changed in response to the stages of follicular development in human postnatal ovaries(24). Here, we present evidence that significant abnormal activation of Hedgehog signaling pathway is identified in PCOS patients. The above results indicats that Hh signaling pathway may be associated with follicular development and PCOS.

## **Materials And Methods**

### **Patients selection**

A prospective case-control study and qPCR analysis were designed including 22 patients with PCOS (PCOS group) and 21 patients with regular menstrual cycles (control group) from December 2016 to March 2017 in Jiangxi Maternal and Child Health Hospital. In addition, RNA-seq analysis was performed on the ovarian granular cell layer tissues from 3 PCOS patients and 4 controls. These patients received intracytoplasmic sperm injection (ICSI) and were between the ages of 20 and 34 years and weighed at least 40 kg. PCOS patients were diagnosed based on the Rotterdam criteria, including oligoovulation or anovulation and PCO(2). In the control group, patients were selected including normal ovulation, absence of hirsutism and acne, absence of PCO on sonography, and normal hormonal parameters. All the patients were also excluded from the study if they had a history of unilateral oophorectomy, recurrent spontaneous abortion (defined as three or more previous spontaneous pregnancy losses), congenital or acquired uterine malformations, abnormal results on parental karyotyping, endometriosis, hyperprolactinemia and thyroid dysfunction. All selected patients followed the voluntary principle and signed informed consent. This experiment conforms to the Helsinki principle and is approved by our ethics committee

### **Blood sampling and sex hormone measurement**

Blood samples were collected on the third day of the menstrual cycle and on the day of HCG injection, subsequently centrifuged at 4000 rpm for 1 min. The serum was used for the quantitative determination of sex hormone (follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol ( $E_2$ ), prolactin (PRL) and testosterone (TES) level by chemiluminescent enzyme immunoassay using Automated Enzyme Immunoassay Analyzer (AIA-2000ST, TOSOH CORPORATION). The above experiment was repeated three times, and the results come from the clinical test results.

### **Controlled ovarian stimulation**

Follicular aspirates were collected during oocyte retrieval following published procedures and ovarian stimulation was the use of a prolonged protocol(25, 26). Briefly, gonadotropin-releasing hormone agonist (GnRH-a, Ipsen, Boulogne-Billancourt, France) was used in the second or three day of menstrual cycle for pituitary down-regulation. Gonadotropin stimulation were started after 28 or 38 days following the

criteria: no ovarian cysts > 8 mm, E<sub>2</sub> < 50 pg/ml, FSH < 5 IU/L, LH < 5 IU/L. Initial, patients received 75-112.5 IU/d of recombinant human FSH (Merck-Serono, Darmstadt, Germany) according to the patient's age, body mass index (BMI), serum basal FSH levels, LH levels, E<sub>2</sub> levels and antral follicle count. The time and dose of recombinant human FSH were adjusted according to ovarian response as monitored by serum E<sub>2</sub> levels and vaginal ultrasound. When the dominant follicle was ≥ 19 mm in diameter or at least 3 follicles were ≥ 17.5 mm in diameter, recombinant human FSH was stopped, and a single injection of 6000–8000 IU of hCG (Merck-Serono, Darmstadt, Germany) was administered. Oocyte-retrieval was performed 36–40 hours later under transvaginal ultrasound guidance.

## Human GCs collection and cell culture

Follicular fluids were centrifuged at 2000 rpm for 5 min. The cells were resuspended with DMEM/F12 (Life Technologies, Carlsbad, CA, USA) medium and transferred to a 50% (volume fraction) Percoll gradient (Sigma-Aldrich, St. Louis, MO, USA); they were centrifuged at 4000 rpm for 20 min to purify human GCs from any red blood cells. After washing and recentrifugation, sheets of human GCs were digested with trypsin at a 1:1 ratio for 4 min to separate them. The GCs were removed using a pipette and washed with phosphate buffered saline (PBS). 10% FBS DMEM / F12 culture medium at a 2:1 ratio were added to terminate the digestion, centrifuged at 1500 rpm for 3 minutes. GCs were resuspended with 1xPBS at a 1:5 ratio, centrifuged at 1500 rpm for 3 minutes. After discarding the supernatant, added 1 ml of 10% FBS DMEM / F12 culture medium, resuspended, inoculated in a dish, and placed in a 37°C, 5% CO<sub>2</sub> incubator. After observing the morphology of the granulocytes under the microscope, changed the fluid per 24 hours. The cells at indicated time were stored at -80 °C for future analysis.

## FSHR immunohistochemical staining in human GCs

After the GCs are cultured for 3 days, the fixed cell slides are washed with PBS for 3 times, and fixed with 4% paraformaldehyde for 30 min, washed with PBS for 3 times. Incubated in 3% H<sub>2</sub>O<sub>2</sub> deionized water for 10 minutes to block the effect of endogenous peroxidase, washed in 1xPBS for 2 times. Drop anti-rabbit anti-human FSHR polyclonal antibody (1:100) dropwise at 4 °C overnight, washed in PBS for 2 times. Added reagent 1 (polymer helper) dropwise, incubated at room temperature for 20 min, and washed in 1xPBS for 2 times. Added reagent 2 (polyperoxidase-anti-mouse / rabbit IgG) dropwise and incubated at room temperature. Finally, DAB was added dropwise for color reaction (control reaction time under microscope). Rinsed thoroughly with tap water, counterstained with hematoxylin for 1 min, dehydrated with conventional gradient alcohol, transparent xylene and seal with neutral resin. As a negative control, 1xPBS was used instead of the primary antibody. The cells with yellow staining on the cell membrane were FSHR positive cells, and the number of positive cells in 10 high-power (× 400) visual fields was randomly counted.

## RNA-seq and qPCR

For RNA-seq, total RNA were extracted from 4 normal tissues and 3 PCOS tissues by using the RNeasy reagent (Qiagen, Crawley, UK). The library was validated on the Agilent Technologies 2100 bioanalyzer subjected to deep sequencing on Illumina HiSeq 2000 (50-bp single-read sequencing), and

analyzed at BGI Genomics Co., Ltd. For RT-qPCR analysis, the GCs total RNA from 22 patients with PCOS and 21 controls were carried out by using the PrimeScript RT reagent Kit (TaKaRa). Standard qPCR was carried out with the following primers: hsa-RT-Ptch1 (5'-GCTGCACTACTTCAGAGACTGG-3' and 5'-CACCAGGAGTTTGTAGGCAAGG-3'), hsa-RT-Gli1 (5'-AGCCTTCAGCAATGCCAGTGAC-3' and 5'-GTCAGGACCATGCACTGTCTTG-3'), hsa-RT-Gli2 (5'-GTCAGAGCCATCAAGACCGAGA-3' and 5'-GCATCTCCACGCCACTGTCATT-3'), hsa-RT-Gli3 (5'-TCAGCAAGTGGCTCCTATGGTC-3' and 5'-GCTCTGTTGTCGGCTTAGGATC-3') and has-RT-actin (5'-ACCTTCTACAATGAGCTGCG-3' and 5'-CCTGGATAGCAACGTACATGG-3'). Real-time PCR was carried out using the FastStart SYBR Green Master mix (Roche) on a 7500 Real-Time PCR System (Applied Biosystems, Grand Island, NY). The actin was used as an internal control. The results were presented as fold change, calculated using the  $2^{-\Delta CT}$  method, and a ratio of expression in the PCOS relative to the Controls less than 1.0 was considered as low.

## Statistical analysis

Statistical analysis was performed by using the SPSS 17.0 software package. Data were shown as mean  $\pm$  SEM. The t-test was used for comparison between measurement data groups and the  $\chi^2$  test was used for count data groups. Each experiment was repeated at least 3 times. \*  $p < 0.05$  was considered as statistically significant.

## Results

### Ihh and Ptch2 were upregulated in PCOS

In order to explore the molecular mechanism of PCOS, we used RNA-seq analysis to detect the difference in gene expression levels between the PCOS (PT) group and the normal control group (NT). The RNA-seq result showed that there were total 673 differentially expression genes(DEG). Among them, 296 genes were up-regulated and 377 genes were down-regulated (Fig. 1). Moreover, pathway functional enrichment results of NT-VS-PT.DEGseqPathway indicated that immune system related genes occupied an important part of DEG (Fig. 2). Interestingly, among DEGs, we found that the Hh pathway member, ligand Ihh and the receptor Ptch2 (also the target genes downstream of the Hh pathway) are highly expressed in the PCOS group (Table.1). Next, we would use more clinical samples for subsequent analysis.

### General conditions

In the general comparison of the two groups of patients(22 PCOS and 21 Controls), the age, infertility, BMI, blood base FSH, E2, PRL of the PCOS group were not statistically significant compared with the control group. Comparing the blood LH between the two groups, the PCOS group ( $7.30 \pm 3.59$  IU / L) was significantly higher than the control group ( $4.43 \pm 2.35$  IU / L), which was statistically significant. The blood TES in the PCOS group ( $38.28 \pm 20.30$  nmol). / L) was significantly higher than that in the control group ( $26.34 \pm 10.79$  nmol / L), the two groups were statistically significant,  $p < 0.05$ (Table.2).

### Clinical outcomes

In the general comparison of IVF-ET treatment between the two groups of patients, the endometrial thickness of HCG on the PCOS group ( $10.6 \pm 1.7$  mm) was significantly lower than that of the control group ( $11.9 \pm 2.0$  mm). Other indicators such as total Gn amount, total Gn days, HH day LH, E2 and P were not statistically significant in the two groups. The MII egg rate of the PCOS group ( $60.7 \pm 28.9\%$ ) was significantly lower than that of the control group ( $80.4 \pm 13.6\%$ ), and the two groups were statistically significant. The 2PN fertilization rate of the PCOS group ( $61.7 \pm 20.8\%$ ) was significantly lower than the control group ( $76.9 \pm 18.6\%$ ), the two groups were statistically significant. Other indicators such as the number of eggs obtained, the number of embryos available and the rate of superior embryos were not statistically significant between the two groups (Table.3).

## Expression of Hh family members in GCs from PCOS group and control Group

As we know that PCOS are often accompanied with abnormal follicular development, therefore, we consider that whether the disordered Hh signaling pathway is contributed to the PCOS-related abnormal follicles. Then we isolated and purified granulosa cells from 22 PCOS and 21 Control samples. We performed FSH staining after 3–5 days of cell culture (Fig. 3). After identification, total RNA was extracted for qPCR detection. Next, we compared the mRNA levels of Gli1, Gli2, Gli3 and Ptch1 in PCOS and non-PCOS groups undergoing IVF treatment to explore the potential role of Hh signaling pathway in PCOS-related abnormal follicles. We tested the expression of Hh family members of GCs in both PCOS and controls. The levels of Gli1 mRNA, Ptch1 mRNA and Gli2 mRNA were significantly higher in PCOS group than those in the control group, while the expression of Gli3 mRNA had no significant difference between the two groups. These results indicate that the abnormality of the Hh pathway has a potential role in promoting the development of PCOS.

## Discussion

In our research, PCOS patients were diagnosed based on the Rotterdam criteria including oligoovulation or anovulation and PCO. This study was a matching study and no statistically significant difference was found in age, infertile period, or BMI in comparing the two groups as we showed in the Table 1. The PCOS group had higher level of Basal LH and T, which were consisted with their abnormal endocrine results (3–6). Previous studies have found that PCOS patients were often accompanied with aberrant follicles and unsatisfactory fertilization rate (25–27). We also analyzed clinical outcomes of PCOS groups and control. As shown in Table 2, the MII oocyte rate and fertilization rate were remarkably reduced in PCOS group when compared with the control group ( $60.7 \pm 28.9$  vs.  $80.4 \pm 13.6$ ,  $P < 0.05$ ;  $61.7 \pm 20.8$  vs.  $76.9 \pm 18.6$ ,  $P < 0.05$ ). In line with previous studies, our data also showed a high rate of immature oocyte and low rate of fertilization in PCOS patients.

Table 1

Expression of a part of DEGs hierarchical clustering analysis results of PCOS patients and control group

Symbol	GeneID	Length	NT	PT	log <sub>2</sub> (PT/NT)	Up/Down	p-value
TNXA	7146	2783	168.4	354.2	1.4	Up	< 0.001
IL4I1	259307	1798	168.2	350.4	1.4	Up	< 0.001
S100B	6285	1135	357	572	1.0	Up	< 0.001
IHH	3549	2074	20	129	3.0	Up	< 0.001
APOD	347	1148	878	1984	1.5	Up	< 0.001
SDS	10993	1620	410	1322	2.0	Up	< 0.001
PTCH2	8643	3840	1803.9	3109.2	1.1	Up	< 0.001
CTSH	1512	1532	1616	2873	1.2	Up	< 0.001
LILRB5	10990	2395	361.4	1231.8	2.1	Up	< 0.001
CCL4	6351	667	2042.2	3323.8	1.1	Up	< 0.001
C4B	721	5444	838.9	1840.7	1.5	Up	< 0.001

Table 2

Comparison of general conditions between PCOS group and control group

Characteristics	PCOS (n = 22)	Control (n = 21)	P
Age (years)	27.64 ± 2.82	28.95 ± 2.77	> 0.05
Infertility duration (years)	4.05 ± 2.44	3.14 ± 2.29	> 0.05
BMI (Kg/m <sup>2</sup> )	22.03 ± 3.25	22.67 ± 3.47	> 0.05
Basal FSH (IU/L)	5.42 ± 1.09	5.93 ± 1.36	> 0.05
Basal LH (IU/L)	7.30 ± 3.59	4.43 ± 2.35	< 0.05
Basal E <sub>2</sub> (pg/mL)	39.05 ± 18.67	40.11 ± 17.00	> 0.05
PRL (ug/L)	19.02 ± 13.24	17.65 ± 9.84	> 0.05
T (nmol/L)	38.28 ± 20.30	26.34 ± 10.79	< 0.05
Note: The data were expressed as mean + SD.			

Table 3

Comparison of clinical outcomes between PCOS group and control group

<b>Variables</b>	<b>PCOS</b>	<b>control</b>	<b>P</b>
Dosage of Gn (IU)	1916 ± 944	2144 ± 925	> 0.05
Duration of Gn (days)	12.7 ± 2.6	11.6 ± 1.7	> 0.05
Endometrial thickness on HCG day (mm)	10.6 ± 1.7	11.9 ± 2.0	< 0.05
LH on HCG day (IU/L)	1.1 ± 0.6	0.9 ± 0.5	> 0.05
E <sub>2</sub> on HCG day (pmol/l)	2269 ± 1155	2510 ± 1110	> 0.05
P on HCG day (nmol/l)	1.1 ± 0.4	1.0 ± 0.5	> 0.05
No of oocytes retrieved	16 ± 9	14 ± 6	> 0.05
MII oocyte rate (%)	60.7 ± 28.9	80.4 ± 13.6	< 0.05
Fertilization rate (%)	61.7 ± 20.8	76.9 ± 18.6	< 0.05
Cleavage rate (%)	97.7 ± 5.9	92.7 ± 12.0.	> 0.05
No of Embryo	3.4 ± 1.6	4.1 ± 2.4	> 0.05
Good quality embryo rate (%)	2.1 ± 1.8	3.1 ± 2.8	> 0.05
Note: The data were expressed as mean + SD.			

Patients with PCOS infertility can usually obtain normal pregnancy through clinical routine treatment methods such as drug promotion, surgery, lifestyle adjustment and weight loss. However, for patients with refractory PCOS or patients with other infertility factors and male factors, they will choose assisted reproductive technology (ART). In the treatment of PCF patients with IVF-ET, the high levels of LH and androgens in the body tend to cause follicular atresia and premature lutealization. The quality of eggs and embryos formed is relatively poor, the pregnancy rate is reduced, and the abortion rate is relatively high. It is also possible to restrict the transfer of embryos by causing the endometrium to express the progesterone receptor prematurely and convert it into the secretory endometrium, thereby reducing the implantation rate and clinical pregnancy rate (25–27). In addition, a large number of small follicles remain in the ovaries of patients with PCOS. For a long period of time under high androgen levels, a high level of gonadotropin is required to start. Once activated, a large number of follicles develop at the same time, and it is easy to form excessive reactions. Lead to the occurrence of ovarian hyperstimulation syndrome (OHSS)(28). The common ovulation-promoting programs of PCOS mainly include long programs, ultra-long programs, antagonist programs, and microstimulation programs (29). In our study, patients in both groups were treated with COH after down-regulation with an ultralong protocol. Studies have shown that prolonged down-regulation can significantly improve the thickness, morphology, and blood flow of the endometrium, thereby increasing the implantation rate and pregnancy rate (27). At the

same time, it can reduce the concentration of pelvic inflammatory cytokines and improve pelvic cavity. The environment is favorable for embryo implantation (30).

In our study comparing IVF-ET treatment in general, the endometrial thickness of HCG in the PCOS group ( $10.6 \pm 1.7$  mm) was significantly lower than that in the control group ( $11.9 \pm 2.0$  mm), and the results were statistically significant. Other indicators such as total Gn amount, total Gn days, HH day LH, E2 and P were not statistically significant in the two groups. Studies have shown [95] that an appropriate thickness of the endometrium is important for embryo implantation. When the endometrial thickness is  $> 14$  mm on the day of hCG injection, the IVF implantation rate and pregnancy rate are reduced, and the endometrial thickness is  $< 6$ . At  $\sim 7$  mm, the embryo implantation rate and pregnancy rate are affected, and the miscarriage rate increases. In this study, the average endometrial thickness of PCOS patients on HCG day was 10.6 mm lower than the normal control group's 11.9 mm, but all were within the normal range and had no obvious clinical significance.

In IVF-ET treatment, the MII egg rate is the ratio of mature eggs. After the sperm and the egg meet, the sperm enters the egg, and the nucleus of the egg and the nucleus of the sperm will form a pronucleus, respectively. The two pronucleus are called "2PN", indicating that the egg is fertilized normally. The 2PN fertilization rate represents the rate of normal fertilization of the egg. In our study, the MII egg rate of the PCOS group ( $60.7 \pm 28.9\%$ ) was significantly lower than that of the control group ( $80.4 \pm 13.6\%$ ), which was statistically significant; the 2PN fertilization rate ( $61.7 \pm 20.8\%$ ) of the PCOS group was significant. Compared with the control group ( $76.9 \pm 18.6\%$ ), the two groups were statistically significant, consistent with the results of previous studies of PCOS. At the same time, other indicators such as the number of eggs obtained, the number of available embryos, and the rate of superior embryos were also consistent with other studies. There was no statistical significance in the two groups.

The Hh signaling pathway is proved to play a crucial role in embryonic development of mammalian including human. GCs from growing follicles in mouse ovary acting as a source of Hh signaling was first reported in 2005(22), which demonstrated that Hh family were expressed on the GCs from primary to antral stages of follicular development in postnatal ovaries of human, respectively(24, 31). These results implied that Hh signaling played an important role in the communication between the cellular compartments that perform gametogenesis, steroidogenesis and ovarian vasculature(22, 24, 32). Activation of Hh signaling regulated follicle growth and GCs proliferation at least one of the potential targets(24). These studies suggested a sequential requirement for Hh signaling pathway in ovarian follicular development. Previous studies also found that patients with PCOS were often accompanied with follicular dysplasia(6, 33). Therefore, we speculate that maybe there is a relationship between Hh components expression and PCOS.

The three Hh ligands (Ihh, Shh and Dhh), the two receptors (Ptch1 and Ptch2) and the mediator of HH signaling (Smo), are expressed in GCs and in corpora lutea from pseudopregnant mice(24). In addition, the transcription factor, Gli1, Gli2 and Gli3 are expressed in all ovarian tissues(34–36). The ovarian Hh signaling system could be involved in the proliferation of GCs under certain conditions(31). Expressions

of a number of Hh genes in GCs that are known to be important for ovulation were no difference between mutants and controls(35). Some studies suggest that an association exists between modulation of the Hh pathway and selection of the dominant follicle(s)(36). In order to discuss the relationship between Hh signaling activity and PCOS which were accompanied with follicular dysplasia, we measured the components of the Hh pathway in GCs. We found that PCOS groups showed higher mRNA levels of Ptch1, Gli1 and Gli2, when compared to control groups; while the level of Gli3 mRNA had no significant difference. Ptch1 is a key component of the Hh signaling pathway, which controls cell fate determination during development(37). Ptch1 mutations cause derepression of target genes, cell fate changes, and excessive growth in some tissues(38). Gli1 lacks the N-terminal repressor domain and functions exclusively as an activator. The Gli1 gene is also a target of Hh signaling and thus acts to amplify the response to the signal (35, 39). Thus, the higher mRNA levels of Ptch1 and Gli1 confirmed that Hh signaling pathway is aberrant activated in the GCs of PCOS patients than control. In addition, Gli2 and Gli3 proteins contain both activator and repressor domains and undergo proteasome-dependent proteolytic cleavage(16). In our research, although Gli3 acting mainly as a repressor was no difference in GCs from these two groups, Gli2 appearing to function mostly as an activator was significantly higher in GCs of PCOS.

Admittedly, the communication between oocytes and GCs is important for the development of follicular, in which GCs secrete various kinds of nutritional factors to promote oocyte growth, simultaneously, oocytes product several factors to regulate GCs development(7, 8, 33). Therefore, the function status of GCs is often considered as the mirror of oocyte quality. It is well known that PCOS patients are often accompanied with aberrant GCs in follicles(7–9). The Hh signaling pathway was higher activated in GCs of PCOS than control, which implied the aberrant activation of Hh signaling pathway was related to abnormal follicular development in PCOS patients.

## Conclusions

Herein, we first demonstrate that aberrant activation of Hh signaling pathway in GCs is related to abnormal follicular development in PCOS patients. These findings provide a basis for future investigations to define cell-specific response to Hh signaling in the follicle and to determine how the pathway modulates follicle development.

## Declarations

### Abbreviations

PCOS: polycystic ovary syndrome

GCs: granulosa cells

Hh: Hedgehog

SMO: smoothened

FSH: follicle-stimulating hormone

LH: luteinizing hormone

E<sub>2</sub>:estradiol

PRL: prolactin

TES: testosterone

GnRH-a: gonadotropin-releasing hormone agonist

### **Ethics approval and consent to participate**

The patient signed a written informed consent form prior to recruitment. This study is in line with the Helsinki Declaration and approved by the Ethics Review Body Committee of the Jiangxi Maternal and Child Health Hospital.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

O.P.H and Y.L were responsible for the experimental design and drafting the manuscript. Y.L and S.D.W were involved in carrying out the experiments. G.H.X and Z.Y.Z were responsible for analyzing and interpreting the data with the assistant of Q.F.W and L.W. J.T performed the statistical analysis and edited the manuscript.

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## Figures

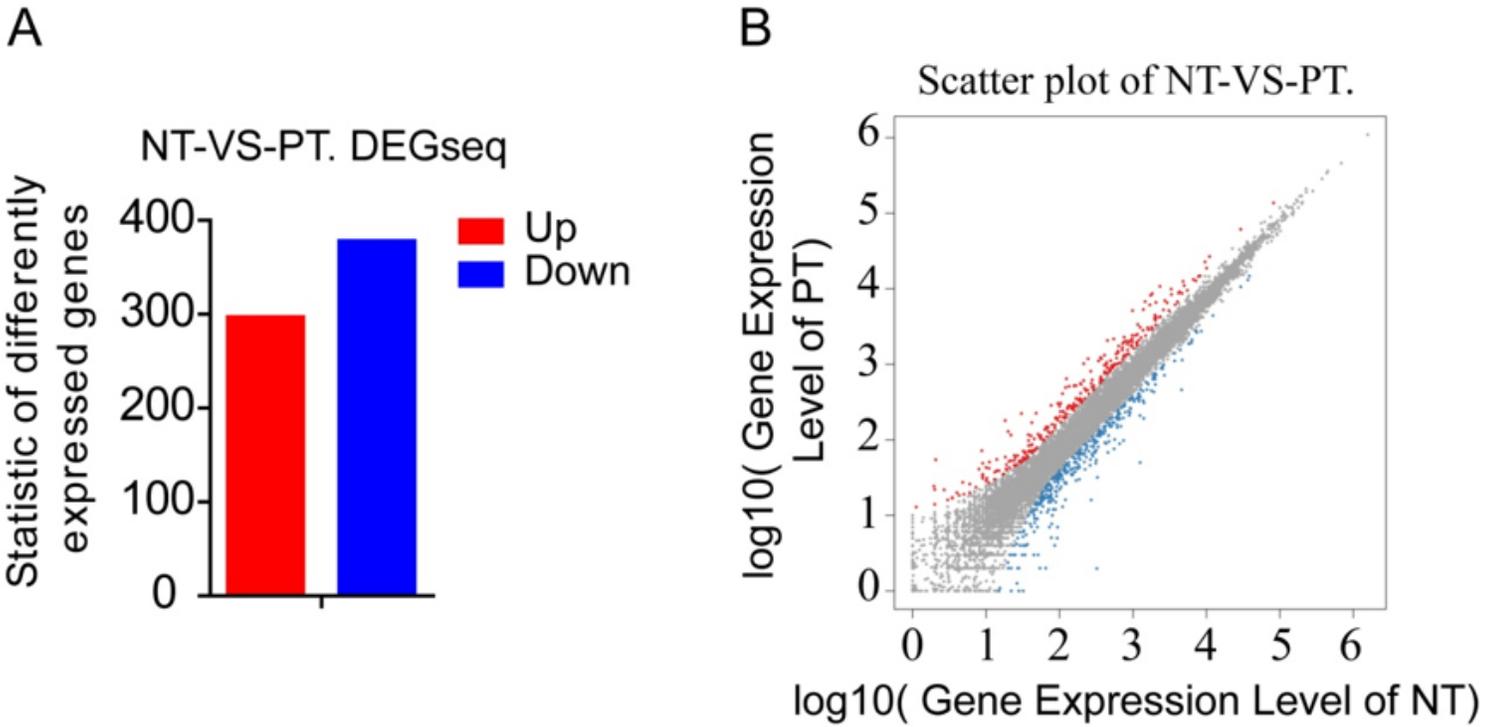
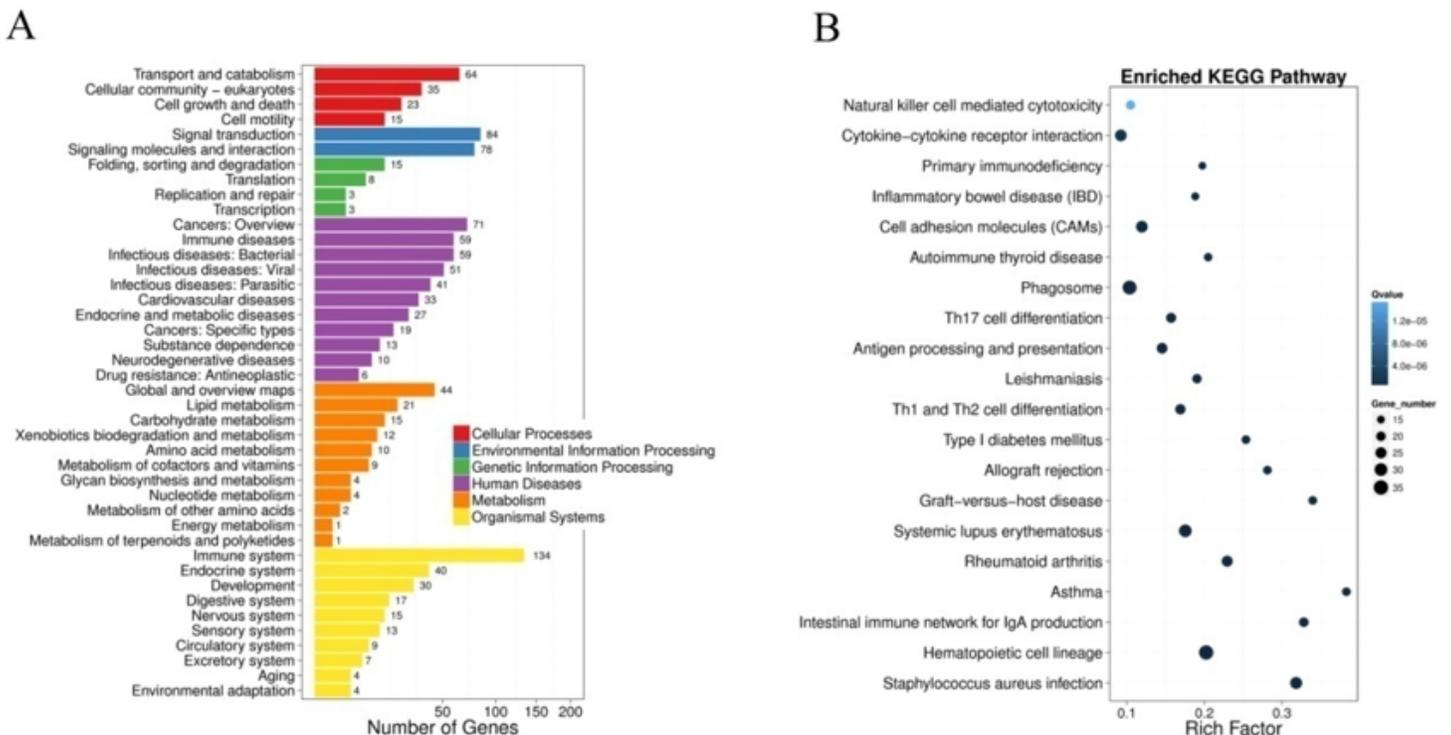


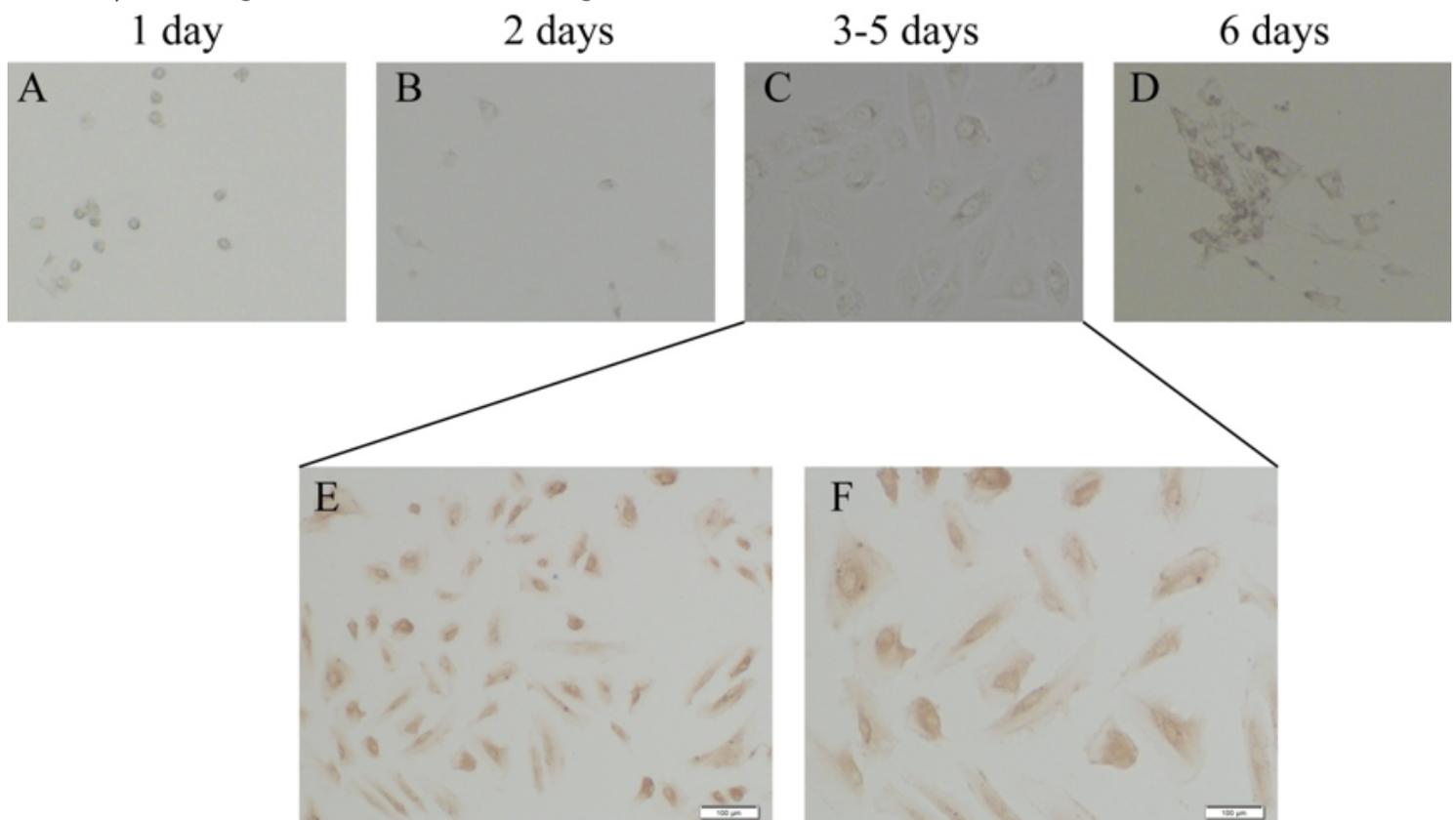
Figure 1

Summary of DEGs by RNA-seq. (A) X axis represents comparison method between each group. Y axis represents DEG numbers. Red color represents up-regulated DEGs. Blue color represents down-regulated DEGs. (B) Scatter plot of DEGs. X Y axis represents log10 transformed gene expression level, red color represents the up-regulated genes, blue color represents the down-regulated genes, gray color represents the non-DEGs.



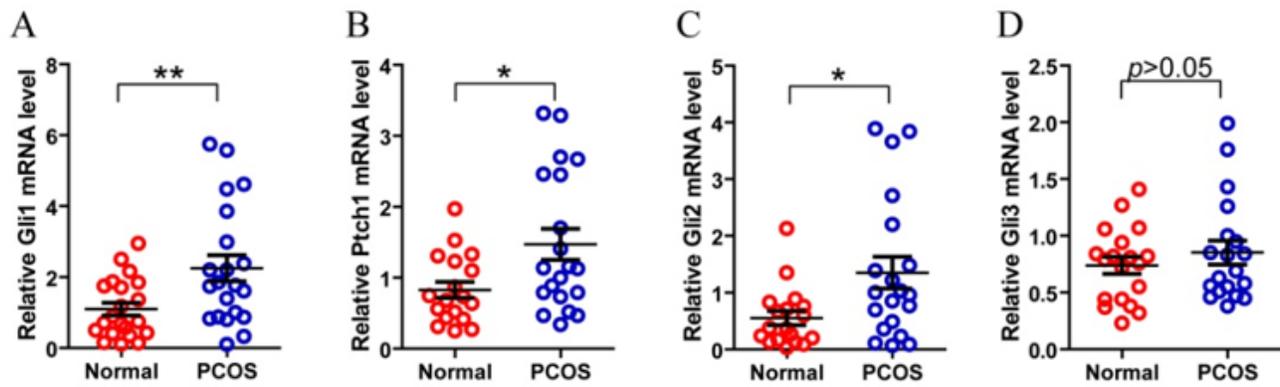
## Figure 2

Pathway functional enrichment results of NT-VS-PT. DEGseq Pathway. (A) X axis represents number of DEG. Y axis represents functional classification of KEGG. There are seven branches for KEGG pathways: Cellular Processes, Environmental Information Processing, Genetic Information Processing, Human Disease (For animals only), Metabolism, Organismal Systems and Drug Development. (B) X axis represents enrichment factor. Y axis represents pathway name. The color indicates the q-value (high: white, low: blue), the lower q-value indicates the more significant enrichment. Point size indicates DEG number (The bigger dots refer to larger amount). Rich Factor refers to the value of enrichment factor, which is the quotient of foreground value (the number of DEGs) and background value (total Gene amount). The larger the value, the more significant enrichment.



## Figure 3

Culture and identification of ovarian GCs in vitro. (A)-(D) represent the situation of GCs cultured in vitro for 1-7 days. (E)-(F) represent the IHC results of GCs staining with FSH antibody during 3-5 days. Scale bar=100  $\mu$ m for (E), Scale bar=50  $\mu$ m for (F).



**Figure 4**

Expression of Hh family members in GCs from PCOS group and control Group. A-D represent Gli, Ptch1, Gli2 and Gli3 mRNA levels from GCs between normal and PCOS. The red circle represents the normal group (Normal), and the blue circle represents the experimental group (PCOS). p-values were determined by Student's t-test, \*p<0.05, \*\*p<0.01.