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

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Abstract

The molecular mechanism that triggers polycystic ovary syndrome is mysterious. Abnormal ovarian granulosa cells are one of the causes of PCOS. Therefore, we carried out RNA-seq in ovarian granulosa cells from patients with PCOS and normal controls and found that Hedgehog signaling pathway members Ihh and ptch2 were abnormally highly expressed in the PCOS group. Granulosa cells from 22 patients with PCOS and 21 controls with normal ovulation were collected. Subsequent qPCR tests also indicated that the expression of ptch1, gli1, and gli2 of other downstream members of Hh in the PCOS group was significantly higher than that in the control group. These results indicate that abnormally activated Hh signaling pathway, especially Ihh signal, may have a profound influence on PCOS.

Background

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women, which affects 10% reproductive age women(Norman et al., 2007). The Rotterdam Criteria requires women to conform to two of three following symptom: oligoovulation or anovulation, clinical or biochemical hyperandrogenism and polycystic ovaries (PCO)(Rotterdam, 2004). In the late development stage of PCOS, the complication associated with metabolic diseases such as diabetes, abdominal adiposity, high cholesterol and hypertension most occur(Japur et al., 2014; Setji and Brown, 2014; Tandulwadkar et al., 2014). Therefore, the PCOS patients need long term therapy to avoid the consequence. But the etiology,especialmolecular mechanismand 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(Diamanti-Kandarakis, 2008). 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, granulose cells (GCs) and theca cells(Parrott and Skinner, 1998; Nilsson and Skinner, 2001; Zhang et al., 2017), which involving several signaling pathways(Jiang et al., 2017; Li et al., 2017; Nasri et al., 2017; Wu et al., 2017; Xing et al., 2017).

The *hedgehog* (Hh) gene was first cloned in Drosophila(Nusslein-Volhard and Wieschaus, 1980). It plays a very important role in the embryonic development and remodeling processes of adult tissues(Lee et al., 2016). Hh signaling regulates cell fate determination, proliferation, and differentiation(King et al., 2008). Moreover, over-activation of Hh signaling is associated with the tumorigenesis(Hirotsu et al., 2010; Galimberti et al., 2012; Lubik et al., 2017; Rennert et al., 2017). In mammals, the Hh pathway consists of three Hh ligands, indian (IHH), desert (DHH), and sonic (SHH); the membrane receptor patched (PTCH) 1 and 2; and the transmembrane signal transducer protein smoothened (SMO)(Wijgerde et al., 2005). In the

absence of ligand binding, PTCHmaintains 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(Hooper and Scott, 2005).

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(Russell et al., 2007). Here, we present evidence that significant abnormal activation of Hedgehog signaling pathwayis identified in PCOSpatients. These results indicated that Hh signaling pathway may be associated with follicular development and PCOS.

Methods

Patients selection

A prospective case-control study was designed including 22 patients with PCOS (PCOS group) and 21 patients with regular menstrual cycles (control group) from December 2016 to March 2017. They 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 and must including oligoovulation or anovulation and PCO(Rotterdam, 2004). 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 of more previous spontaneous pregnancy losses), congenital or acquired uterine malformations, abnormal results on parental karyotyping, endometriosis, hyperprolactinemia and thyroid dysfunction.

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 4000r 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).

Controlled ovarian stimulation

Follicular aspirates were collected during oocyte retrieval following published procedures and ovarian stimulation was the use of a prolonged protocol(Ren et al., 2014; Tan et al., 2016). 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_2< 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_2 levels and antral follicle count. The time and dose of recombinant human FSH were adjusted according to ovarian response as monitored by serum E_2 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, Darm-stadt, Germany) was administered. Oocytere-trieva l was perfor med 36-40 hours later under transvaginal ultrasound guidance.

Human GCs collection

Follicular fluids were centrifuged at 2000 rpm for 5 min. The cells were resuspended with DMEM/F 12 (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 hyaluronidase at a 1:1 ratio for 2 min to separate them. The GCs were removed using a pipette and washed with phosphate buffered saline (PBS). The cells were stored at -80°C for future analysis.

RNA-seq and qPCR

Total RNAs were extracted from 4 normal tissues and 3 PCOS tissues by using the RNAiso reagent (TaKaRa, Shiga, Japan). The Library was validated on the Agilent Technologies 2100 bioanalyzersubjected to deep sequencing onIllumina HiSeq 2000 (50-bp single-read sequencing), and analyzedatBgi Genomics Co., Ltd. RT-PCR and reverse transcription was carried out using the PrimeScript RT reagent Kit (TaKaRa). Standard RT-PCR 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-R-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).

Statistical analysis

The software package SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for all data analysis. In general, results among experimental groups were analyzed by student's t-test or one-way ANOVA or two-tail student's t-test. For all tests, *P*-value < 0.05 was considered 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 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 POCS group (Table 1). Next, we would use more clinical samples for subsequent analysis.

General conditions

According to the standard, we have 22 PCOS and 21 Control in our research. In this study, and no statistically significant difference was found in age, infertile period, or BMI between these two groups (Table 2). The basal serum hormone levels were also comparable between the patient cohorts, except for the LH and TES levels. Patients in PCOS group showed a statistically significantly higher basal LH and TES levels.

Clinical outcomes

Table 3 presents the comparisons between clinical outcomes of the two groups. The rates of MII oocyte and fertilization were significantly lower in PCOS group than those in control group (P < 0.05). No difference was observed for the rate of embryo cleavage and good-quality embryo in these two groups. Endometrial thickness on HCG day was significantly thinner in the group B. Other parameters included: dosage of Gn, duration of Gn, levels of LH, E2 and P on the day of hCG, number of oocytes retrieved and embryo, all of which exhibited no significant differences.

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 (Diamanti-Kandarakis, 2008; Japur et al., 2014; Setji and Brown, 2014; Tandulwadkar et al., 2014).

Previous studies have found that PCOS patients were often accompanied with aberrant follicles and unsatisfactory fertilization rate(Ren et al., 2014; Gong et al., 2015; Tan et al., 2016). 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 ± 28.9 vs. 80.4 ± 13.6, $P < 0.05$; 61.7 ± 20.8 vs. 76.9 ± 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.

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(Wijgerde et al., 2005), which demonstrated that Hh family were expressed on the GCs from primary to antral stages of follicular development in postnatal ovaries of human, respectively(Russell et al., 2007; Spicer et al., 2009). These results implied that Hh signaling played an important role in the communication between the cellular compartments that perform gametogenesis, steroidogenesis and ovarian vasculature(Wijgerde et al., 2005; Russell et al., 2007; Ren et al., 2012). Activation of Hh signaling regulated follicle growth and GCs proliferation at least one of the potential targets(Russell et al., 2007). 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(Franks et al., 2006; Diamanti-Kandarakis, 2008). 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(Russell et al., 2007). In addition, the transcription factor, GLI1, GLI2 and GLI3 are expressed in all ovarian tissues(Ren et al., 2009; Aad et al., 2012; Migone et al., 2012). The ovarian Hh signaling system could be involved in the proliferation of GCs under certain conditions(Spicer et al., 2009). Expressions of a number of Hh genes in GCs that are known to be important for ovulation were no difference between mutants and controls(Ren et al., 2009). Some studies suggest that an association exists between modulation of the Hh pathway and selection of the dominant follicle(s)(Aad et al., 2012). 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(Hammerschmidt et al., 1997). PTCH1 mutations cause derepression of target genes, cell fate changes, and excessive growth in some tissues(Ingham et al., 1991). 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 (Ren et al., 2009; Ingham et al., 2011). 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(Lee et al., 2016). 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(Parrott and Skinner, 1998; Nilsson and Skinner, 2001; Franks et al., 2006). 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(Parrott and Skinner, 1998; Nilsson and Skinner, 2001; Zhang et al., 2017). 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.

The date presented here 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

Ethics approval and consent to participate

The patient signed a written informed consent form prior to recruitment. This study is in line with theHelsinki 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.Wand L.W. J.T performed the statistical analysis and edited the manuscript.

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Tables

Table 1. 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^2)	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_2 (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 date were expressed as mean + SD.

Table 2. Comparison of clinical outcomes between PCOS group and control group

Variables	PCOS	control	*P*
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_2 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 date were expressed as mean + SD.

Table 3. Expression of a part of DEGs hierarchical clustering analysis results of PCOS patients and control group

Symbol	GeneID	Length	NT	PT	log2(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

Figures

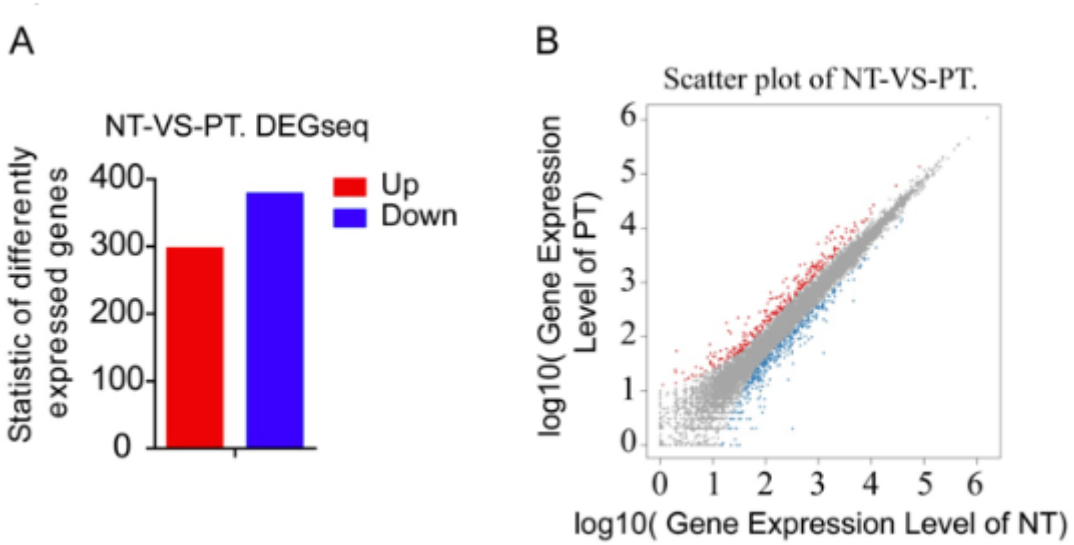

Figure 1

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.

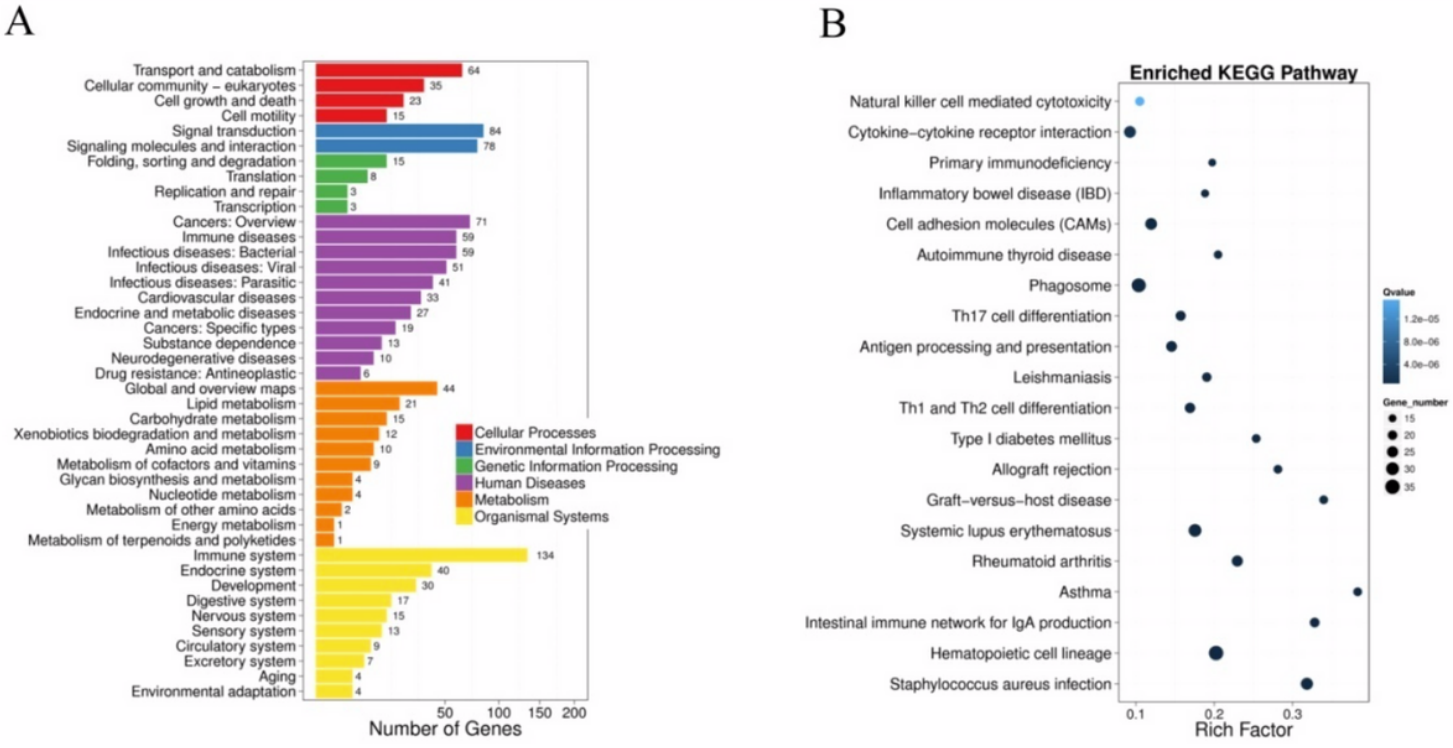

Figure 2

Pathway functional enrichment results of NT-VS-PT.DEGseqPathway. (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.

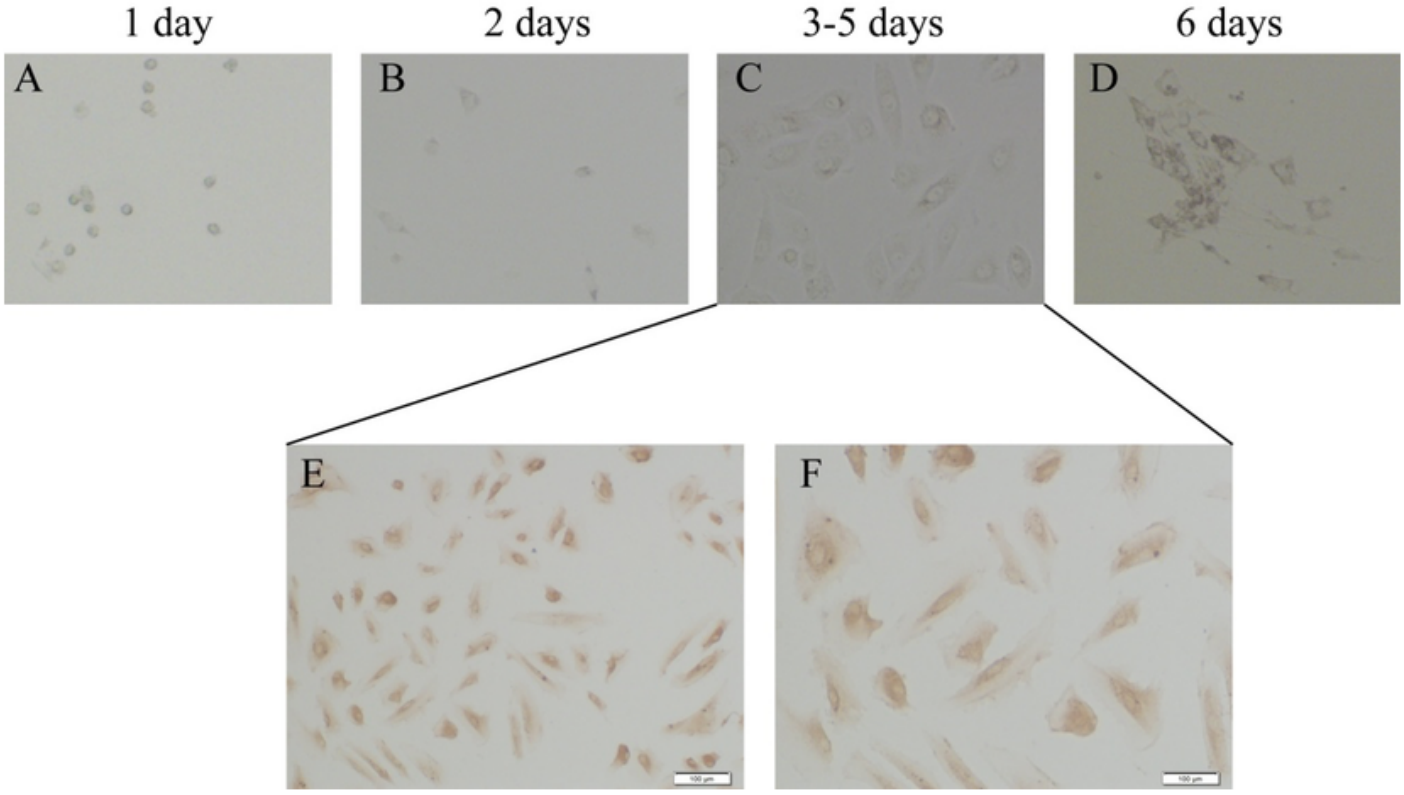

Figure 3

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

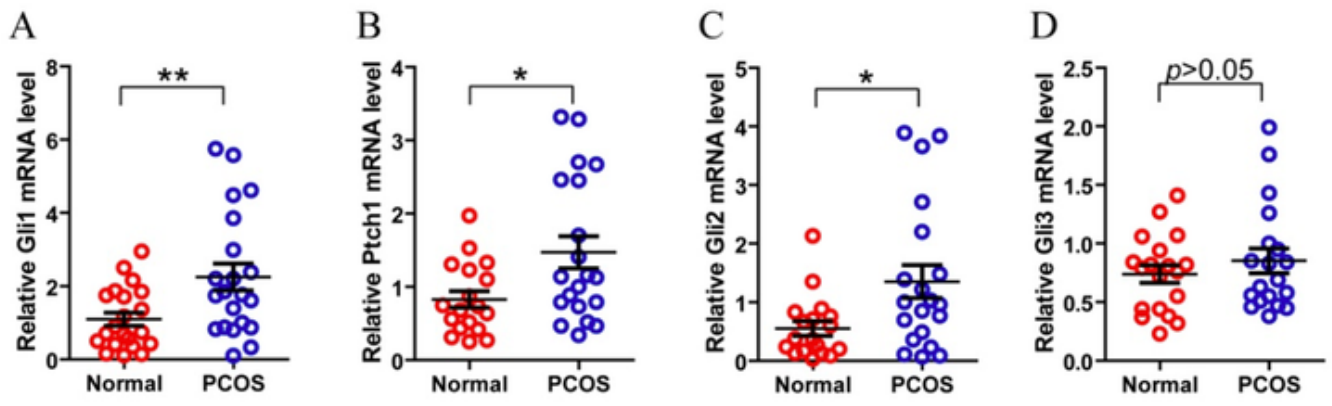

Figure 4

Expression of Hh family members in GCs from PCOS group and control Group. The levels of GLI1 mRNA, PTCH1 mRNA and GLI2 mRNA were significantly higher in PCOS group than those in the control group, while the level of GLI3 mRNA had no significant difference between the two groups. p-values were determined by Student's t-test, *p<0.05, **p<0.01.