

# Protective Effect of SCUBE1 on Oxidative Stress-induced Apoptosis in Human Ovarian Granulosa Cells

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## Research

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# Abstract

**Background:** Apoptosis of ovarian granulosa cells (GCs) is a sign of follicular atresia. This study aimed to explore the role and mechanism of signal peptide, CUB domain, epidermal growth factor-like protein1 (SCUBE1) in protecting GCs from apoptosis induced by hydrogen peroxide ( $H_2O_2$ ).

**Methods:** Firstly, the expression of SCUBE1 on the ovaries of humans and mice was analyzed by qRT-PCR, western blot and immunohistochemistry. Subsequently, the  $H_2O_2$  treated GCs were pretreated with SCUBE1 recombinant protein, and their cell viability and proliferation were detected by Cell Counting Kit-8 (CCK-8) assay. The levels of reactive oxygen species (ROS) and mitochondrial membrane potential ( $\Delta\Psi_m$ ) in the cells were determined by DCFH-DA and rhodamine 123, respectively. The percentage of apoptotic cells was analyzed by flow cytometry after staining with Annexin V/PI. The expression levels of pathway related proteins, such as Bcl-2, Bax, p53, caspase-3, were determined by western blot analysis. Finally, the pathogenicity of SCUBE1 (c.1169C>G, p.P390R) were analyzed based on the software.

**Results:** SCUBE1 was expressed in women of all ages and had the highest expression level in the ovaries in multiple organs and tissues of KM mouse. In vitro cell experiments show that SCUBE1 pretreatment reduced  $H_2O_2$ -induced apoptosis and improved cell viability. SCUBE1 also blocked the production of ROS in cells and improved mitochondrial membrane potential. After SCUBE1 pretreatment, anti-apoptotic protein Bcl-2 expression was upregulated, whereas the expression of the pro-apoptotic proteins Bax, Bax/Bcl-2, Caspase-3, and p53 were downregulated. Analysis of the impact of SCUBE1 (c.1169C >G, p.P390R) mutation from the aspect of mutation pathogenicity; protein stability; and gene haplotype insufficiency, indicated that the p.P390R mutation is significantly pathogenic.

**Conclusions:** This is the first time that the potential role of SCUBE1 in protecting GCs from  $H_2O_2$ -induced damage through the mitochondrial pathways, attributing to POI, is studied. SCUBE1 (c.1169C >G, p.P390R) mutation has significant pathogenicity but the specific harm needs to be confirmed by further studies.

**Trial registration:** Not applicable.

## Background

Premature ovarian insufficiency (POI) is a clinical syndrome of women with ovarian dysfunction before the age of 40, including infertility, menstrual disorders, amenorrhea, hypoestrogenic symptoms, cardiovascular damage, and other short- and long-term symptoms [1], which can greatly endanger a women's physical and mental health. At present, the global incidence of POI is 1–2% [2], but it has been increasing in recent years. The essence of POI is the acceleration of follicular atresia to premature exhaustion, with the apoptosis of ovarian granulosa cells (GCs) being the initiating factor of follicular atresia [3]. Studies have shown that oxidative stress (OS) is closely related to POI and apoptosis in GCs [4-5].

OS refers to the imbalance of oxidative and antioxidant systems in the body, resulting in an abnormal increase in reactive oxygen species (ROS) [6]. High ROS levels induce changes in mitochondrial DNA, leading to mitochondrial dysfunction. This leads to a decrease in ATP production and impaired oxidative phosphorylation, which damages oogenesis, promotes GCs to accelerate apoptosis, affects embryo quality and embryo

development, and leads to a decline in female fertility. ROS play an important role in various female reproductive diseases including POI, endometriosis, polycystic ovary syndrome, spontaneous abortion, and moles [6].

Previously, this research group used exon sequencing technology to screen out 12 genes in 10 sporadic POI patients, including 22 mutation sites, as candidate pathogenic mutations for POI, of which the rare gene mutation SCUBE1 (c.1169C>G, p.P390R) was confirmed to have a mutation frequency of 0.15 in 20 POI samples (3/20) [7]. The SCUBE1 gene is located on human chromosome 22q13.3, which mainly encodes cell surface proteins and secreted proteins [8], and participates in inflammatory responses [9], cell proliferation repair [10], ischemia, hypoxia, and other pathophysiological processes, while OS plays an important role in the pathophysiological processes of inflammation, ischemia, and hypoxia. Recent studies have confirmed the involvement of the SCUBE1 gene in regulating the differentiation of porcine ovarian GCs, with subsequent verification by qRT-PCR showing SCUBE1 mRNA expression in porcine ovarian GCs [11]. SCUBE1 is widely expressed in human tissues and the highest expression levels are in ovarian tissues (The Human Protein Atlas: <https://www.proteinatlas.org/ENSG00000159307-SCUBE1>). However, it is unclear whether SCUBE1 can protect human GCs from the damage caused by OS.

The use of hydrogen peroxide ( $H_2O_2$ ) to induce oxidative damage in ovarian GCs in vitro and thus induce cell apoptosis, is a common, reasonable, and effective method for exploring the factors and mechanisms that affect follicular development and ovarian function [12]. This study aimed to investigate the expression of SCUBE1 in the ovaries and the effects of SCUBE1 on ROS, mitochondrial membrane potential ( $\Delta\Psi_m$ ), and related apoptosis factors in GCs treated with  $H_2O_2$ , to analyze the role of SCUBE1 in human GC apoptosis and provide new ideas for the prevention and treatment of POI.

## Materials And Methods

All manufacturer guidelines were followed when using kits in this investigation unless otherwise specified.

### *Tissue samples*

### *Patients*

The study protocol was approved by the ethics committee of Zhujiang Hospital of Southern Medical University, and all subjects signed an informed consent form before surgery. Participants were divided into a young group ( $18 \leq \text{group 1} \leq 36$  years old), middle-aged group ( $36 < \text{group 2} \leq 54$  years old), and elderly group ( $\text{group 3} > 54$  years old), with three cases in each group. The clinical characteristics of the study participants are summarized in Table 1. Normal ovarian tissues were surgically removed and collected from the Department of Obstetrics and Gynecology, Zhujiang Hospital of Southern Medical University. The criteria for selected ovarian tissues included the exclusion of ovarian diseases, including ovarian cysts, ovarian benign tumors, and ovarian cancer. Postoperative pathology was confirmed by two professional pathologists. The ovarian tissue was placed in a liquid nitrogen tank within 30 min of being isolated until further processing.

### Table 1. Characteristics of the study participants

Patient no.	Age (year)	Preoperative diagnosis	Postoperative pathology	Ovarian pathology	Complications
1	30	Cervical cancer	Cervical adenocarcinoma, moderately differentiated	No abnormalities in both ovaries	None
2	32	Cervical adenocarcinoma	Cervical adenocarcinoma, moderately differentiated	No abnormalities in both ovaries	None
3	36	Cervical cancer	Cervical adenocarcinoma, moderately differentiated	No abnormalities in both ovaries	None
4	50	Adenomyosis; uterine fibroids	Adenomyosis; uterine fibroids	No abnormalities in both ovaries	None
5	51	Cervical squamous cell carcinoma	Cervical squamous cell carcinoma	No abnormalities in both ovaries	Tricuspid regurgitation
6	53	Endometrium adenocarcinoma	Focal endometrial hyperplasia with atypical, no invasive cancer tissue	No abnormalities in both ovaries	None
7	63	Pelvic mass	Epidermoid cyst of the right ovary with multinucleated giant cell reaction (teratoma)	Normal left ovary	None
8	64	Abnormal uterine bleeding	Cervical adenocarcinoma in situ	No abnormalities in both ovaries	None
9	71	Cervical intraepithelial neoplasia II	Cervical intraepithelial neoplasia II	No abnormalities in both ovaries	None

### *Animals*

The study protocol was approved by the Ethics Committee of the Zhujiang Hospital of Southern Medical University. Kunbai strain healthy female mice (KM mouse) aged 3–5 weeks and 10–11 weeks were purchased from the Experimental Animal Center of Southern Medical University. They were reared in a single cage in a conventional manner under a 14/10 h light/dark environment at a room temperature of 22 °C. For the experiment, 6-week-old and 12-week-old mice were selected, with 5 mice per week. After the mice were sacrificed, both ovaries were immediately removed. One ovary was fixed with 4% paraformaldehyde for 24–48 h. The other

ovary and the removed brain tissue, heart, lung, liver, spleen, stomach, small intestine, and muscle were placed in a liquid nitrogen tank.

#### *Hematoxylin and eosin (HE) staining and immunohistochemistry*

Fixed KM mouse ovaries were paraffin-embedded. Serial sections of the wax block (4 µm thick) were mounted on a glass slide, and four sets of sections were prepared for HE staining (tissue location control), SCUBE1 immunohistochemical analysis (observation of SCUBE1 distribution), PBS replaces the primary antibody (blank control), and one sheet for standby application. The sections were deparaffinized in xylene, hydrated in gradient alcohol, and then immersed in citric acid-EDTA antigen retrieval solution (Beyotime, Shanghai, China); the mixture was placed in a microwave oven on high heat for 5 minutes, left to rest for 3 minutes, returned to a medium heat for 5 minutes, and then naturally cooled to room temperature. After blocking with endogenous peroxidase, the sections were incubated with an anti-SCUBE1 antibody (1:400, bs-9903R, Bioss, Beijing, China), followed by

incubated the secondary antibody conjugated with biotin and the horseradish enzyme-labeled streptavidin working solution (ZSGB-BIO, Beijing, China) for 1 h at room temperature. The immunoreactivity of SCUBE1 in ovarian cells was then analyzed and scored independently by three observers. The immune response to SCUBE1 was evaluated according to the intensity of the brown color. The immunoreactivity corresponding to the degree of brown color from light to dark was weak, medium, and strong.

#### *Quantitative real time PCR (qRT-PCR)*

A TRIzol reagent kit (Takara, Japan) was used to extract total RNA from human ovarian tissue and different organs and tissues of KM mouse, according to the manufacturer's instruction. The absorbance at 260/280 nm was measured using an ultra-micro high-precision spectrophotometer Nanodrop ND-2000 (Thermo, USA) ratio to determine the concentration and purity of RNA. After reverse transcription of total RNA (1 µg), FastFire qPCR PreMix (SYBR Green) (Tiangen Biotech, Beijing, China) was used for real-time PCR analysis. The primer sequences for SCUBE1 and GAPDH are listed in Table 2.

**Table 2. Primers for SCUBE1 and GAPDH used for qRT-PCR**

<b>Genes</b>	<b>Primer</b>
SCUBE1	Forward 5'-GAGGATGAGTGCGGCGATGTTC-3'
	Reverse 5'-CTCTCGTAGGTCTGGCAGGTCTC-3'
GAPDH Human	Forward 5'-CAGGAGGCATTGCTGATGAT -3'
	Reverse 5'-GAAGGCTGGGGCTCATTT-3'
GAPDH Mice	Forward 5'-GGTTGTCTCCTGCGACTTCA-3'
	Reverse 5'-TGGTCCAGGGTTTCTTACTCC-3'

### *Cell culture*

A granulosa-like tumor cell line, KGN cells, was used in this experiment and was purchased from Wuhan Pu Nuosai Life Technology Co., Ltd., China. Cells were prepared with DMEM/F12 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (HyClone, USA) at 37 °C in a 5% CO<sub>2</sub> atmosphere, and cultivated in an incubator with 95% humidity.

### *Cell treatment*

Cells were seeded in a 6-well plate or Petri dish and divided into three groups (control, model, and experimental groups). Twenty-four hours after cell inoculation, the experimental group was treated with 5 ng/mL SCUBE1 human recombinant protein (rhSCUBE1, Abnova, USA), and the remaining two groups were replaced with the same volume of serum-containing DMEM/F12 medium. After another 24 h, the model and experimental groups replaced the medium with 0.3 mmol/L (mM) H<sub>2</sub>O<sub>2</sub>, and the control group replaced the same volume of serum-containing DMEM/F12 medium. After incubation at 37 °C in an incubator with a 5% CO<sub>2</sub> atmosphere for 24 h, one step of processing was performed.

### *Analysis of cell viability*

Cells were inoculated in 96-well plates (5000 cells/100 µL/well), incubated at 37 °C in an incubator with a 5% CO<sub>2</sub> atmosphere for 24 h, the culture medium was discarded, H<sub>2</sub>O<sub>2</sub> (0.01, 0.1, 0.3, 0.5, 0.8, 1.0, and 1.5 mmol/L) and an equal volume of serum-containing DMEM/F12 medium was added to the control group. Cells were incubated for 24 h, the medium was aspirated, and 100 µL of medium containing 10% CCK-8 solution (Apexbio, USA) was added to each well, with each plate incubated at 37 °C in an incubator with a 5% CO<sub>2</sub> atmosphere for 3 h. A microplate reader was used to measure the optical density (OD) of each well at a 450 nm wavelength. All experiments were repeated three times, analyzed, and graphed using GraphPad Prism 8 software. The concentration of H<sub>2</sub>O<sub>2</sub> was determined based on the half-maximal inhibitory concentration (IC<sub>50</sub>). The inhibition rate of cell proliferation (%) = (mean OD value of control group - mean OD value of experimental group) / (mean OD value of control group - mean OD value of blank group) × 100.

The protective effect of rhSCUBE1 on KGN cells was determined by CCK-8 assay according to the manufacturer's instructions. Cells were seeded in 96-well plates and divided into eight groups: control group (DMEM medium containing serum), model group (0.3 mmol/L H<sub>2</sub>O<sub>2</sub>), low concentration group (5 ng/mL rhSCUBE1), medium concentration group (20 ng/mL rhSCUBE1), high concentration group (40 ng/mL rhSCUBE1), low concentration pretreatment group (5 ng/mL rhSCUBE1 + 0.3 mmol/L H<sub>2</sub>O<sub>2</sub>), medium concentration pretreatment group (20 ng/mL rhSCUBE1 + 0.3 mmol/L H<sub>2</sub>O<sub>2</sub>), and high concentration pretreatment group (40 ng/mL rhSCUBE1 + 0.3 mmol/L H<sub>2</sub>O<sub>2</sub>). Twenty-four hours after cell inoculation, the recombinant protein treatment groups and the pretreatment groups were added with the corresponding concentrations of rhSCUBE1, with the remaining groups replaced with the same volume of serum-containing DMEM. After 24 h, the medium was changed in the pretreatment groups to 0.3 mM H<sub>2</sub>O<sub>2</sub>, and in the rest of the groups it was changed using the same volume of serum-containing DMEM medium. These groups were all incubated at 37 °C in an incubator with a 5% CO<sub>2</sub> atmosphere for 24 h, with the respective culture media discarded, and the cells tested using the CCK-8 assay.

### *Determination of intracellular ROS level*

Cells were washed three times with PBS and incubated with DCFH-DA (10  $\mu\text{mol/L}$ ) (Beyotime, Shanghai, China) in a 37 °C incubator for 20 min. Cell culture medium was washed 3 times to remove excess DCFH-DA, followed by an analysis of cells fluorescence intensity with a flow cytometer (BD Biosciences, USA).

### *Measurement of mitochondrial membrane potential*

The changes in mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ) were measured using the fluorescent dye rhodamine123 (Rh123, Beyotime, Shanghai, China). Cells were digested and collected by trypsin (Gibco, USA), washed twice with PBS, with Rh123 added at a final concentration of 10  $\text{mmol/L}$ . Cells were, incubated at 37 °C for 30 min, followed by washing with PBS three times. Immediately after, flow cytometry was performed to measure the average fluorescence intensity of the cells.

### *Analysis of apoptosis by flow cytometry*

Treated cells were collected, washed, centrifuged, and resuspended in Binding Buffer, and then 5  $\mu\text{L}$  Annexin V-FITC and 10  $\mu\text{L}$  PI (CW BIO, Jiangsu, China) were added, mixed, and incubated at room temperature for 15 min in the dark for analysis with flow cytometry.

### *Western blotting*

Total protein extract was obtained from human ovarian tissues or cultured KGN cells. The BCA protein quantification kit (CW BIO, Jiangsu, China) was used to detect the protein concentration and adjust it to the same level. The protein sample (20  $\mu\text{g}$ ) was loaded onto an SDS-polyacrylamide gel for electrophoresis and then transferred to a PVDF membrane. After blocking non-specific protein binding sites with 5% skimmed milk for 1.5 h, they were combined with the corresponding primary antibodies [ $\beta$ -actin (1:2000, Servicebio, Wuhan, China), SCUBE1 (1:500, Bioss, Beijing, China), Bax 1:1000, Servicebio, Wuhan, China), Bcl-2 (1:1000, Proteintech, Wuhan, China), Caspase-3 (1:500, Proteintech, Wuhan, China) and p53 (1:1000, Servicebio, Wuhan, China)] overnight at 4 °C. They were then incubated with a secondary antibody (1:3000, Servicebio, Wuhan, China) for 1 h at room temperature. The bands were visualized using an ECL detection reagent (Merck Millipore, USA).

### *SCUBE1 (c.1169C>G, p.P390R) pathogenicity and stability analysis*

A variety of silicon prediction tools, including Polyphen-2 [13], SIFT, Mutation Taster [14], LRT, SNAP, and PANTHER, were used to analyze the pathogenicity of SCUBE1 (c.1169C>G, p.P390R).

According to the Exome Aggregation Consortium (ExAC) PLI (loss-intolerance) [15] the score used to evaluate the possibility of SCUBE1 loss of function (LOF) mutation ranges from 0 to 1. The higher the score, the lower the tolerance.

Align-GVGD [16] is a free online tool for the physical and chemical characterization of mutations. It combines the GVs and GDs for the prediction. The predicted categories range from C0 to C65, and the higher the level, the greater the probability that the mutation will interfere with protein function.

iStable [17] is an integrated prediction tool that predicts the results of MUPRO and I-Mutant2.0. Using the sequence of the protein, we predicted the changes in stability due to specific substitutions.

PyMol software was used to analyze the local spatial configuration changes in the wild-type and mutant-type SCUBE1 models.

### *Statistical analysis*

The experiments were repeated at least three times. Data are expressed as mean  $\pm$  SD, and SPSS software (Version 23.0, SPSS, Inc., Chicago, USA) was used for analysis. One-way analysis of variance was used to determine the statistical differences. Non-normally distributed data were analyzed with a non-parametric Kruskal-Wallis test. A P value  $< 0.05$  was considered statistically significant (\* P  $< 0.05$ , \*\*P  $< 0.01$ ).

## **Results**

### *Localization of SCUBE1 in KM mouse ovaries*

The expression and localization of SCUBE1 in the ovaries of the KM mice was determined first. HE staining (Fig. 1 A, B) indicated that hematoxylin resulted in a purple-blue nucleus while eosin gave the cytoplasm and extracellular matrix components a red color. The positive reaction in immunohistochemistry showed yellow, brown, and dark brown particles. No reaction particles were observed in the blank control section, which appeared colorless or light yellow (Fig. 1 a4).

Results showed that SCUBE1 had similar expression patterns in the ovaries of KM mice at 6 and 12 weeks (corresponding to puberty and sexual maturity, respectively) (Fig. 1 a, b). SCUBE1 was strongly expressed in stromal cells, ovarian surface epithelial cells, and the surrounding area rich in blood vessels, moderately expressed in the corpus luteum, and there was no obvious expression in the follicles (Fig. 1 a1-a3, b1-b4).

### *Expression of SCUBE1*

Subsequently, the total RNA and protein of the human ovaries in the young, middle-aged, and old groups were extracted, and the expression levels of SCUBE1 mRNA and protein in each group were detected by qRT-PCR and western blotting, respectively. Results showed that SCUBE1 was expressed in women of all ages, and the expression level was higher in the elderly group (Fig. 1 D), consistent with the qRT-PCR results (Fig. 1 C). SCUBE1 mRNA expression levels in the different organs and tissues of the mice were as follows: ovary > lung > brain > stomach > spleen > small intestine > liver > heart > thigh muscles (Fig. 1 E).

### *Effect of rhSCUBE1 treatment on KGN cell viability and apoptosis*

Considering that the apoptosis of GCs is a sign of follicular atresia, KGN cells were used as the research object to explore the effect and mechanism of SCUBE1 on the apoptosis of human GCs cultured in vitro. When KGN cells were exposed to different concentrations of H<sub>2</sub>O<sub>2</sub> for 24 h, cell viability decreased in a concentration-dependent manner (Fig. 2 A). When the concentration of H<sub>2</sub>O<sub>2</sub> reached 0.3 mM, the cell proliferation inhibition rate dropped to 54.74  $\pm$  0.06% (P = 0.006), which was established as the best IC<sub>50</sub> value, with subsequent experiments therefore all treated with 0.3 mM H<sub>2</sub>O<sub>2</sub>.

To determine the effect of SCUBE1 on H<sub>2</sub>O<sub>2</sub>-induced KGN cell damage, cells were pretreated with rhSCUBE1 for 24 h before exposure to H<sub>2</sub>O<sub>2</sub>. 5 ng/mL rhSCUBE1 was sufficient to significantly increase cell viability (P =



0.002), and at the same time, it could significantly reduce the damage of KGN cells induced by H<sub>2</sub>O<sub>2</sub> (P = 0.000) (Fig. 2 B). Therefore, in subsequent experiments, cells were pretreated with 5 ng/mL rhSCUBE1.

Annexin V binds to FITC and specifically binds to phosphatidylserine residues in apoptotic cells. Results showed that the apoptosis of KGN cells increased after exposure to 0.3 mM H<sub>2</sub>O<sub>2</sub> (P = 0.001), while 5 ng/mL rhSCUBE1 pretreatment reduced H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis (P = 0.018) (Fig. 2 C and D).

#### *rhSCUBE1 reduced the generation of intracellular ROS and increased the mitochondrial membrane potential*

Classic DCFH-DA staining was used to determine the changes in ROS levels in KGN cells. Results showed that 0.1 mM and 0.3 mM H<sub>2</sub>O<sub>2</sub> could induce intracellular ROS to increase to 2.40 and 3.40 times that of the control, respectively (P = 0.003, P = 0.015) (Fig. 3 A and B), suggesting a concentration-dependent increase in ROS, and the rhSCUBE1 pretreatment could significantly reduce intracellular ROS levels (P = 0.000) (Fig. 3 C and D).

Early stages of apoptosis caused a drop in mitochondrial membrane potential. It was found by Rh123 staining that 0.1 mM and 0.3 mM H<sub>2</sub>O<sub>2</sub> could cause the mitochondrial membrane potential to drop to 0.86 and 0.53 times that of the control, respectively (P = 0.008, P = 0.000) (Fig. 3 E and F), indicating the concentration-dependent loss of the mitochondrial membrane potential. rhSCUBE1 pretreatment reversed this loss (P = 0.022) (Fig. 3 G and H).

#### *rhSCUBE1 exerts cytoprotective effects via the mitochondrial pathway*

To study the protective mechanism of rhSCUBE1, the expression of pathway-related proteins in KGN cells were detected by western blotting (Fig. 4 A and B). We found that SCUBE1 was expressed on KGN cells. It was clear that H<sub>2</sub>O<sub>2</sub> could downregulate the expression of SCUBE1, and SCUBE1 expression increased after rhSCUBE1 pretreatment. Results from the investigation of the mitochondrial apoptosis pathway showed that H<sub>2</sub>O<sub>2</sub> upregulated the expression of the pro-apoptotic protein Bax, while downregulating the expression of the anti-apoptotic protein Bcl-2, resulting in a significant increase in the ratio of Bax/Bcl-2 (P = 0.000); rhSCUBE1 pretreatment could reverse this effect. H<sub>2</sub>O<sub>2</sub> treatment upregulated p53 protein expression, however, the difference was not statistically significant (P = 0.092), but the rhSCUBE1 pretreatment downregulated p53 protein expression (P = 0.033).

Caspase-3 protein expression, one of the key reaction proteins downstream of the mitochondrial apoptosis pathway, was significantly increased under the induction of H<sub>2</sub>O<sub>2</sub> (P = 0.000), while the rhSCUBE1 pretreatment significantly downregulated expression of Caspase-3 (P = 0.000) and cleaved Caspase-3 (P = 0.011).

#### *SCUBE1(c.1169C>G, p.P390R) pathogenicity and stability analysis*

Based on software analysis of the bioinformatic changes before and after gene point mutations, the various effects of the p.P390R mutation on SCUBE1 could be more clearly understood. Sanger sequencing was performed on 20 POI samples [5]. Three patients were consequently found to have heterozygous mutations in SCUBE1 (c.1169C>G, p.P390R) (Fig. 5 A). The p.P390R mutation resulted in a proline mutation (Fig. 5 B, a) to arginine (Fig. 5 B, b) at position 390. Based on PyMol software, analysis of the SCUBE1 homologous protein model (Template PDB Code 4xbmB), there were changes from proline to arginine in the local space of SCUBE1 (Fig. 5 B, c, and d).

Data from SIFT, LRT, SNAP, and PANTHER software also suggested that this mutation seriously affects protein function (Table 3). According to ExAC, the PLI score of SCUBE1 was 0.96. SCUBE1 cannot tolerate LOF mutations, that is, SCUBE1 has a single dose under-dose, which further supports the pathogenicity of the p.P390R mutation.

**Table 3. Pathogenic predictions about SCUBE1 (c.1169C>G→p.P390R)**

Variants	Polyphen-2	SIFT	Mutation Taster	LRT	SNAP	PANTHER
P390R	Probably damaging	Deleterious	Disease causing	Deleterious	Deleterious	Deleterious

Both iStable and i-Mutant2.0 in the iStable tool predicted that p.P390R is a destabilizing activity (Table 4). The Align-GVGD server was further used to predict the destabilizing effect of the p.P390R mutation on SCUBE1 and the results showed that the mutation was classified as Class C65, which significantly hindered protein function (Table 5).

**Table 4. Stability prediction analysis of SCUBE1 mutations using the iStable consensus tool**

Variant	i-Mutant2.0 SEQ	DDG	MUpro	Conf. score	iStable	Conf. score
P390R	Decrease	-0.81	Increase	0.093876871	Decrease	0.511507

**Table 5. Biophysical characterization using Align-GVGD**

Variant	GV	GD	Prediction
P390R	0.00	102.71	Class C65

## Discussion

We studied the expression of SCUBE1 in ovarian tissue for the first time and found that SCUBE1 is expressed in human and mouse ovaries. Although SCUBE1 is mainly expressed in several highly vascularized tissues such as liver, kidney, lung, spleen and brain [18], its expression levels in other organs were lower than those of the ovaries. It is speculated that the continuous expression of SCUBE1 is likely to be an important guarantee for maintaining normal ovarian function. As a secreted protein, it may regulate the growth and development of follicles through paracrine and autocrine functions.

At present, one of the focuses of the etiology of POI is the molecular biological mechanism of granulosa cell apoptosis during follicular atresia. Studies have shown that ROS levels in POI patients with normal cytogenetics are significantly higher than those in normal controls [19], and ROS plays an important role in antral follicular apoptosis [20]. According to reports, SCUBE1 may be a potential serological marker of inflammatory diseases (acute appendicitis [21]), ischemic hypoxic diseases (acute mesenteric ischemia [22], acute coronary syndrome and ischemic stroke [23], ovarian torsion [24]). In rats with ischemia-reperfusion kidney injury, SCUBE1 can promote the proliferation of renal tubular epithelial cells and antagonize apoptosis [25–26]. However, whether SCUBE1 can protect human GCs from damage by H<sub>2</sub>O<sub>2</sub> has not yet been reported.

The key to H<sub>2</sub>O<sub>2</sub> inducing apoptosis of GCs lies in the abnormal increase of ROS, which causes mitochondrial dysfunction, and the decrease in mitochondrial membrane potential is a landmark event in the early stage of apoptosis [27]. Nishi et al. [28] first established a new ovarian granular tumor cell line KGN from granular cell tumors in 2001. KGN cells have steroid-producing activity similar to normal granule cells and express functional FSH receptors. This cell line is expected to enable us to study various aspects of the physiological regulation of human ovarian GCs. This study found 0.3 mM H<sub>2</sub>O<sub>2</sub>, used for 24 h as the IC<sub>50</sub> value for inducing apoptosis of KGN cells; as the cell morphology changed accordingly, the intracellular ROS level increased, and the mitochondrial membrane potential was reduced. rhSCUBE1 antagonized these changes after pretreatment. This suggests that SCUBE1 can protect KGN cells from OS damage, and this protection is closely related to the normal operation of mitochondrial functions.

The mitochondrial apoptotic pathway is one of the key pathways by which OS promotes the apoptosis of GCs. It mediates the expression of Bcl-2 and Bax, leading to a decrease in mitochondrial membrane potential and the opening of mitochondrial permeability transition pores, releasing cytochrome C (Cyt-c), and activating the caspase family cascade [29]. These results showed that after H<sub>2</sub>O<sub>2</sub> cell treatment, the apoptosis rate was increased by flow cytometry, while the apoptosis rate decreased after rhSCUBE1 pretreatment. At the same time, apoptosis-related proteins Bax, Bax/Bcl-2 ratio, and Caspase-3 showed similar trends. This suggested that H<sub>2</sub>O<sub>2</sub> activates the ROS-mitochondrial-Caspase-3 pathway to induce KGN cell apoptosis, and rhSCUBE1 can protect KGN cells from OS damage through the mitochondrial pathways. Additionally, OS activates p53 protein, which activates the Bcl-2 family and plays a role in promoting apoptosis and anti-proliferation [30]. In this study, the upregulation of p53 expression after H<sub>2</sub>O<sub>2</sub> treatment was not significant, but pretreatment with rhSCUBE1 significantly downregulated the p53 protein. This showed that the p53 protein is also a regulatory target of rhSCUBE1 used to protect KGN cells.

Finally, analysis of the impact of SCUBE1 (c.1169C >G, p.P390R) mutation from the aspect of mutation pathogenicity; protein stability; and gene haplotype insufficiency, indicated that the p.P390R mutation is significantly pathogenic. In this study, the direct effect of the p.P390R mutation on GCs or the effect on phenotypic changes in animals and mice was not determined, however, this study remains significant as there is limited research on SCUBE1 and GCs, and the mechanisms regulating the proliferation and apoptosis of GCs have not previously been reported. These findings will lay a theoretical and practical foundation for further in-depth research, and at the same time provide new ideas for POI research.

In summary, we speculate that the continuous expression of SCUBE1 is an important guarantee for maintaining normal ovarian function, as SCUBE1 can protect ovarian (KGN) cells from OS damage. The role of p.P390R

mutation of SCUBE1 in causing local spatial conformation changes, weakening protein stability, hindering protein function, and causing high pathogenicity, is indicative of the significant role that SCUBE1 can play as a biomarker for genetic manipulation in POI research studies. However, further rigorous and scientific experiments are required for verification.

## Abbreviations

GCs: Granulosa cells; SCUBE1: Signal peptide, CUB domain, epidermal growth factor-like protein1; H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide; CCK-8: Cell Counting Kit-8; ROS: Reactive oxygen species;  $\Delta\Psi_m$ : Mitochondrial membrane potential; POI: Premature ovarian insufficiency; OS: Oxidative stress; HE: Hematoxylin and eosin; rhSCUBE1: SCUBE1 human recombinant protein; OD: Optical density; IC<sub>50</sub>: Half-maximal inhibitory concentration; Rh123: Rhodamine123; ExAC: Exome Aggregation Consortium; LOF: Loss of function

## Declarations

### Ethics approval and consent to participate

Experimental procedures for human (authorization reference number: 2020-KY-061-02) and mice (authorization reference number: 2019-008) were respectively approved by the ethics committee and animal experiment committee of Southern Medical University.

### Consent for publication

Not applicable.

### Availability of data and materials

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

### Competing interests

The authors declare that they have no conflict of interest.

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### Authors' contributions

HF and XW designed the study. HF and XC conducted laboratory experiments and made significant contributions to data acquisition, collation, analysis and interpretation. QL and WY participated in the maintenance of mice, the acquisition of data and construction of the tables and figures. HF and XW wrote the manuscript. XC, QL, and WY revised the manuscript. All authors read and approved the final version of the manuscript, and HF and XW approved the submission of the latest version.

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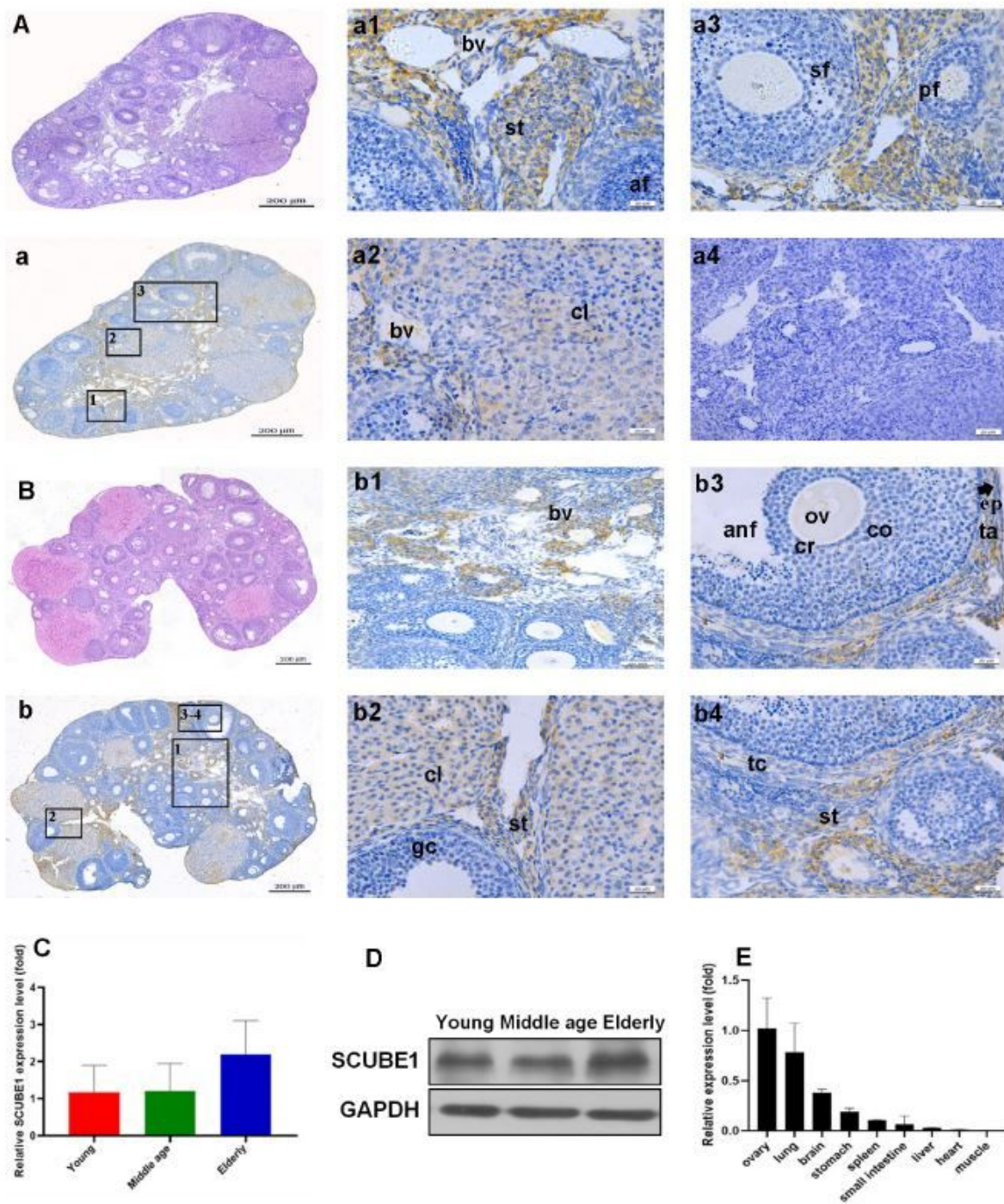
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## Figures



**Figure 1**

Immunohistochemical localization of SCUBE1 in the mouse ovaries at 6 and 12 weeks of ages (a & b). HE staining of ovarian tissue at 6 and 12 weeks (A & B). Negative control (a4). Abbreviations: anf, antral follicle; pf, primary follicle; sf, secondary follicle; af, atresia follicle; cl, corpus luteum; ov, oocyte; gc, granulosa cells; cr, corona radiata; co, cumulus oophorus; tc, theca cells; st, stroma cells; ta, tunica albuginea; ep, epithelium germinal; bv, blood vessel. The scale bar represents 200  $\mu$ m for A, a, B, & b, 50  $\mu$ m for a4 & b1, and 20  $\mu$ m for a1-a3 & b2-b4. Expression of SCUBE1 mRNA of human ovaries was detected by RT-qPCR (n=3 per group) (C). The



expression of SCUBE1 protein detected by western blot in human ovaries (n=3 per group) (D). SCUBE1 mRNA has the highest expression in the ovarian tissue of KM mouse (n=5 per group) (E).

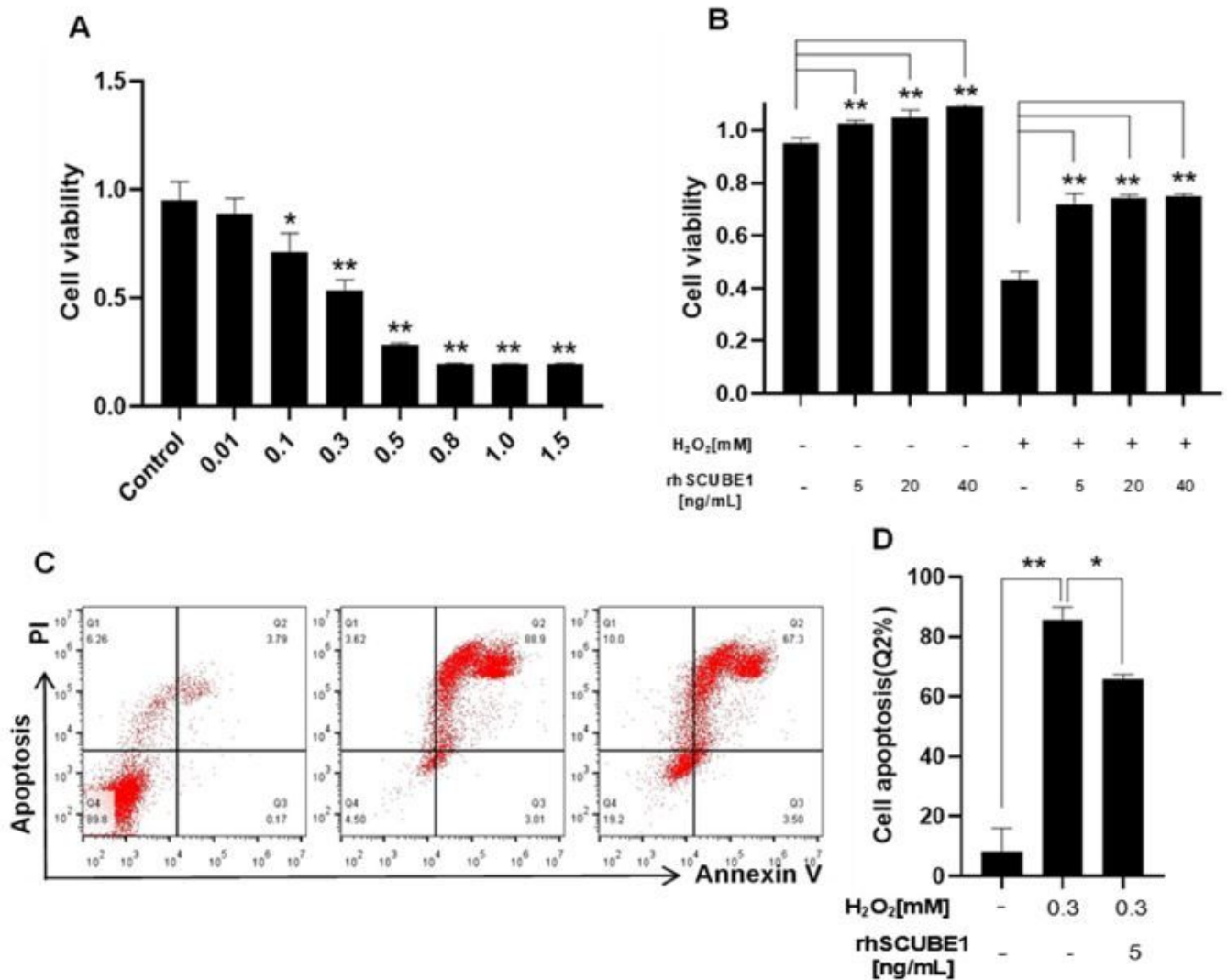
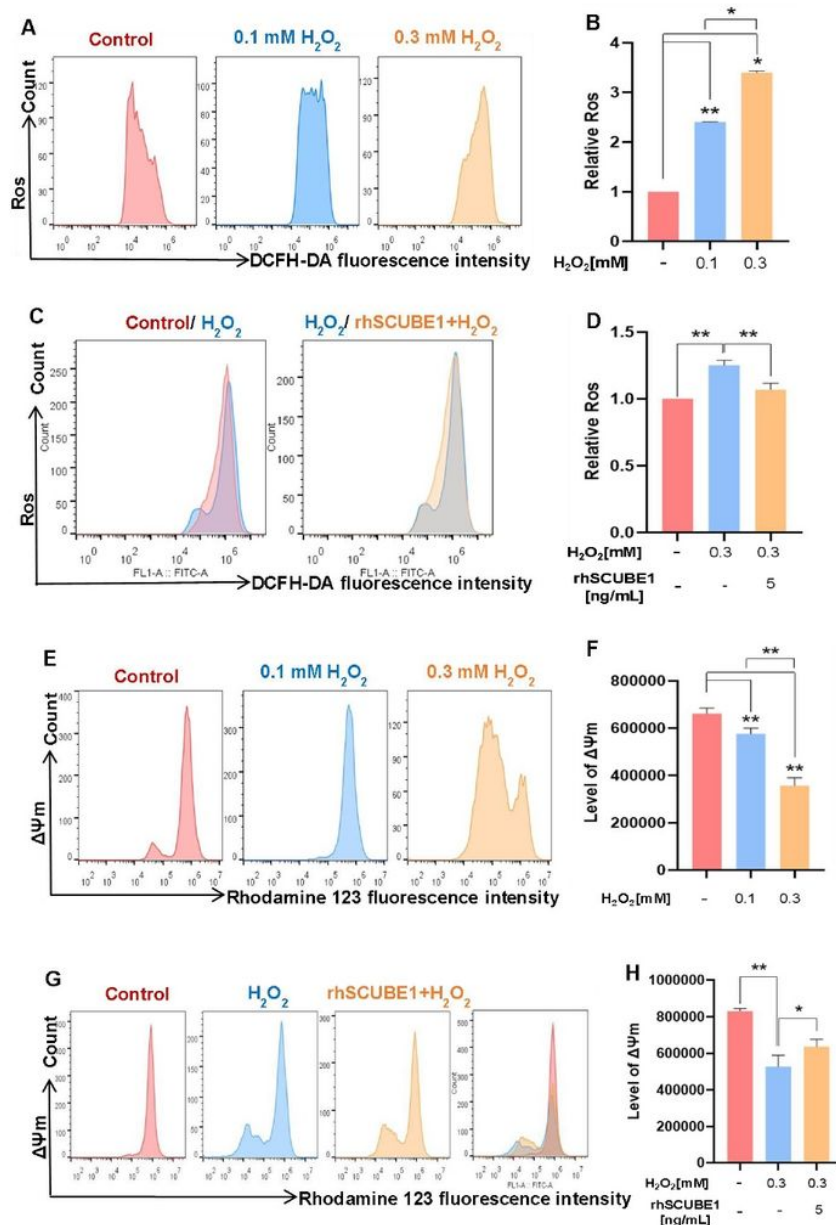


Figure 2

H<sub>2</sub>O<sub>2</sub> inhibited the viabilities of KGN cells and triggered cell death (A). rhSCUBE1 enhances KGN cell viability. KGN cells were exposed to the increasing doses of rhSCUBE1 (5 ng/mL or 20 ng/mL or 40 ng/mL) for 24h with or without H<sub>2</sub>O<sub>2</sub> (0.3 mM) for another 24h (B). Flow cytometry with Annexin V/PI double staining confirmed that 5 ng/mL rhSCUBE1 rescued H<sub>2</sub>O<sub>2</sub>-induced KGN cells death (C & D). Bars represent three independent experiments. The ratio between each treatment and the control is plotted as mean ±SD (\*P<0.05; \*\*P<0.01).



**Figure 3**

H<sub>2</sub>O<sub>2</sub> could promote the intracellular ROS accumulation of KGN cells (A & B). rhSCUBE1 can reduce the accumulation of H<sub>2</sub>O<sub>2</sub>-induced ROS in KGN cells (C & D). H<sub>2</sub>O<sub>2</sub> could reduce the mitochondrial membrane potential of KGN cells (E & F). rhSCUBE1 could prevent the decrease of mitochondrial membrane potential in H<sub>2</sub>O<sub>2</sub>-induced KGN cells (G & H). Bars represent three independent experiments. The ratio between each treatment and the control is plotted as mean ± SD (\*P<0.05; \*\*P<0.01).

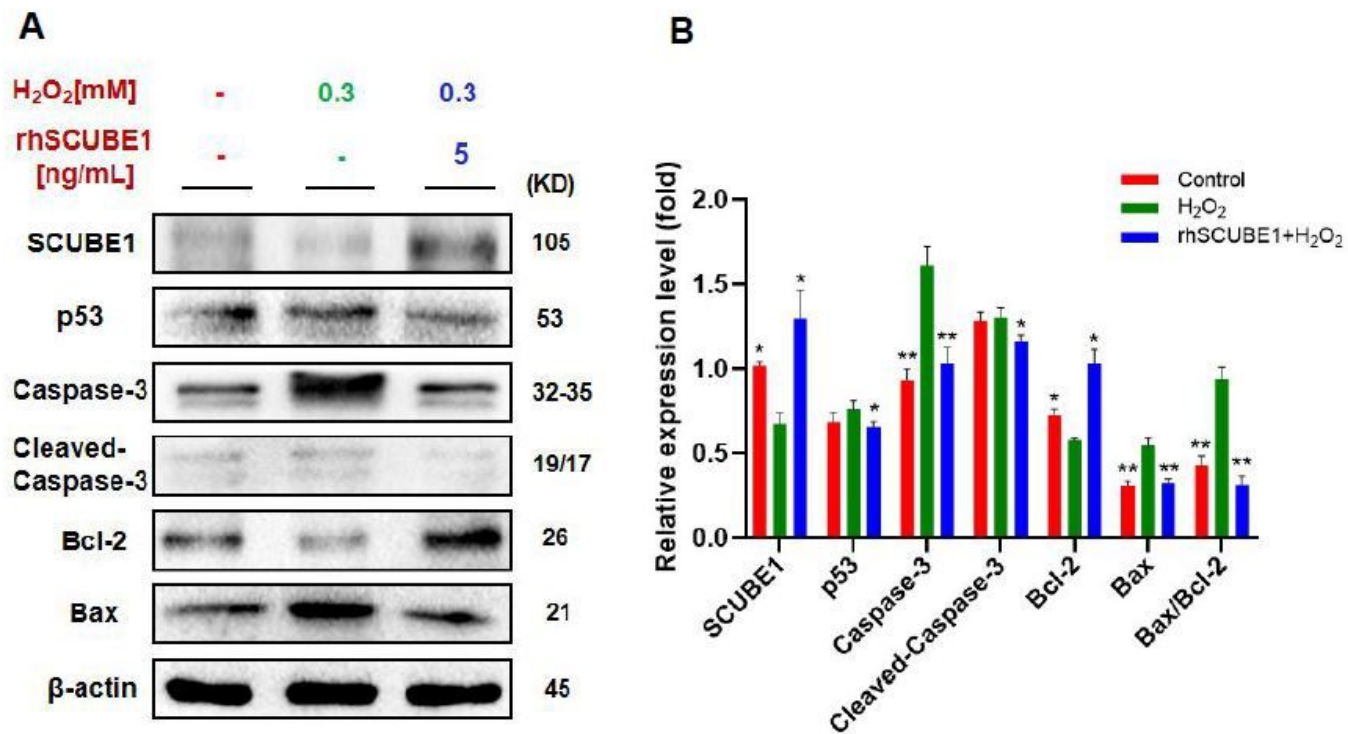


Figure 4

The expression level of SCUBE1, p53, Caspase-3, Bax, Bcl-2 was detected by western blot; β-actin was used as internal control. Bars represent three independent experiments. The ratio between each treatment and the control is plotted as mean ±SD (\*P<0.05; \*\*P<0.01; significantly different from model value).

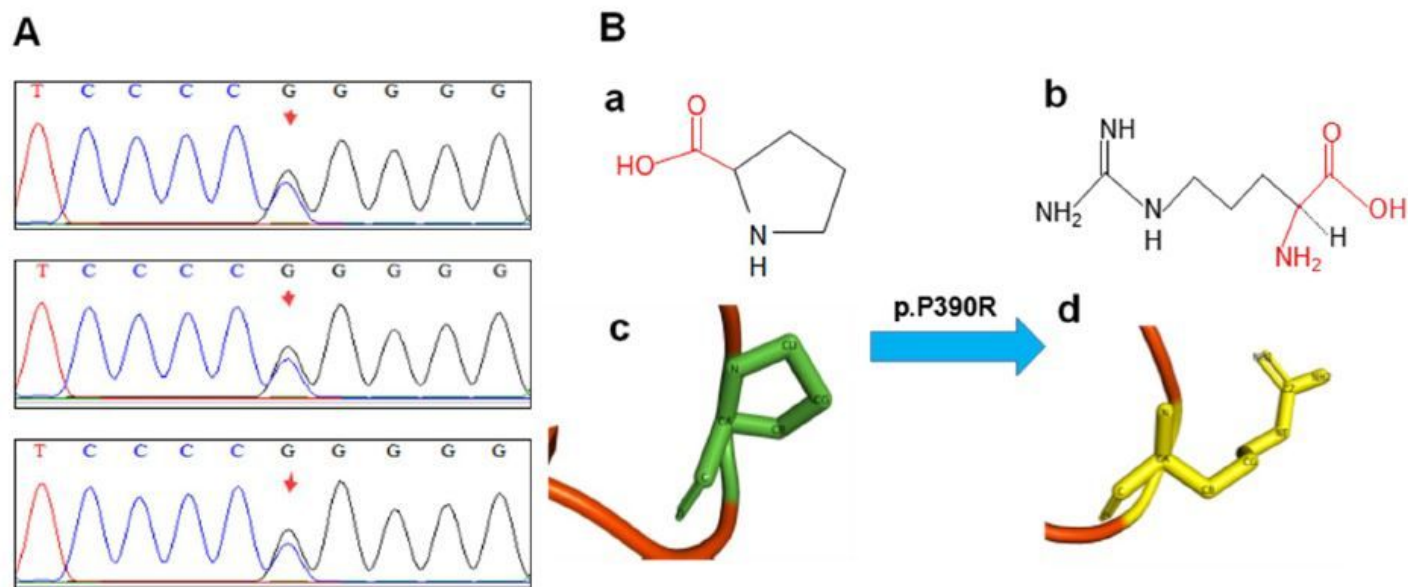


Figure 5

Sanger sequencing confirmed that 3 POI patients had heterozygous missense mutations (c.1169C>G, p.P390R) of SCUBE1 genes, and the red arrow indicated the mutation position (A). The schematic structure of the amino acids before and after the mutation of P390R, the red part is the main chain of the amino acid, and the black part is the unique side chain of each amino acid (B, a & b). The local spatial structure of proline (green part) and arginine (yellow part) displayed by PyMol software (B, c & d).