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Proanthocyanidins delaying the premature ovarian insufficiency through regulatory sirt1-p53-p21 signaling pathway in female germline stem cells

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Abstract

Background As women age, their ovarian follicle pool naturally declines. However, female germline stem cells (FGSCs) possess a unique ability to differentiate into oocytes and continuously self-renew, providing an effective means of delaying ovarian aging by replenishing the primordial follicle pool. Therefore, activating FGSCs is critical in reshaping and safeguarding ovarian function.

Methods In this study, we investigated the biological activity of proanthocyanidins (PACs), natural antioxidants that exhibit anti-aging and anti-inflammatory properties beneficial for both male and female reproduction. Our in vivo and in vitro experiments demonstrate that PACs promote FGSCs proliferation while delaying ovarian aging.

Results PACs increase the number of primordial follicles, primary follicles, corpus luteum while reducing cystic follicles, and elevate estradiol (E_2) levels along with anti-mullerian hormone (AMH) concentration levels in mice. Additionally, PACs significantly boost FGSCs proliferation time- and dose-dependently by upregulating mRNA & protein expressions for FGSCs-specific markers such as MVH and OCT4 while downregulating p53/p21 via activation of silent information regulator 1(Sirt1) signaling pathway. The effects of PACs on FGCSs were found to be impeded by the Sirt1 inhibitor EX527.

Conclusion PACS delay premature ovarian insufficiency (POI) through regulating the Sirt1-p53-p21 signaling pathway involving FGSCs.

Keywords Proanthocyanidins, Female germline stem cells, Silent information regulator 1, Premature ovarian insufficiency, Mice

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Introduction

The incidence of POI has been on the rise due to environmental degradation, increased life stressors, and unhealthy lifestyle habits. POI not only results in decreased fertility, but also hormonal imbalances that may lead to various diseases such as osteoporosis, ovarian cancer, and arteriosclerosis [1–3]. Therefore, finding safe and effective means to reduce the incidence of POI is an urgent concern in the field of reproductive medicine. Since Johnson J's group groundbreaking discovery that female mammals can generate new oocytes from birth until adulthood to replenish their follicle pool has challenged the long-held notion of a fixed follicle pool monopolizing the reproductive medical community for over a century [4]. Multiple research teams have confirmed that postnatal ovaries contain FGSCs through methods such as stem cell culture and transplantation, transgenesis, lineage tracing in various mammals including humans (including menopausal women), sheep, pigs and mice [5–8]. These studies have observed that FGSCs are capable of self-renewal, clonal expansion with directed differentiation into oocytes; continuous renewal of the follicle pool; restoration of fertility in infertile animal models after transplantation. While some scholars failed to replicate FGSCs experiments possibly due to experimental conditions or technical limitations like Wagner's single-cell sequencing study which did not find any FGSCs in human ovaries but attributed it to too few cells being examined [9]. Zheng Ping's team provided first-in-vivo evidence supporting physiological activity for FGSCs existence within mammalian ovary by utilizing intravital cell tracing techniques [10]. More recently Professor Wu Ji's group successfully established 3D organoid models derived from mouse FGSCs showing they could develop into mature oocyte vivo which upon fertilization produced normal offspring [11]. These findings suggest that FGSCs play a crucial role in determining ovarian reserve function and lifespan, and are important tools for reshaping and preserving ovarian function, making them a key factor behind ovarian aging and dysfunction. According to Sharma D et al. [12–14] identified two types of stem cells in ovary surface epithelial (OSE) of mammals: very small embryonic-like stem cells (VSELs) and ovarian germline stem cells (OGSCs). These cells can undergo long-term in vitro expansion and ultimately differentiate into an oocyte-like structure, resulting in the production of fertile pups. OGSCs are strongly correlated with the FGSCs that we are currently studying. Sharma D found OGSCs do not disappear due to chemotherapy or aging, and data showed that a certain number of VSELs survived in ovarian tissue after chemoablation and retained the ability to differentiate into oocyte-like structures [15]. In future experimental manipulations, usable cells from OSE will also be isolated for further studies on stem cells [16]. As such finding ways to activate these cells represents an important strategy for protecting the ovary against age-related decline or damage caused by external factors!

PACs are natural antioxidants widely recognized for their anti-aging and anti-inflammatory properties, and are commonly used in various fields such as food, medicine, environmental protection, cosmetics, and agriculture. PACs are water-soluble phenolic compounds that typically exist in fruits, vegetables, nuts and plants. Their antioxidant capacity is 40 times greater than that of vitamin C or E [17, 18]. Due to their natural antioxidant properties, PACs have been suggested as a possible protector against oxidative stress damage to various organs and tissues, including the ovaries [19–21]. Research has shown that PACs can effectively mitigate pathological changes in granulosa cells by inhibiting autophagy and apoptosis, which ultimately contributes towards delaying ovarian aging [22–24]. However, studies investigating the effects of PACs on FGSCs remains unreported.

Sirt1 is a highly conserved NAD+-dependent protein deacetylase found in mammals, is closely related to the silent information regulator 2(Sir2) family of enzymes involved in regulating reproductive system function [25]. This enzyme has been implicated in several female reproductive disorders such as endometriosis, polycystic ovary syndrome, and age-related infertility [26–28]. Sirt1 has demonstrated the ability to improve primordial follicle quality, oocyte and granulosa cell function while enhancing ovarian function through various mechanisms including inhibiting mitochondrial dysfunction, reactive oxygen species (ROS) accumulation and spindle defects; suppressing apoptosis; regulating epigenetic changes among others [29–32]. While Sirt1 regulates the proliferation, survival, and self-renewal of hematopoietic stem cells [33], it remains unclear whether it can regulate or how it regulates ovarian stem cell function. Studies have shown that Sirt1 can regulate the p53/p21 signaling pathway in aging mice subjected to oxidative stress injury and apoptosis [34, 35]. Additionally, melatonin, resveratrol are examples of antioxidants known for their ability to enhance ovarian function by upregulating Sirt1 expression which helps inhibit mitochondrial damage while promoting antioxidant and anti-inflammatory systems [27, 36]. This study aims at investigating whether PACs-with potent antioxidant effects-could improve FGSCs functionality while delaying ovarian aging by regulating the Sirt1-p53-p21 pathway.

Materials and methods

Mice and treatment

Female KM mice, aged 6 weeks and weighing between 20 and 25 g, were procured from the Department of Zoology at Jiangxi University of Traditional Chinese

Medicine. After a seven-day acclimation period, the mice were housed on a 12-hour light/dark cycle at (22 ± 1) °C with free access to both food and water. The mice were then randomly divided into following groups, namely the Control group, POI model group, and PACs-treated groups (n = 20 each).

To establish the POI model, all the mice, except those in the control group, were intraperitoneal injections cyclophosphamide/busulfan (CY/BUS) for 21 days. The PACs-treated groups received intragastric administration of PACs in various doses (100 mg/kg.d, 200 mg/kg.d, and 400 mg/kg.d, respectively) for 28 days, while the control group was administered the same volume of normal saline. 3–5 days-old suckling female mice were utilized for the primary FSGCs cell extraction experiments. All animal experiments adhered to the guidelines set forth by the Animal Center of Nanchang University and were approved by the Animal Care and Use Committee of Nanchang University (license number: SYXK2021-0004).

Ovarian index

After sacrificing the mice, the ovaries were aseptically removed and cleaned using a phosphate-buffered saline (PBS) solution. Following cleaning, the ovaries were weighed after removing any surface fluid with absorbent paper. The ovary index was then calculated using the following formula:

Ovary index (‰) = ovary weight / body weight * 1000.

HE staining

HE staining was conducted following standard protocols. Briefly, tissue blocks were fixed, dehydrated, embedded in paraffin, and cut into 4–5 μm slices, which were placed on glass slides. All sections were then de-waxed using xylene, subjected to HE staining, and subsequently observed under a light field microscope.

Measurement of E_2 and AMH levels

Eyeball blood from mice was collected and allowed to stand at room temperature for 20 min. The sample was then centrifuged at 12,000 g for 10 min. The supernatant was collected after tilting the tube wall, and the serum E_2 and AMH contents were measured using Electrochemiluminescence Immunoassay (ECLIA).

Culture of FGSCs in vitro

The mouse FGSCs were isolated in vitro, following our previous study [37–39]. The FGSCs culture medium was passed through a 70 μm nylon filter. The resulting cell suspension was added to a 48-well culture plate that had been coated with 0.5% (w/w) gelatin and then incubated under 5% CO2 at 37 ℃. After 4 h of culture, the supernatant was collected followed by careful removal of any adherent cells. The collected supernatant was then transferred to another 48-well culture plate that had also been incubated with 0.5% (w/w) gelatin. The culture medium was replaced every 12 h, and subculturing was performed as per the state of the cells.

Alkaline phosphatase staining

The cells were fixed with 4% paraformaldehyde (PFA), washed with PBS, and subjected to alkaline phosphatase incubation solution for 15–20 min in the dark. The cells were then washed thrice with PBS, and images were captured and analyzed using a microscope.

Immunofluorescence staining

Ovaries and cultured cells were fixed with 4% PFA, washed with PBS, and incubated for 10 min in 0.5% Triton X-100 (prepared with PBS) at 4℃. Subsequently, the ovaries and cells were sealed with 5% BSA (prepared with TBST) for 30 min at room temperature. The first antibody was then prepared according to the optimal proportion and incubated overnight in a wet box at 4℃. After discarding the PBS solution, the second antibody was added and incubated for 1 h at room temperature. Then, the liquid was removed and cleaned with PBS while the entire process was protected from light. Subsequently, the cells were stained with 4',6-diamidino-2-phenylindole for 10 min at room temperature and then cleaned with PBS. Finally, the fluorescent anti-quenching agent was added, and the cells were photographed using a fluorescence microscope and analyzed.

CCK-8 assay

FGSCs were seeded into 96-well plates and incubated with different concentrations of 5, 10, 20 mg/L PACs and EX527 (2.487 mg/L) for 24, 48 and 72 h. Following treatments, 10 μL of CCK8 solution (Geno Meditech, Shanghai, China) was added to each well, and the cells were cultured at 37 °C for 1 h. Absorption values were measured at a wavelength of 450 nm using a Bio-Tek microplate reader Instruments, Thermo Fisher Scientific, Winooski, VT, USA).

Western blotting

The cells were harvested and rinsed twice with PBS for protein extraction, and proteins were extracted in 1× SDS–PAGE sample loading buffer. Total proteins were resolved by SDS–PAGE and transferred to a 0.22 μm polyvinylidene fluoride membrane using a constant-voltage 40-V ice bath for 1.5 h. After blocking, the membranes were incubated overnight at 4 °C with the appropriate primary antibody, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive proteins were detected with the Thermo Scientific Pierce enhanced chemiluminescence Western blot substrate

Table 1 Primers used for quantitative-PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
GAPDH	CAGGTGGTCTCCTCGACTT	CCAAATTCGTTGTCATACCA
MVH	GTGTATTATTGTAGCACCAACTCG	CACCCTTGTACTATCTGTCGAACT
Fragilis	CTGGTCCCTGTTCAATACACTCTT	CAGTCACATCACCCACCATCTT
OCT-4	AGCTGCTGAAGCAGAAGAGG	GGTTCTCATTGTTGTCGGCT
Stella	CCCAATGAAGGACCCTGAAAC	AATGGCTCACTGTCCCGTTCA
Dazl	GTTAGGATGGATGAAACCGAAAT	ATGCCTGAACATACTGAGTGATA
c-Kit	CGCCTGCCGAAATGTATG	TCAGCGTCCCAGCAAGTC
BMP15	GAGCATGATGGGCCTGAAAG	TAAGGGACACAGGAAGGCTG
GDF9	TTATTTAAGGACCACGCCAGGG	CTCCTCGTGCCAGTCTTCTT
Figla	CCAAAGAGCGTGAACGGATAA	TCTTCCAGAACACAGCCGAGT

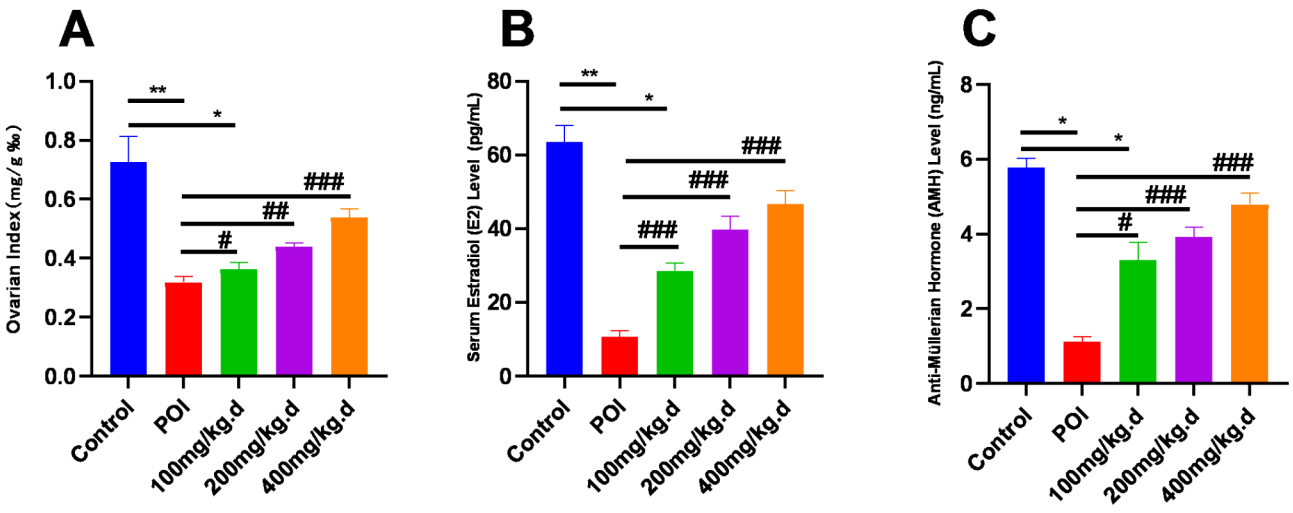

Fig. 1 (**A**) Effect of PACs on mouse ovarian index; (**B**) Effect of PACs on E_2 levels in the peripheral blood of mice; (**C**) Effect of PACs on AMH levels in the peripheral blood of mice; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared with the POI group;

(Thermo Scientific). The results were analyzed using the Tanon-410 automatic gel imaging system (Tanon Corporation, China).

Quantitative real-time PCR

RNA isolation was performed on freshly isolated cells using miRNA easy Micro kit following the manufacturer's protocol. RNA quantification was performed using QIAxpert. cDNA was obtained using Superscripts VILO cDNA Synthesis kit, and quantitative reverse transcriptase PCR (qRT-PCR) was performed using SYBR Green PCR Master Mix. Items used for the RT-PCR are listed in Table 1(Table 1. Primers used for quantitative-PCR).

Statistical analysis

Each group of experiments was repeated at least three times. The data were analyzed using GraphPad Prism5.0 software and statistical analyses were performed using analysis of variance and t-tests (with p values <0.05, <0.01, <0.001, respectively).

Results

PACs can improve the ovarian endocrine and reproductive functions in mice with POI

Intragastric administration of PACs for 28 days in mice resulted in a significant increase in ovarian index (Fig. 1A). Moreover, levels of E_2 and AMH in the POI-treated group were significantly reduced compared to those in the control group. However, in mice treated with 100, 200, and 400 mg/kg.d PACs, there was a dose-dependent increase in these hormone levels, though none returned to the control group levels. Notably, the 400 mg/kg.d PACs-treated group demonstrated the most efficacious recovery of E_2 and AMH levels (Fig. 1B, C). Ovarian morphology was evaluated by HE staining in Control, POI model and PACs-treated groups (Fig. 2A). The ovarian structure was significantly disrupted in the POI group, but its recovery varied following treatment with different PACs concentrations in POI mice (Fig. 2B-F). Although the number of atretic follicles was not significantly different between PACs-treated and POI groups, we found that the number of primordial follicles, antral follicles, and corpus luteum was significantly higher in the 400 mg/kg.d PACs-treated groups

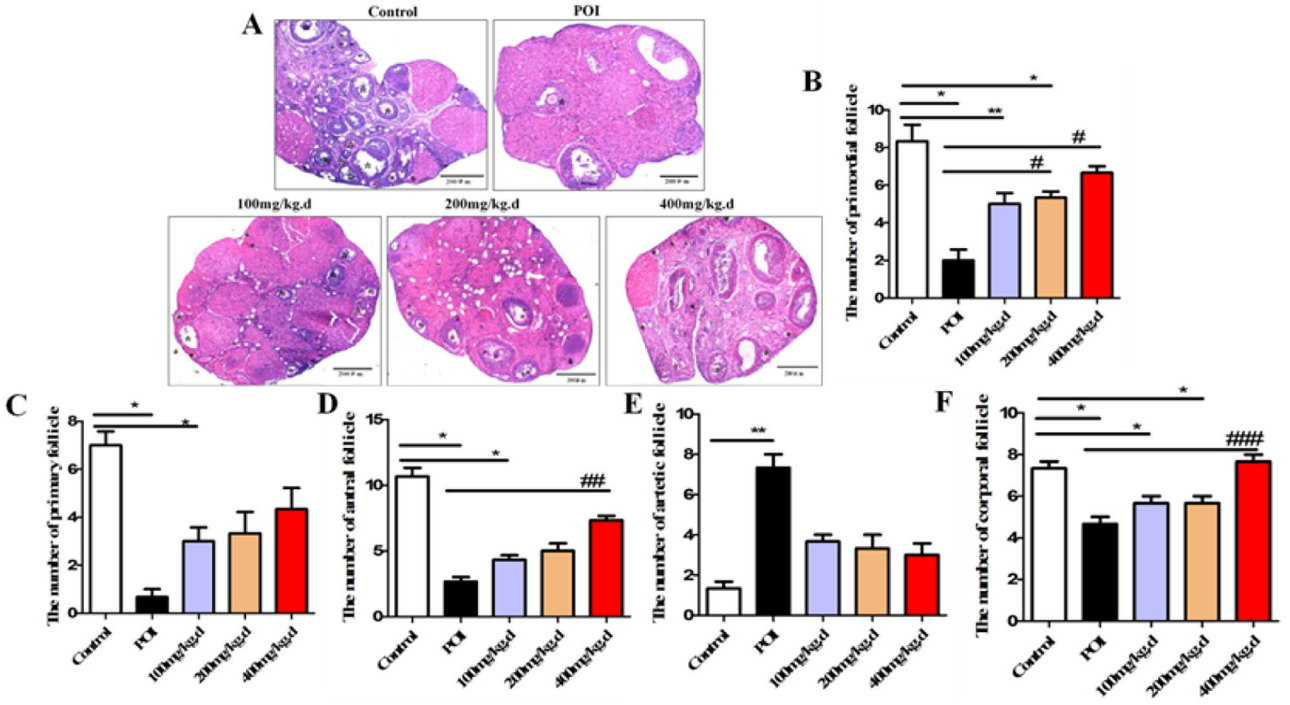

Fig. 2 Ovarian histology of PACs-treated mouse ovaries (**A**) Ovarian HE staining of PACs-treated ovaries with various PACs concentrations. Control group; POI group; 100 mg/kg.d PACs-treated mouse ovaries; 200 mg/kg.d PACs-treated mouse ovaries; 400 mg/kg.d PACs-treated mouse ovaries (**B-F**) The number of ovarian follicles and corpus luteum in different groups. Bar 200 μm (**B**) Number of primordial follicles; (**C**) Number of primary follicles; (**D**) Number of antral follicles; (**E**) Number of atretic follicles; (**F**) Number of corpus follicles; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, comparison with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, comparison with the POI group;

compared with the POI group ($p < 0.05$). However, the number of primary follicles was significantly higher in the 100 mg/kg.d PACs-treated group compared with the control group ($p < 0.05$).

PACs can increase the number of FGSCs in ovarian surface epithelium of mice with POI

Intragastric administration of 400 mg/kg.d PACs via intragastric route for 28 days significantly upregulated MVH and OCT4 protein expression in the ovarian surface epithelium of mice with POI, as determined by protein relative content in ovaries (Fig. 3A, B). Dual immunofluorescence assays further confirmed that PACs induce a significant increase in MVH and OCT4 protein expression in the ovarian surface epithelium of POI (Fig. 3C). These results suggest that PACs may serve as a promising therapeutic agent for augmenting the number of FGSCs in the ovarian surface epithelium of mice with POI.

FGSCs identification

The FGSCs that were isolated had a round shape with a diameter of approximately 10–20 μm, and their cell proliferation cycle was around 1–2 days. After being cultured for the 4th to 5th generations, they exhibited significant proliferation and were either beaded or colony-shaped at 24 h; after logarithmic growth at 48 h, cells could be observed in bead-like structures at 32 or more consistent with those reported in literature (Fig. 4A). RT-qPCR results demonstrated that FGSCs expressed five genes specific for stem cells and reproductive cells: MVH, OCT4, Fraglis, Dazl and Stella. but no GDF9, BMP15, C-kit nor Figla. However, these genes are found in ovary. (Fig. 4B). Dual immunofluorescence assays revealed that both the reproductive marker MVH and the stem cell-specific protein OCT4 were present in FGSCs (Fig. 4C), while alkaline phosphatase testing also returned positive results (Fig. 4D). These findings suggest that the extracted cells have been preliminarily identified as FGSCs.

PACs promote FGSCs proliferation in a time- and dose-dependent manner

The results of the CCK-8 analysis indicated that PACs, at concentrations of 5, 10 and 20 mg/L, significantly promoted the proliferation of FGSCs in a time- and dose-dependent manner. At 48 h post-treatment, there were significant differences observed in optical density (OD) values between the groups treated with 5 mg/L or 20 mg/L compared to the control group (Fig. 5A). Furthermore, RT-PCR analyses revealed that FGSCs treated with PACs at a concentration of 10 mg/L exhibited a significant increase in MVH and OCT4 mRNA expression

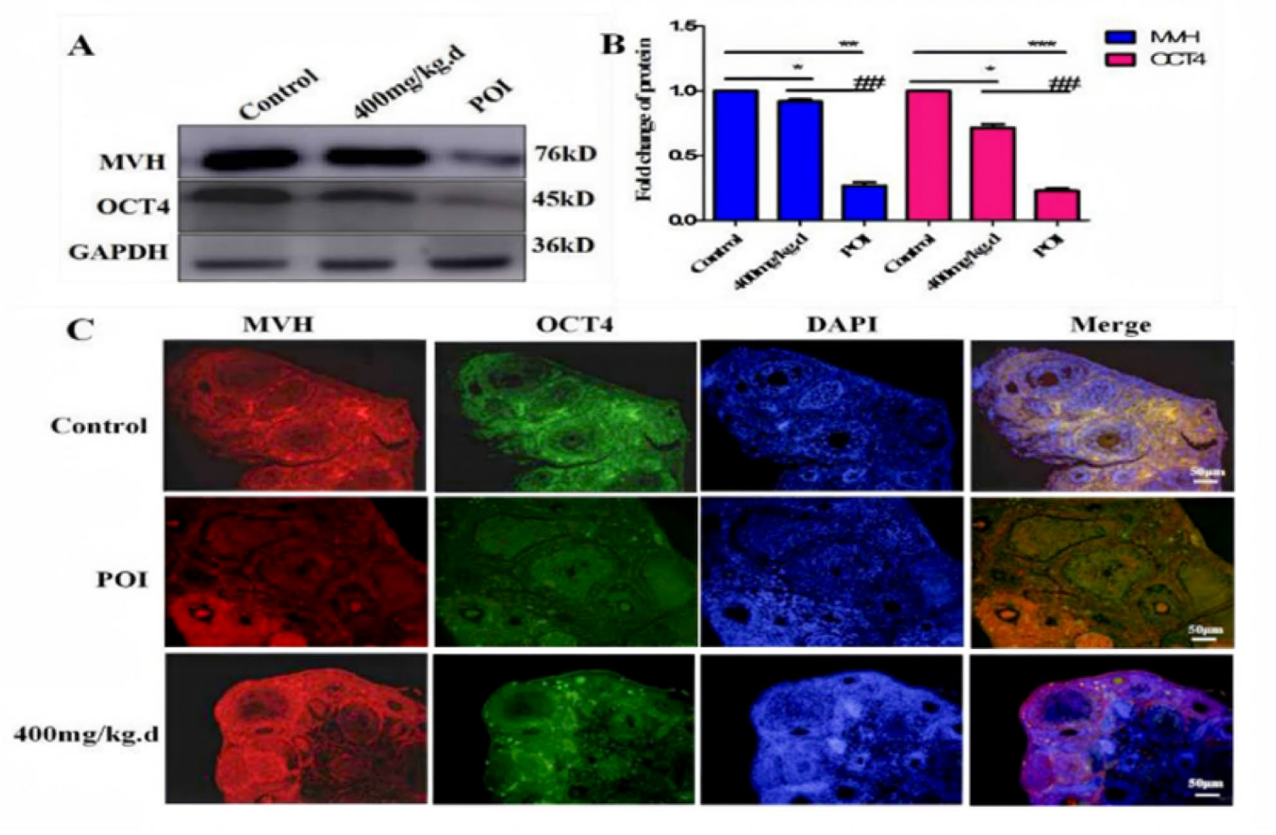

Fig. 3 (**A**) Effects of PACs on MVH and OCT4 protein; (**B**) Expression of MVH and OCT4 protein and their relative content in ovaries; (**C**) Dual immunofluorescence of MVH and OCT4 in ovarian tissue; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared with the 400 mg/kg.d group

levels (Fig. 5B). Western blotting also demonstrated an elevation of MVH and OCT4 protein expression levels following treatment with PACs at a concentration of 10 mg/L (Fig. 5C, D). These findings suggest that PACs can promote the proliferation of FGSCs through both transcriptional and translational regulation mechanisms.

PACs promote FGSCs proliferation through the Sirt1-p53-p21 signaling pathway

The mRNA levels of Sirt1 were upregulated in the PACs-treated group compared to the control group, while the mRNA levels of p53 and p21 were downregulated (Fig. 6A). These findings were further supported by protein expression analysis that was consistent with the mRNA results (Fig. 6B, C). CCK-8 assay results demonstrated that EX527 (a Sirt1 inhibitor) significantly inhibited the proliferative effect of PACs on FGSCs (Fig. 6D) accompanied by a decrease in both Sirt1 and MVH/OCT4 mRNA levels and an increase in both p53 and p21mRNA levels (Fig. 6G-I). These findings further confirm that PACs promote FGSCs proliferation through activation of the Sirt1-p53-p21 signaling pathway.

Discussion

Ovarian aging is a multifaceted process that involves various factors, including natural aging and pathological premature senescence. This process is characterized by a gradual loss of follicles and diminished quality of remaining follicles, which ultimately leads to infertility. These factors can include genetic factors, immune diseases, inflammation, and treatments such as radiotherapy and chemotherapy for cancer. As the global cancer rate increases yearly, POI caused by chemotherapy has become one of the major causes of infertility in young women [40]. Currently, the primary approach to solve fertility problems in cancer patients is to cryopreserve their normal eggs and ovarian tissues, but these methods cannot reverse the ovarian damage caused by chemotherapy. Hence, the objective of this study was to investigate whether PACs could serve as a potential therapy for ovarian aging. To achieve this goal, we first established a pathological ovarian aging model using CY/BUS intraperitoneal injection, which is widely recognized as the most commonly used animal model for POI.

The ovary's structure can be categorized into epithelium, cortex, and medulla, with resting follicles

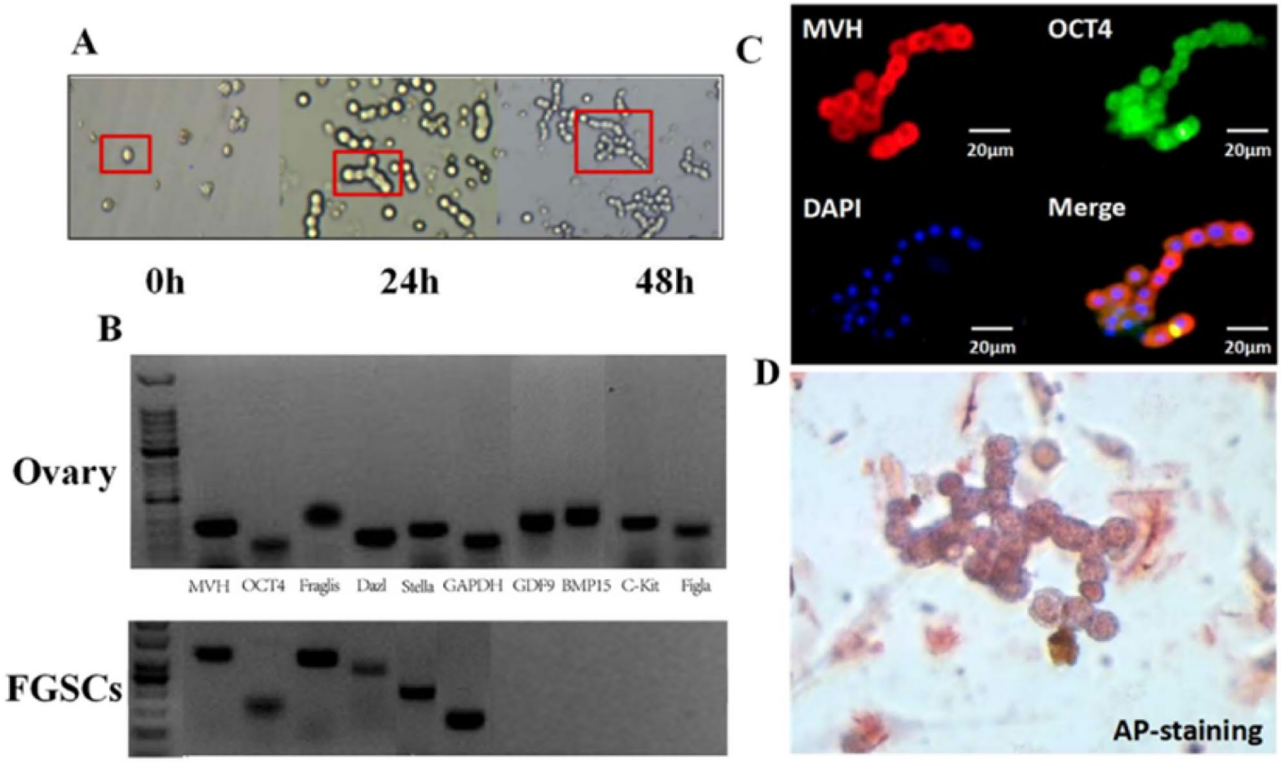

Fig. 4 Identification of FGSCs (**A**) Observation under a light microscope at different times of culture; (**B**) RT-PCR identification of FGSCs; Amplicon sizes: MVH (189 bp), OCT4 (235 bp), Fragilis (172 bp), Dazl (210 bp), Stella (156 bp), GAPDH (254 bp), GDF9 (278 bp), BMP15 (242 bp), C-kit (305 bp), and Figla (195 bp) (**C**) Dual immunofluorescence assay of MVH and OCT4; (**D**) Alkaline phosphatase staining; (MVH, a specific marker of reproduction, and OCT4, a specific marker of stem cells.)

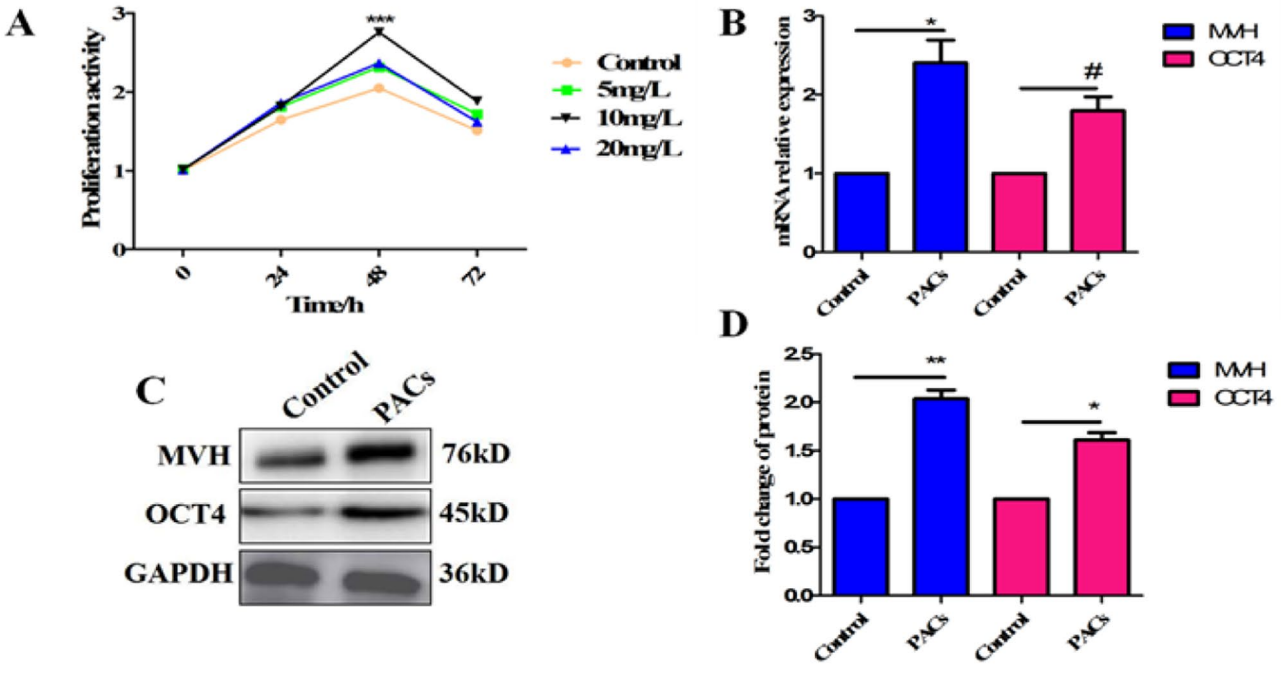

Fig. 5 Effects of PACs on the proliferation of FGSCs (**A**) Effects of different concentrations of PACs on the proliferation of FGSCs, **P*<0.05, 48 h comparison with the control group; (**B**) Effect of PACs on the expression of mRNA related to FGSCs; (**C**, **D**) Effect of PACs on the expression of protein related to FGSCs, **P* <0.05, MVH comparison with the control group; #*P*<0.05, OCT4 comparison with the control group

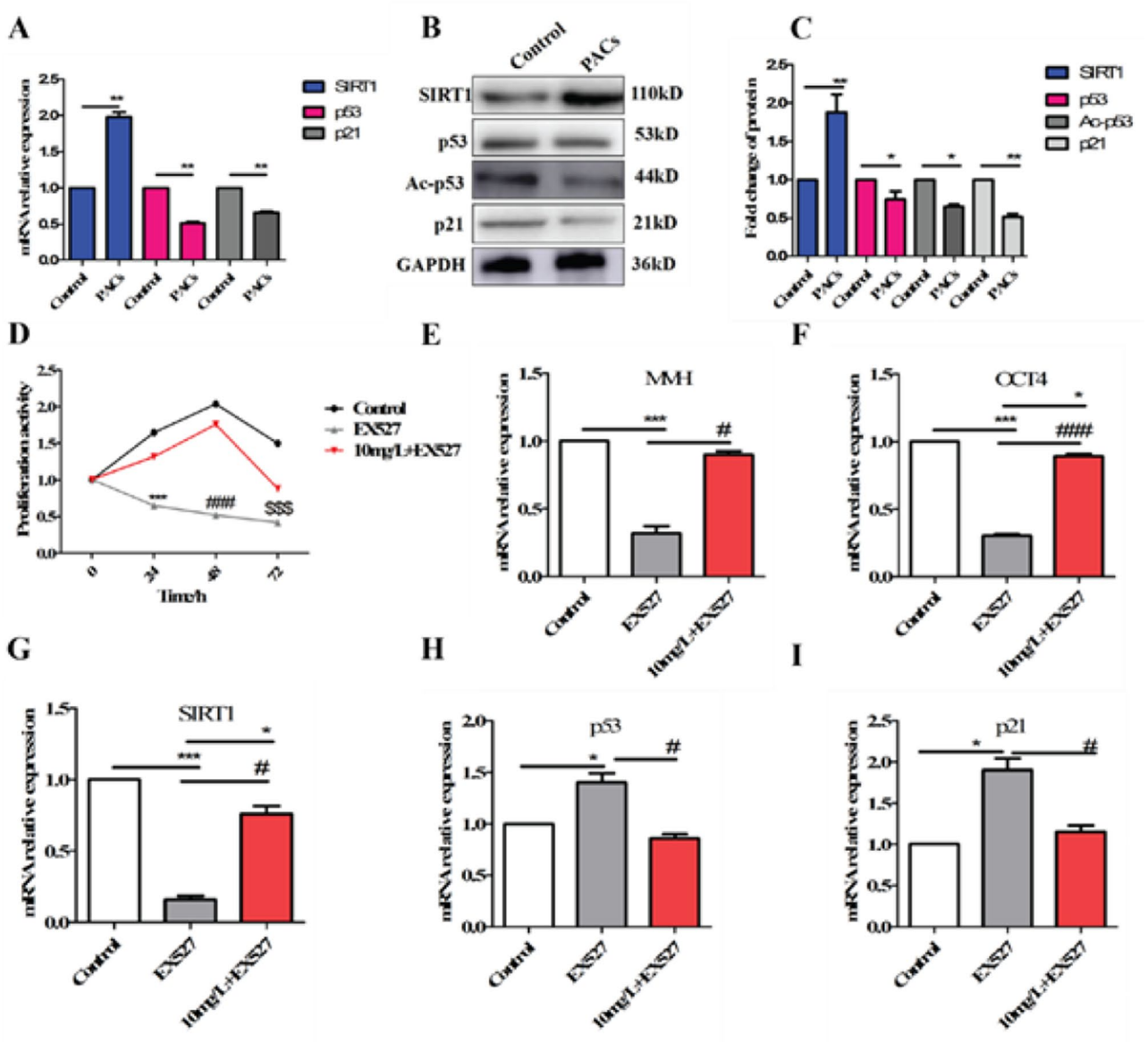

Fig. 6 Effects of PACs on Sirt1-p53-p21 mRNA and protein (**A**) Effect of PACs on the expression of Sirt1-p53-p21 mRNA; $^{**}P<0.01$, compared with the control group; (**B**, **C**) Effect of PACs on the expression of Sirt1-p53-p21 protein and their relative content; $^{*}P<0.05$, $^{**}P<0.01$, compared with the control group; (**D**) EX527, a Sirt1 inhibitor, had a negative effect on FGSCs proliferation, *** $P<0.001$, 24 h comparison with the control group; $^{\#\#\#}P<0.001$, 48 h comparison with the control group; $^{\$\$\$}P<0.001$, 72 h comparison with the control group; (**E-H**) Effect of PACs on the expression of MVH/OCT4 and Sirt1-p53-p21 mRNA; $^{**}P<0.01$, compared with the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared with the EX527 group

and FGSCs in the cortex. Each follicle contains a single oocyte supported by granulosa cells and follicular cells. Poor-quality oocytes after fertilization lead to aneuploidy, embryonic development stagnation, and abortion, so the number and quality of follicles can evaluate ovarian function. The histological examination revealed a notable reduction in the growth of ovarian follicles in the POI group compared to the control group. Additionally, there was an increase in atretic follicles and evident abnormalities in ovarian structure observed. The results of the ovarian index showed that degenerative atrophy occurred in the POI group. A significant reduction in ovarian follicle quantity and quality leads to lower estrogen levels, potentially causing health problems such as bone loss and hot flashes. Chemotherapy damages follicular cells (granulosa and theca cells), causing a continued decline in female steroid secretion and a disorder of female endocrine balance. AMH, secreted by antral and preantral follicular granulosa cells, reflects ovarian function and reserve [41]. Its level is less affected by pregnancy, oral contraceptives, and female physiological cycles, and its decline may indicate a decrease in ovarian

follicular cistern reserve. Consequently, serum levels of E_2 and AMH in mice were measured to assess ovarian function. The results showed that serum AMH and E_2 levels in the POI group were significantly lower than those in the control group. These findings confirm the successful construction of a pathological ovarian aging model.

The discovery and study of FGSCs have provided a ray of hope for the complex issue of POI and female infertility. Our experimental results revealed a significant downregulation of ovarian stem cell markers in the chemotherapy-induced POI mouse model, suggesting that stem cell depletion may be a key contributing factor to ovarian aging. Recent years have witnessed significant progress in the matured techniques employed in the extraction and in vitro cultivation of FGSCs, which can be attributed to the extensive research on their biological properties [42]. In our laboratory, we have managed to steer clear of long-term exposure of the cells to non-cultured environments, ensuring both purity and survival rate of the cells by eliminating the magnetic beads and exploiting the biological characteristics of FGSCs for artificial screening. Impressively, within 12 h of observation, we have observed the occurrence of mitosis, and the membrane structure and formation of FGSCs are found to resemble that of primordial germ cells. Furthermore, our experimental results demonstrate that the stem cells we extracted possess the distinguishing characteristics of reproductive and stem cell genes while not expressing the meiotic cell markers BMP15, GDF9, Figla, and differentiation marker gene C-kit. Additionally, immunofluorescence staining reveals that both the reproductive feature protein MVH and the stem cell characteristic protein OCT4 are positively expressed, along with a high expression of alkaline phosphatase, which strongly suggests the stem cell characteristics of our cells.

FGSCs possess the remarkable ability to self-renew and differentiate into oocytes. Upon migration to the ovarian region, FGSCs exhibit homing ability and differentiate into early-stage oocytes. In vitro differentiation studies have successfully produced high-quality reproductive cyst oocytes from FGSCs under the regulation of granulosa cells, estrogen, progesterone, and retinoic acid treatment, offering novel avenues for IVF technology. Researchers suggest that the loss of FGSCs is responsible for ovarian aging and that promoting their proliferation could delay the onset of ovarian aging. It is worth noting, however, that little is known about the mechanisms of FGSCs aging and its relationship with physiological and pathological ovarian aging. Therefore, understanding the mechanisms of FGSCs aging and identifying proliferation strategies is critical for preventing and treating ovarian aging.

Sirt1, a highly conserved NAD+-dependent deacetylase, regulates the activity of various proteins, including p53, NF-κB, and FOXO1 by deacetylating them, and plays a multifaceted role in inflammation, oxidative stress, aging, cell proliferation, apoptosis, and other biological processes [43, 44]. Specifically, Sirt1 mediated deacetylation of lysine 382 on p53 reduces the transactivation of p21 and the cell cycle arrest induced by DNA damage, thereby inhibiting apoptosis [45, 46]. Examples of known antioxidants include melatonin and resveratrol, both of which have been shown to enhance ovarian function by upregulating Sirt1 expression. This phenomenon helps to suppress mitochondrial damage while also promoting antioxidation and anti-inflammatory systems [27, 36]. Our in vitro observations of the antioxidant PACs on FGSCs indicate that PACs significantly induce the mRNA and protein expression of Sirt1, in a dose- and time-dependent manner, which promotes FGSCs proliferation while reducing p53 and p21. The effects of PACs on FGSCs were inhibited by the Sirt1 inhibitor EX527. Therefore, we suggest that PACs regulate FGSCs proliferation through the Sirt1-p53-p21 signaling pathway.

PACs are important flavonoid compounds found in the human diet, typically extracted from grape seeds and skins, and have been shown to possess antibiotic, antioxidant, and anticancer properties [47]. The ovarian indices of the PACs-treated group increased in a dose-dependent manner and were statistically significant compared to the model group. The levels of AMH and E_2 in the PACs-treated group were also dose-dependently higher than those in the POI group. These findings suggest that PACs can improve ovarian function in chemotherapy-induced premature aging mice. We observed a significant increase in MVH and OCT4 protein expression after 28 days of intragastric administration with 400 mg/kg.d PACs, which was confirmed by Western blot. Dual immunofluorescence detection also showed that PACs increased the protein expression of MVH and OCT4 in the POI ovarian epithelium, indicating that PACs can increase the number of FGSCs in the POI ovarian epithelium. In recent years, our team and other researchers have suggested that uncontrolled chronic low-grade inflammation plays a crucial role in ovarian aging and contributes significantly to FGSCs aging [48–52]. Therefore, PACs hold promise as important anti-inflammatory and oxidative stress agents that can reshape female reproductive stem cell function and delay ovarian aging.

Conclusion

In conclusion, the results of both in vitro and in vivo experiments demonstrate that PACs significantly enhance the proliferation of FGSCs. The underlying mechanism has been shown to be associated with the Sirt1-p53-p21 signaling pathway. Furthermore, it has been found that

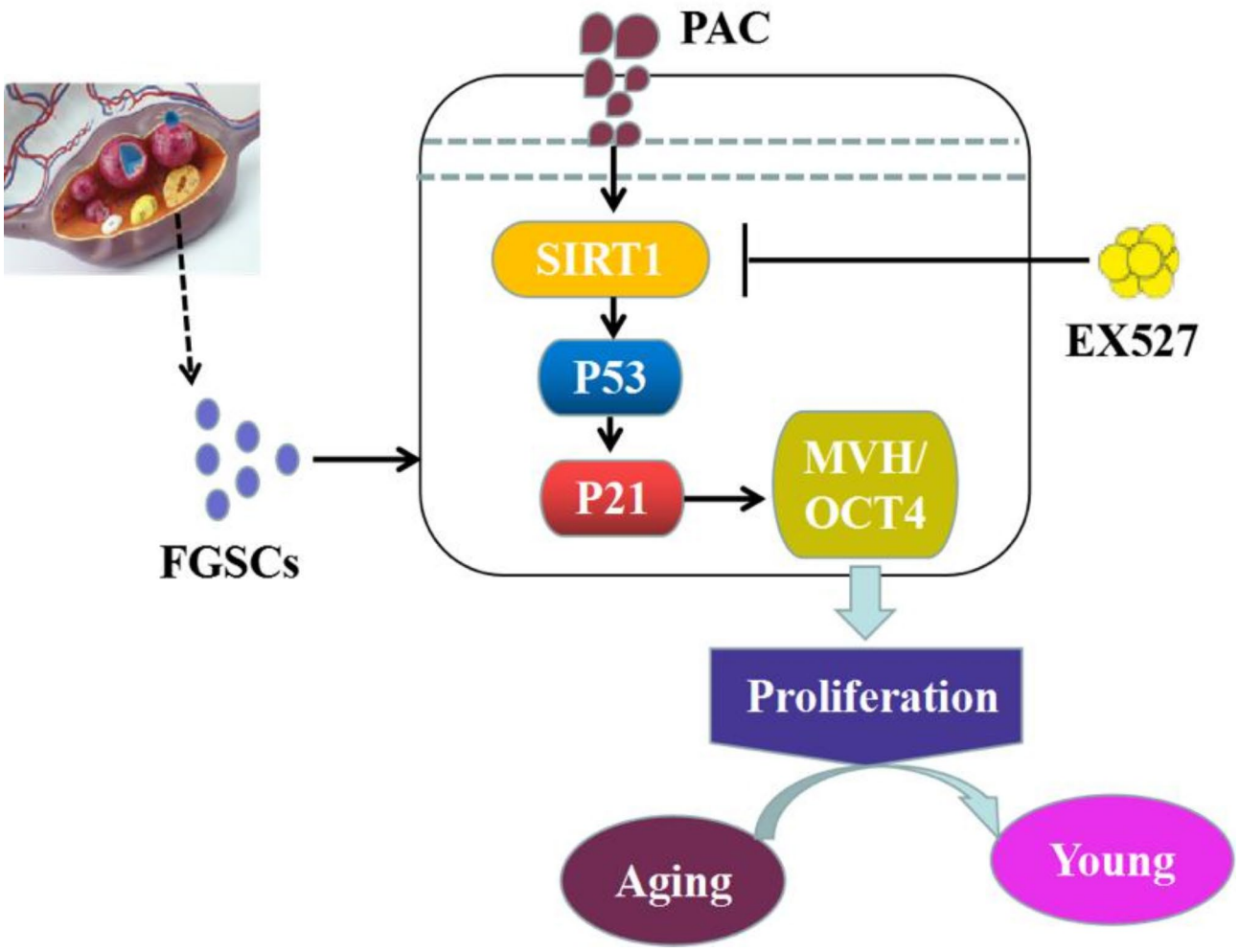

Fig. 7 The role of PACs in delaying POI through the regulatory Sirt1-p53-p21 signaling Pathway in FGSCs

PACs play a protective role against pathological ovarian aging by promoting FGSCs proliferation (Fig. 7). Our study has identified potential therapeutic strategies that can extend reproductive lifespan of aged women.

Abbreviations

FGSCs	Female Germline Stem Cells
PACs	Proanthocyanidins
E_2	Estradiol
AMH	Anti-Mullerian Hormone
Sirt1	Silent Information Regulator 1
POI	Premature Ovarian Insufficiency
OSE	Ovary Surface Epithelial
VSELs	Very Small Embryonic-Like Stem Cells
OGSCs	Ovarian Germline Stem Cells
Sir2	Silent Information Regulator 2
ROS	Reactive Oxygen Species
OD	Optical Density
CY/BUS	Cyclophosphamide/Busulphan
PBS	Phosphate-Buffered Saline
ECLIA	Electrochemiluminescence Immunoassay
PFA	Paraformaldehyde
qRT-PCR	Quantitative Reverse Transcriptase PCR

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13048-025-01661-y.

Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4
Supplementary Material 5
Supplementary Material 6
Supplementary Material 7
Supplementary Material 8

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Author contributions
W.B.W. and M.Y.B. and S.Y.L. conceived and coordinated the study. W.L.H. and Y.L. conducted cytological and animal experiments. C.Y.R. and H.F.W. consult the relevant information. Y.Q.D and Z.Q.Z. collected data. R.X.W. and

Z.S.Z. analyzed the data. X.X.F. and Y.H.Z. revised the manuscript. All authors contributed to reading and approving of this manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All animal procedures were conducted in accordance with the recommendations of the Animal Center of Nanchang University guidelines and approved by the Animal Care and Use Committee of Nanchang University (Permit Number: SYXK2021-0004).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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