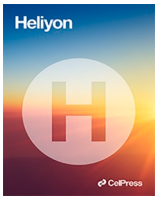
Heliyon 10 (2024) e32802

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Investigating the potential role of α-SNAP in preventing chemotherapy-induced ovarian dysfunction: Insights from cellular and animal models

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ARTICLE INFO

Keywords:
Primordial follicle
α-SNAP
Depletion
PI3K/Akt/mTORC1
Cisplatin

ABSTRACT

Background: The phosphoinositide 3-kinase/Akt/mammalian target of rapamycin complex 1 (PI3K/Akt/mTORC1) pathway plays a crucial role in the activation of primordial follicles. However, excessive activation and the loss of primordial follicles can lead to ovarian dysfunction. The alpha-soluble N-ethylmaleimide sensitive factor attachment protein (α-SNAP) protein has been implicated in PI3K/Akt/mTORCl signaling, suggesting its potential involvement in follicle activation. Thus, this study aimed to explore the role of α-SNAP in the activation of the PI3K/Akt/mTORC1 signaling pathway and its ability to mitigate the effects of cisplatin on ovarian function, using both in vitro and in vivo models.

Methods: We transfected KGN human ovarian granulosa cells (GCs) with small interfering RNA (siRNA) targeting α-SNAP to investigate the effects of α-SNAP inhibition on GC proliferation and apoptosis, as well as on the activity of the PI3K/Akt/mTORC1 pathway. In a mouse model, α-SNAP siRNA was delivered via an adeno-associated virus before treatment with cisplatin to assess its effects on follicle activation and ovarian function. Follicle counts at various growth stages, western blotting, and immunohistochemistry analyses were conducted to detect the expression of cleaved caspase-3, Ki67, α-SNAP, and p-mTOR. Additionally, the serum concentrations of anti-Müllerian hormone (AMH) were measured through an enzyme-linked immunosorbent assay.

Results: In vitro, α-SNAP depletion prevented GC proliferation by inhibiting the PI3K/Akt/mTORC1 pathway, thereby indicating its role in the regulation of cell growth. In vivo, α-SNAP knockdown attenuated the cisplatin-induced overactivation of primordial follicles by suppressing the PI3K/Akt/mTORC1 signaling pathway and partially restoring AMH levels. In addition, the expression and distribution patterns of cleaved caspase-3, Ki67, α-SNAP, and p-mTOR varied across different follicular growth stages, suggesting a protective effect against chemotherapy-induced ovarian damage.

Conclusions: Inhibiting α-SNAP may attenuate GC proliferation by suppressing the PI3K/Akt/mTORC1 pathway, thereby mitigating the overactivation and loss of primordial follicles induced by cisplatin. Targeting α-SNAP may emerge as a novel strategy to prevent ovarian damage

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https://doi.org/10.1016/j.heliyon.2024.e32802
Received 15 November 2023; Received in revised form 6 June 2024; Accepted 10 June 2024
Available online 10 June 2024
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resulting from chemotherapy. However, these conclusions warrant repeated testing, and the mechanistic underpinnings of α-SNAP must be further elucidated in the future.

1. Introduction

Ovarian function is closely associated with the quantity of primordial follicles in the ovarian reserve. Overactivation of primordial follicles leads to a depletion of the ovarian reserve [1], thereby inducing ovarian dysfunction and potentially leading to amenorrhea. Unlike antral follicles, whose development relies on gonadotropins, the activation of primordial follicles and the development of primary and secondary follicles occur independently of gonadotropins. Instead, these processes are regulated by factors such as bone morphogenetic protein [2], growth differentiation factor 9 [3], anti-Müllerian hormone (AMH) [4], and inhibin (INH) [5], as well as the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin complex 1 (PI3K/Akt/mTORC1) signaling pathway [6,7]. However, many regulatory factors and mechanisms underlying primordial follicle activation remain unknown. An exploration of these mechanisms holds potential to enhance our understanding of ovarian physiology and pathology, facilitate effective treatment and prevention measures for patients with premature ovarian failure (POF), and advance the field of in vitro follicular activation and ovarian tissue culture technology.

Of the three branches in the PI3K signaling network, the type I PI3K pathway is the most extensively studied. Activated PI3K can catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) on the plasma membrane, thereby generating the second messenger, phosphatidylinositol 3,4,5-triphosphate (PIP3). This molecule, in turn, activates Akt by facilitating its phosphorylation at Ser308 via phosphoinositide-dependent kinase 1 (PDK1). Once activated, Akt regulates cell function, promotes cell proliferation by phosphorylating various downstream factors, such as transcription factors, enzymes, and kinases.

The mammalian target of rapamycin (mTOR) is a crucial serine/threonine kinase downstream of PI3K/Akt signaling. It belongs to the PI3K-related protein kinase family because its C-terminus exhibits homology with the PI3K catalytic domain. In mammalian cells, mTOR predominantly exists in two complexes, namely mTORC1 and mTORC2, with the former playing a more seminal role [8]. mTORC1 is composed of mTOR, proline-rich Akt substrate of 40 kDa (PRAS40), Raptor, mLST8, and Deptor proteins. mTORC1 signaling is implicated in processes such as proliferation, differentiation, and autophagy, thereby playing a crucial role in the progression of various diseases. Phosphorylated Akt can directly phosphorylate PRAS40 to block its inhibitory effect on mTORC1, thereby activating the mTORC1 pathway. The phosphorylation of mTOR at Ser2448 represents another recognized mechanism for mTORC1 activation. Once activated, mTORC1 can activate the p70 ribosomal protein S6 kinase 1 (S6K1), a downstream effector, by phosphorylating it. This, in turn, phosphorylates ribosomal protein S6 to initiate mRNA translation at the 5′ end, promoting the synthesis of ribosomal protein and elongation factors, ultimately sustaining cell growth and proliferation [9].

The number of primordial follicles in the female ovaries during dormancy reflects the ovarian reserve capacity. In the ovaries of women of childbearing age, most oocytes within the primordial follicles remain in a dormant state, surrounded by a single layer of flat granular cells called granulosa cells (GCs) of the primordial follicles (pfGCs). Although studies have demonstrated that the maturation and differentiation of pfGCs play important roles in activating the oocytes within the resting primordial follicles, the specific mechanisms involved remain unclear. In a Raptor-knockout mouse model, the inhibition of mTORC1 signaling precludes the differentiation of pfGCs into mature columnar GCs. On the contrary, overactivation of mTORC1 signaling accelerates the differentiation of pfGCs into GCs, thus inducing the premature activation of resting oocytes and the premature maturation of primordial follicles [10]. Studies have detected mTOR expression in the oocytes and GCs of follicles at all stages, with elevated levels observed in growing follicles compared to primordial follicles. Furthermore, mTOR knockout affects the growth of oocytes within primordial follicles by inhibiting gene transcription [11].

The activation of primordial follicles for their development into primary follicles induces the downregulation of phosphatase and tensin homolog (PTEN), a cytoplasmic protein found in oocytes and GCs. Deletions or mutations in PTEN may enhance PI3K signaling, elevate PIP3 levels, and bolster Akt phosphorylation, eventually activating the mTORC1 pathway. Furthermore, Akt phosphorylation promotes the nuclear transportation of the transcription factor forkhead box O3a, thereby triggering the overactivation of primordial follicles and the development of primary and secondary follicles. Therefore, the activation of PTEN-PI3K/Akt/mTORCl signaling induces the premature overactivation of the primordial follicle pool [12]. Given the irreversibility of follicular activation and growth, as well as the follicular recruitment and dominance selection mechanisms during the growth stage, most follicles fail to progress to maturity and ultimately undergo atresia. This leads to the loss of a significant number of primordial follicles and the onset of POF. Therefore, the inhibition of PI3K/Akt/mTORCl signaling is expected to effectively prevent the premature overactivation of the primordial follicle pool and preserve the primordial follicle reserve [13].

The soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptors (SNAREs) are a vast protein superfamily comprising 60 members in mammalian cells. SNAREs regulate the intracellular transport of cargo and neurotransmitter release by mediating membrane fusion of vesicles. The SNARE core complex comprises a stable, equimolar trimer consisting of synaptobrevin/vesicle-associated membrane protein located on the vesicle membrane (v-SNARE), and syntaxin and SNAP-25, which are anchored to the membrane of the target cell/organelle (t-SNAREs). v-SNAREs and t-SNAREs combine to initiate vesicular membrane fusion [14]. NSF and α-SNAP facilitate the recycling of the SNARE core complex by assisting in its depolymerization. NSF exhibits ATP hydrolase activity, which provides the energy required for depolymerizing the SNARE core complex subsequent to binding. Additionally, the recruitment of NSF to the SNARE complex requires α-SNAP [15]. At present, the precise molecular mechanisms of SNARE proteins remain unclear. Some studies have reported that the polarity and proliferation of *Drosophila* epithelial cells are regulated by the

endocytosis of apical proteins, with endosomal SNARE being polarized into distinct endosomal networks at the apical and basolateral domains. Modulating the sorting of apical SNARE can regulate the rate of endocytosis of apical proteins, thereby affecting signal transduction and epithelial cell proliferation [16,17]. A study elucidated that mutations in α-SNAP could activate the AMP-activated protein kinase (AMPK) pathway in neural stem/progenitor cells, thereby diminishing cell proliferation activity [18]. Some studies have reported a correlation between α-SNAP expression and the activation of PI3K/Akt/mTORCl signaling. Akt phosphorylation at Ser473 is significantly attenuated in $CD4^+$ T cells isolated from the peripheral blood of α-SNAP mutant mice [19]. Inhibiting the expression of α-SNAP in human epithelial cells results in decreased levels of mTOR, p-mTOR, and phosphorylation of the downstream substrate S6K1, thus inhibiting the mTORC1 signaling pathway [20]. Therefore, α-SNAP may be associated with the activation of primordial follicles.

In a previous study, we successfully established a model of apoptotic ovarian GCs and an animal model of cisplatin-induced POF [21]. This study aimed to explore the correlation between α-SNAP and PI3K/Akt/mTORC1 signaling and their correlation with the activation of primordial follicles, using cisplatin-treated cellular and animal models.

2. Materials and methods

2.1. Cell lines

KGN human ovarian GCs served as the cellular model in this study. KGN human ovarian GCs are derived from ovarian GC tumors and possess gonadotropin receptors. KGN cells can synthesize and secrete steroid hormones and various follicle growth-related factors. Furthermore, they can be transfected with small interfering RNA (siRNA) [22,23]. A preliminary experiment showed that these cells expressed α-SNAP. Subsequently, we transfected them with α-SNAP siRNA (lentivirus) to induce stable downregulation of α-SNAP expression. A scrambled siRNA served as the negative control.

2.2. Plasmid construction for RNA interference

The siRNA-mediated knockdown of α-SNAP was performed. α-SNAP was downregulated using a specific siRNA, ATCA-GAAGGCCATTGACAT. An siRNA duplex, TTCTCCGAACGTGTCACGT, served as the negative control. Subsequently, primers were designed based on these siRNA sequences and annealed to produce DNA oligos. These DNA oligos were then inserted into enzyme-digested and linearized lentiviral vectors (#GV248, Genechem Co., Ltd., Shanghai, China) to generate recombinant lentiviral plasmids. Conversely, for the generation of recombinant adeno-associated virus (AAV) plasmids, α-SNAP was downregulated using another siRNA, GCTCGCTGTTCAGAAGTATGA. An siRNA duplex, CGCTGAGTACTTCGAAATGTC, was employed as the negative control. Similarly, primers designed based on these siRNA sequences were annealed to form DNA oligos, which were inserted into enzyme-digested and linearized AAV9 vectors (#GV478, Genechem Co., Ltd., Shanghai, China). All siRNA sequences used were designed by Genechem Company in Shanghai. Subsequently, we used $CaCl_2$ to prepare *Escherichia coli* competent cells. The transformed competent cells were transferred to LB agar medium supplemented with antibiotics for cloning. Positive clones were identified through PCR and subjected to sequencing analysis to identify the correct clones that matched the target siRNA sequence. Finally, the correctly cloned bacteria were cultured, and high-purity plasmids were extracted.

2.3. Cell culture and transfection

HEK293T, AAV-293, and KGN cells were individually cultured in RPMI-1640 medium (72400047, Gibco) supplemented with 10 % fetal bovine serum (FSP500, ExCell Bio). HEK293T cells, cultured in a 10-cm dish, were cotransfected with 20 μg of siRNA-expressing lentiviral plasmids, 15 μg of pHelper 1.0, and 10 μg of pHelper 2.0 (Genechem Co., Ltd., Shanghai, China) using Lipofectamine 2000 (11668-019, Invitrogen). Virus-containing supernatants were collected 48 h post-transfection and subsequently employed to infect KGN cells. Cells were infected in a culture medium supplemented with HitransGP infection enhancer (Genechem Co., Ltd., Shanghai, China) at the optimal multiplicity of infection (MOI = 50). Green fluorescent protein-positive cells were analyzed on the third day post-infection. Subsequently, an α-SNAP siRNA-KGN cell line was established by subjection cells to selection using 2 μg/mL puromycin (631305, Clontech) for an additional 48 h, until cellular mortality ceased. Afterward, the infected cells were expanded. Subsequently, the concentration of puromycin was decreased to 1 μg/mL, and the cells were further expanded. Concurrently, cell samples were collected for the assessment of target gene expression through quantitative real-time PCR. Cells exhibiting normal identification results were cryopreserved for seed preservation, while a pool of stably transduced cell clones was generated. In parallel, AAV-293 cells cultured in a 10-cm dish were cotransfected with 10 μg of siRNA-expressing AAV9 plasmids, 10 μg of pHelper, and 10 μg of pAAV-RC (Genechem Co., Ltd., Shanghai, China). Virus-containing supernatants were obtained 72 h post-transfection.

2.4. Cell experiments

KGN cells, negative control KGN cells, and α-SNAP siRNA-KGN cells were cultured separately in RPMI-1640 medium supplemented with 10 % fetal bovine serum, 100 IU/mL penicillin, and 100 g/mL streptomycin (15140-122, Gibco) in six-well plates (10^6 cells/well) at 37 °C in a humidified atmosphere with 5 % CO_2. Upon reaching 80 % confluency, the cells were transitioned to serum-free medium and cultured for 24 h to enhance their sensitivity to chemotherapy drugs. Subsequently, we replaced the medium with either cisplatin-containing or cisplatin-free medium (HY-17394, MCE, USA), followed by further incubation of the cells. PI3K/AKT signaling agonist

740Y–P was purchased from MCE (HY–P0175, UK). Cells treated with cisplatin were co-incubated with 740Y–P (20 μM) for 48 h. Protein extraction for western blotting was then performed.

2.5. Quantitative real-time reverse transcription-PCR

Total RNA was isolated using a TRIzol kit (Pufei, China) and treated with DNase to eliminate traces of genomic DNA. Subsequently, total RNA (2 μg) was reverse transcribed into cDNA using an M-MLV kit (Promega). Quantitative real-time PCR was performed on 1.6 μL of cDNA using the SYBR Master Mixture (TAKARA, DRR041B). The relative gene expression in each sample was determined employing the comparative threshold cycle method. The following primers were utilized: for human β-actin, forward primer GCGTGACATTAAGGAGAAGC and reverse primer CCACGTCACACTTCATGATGG; for α-SNAP, forward primer GCATTCAA-GAAAGCCGACCC and reverse primer CAGCAATGGAGATGTGGTGC.

2.6. Cell proliferation assay

The proliferation of KGN cells was assessed through the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan). A cell suspension containing approximately 5,000 cells in 100 μL per well was seeded into 96-well plates. The plates were then incubated at 37 °C with 5 % CO_2 for 12 h to facilitate cell attachment. Subsequently, varying concentrations of cisplatin were introduced into the wells, and the cells were further incubated for an additional 48 h. Following drug treatment, 100 μL of 10 % CCK-8 solution was added to each well, and the plates were incubated for 2 h at 37 °C. The absorbance was then measured at 450 nm using a microplate reader. All conditions were performed in triplicate wells, and the data are presented as the mean of three independent experiments.

2.7. Animal experiments

To assess the effect of downregulating α-SNAP on cisplatin-induced ovarian damage, we employed female CD-1 (ICR) mice, which are recognized for their reproductive capacity, in this study. CD-1 (ICR) mice were procured from the Guangdong Medical Laboratory Animal Center. Eight-week-old female mice (n = 5 per group) were housed in cages under a 12-h light/12-h dark cycle. Following complete anesthesia, their abdominal cavities were opened, and an equivalent volume of phosphate-buffered saline (PBS), negative control AAV9 solution (at 1E12 viral particles per mouse), or α-SNAP-siRNA-AAV9 solution (at 1E12 viral particles per mouse) was injected into their bilateral ovarian tissues at multiple sites [24,25]. The tissues were sutured, and the animals were injected with anti-inflammatory and analgesic drugs post-operation. They were then fed for 3 weeks. In the fourth week, they were intraperitoneally injected with saline or cisplatin (15 mg/kg body weight) (Fig. 1). One week later, mice were euthanized, and their ovaries were excised for western blotting, histological examination, and immunohistochemistry (IHC) analyses. Blood samples were collected from their hearts to measure AMH concentrations through enzyme-linked immunosorbent assay (ELISA). All animal experiments conformed to and were approved by the Nanfang Hospital Animal Ethic Committee.

2.8. Hematoxylin–eosin staining and follicle counting

The ovaries extracted from treated mice were fixed in 4 % paraformaldehyde for 24 h, followed by dehydration, embedding in paraffin blocks, and slicing into 5-μm-thick serial sections. Every 20th section was selected for hematoxylin–eosin staining (G1004, Servicebio). Approximately 12–16 sections were obtained from each mouse ovary and examined under a light microscope (DS-U3, Nikon) for follicle quantification.

Follicles were classified into four stages according to the following criteria: primordial follicles were defined as those with one layer of flattened GCs; primary follicles were considered when the follicles contained one layer of cuboidal GCs, with or without flattened GCs; secondary follicles were defined as those with two or more layers of cuboidal GCs without a visible antrum; and antral follicles were characterized by the presence of an antral space filled with follicular fluid [26].

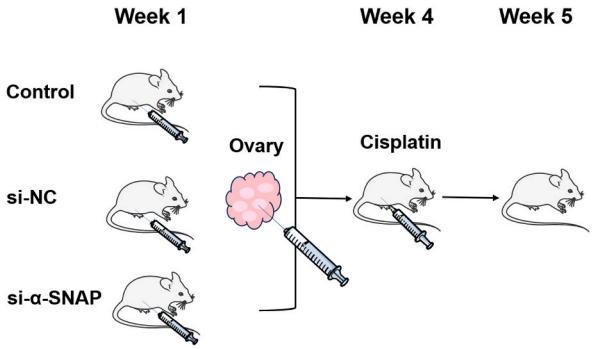


Fig. 1. Animal experimental schema.

2.9. IHC

The extent of apoptosis was assessed by measuring the levels of cleaved caspase-3 (CC3) via IHC. The proliferation of follicles was evaluated by detecting Ki67 through IHC. Additionally, IHC was employed to analyze the expression of α-SNAP and p-mTOR across the four stages of follicular development. Ovarian sections marked with the middle number of all the serial sections in each ovary were selected for IHC. Given that each group comprised five mice, five slices from each group were used to detect the expression of each protein via IHC. After deparaffinization and rehydration, the selected sections were immersed in EDTA antigen repair buffer and heated in a microwave to facilitate antigen retrieval. Afterward, they were incubated with 0.3 % H_2O_2 for 25 min and then incubated with a blocking solution (3 % bovine serum albumin) at room temperature for 30 min. Subsequently, the sections were probed overnight at 4 °C with the following primary antibodies: anti-α-SNAP (1:600, ab133673, Abcam, USA), anti-phospho-mTOR (1:100, ab109268, Abcam, USA), anti-cleaved caspase-3 (1:100, AF7022, Affinity, USA), and anti-Ki67 (1:100, AF0198, Affinity, USA). Afterward, they were washed in PBS and incubated with horseradish peroxidase-labeled secondary antibodies at room temperature for 60 min. Subsequently, the sections were stained with 3,3′-diaminobenzidine and finally counterstained with hematoxylin to indicate positive results.

We employed a scoring system and calculated the average intensity to evaluate positive results. The scoring system was based on the following criteria: the percentage of positive GCs in a follicle was categorized as follows: 0 %, 0; 0–10 %, 1; 10–50 %, 2; and 50–100 %, 3; and oocyte positive density, negative = 0, weak = 1, and strong = 2. Finally, we calculated the score for each follicular stage in each section and divided it by the number of follicles in the corresponding stage to derive the average positive intensity for each follicular stage. For example, the average positive intensity of primordial follicles in each slice equaled the sum of scores for primordial follicles divided by the number of primordial follicles.

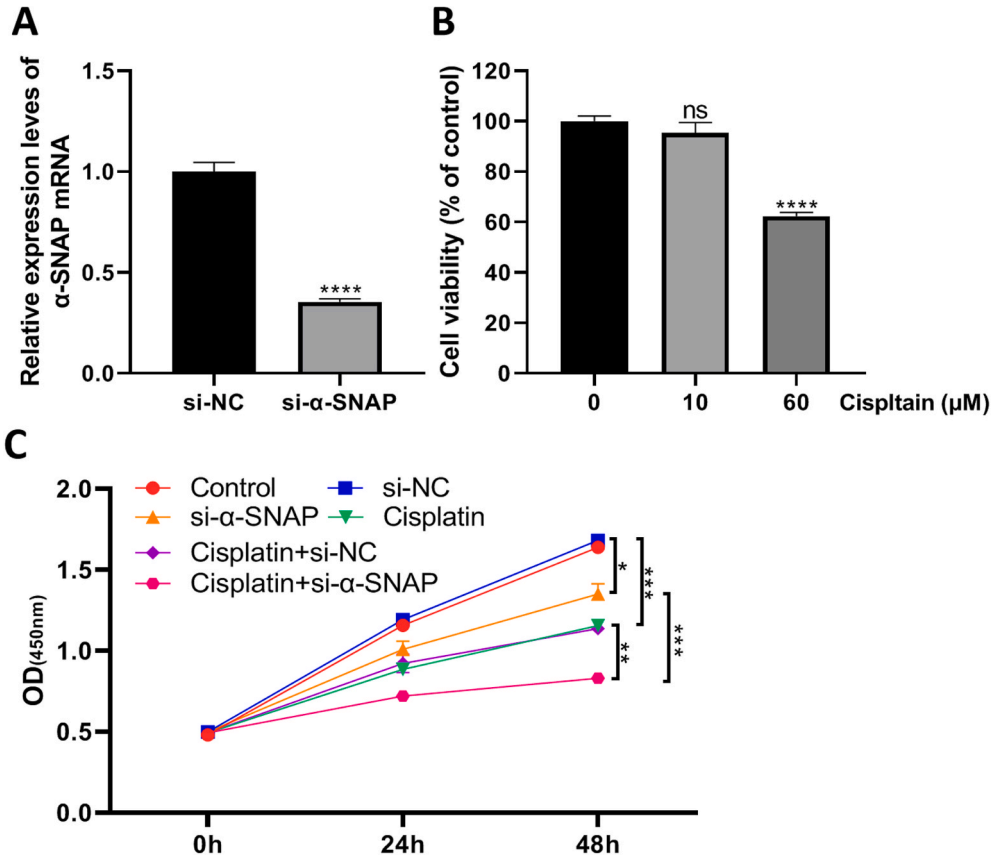


Fig. 2. Depletion of α-SNAP inhibited the proliferation of human ovarian GCs in vitro. (A) RT-PCR showed that mRNA levels of α-SNAP were decreased in α-SNAP-siRNA transfected KGN cells. (B) A CCK-8 assay was performed to measure the rate of KGN cell proliferation treated with different concentrations of cisplatin for 48 h. The results indicated that treatment with 60 μM cisplatin significantly inhibited cell viability. (C) The effect of α-SNAP knockdown and cisplatin treatment on the proliferation of KGN cells was determined via a CCK-8 assay. Ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

2.10. Western blotting

Total protein was isolated from cells and ovarian tissues using RIPA lysis buffer (P0013, Beyotime, China) and quantified employing a BCA detection kit (KeyGEN, KGPBCA, China) according to the manufacturer's instructions. After quantifying the protein concentrations, we separated equal amounts of protein (20–40 μg) on 10 % SDS polyacrylamide gels and then electrotransferred them to polyvinylidene fluoride membranes (WGPVDF22, Servicebio, China). Post-blocking with 5 % bovine serum albumin for 1 h at room temperature (approximately 20 °C), the membranes were incubated overnight at 4 °C with the following primary antibodies: anti-α-SNAP (1:1000, ab133673, Abcam, USA), anti-Akt (1:1000, ab179463, Abcam, USA), anti-phospho-Akt (1:1000, ab192623, Abcam, USA), anti-mTOR (1:1000, 66888-1-Ig, Proteintech, China), anti-phospho-mTOR (1:1000, ab109268, Abcam, USA), anti-PI3KB (1:500, DF6164, Affinity, USA), anti-cleaved caspase-3 (1:500, AF7022, Affinity, USA), anti-S6K1 (1:500, AF6226, Affinity, USA), anti-phospho-S6K1 (1:500, AF3228, Affinity, USA), anti-PCNA (1:500, ab29, Abcam, USA), and anti-β-actin (1:1000, GB15001, Servicebio, China). The membranes were then washed thrice with TBST solution and incubated with horseradish peroxidase-labeled goat anti-rabbit IgG antibody (1:5000, 4050-05, Southern biotech) at room temperature for 2 h. The target bands were visualized using an enhanced chemiluminescent reagent (Vazyme, Nanjing, China) and quantified using ImageJ software (version 1.8.0, National Institutes of Health, USA). β-actin served as the internal reference. Western blotting was performed with a minimum of three independent biological replicates to ensure the reproducibility and reliability of the results.

2.11. ELISA

We assessed the serum levels of AMH using mouse AMH (MM-44204M1) ELISA kits (Meimian Biotechnology), according to the manufacturer's instructions.

2.12. Statistical analysis

The Student's t-test, or Mann–Whitney *U* test, was used to determine statistically significant differences between two groups. A one-way analysis of variance was used to detect statistically significant differences among more than three groups. Values are expressed as the mean ± standard error of the mean (SEM). A statistically significant difference was deemed to exist when the p-value was less than 0.05. SPSS (Chicago, IL, USA) was used for statistical analysis.

3. Results

3.1. Depletion of α-SNAP inhibits the proliferation of human ovarian GCs in vitro

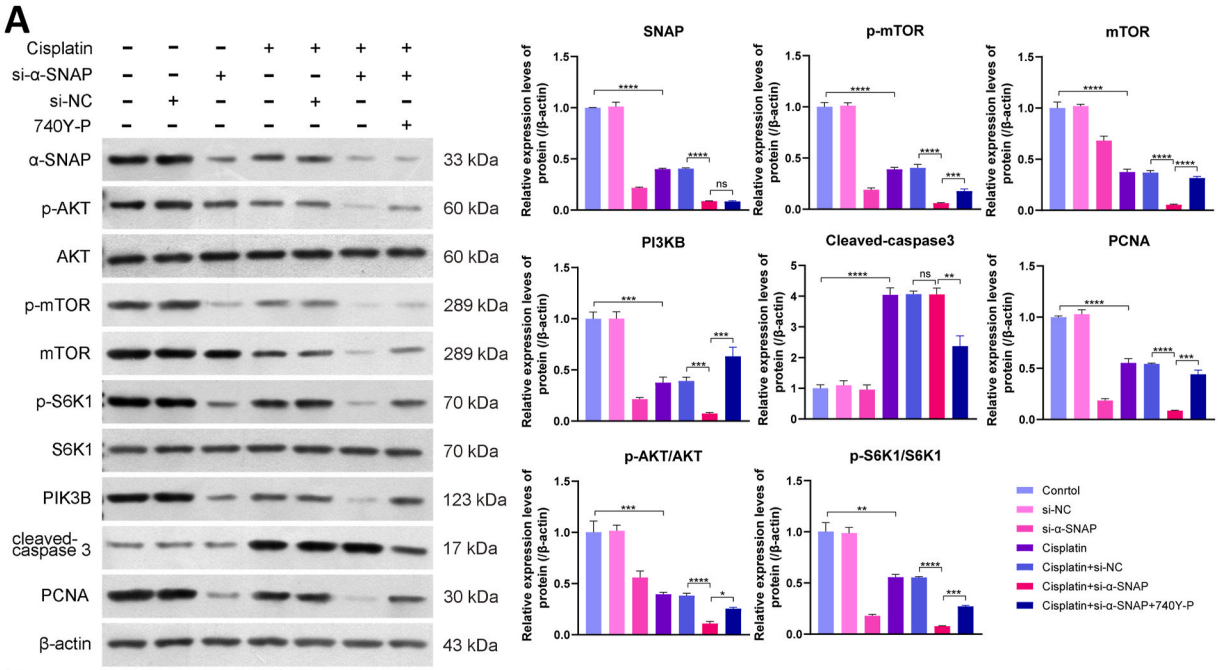
To validate the efficient knockdown of α-SNAP by siRNA, we performed RT-PCR to examine the mRNA levels of α-SNAP in KGN cells transfected with either a negative control or α-SNAP targeting siRNA. Quantitative analysis of the results shown in Fig. 2A revealed that α-SNAP siRNA transfection resulted in a 64 % reduction in α-SNAP mRNA levels compared to control siRNA. This confirmed the successful knockdown of α-SNAP at the mRNA level. Subsequently, both α-SNAP siRNA and negative control siRNA were subcloned into lentiviral vectors. These lentiviral vectors were then used to transfect KGN cells, facilitating the establishment of stable cell lines that expressed the siRNAs through puromycin selection.

To determine the optimal cisplatin concentration, we initially reviewed existing literature detailing the utilization of cisplatin in KGN cells. Across these studies, concentrations ranging from 10 to 50 μM were utilized [27–29]. However, initial pilot experiments conducted in our laboratory, testing cisplatin within this concentration range, did not yield a robust effect on KGN cells. We therefore investigated higher cisplatin concentrations and identified 60 μM as an effective dose, which reduced KGN viability to approximately 62 % ($p < 0.0001$) after 48 h of treatment compared to untreated controls, as assessed via the CCK-8 assay (Fig. 2B). Based on these preliminary results, a cisplatin concentration of 60 μM with a treatment duration of 48 h was selected for all subsequent experiments. This dosage exhibited adequate cytotoxicity while preserving some viability for subsequent mechanistic studies. Nevertheless, further optimization and validation of the cisplatin treatment conditions may be warranted in future studies.

We next explored the effects of α-SNAP knockdown on cisplatin-induced inhibition of KGN cell proliferation. Cell viability was assessed through a CCK-8 assay in siRNA α-SNAP and si-NC cells treated with or without cisplatin. As shown in Fig. 2C, in the absence of cisplatin, si-α-SNAP cells exhibited a significant decrease in viability compared to si-NC cells ($p < 0.05$). Upon cisplatin treatment, si-α-SNAP cells again displayed significantly lower viability compared to si-NC cells ($p < 0.01$). Moreover, compared to si-α-SNAP alone, the combination of cisplatin treatment and si-α-SNAP further significantly reduced cell viability ($p < 0.001$). Overall, these results demonstrate that α-SNAP knockdown inhibits KGN cell proliferation, with this effect being enhanced when combined with cisplatin treatment. Depletion of α-SNAP appears to sensitize ovarian GCs to the antiproliferative effects of cisplatin chemotherapy.

3.2. α-SNAP knockdown sensitizes ovarian GCs to cisplatin by blocking PI3K/Akt/mTOR signaling

To evaluate the role of α-SNAP in the PI3K/Akt/mTORC1 signaling pathway, we performed Western blot analysis of pathway proteins. Western blot results revealed that following cisplatin treatment, the levels of α-SNAP, PI3K, mTOR, p-mTOR, p-Akt/Akt, p-S6K1/S6K1, and proliferating cell nuclear antigen (PCNA) were significantly reduced, while the level of CC3 was significantly increased compared with the control groups (no cisplatin). Regardless of cisplatin presence, transfection with α-SNAP siRNA led to



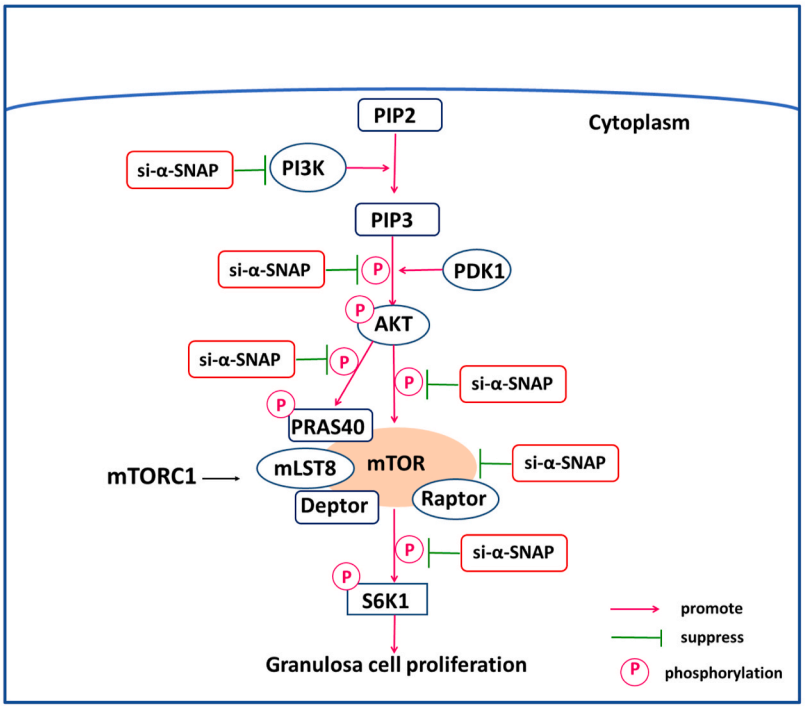
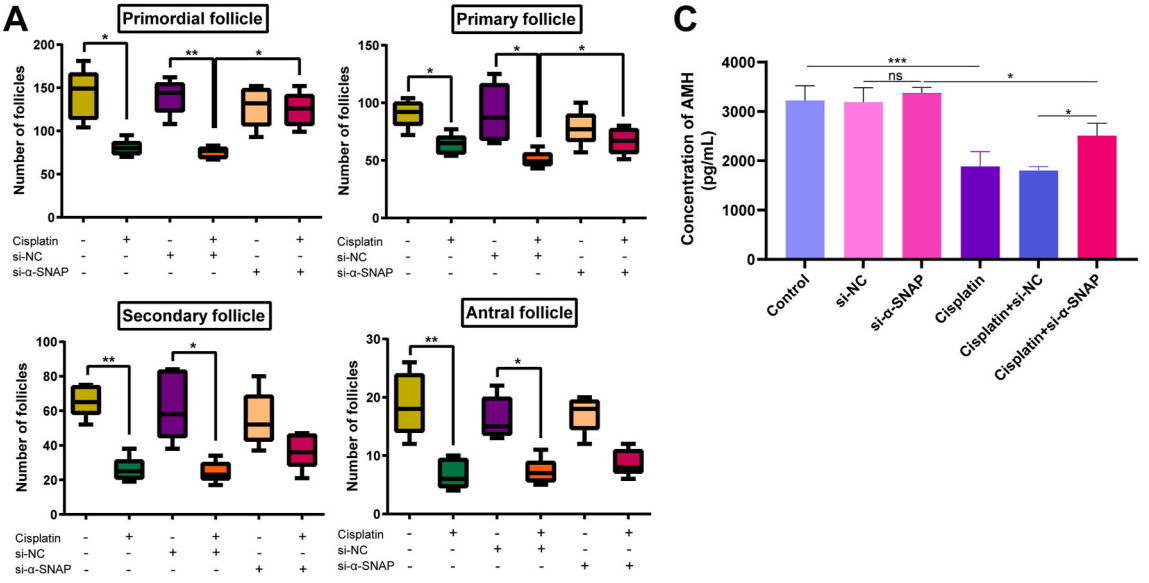


Fig. 3. α-SNAP can regulate GC proliferation via the PI3K/Akt/mTORC1 pathway. (A) KGN cells, negative control KGN cells, and α-SNAP siRNA-KGN cells were treated with or without 60 μM cisplatin. Western blotting was performed to quantify the levels of α-SNAP, PI3K, p-mTOR, mTOR, p-Akt,Akt, p-S6K1, S6K1, PCNA, and CC3 in cells. The full, uncropped version of this image is available in the supplementary material (Supplementary Materials). (B) Si-α-SNAP inhibited GC proliferation by inhibiting the PI3K/Akt/mTORC1 pathway. Si-α-SNAP inhibited the PI3K/Akt/mTORC1 signaling pathway by decreasing the levels of PI3K, p-mTOR, and mTOR, as well as the phosphorylation of Akt and S6K1, thereby inhibiting the proliferation of ovarian GCs.



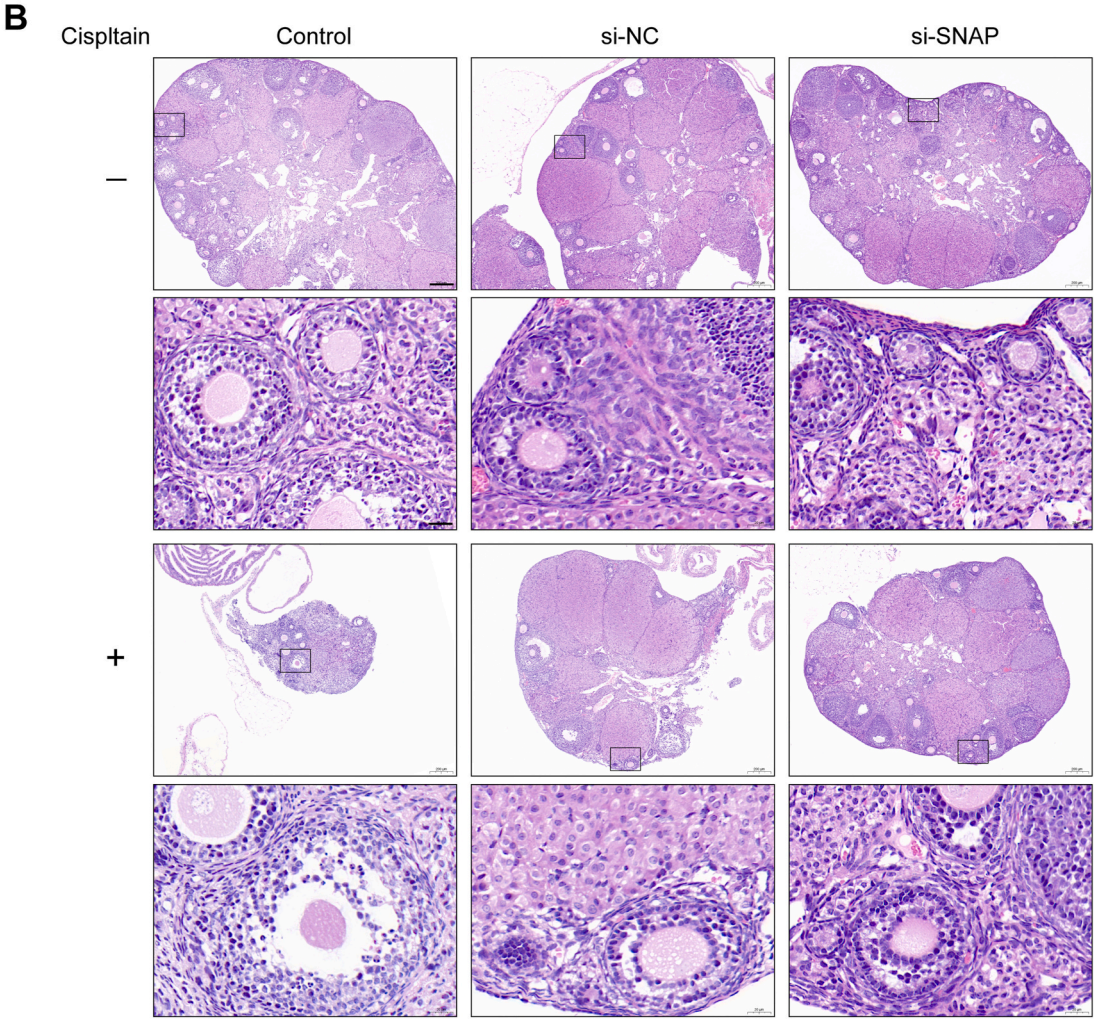


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Fig. 4. Inhibiting α-SNAP can prevent the cisplatin-induced loss of primordial follicles and ovarian dysfunction in mice. (A, B) Follicle counting showed that the number of primordial, primary, secondary, and antral follicles significantly decreased compared with their respective control groups following cisplatin injection. In the presence of cisplatin, mice treated with α-SNAP siRNA showed a significant recovery in the number of primordial and primary follicles compared with mice treated with negative control siRNA. Bar, 500 μm. Mean ± SEM, *, $p < 0.05$, **, $p < 0.01$. (C) ELISA results indicated that in the presence of cisplatin, the concentrations of AMH in mice sera were significantly reduced compared with their respective control groups (without cisplatin). Concurrently, in the presence of cisplatin, the concentration of AMH increased in mice treated with α-SNAP siRNA compared with those treated with negative control siRNA. Mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

diminished levels of α-SNAP, PI3K, mTOR, p-mTOR, p-Akt/Akt, p-S6K1/S6K1, and PCNA, with CC3 levels remaining unchanged compared to cells transfected with negative control siRNA (Fig. 3A).

To further explore the impact of α-SNAP on KGN cell function mediated by the PI3K/Akt/mTORC1 signaling pathway, cells transfected with si-α-SNAP were subjected to treatment with cisplatin in combination with 740Y–P, an activator of the PI3K/AKT pathway. Notably, intervention with 740Y–P effectively reversed the inhibitory effects observed in the group treated with cisplatin and si-α-SNAP, suggesting a potential role of α-SNAP in modulating GC proliferation and PI3K/Akt/mTORC1 signaling under these conditions (Fig. 3A).

These results indicate that cisplatin inhibits GC proliferation by inhibiting PI3K/Akt/mTORC1 signaling while promoting apoptosis. Depletion of α-SNAP may also suppress GC proliferation by inhibiting the same signaling pathway (Fig. 3B), albeit without apparent exacerbation of apoptosis.

3.3. *Inhibiting α-SNAP can prevent the cisplatin-induced loss of primordial follicles and ovarian damage*

Follicle counting revealed a significant decrease in the number of primordial, primary, secondary, and antral follicles following cisplatin injection compared with the control groups (no cisplatin). In contrast, in α-SNAP knockdown mice treated with cisplatin, the number of primordial and primary follicles significantly increased. Moreover, the number of secondary follicles recovered slightly but insignificantly ($p = 0.1012$), while the number of antral follicles remained unaltered compared with the negative control siRNA-transfected mice (Fig. 4A and B).

The concentrations of AMH were significantly reduced in cisplatin-treated mice compared with control mice (no cisplatin), as assessed through ELISA. However, these concentrations were restored to some extent in α-SNAP knockdown, cisplatin-treated mice compared with negative control siRNA-transfected mice (Fig. 4C).

3.4. *α-SNAP regulates the proliferation of primordial follicles in mouse ovaries through the PI3K/Akt/mTORC1 signaling pathway*

Western blotting demonstrated that the levels of α-SNAP, PI3K, mTOR, p-mTOR, p-Akt/Akt, p-S6K1/S6K1, and PCNA were decreased, while the level of CC3 was significantly increased in the ovaries of cisplatin-injected mice compared with control mice (no

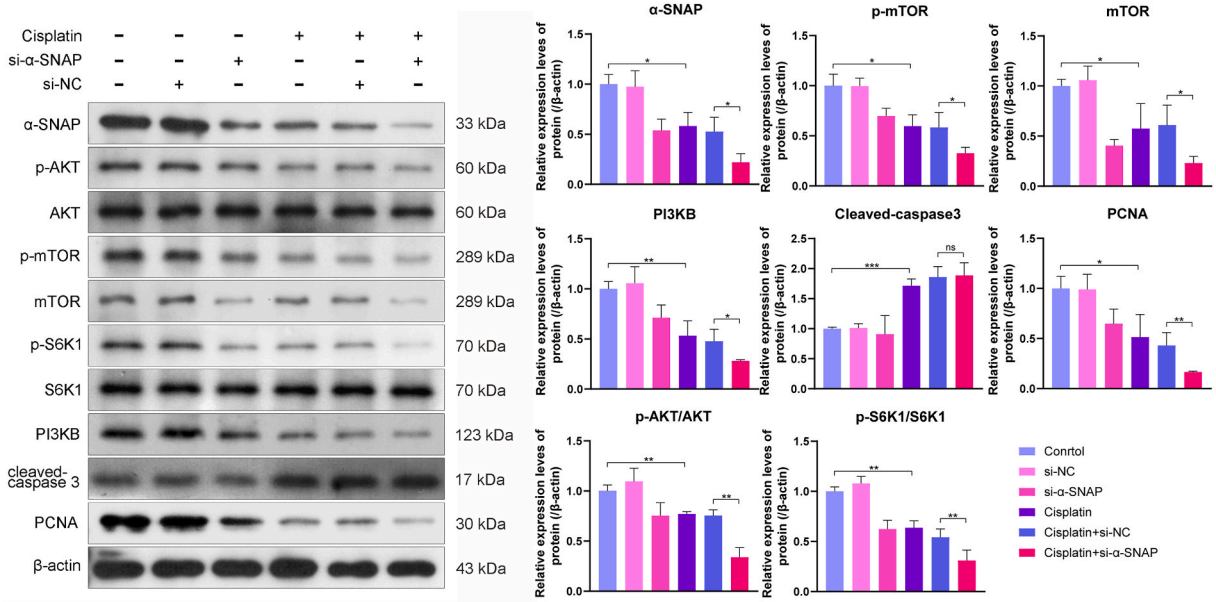

Fig. 5. α-SNAP can regulate the PI3K/Akt/mTORC1 pathway in mouse ovaries. Western blotting was performed to assess the levels of α-SNAP, PI3K, p-mTOR, mTOR, p-Akt, Akt, p-S6K1, S6K1, PCNA, and CC3 in mouse ovaries. The full, uncropped version of this image is available in the supplementary material (Supplementary Materials).

cisplatin). Regardless of cisplatin treatment, knockdown of α-SNAP decreased the levels of α-SNAP, PI3K, mTOR, p-mTOR, p-Akt/Akt, p-S6K1/S6K1, and PCNA compared with negative control siRNA-injected mice. However, in the presence of cisplatin, the level of CC3 did not exhibit significant changes in α-SNAP knockdown mice compared with negative control siRNA-injected mice (Fig. 5). These results are consistent with those of the cellular experiment (Fig. 3A). However, it is important to note that since the western blotting analysis of the ovary elucidated the extent of proliferation of all cells in the ovary, the observed results may indicate that high-dose cisplatin could potentially induce the inhibition of overall cell proliferation in the ovary through the mTORC1 pathway. However, this does not necessarily imply inhibition of primordial follicle proliferation. Therefore, we subsequently analyzed the expression changes in proliferation signals on primordial follicles through IHC experiments. We elaborate on the over-recruitment mechanism [24] of primordial follicular in the Discussion section.

In the negative control and α-SNAP knockdown groups, CC3 was insignificantly expressed in the primordial follicles and only occasionally weakly expressed in the GCs of primary, secondary, and antral follicles, as evidenced by IHC. The injection of cisplatin did not alter the expression of CC3 in the primordial follicles but significantly increased it in the primary, secondary, and antral follicles compared with the negative control group (no cisplatin). Moreover, CC3 was more strongly expressed in GCs than in oocytes. The expression of this protein remained unchanged in the primordial follicles of α-SNAP siRNA-treated mice compared with negative control siRNA-treated mice in the presence of cisplatin. However, its expression was significantly reduced in the primary follicles, slightly decreased in the secondary follicles ($p = 0.0590$), and unchanged in the antral follicles of α-SNAP knockdown mice (Fig. 6A and B).

IHC revealed Ki67 expression in all follicular stages, as well as in the GCs and oocytes of the negative control and α-SNAP knockdown groups. Ki67 exhibited weak expression in the primordial and primary follicles, while its expression was remarkable in the

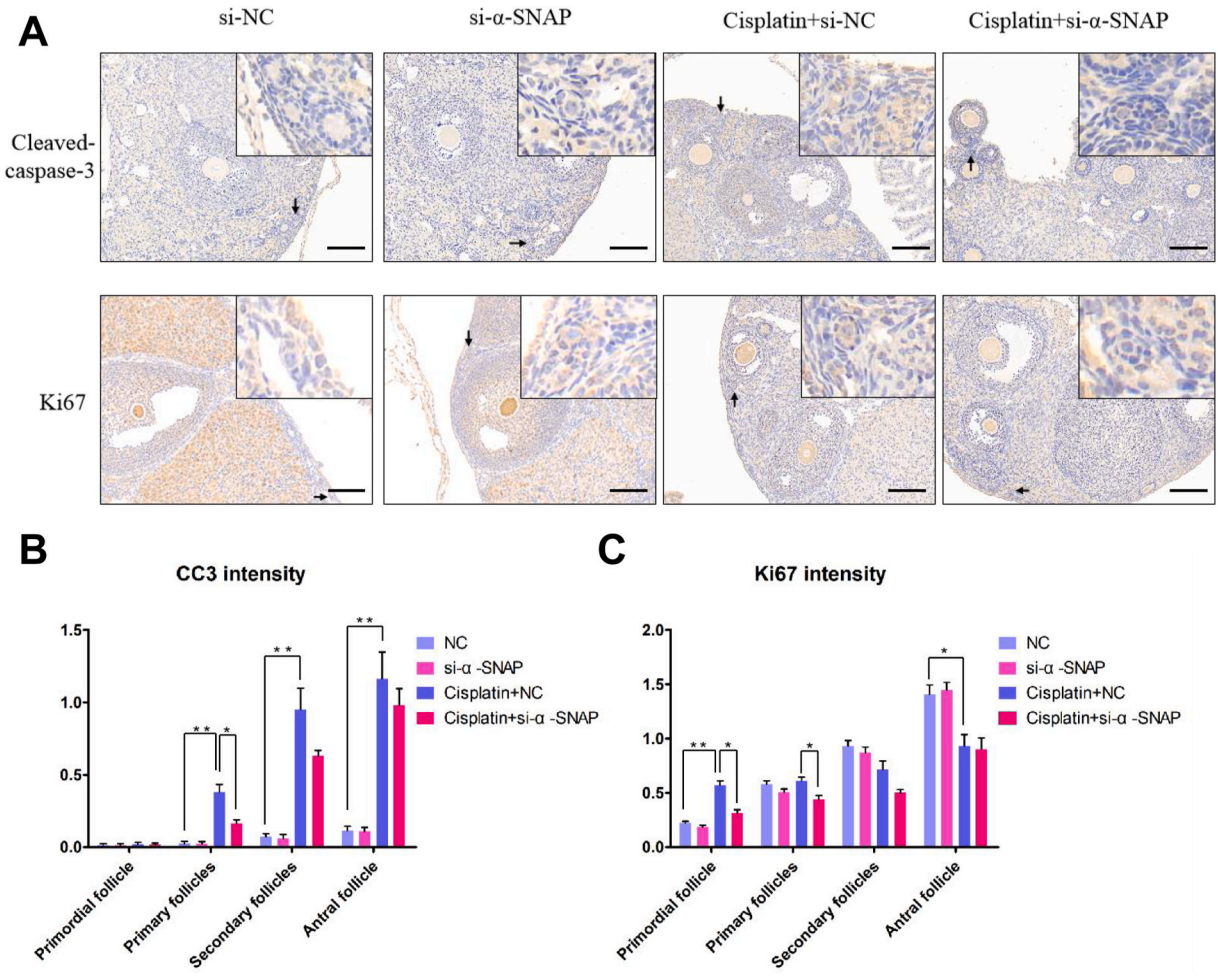

Fig. 6. Expressions of CC3 and Ki67 in each stage of follicular growth in mice. (A) The results of IHC, elucidating the expression of CC3 and Ki67 in the follicles of mice treated with α-SNAP siRNA or negative control siRNA in the absence or presence of cisplatin. The black arrow indicates primordial follicles. Bar, 50 μm. (B) The average immunohistochemical intensity of CC3 in each stage of follicular growth was measured and calculated. Mean ± SEM, *, $p < 0.05$, **, $p < 0.01$. (C) The average immunohistochemical intensity of Ki67 in each stage of follicular growth was determined and calculated. Mean ± SEM; *, $p < 0.05$; **, $p < 0.01$.

secondary and antral follicles. Following the injection of cisplatin, the expression of Ki67 was significantly increased in the primordial follicles, with no effect on its expression in the primary follicles, a slight reduction in its expression in the secondary follicles ($p = 0.0754$), and a significant reduction in its expression in the antral follicles compared with control mice (no cisplatin). Conversely, in the presence of cisplatin, the knockdown of α-SNAP significantly downregulated Ki67 in the primordial and primary follicles, slightly decreasing its expression in the secondary follicles ($p = 0.0872$), while not affecting its expression in the antral follicles compared with control mice (negative control siRNA) (Fig. 6A–C).

α-SNAP and p-mTOR were found to be expressed at all follicular stages as well as in the GCs and oocytes of negative control and α-SNAP knockdown mice, as observed through IHC. They exhibited weak expression in the primordial follicles, while their expression progressively increased from the primary to the antral follicles. Injection of cisplatin significantly increased the expression of α-SNAP and p-mTOR in the primordial follicles, had no impact on their expression in the primary follicles, and slightly reduced their expression in the secondary follicles ($p = 0.0703$ and $p = 0.0874$, respectively), with a significant decrease observed in the antral follicles compared with the control mice (no cisplatin). In contrast, in the presence of cisplatin, the knockdown of α-SNAP significantly downregulated α-SNAP and p-mTOR expression in the primordial and primary follicles, mildly downregulating their expression in the secondary follicles ($p = 0.0678$ and $p = 0.0782$, respectively), while leaving their expression unaffected in the antral follicles compared with control mice (negative control siRNA) (Fig. 7A–C).

4. Discussion

Ovarian dysfunction can arise from various factors, including aging, chemotherapy, radiation therapy, abnormal immune

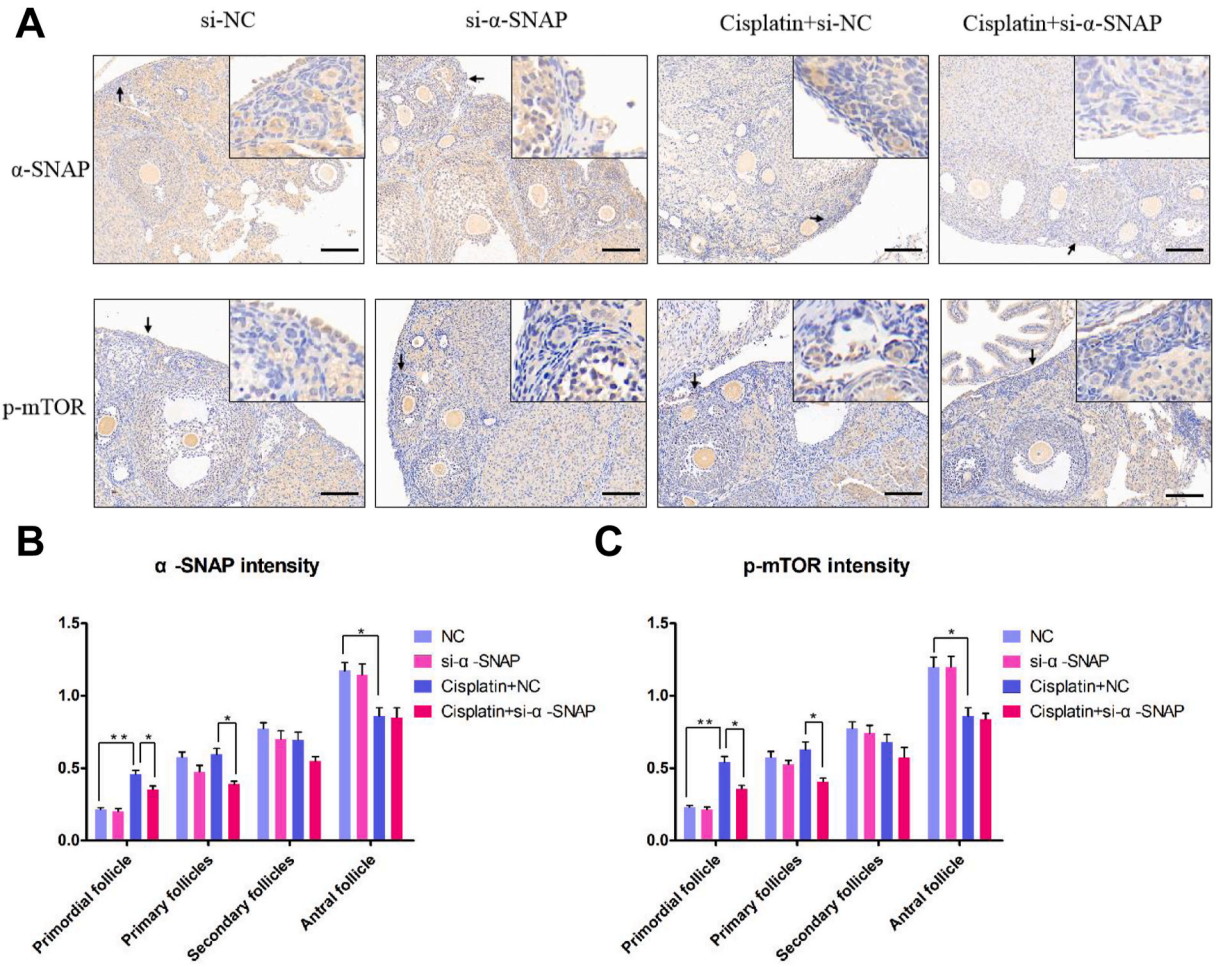

Fig. 7. Expression of α-SNAP and p-mTOR in each stage of follicular growth in mice. (A) The results of IHC, elucidating the expression of α-SNAP and p-mTOR in the follicles of mice treated with α-SNAP siRNA or negative control siRNA in the absence or presence of cisplatin. The black arrow indicates primordial follicles. Bar, 50 μm. (B) The average immunohistochemical intensity of α-SNAP in each stage of follicular growth was measured and calculated. Mean ± SEM, *, $p < 0.05$, **, $p < 0.01$. (C) The average immunohistochemical intensity of p-mTOR in each stage of follicular growth was assessed and calculated. Mean ± SEM; *, $p < 0.05$; **, $p < 0.01$.

mechanisms, and surgical injuries. The pathophysiology of ovarian dysfunction involves the excessive activation of primordial follicles, resulting in marked depletion of the primordial follicle reserve. Previous studies have demonstrated that the activation of the PI3K/Akt/mTORC1 signaling pathway can promote the proliferation and differentiation of GCs in primordial follicles, thereby facilitating their maturation into primary follicles [10,11]. However, overactivation of PI3K/Akt/mTORC1 signaling may lead to a significant loss of primordial follicles. Moreover, the downregulation of α-SNAP has been shown to inhibit mTORC1 signaling [20]. In addition, previous studies have reported that chemotherapy drugs, such as cisplatin, can induce the overactivation and subsequent loss of primordial follicles [30]. The findings of this study substantiate that the inhibition of α-SNAP expression suppresses the PI3K/Akt/mTORC1 pathway and alleviates the cisplatin-induced loss of primordial follicles and ovarian damage. Moreover, building upon insights from previous research [24,25], we successfully delivered α-SNAP siRNA into mouse ovarian tissue utilizing AAV9. Therefore, we infer that the AAV9 vector can cross the blood-follicle barrier and infect GCs and theca cells. Moreover, AAV9 was found not to infect oocytes, thereby ensuring its safety to a certain extent [25]. In addition, our results indicated no significant difference in both the number and morphology of follicles between the negative control group injected with AAV9 and the control group, which was not injected with the virus. This suggests that AAV9 may not induce follicular damage and affect follicle growth, thus presenting a potential avenue for gene therapy in cases of POF. However, we still need to repeatedly verify its effectiveness and safety.

Firstly, through in vitro experiments, we found that the inhibition of α-SNAP led to a decrease in the levels of PI3K, p-mTOR, and mTOR, as well as reduced phosphorylation of Akt and S6K1. In other words, the PI3K/Akt/mTORC1 pathway was inhibited, subsequently suppressing GC proliferation. Meanwhile, PI3K/AKT signaling agonist can weaken the inhibition of α-SNAP siRNA on PI3K/Akt/mTORC1 pathway and GC proliferation. As a member of the SNARE family, α-SNAP facilitates the intercellular transfer of cargo and energy, thereby promoting cell growth. Previous studies have reported a positive correlation between the expression of α-SNAP and mTORC1 pathway proteins [19,20]. Additionally, the activation of mTORC1 signaling has been confirmed to be associated with cell proliferation [31]. Therefore, our findings are consistent with those of previous studies. It can be inferred that α-SNAP siRNA may inhibit GC proliferation by regulating PI3K/Akt/mTORC1 pathway.

Moreover, we discovered that cisplatin suppresses GC proliferation by inhibiting the PI3K/Akt/mTORC1 pathway and inducing apoptosis. Likewise, the inhibition of α-SNAP attenuates GC proliferation by suppressing PI3K/Akt/mTORC1 signaling, albeit without apparent impact on the apoptosis of GC. Further exploration may be required to determine whether α-SNAP affects the apoptosis of GC and whether its effects are contingent upon factors such as cell culture duration.

In our in vivo experiments, we treated mice with cisplatin and injected AAV9 harboring α-SNAP siRNA into their ovarian tissue. Western blotting of the ovarian tissues revealed that cisplatin increased the levels of apoptotic proteins, consistent with the findings of previous studies [32]. However, cisplatin reduced the levels of α-SNAP, PI3K/Akt/mTORC1 signaling proteins, and proliferation-related proteins. Previous studies have reported a significant decrease in the levels of PI3K and mTOR in the ovaries of rats injected with cisplatin compared to the control group, which is consistent with our experimental findings [33]. The knockdown of α-SNAP further decreased the levels of PI3K/Akt/mTORC1 signaling proteins and proliferation-related proteins but had no significant effect on the levels of apoptotic proteins in the ovaries, consistent with the in vitro findings. The interaction among follicles at different stages and the involvement of endocrine regulatory feedback in mice render the influence of α-SNAP and cisplatin on follicular growth more complex than that on GCs alone. Therefore, to further understand their effects on follicular proliferation, development, and apoptosis across various stages of maturation, we employed IHC to quantify and localize the relevant proteins.

The immunohistochemical analysis elucidated that the expression of Ki67, a cell proliferation marker, increased sequentially as the follicles progressed from primordial to antral stages, indicating that the proliferative ability of follicles increases with their growth, which is consistent with the findings of some previous studies [34–36]. Upon injecting cisplatin into the mice, we observed that the proliferative ability of primordial follicles was significantly enhanced, that of primary follicles was unaltered, that of secondary follicles was slightly attenuated ($p = 0.0754$), and that of antral follicles was significantly attenuated. Moreover, previous research has shown that chemotherapy drugs primarily inhibit follicle proliferation in secondary and antral follicles, a finding consistent with our own research findings [37]. Prior studies have indicated that chemotherapy drugs can induce the activation of primordial follicles and increase proliferation signals within them [30,38,39]. Our study also demonstrated cisplatin-induced enhancement of primordial follicle proliferation. Previous studies suggest a mechanism whereby chemotherapy drugs induce apoptosis in GCs of primary, secondary, and antral follicles, leading to diminished secretion of AMH and INH B by GCs. This may ultimately activate primordial follicles. This negative feedback is also known as the over-recruitment mechanism of primordial follicles [24,40]. The expression and distribution patterns of α-SNAP and p-mTOR, both positive regulators of cell proliferation in follicles at varying stages, were consistent with those of Ki67, implying an increase in their expression alongside follicular growth. Upon injecting mice with cisplatin, the expression of α-SNAP and p-mTOR was significantly enhanced in primordial follicles, unchanged in primary follicles, slightly suppressed in secondary follicles ($p = 0.0703$ and $p = 0.0874$, respectively), and significantly decreased in antral follicles. The preceding in vitro experiments suggested that cisplatin can inhibit the proliferation of GCs as well as the PI3K/Akt/mTORC1 pathway, which explains the diminished proliferation capacity of secondary and antral follicles. α-SNAP knockdown significantly inhibits cisplatin-induced expression of Ki67 and p-mTOR in primordial follicles. Additionally, it significantly inhibits the proliferation of primary follicles and slightly inhibits that of secondary follicles ($p = 0.0872$ and $p = 0.0782$, respectively). The expression of Ki67 and p-mTOR in antral follicles, however, remains unaffected. In summary, on the basis of the IHC findings, we infer that α-SNAP regulates the proliferation and growth of follicles via the mTORC1 signaling pathway. Moreover, our findings indicate variations in the inhibitory effect of si-α-SNAP on the proliferation of follicles across different stages. Thus, we speculate that the inhibitory effect of si-α-SNAP on proliferation may be associated with target cell proliferation activity, while also considering the potential involvement of feedback regulation mechanisms among the different follicular stages. However, we still need to explore and validate the specific mechanisms through further research.

Conversely, upon analyzing the expression of CC3 through IHC, we found that cisplatin primarily promoted GC apoptosis in primary, secondary, and antral follicles, and the extent of apoptosis increased with the growth stage of the follicles. However, cisplatin did not induce apoptosis in primordial follicles. Hence, we infer that as follicles grow, their sensitivity to chemotherapy-induced damage increases, which corroborates the findings of some previous studies [41,42]. The knockdown of α-SNAP did not significantly affect the apoptosis of primordial follicles but significantly mitigated the average extent of cisplatin-induced apoptosis in primary follicles, slightly reduced the average extent of apoptosis in secondary follicles ($p = 0.0590$), and did not affect apoptosis in antral follicles. As follicles grow, the sensitivity of GCs within them to chemotherapy damage increases. Therefore, on the basis of the findings of the cell experiments, we speculate that although si-α-SNAP may not directly affect the apoptosis sensitivity of GCs, it may induce a decrease in the number of apoptotic GCs by inhibiting the transformation of primary and secondary follicles into antral follicles. Furthermore, the levels of apoptotic proteins observed in the results of western blotting serve as an indication of the total apoptotic status in all cells within the ovary, including follicular and stromal cells. Thus, arriving at a definitive conclusion regarding the regulatory effect of si-α-SNAP on follicular cell apoptosis may pose challenges.

Follicle counting at each stage established that high-dose cisplatin administration induced a reduction in the number of primordial, primary, secondary, and antral follicles. Similar findings have been reported by previous studies [29,43]. α-SNAP knockdown significantly restored the number of primordial and primary follicles depleted by cisplatin, mildly restored the number of secondary follicles ($p = 0.1012$), but failed to replenish the number of antral follicles. We analyzed the changes in follicle count based on the results of western blotting and IHC. (1) Primordial follicles: cisplatin primarily promotes GC apoptosis in primary, secondary, and antral follicles, with a mild inhibitory effect on the proliferation of GCs in secondary and antral follicles, resulting in a significant decrease in the number of these follicles. AMH and INH B, primarily secreted by GCs in primary, secondary, and antral follicles, can inhibit the activation of primordial follicles. Therefore, a decline in the number of these growing follicles induces the transformation of primordial follicles into primary follicles, resulting in the loss of primordial follicles [24]. α-SNAP knockdown can mitigate this loss by inhibiting the proliferation and transformation of primordial follicles into primary follicles. (2) Primary follicles: although cisplatin can induce the transformation of primordial follicles into primary follicles, high doses of the drug may also trigger apoptosis in a subset of primary follicles, thereby reducing their number. α-SNAP knockdown mitigates the cisplatin-induced decrease in primary follicle count. On the basis of the IHC results, we speculate that α-SNAP knockdown can inhibit the transformation of certain healthy primary follicles with proliferative ability into secondary follicles, thus restoring the number of primary follicles compared with the cisplatin group. (3) Secondary follicles: cisplatin induced significant apoptosis and led to a significant decrease in the number of secondary follicles. α-SNAP knockdown mildly alleviated the cisplatin-induced decline in secondary follicle number, albeit the difference was not statistically significant. On the basis of the IHC results, we speculate that although α-SNAP knockdown primarily inhibits the proliferation of primordial and primary follicles, it may also inhibit the transformation of select healthy secondary follicles, endowed with proliferative ability, into antral follicles, thereby slightly restoring the number of secondary follicles. (4) Antral follicles: cisplatin induced significant apoptosis and led to a significant decrease in the number of antral follicles, with α-SNAP knockdown failing to alleviate this phenomenon. The IHC results validated that α-SNAP knockdown did not affect the apoptosis or proliferation of antral follicles.

Presently, ovarian dysfunction induced by chemotherapy drugs can be mitigated through oocyte cryopreservation, transplantation following ovarian tissue cryopreservation and revival, administration of gonadotropin-releasing hormone (GnRH) agonists, employment of sphingosine 1-phosphate (S1P), utilization of melatonin, and stem cell therapy. While the technology for oocyte cryopreservation has reached a relatively advanced stage, it requires approximately 2 weeks of ovulation induction and is not suitable for patients with hormone-dependent tumors. Conversely, ovarian tissue cryopreservation and transplantation technology have also progressed significantly. Successful pregnancies have been reported subsequent to the transplantation of ovarian tissue back into the patient's body after chemotherapy; however, such cases remain relatively few. Moreover, ischemia–reperfusion injury during transplantation may lead to the loss of primordial follicles and ovarian damage [44]. GnRH agonists are currently undergoing clinical trials to thoroughly examine their ability to prevent ovarian damage induced by chemotherapy drugs. Studies have shown that the administration of GnRH agonists before and during chemotherapy can alleviate chemotherapy-induced ovarian damage and increase pregnancy rates [45,46]. Researchers speculate that GnRH agonists may inhibit the transition of primordial follicles into growth-stage follicles, thereby reducing their sensitivity to the toxic effects of chemotherapy drugs [47,48]. The clinical application of GnRH agonists, however, remains controversial [49]. Conversely, although S1P and melatonin have been proven to be effective in animal experiments, their application in clinical trials has yet to be realized. Researchers speculate that S1P and melatonin, via antioxidant mechanisms, can ameliorate oxidative damage and apoptosis in oocytes and GCs during follicular growth stages, thereby increasing AMH secretion by GCs and inhibiting the overactivation of primordial follicles [26,33,42,50]. Finally, some studies have reported that bone marrow mesenchymal stem cells (BMSCs) can mitigate cisplatin-induced GC apoptosis and reactivate follicular cells in a mouse model of POF [51,52]. However, stem cell transplantation exhibits a low survival rate and is hindered by limited differentiation ability. It has not yet been widely tested in clinical practice, and its therapeutic effectiveness warrants further investigation [53].

5. Conclusion

This study demonstrates that α-SNAP knockdown can inhibit cisplatin-induced overactivation and loss of primordial follicles by inhibiting the PI3K/Akt/mTORC1 pathway. In vitro experiments showed that α-SNAP knockdown did not affect cisplatin-induced apoptosis or the inhibition of tumor GC proliferation. Some studies have reported that mTOR inhibitors can attenuate cisplatin resistance [54–56], implying that α-SNAP knockdown may enhance the anticancer effect of cisplatin by inhibiting the mTORC1 pathway. Therefore, knocking down α-SNAP could offer a novel strategy for preventing ovarian damage induced by chemotherapy.

Moreover, α-SNAP may emerge as a novel target within the PI3K/Akt/mTORC1 pathway for in vitro activation of primordial follicles. However, owing to variations in follicular growth regulatory mechanisms across different stages and the presence of complex interactions between follicles, our conclusions warrant repeated validation and necessitate further elucidation of the mechanism of α-SNAP in future studies.

Funding statement

This work was supported by the Basic Research Project 202102010293 to Ying Qin from the Guangzhou Municipal Science and Technology Bureau, and the Doctor's Fund 2019BS034 to Ying Qin from the Guangzhou Women and Children's Medical Center, China.

Data availability statement

Data will be made available on request.

Ethics approval

This study was approved by the Ethics Committee of the Nanfang Hospital Animal Ethics Committee.

CRediT authorship contribution statement

Ying Qin: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Canliang Wen:** Resources. **Bilan Hu:** Software, Resources, Formal analysis, Conceptualization. **Huijiao Wu:** Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32802.

References

[1] K.R. Hansen, N.S. Knowlton, A.C. Thyer, J.S. Charleston, M.R. Soules, N.A. Klein, A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause, Hum. Reprod. 23 (3) (2008) 699–708.
[2] M.N. Liu, K. Zhang, T.M. Xu, The role of BMP15 and GDF9 in the pathogenesis of primary ovarian insufficiency, Hum. Fertil. 24 (5) (2021) 325–332.
[3] F.C. de Castro, M.H. Cruz, C.L. Leal, Role of growth differentiation factor 9 and bone morphogenetic protein 15 in ovarian function and their importance in mammalian female fertility - a review, Asian-Australas. J. Anim. Sci. 29 (8) (2016) 1065–1074.
[4] H.W.R. Li, S.M. Nelson, Clinical application of AMH measurement in assisted reproduction, Front. Endocrinol. 11 (2020) 606744.
[5] N.V. Guerrero, R.H. Singh, A. Manatunga, G.T. Berry, R.D. Steiner, L.J. Elsas, 2nd. Risk factors for premature ovarian failure in females with galactosemia, J. Pediatr. 137 (6) (2000) 833–841.
[6] M. Liu, Y. Qiu, Z. Xue, R. Wu, J. Li, X. Niu, J. Yuan, Y. Wang, Q. Wu, Small extracellular vesicles derived from embryonic stem cells restore ovarian function of premature ovarian failure through PI3K/AKT signaling pathway, Stem Cell Res. Ther. 11 (1) (2020) 3.
[7] W. Dai, H. Yang, B. Xu, T. He, L. Liu, X. Ma, J. Ma, G. Yang, R. Si, X. Pei, X. Du, X. Fu, Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) alleviate excessive autophagy of ovarian granular cells through VEGFA/PI3K/AKT/mTOR pathway in premature ovarian failure rat model, J. Ovarian Res. 16 (1) (2023) 198.
[8] A.M. Martelli, C. Evangelisti, F. Chiarini, J.A. McCubrey, The phosphatidylinositol 3-kinase/Akt/mTOR signaling network as a therapeutic target in acute myelogenous leukemia patients, Oncotarget 1 (2) (2010) 89–103.
[9] A. Brech, T. Ahlquist, R.A. Lothe, H. Stenmark, Autophagy in tumour suppression and promotion, Mol. Oncol. 3 (4) (2009) 366–375.
[10] H. Zhang, S. Risal, N. Gorre, K. Busayavalasa, X. Li, Y. Shen, B. Bosbach, M. Brannstrom, K. Liu, Somatic cells initiate primordial follicle activation and govern the development of dormant oocytes in mice, Curr. Biol. 24 (21) (2014) 2501–2508.
[11] J. Guo, T. Zhang, Y. Guo, T. Sun, H. Li, X. Zhang, H. Yin, G. Cao, Y. Yin, H. Wang, L. Shi, X. Guo, J. Sha, J.J. Eppig, Y.Q. Su, Oocyte stage-specific effects of MTOR determine granulosa cell fate and oocyte quality in mice, Proc. Natl. Acad. Sci. U. S. A. 115 (23) (2018) E5326–E5333.
[12] P. Reddy, L. Liu, D. Adhikari, K. Jagarlamudi, S. Rajareddy, Y. Shen, C. Du, W. Tang, T. Hamalainen, S.L. Peng, Z.J. Lan, A.J. Cooney, I. Huhtaniemi, K. Liu, Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool, Science 319 (5863) (2008) 611–613.
[13] D. Adhikari, S. Risal, K. Liu, Y. Shen, Pharmacological inhibition of mTORC1 prevents over-activation of the primordial follicle pool in response to elevated PI3K signaling, PLoS One 8 (1) (2013) e53810.
[14] R. Jahn, T.C. Sudhof, Membrane fusion and exocytosis, Annu. Rev. Biochem. 68 (1999) 863–911.
[15] R. Jahn, R.H. Scheller, SNAREs–engines for membrane fusion, Nat. Rev. Mol. Cell Biol. 7 (9) (2006) 631–643.
[16] H. Lu, D. Bilder, Endocytic control of epithelial polarity and proliferation in Drosophila, Nat. Cell Biol. 7 (12) (2005) 1232–1239.
[17] A.E. Rodriguez-Fraticelli, J. Bagwell, M. Bosch-Fortea, G. Boncompain, N. Reglero-Real, M.J. Garcia-Leon, G. Andres, M.L. Toribio, M.A. Alonso, J. Millan, F. Perez, M. Bagnat, F. Martin-Belmonte, Developmental regulation of apical endocytosis controls epithelial patterning in vertebrate tubular organs, Nat. Cell Biol. 17 (3) (2015) 241–250.

[18] F.A. Bustamante-Barrientos, M. Mendez-Ruette, L. Molina, T. Koning, P. Ehrenfeld, C.B. Gonzalez, U. Wyneken, R. Henzi, L.F. Batiz, Alpha-SNAP (M105I) mutation promotes neuronal differentiation of neural stem/progenitor cells through overactivation of AMPK, Front. Cell Dev. Biol. 11 (2023) 1061777.
[19] Y. Miao, J. Bhushan, A. Dani, M. Vig, Na(+) influx via Orai1 inhibits intracellular ATP-induced mTORC2 signaling to disrupt CD4 T cell gene expression and differentiation, Elife 6 (2017).
[20] N.G. Naydenov, G. Harris, V. Morales, A.I. Ivanov, Loss of a membrane trafficking protein alphaSNAP induces non-canonical autophagy in human epithelia, Cell Cycle 11 (24) (2012) 4613–4625.
[21] Y. Qin, A. Iwase, T. Bayasula Murase, C. Ishida, N. Kato, T. Nakamura, S. Osuka, S. Takikawa, M. Goto, T. Kotani, F. Kikkawa, Protective effects of mangafodipir against chemotherapy-induced ovarian damage in mice, Reprod. Biol. Endocrinol. 16 (1) (2018) 106.
[22] Y. Nishi, T. Yanase, Y. Mu, K. Oba, I. Ichino, M. Saito, M. Nomura, C. Mukasa, T. Okabe, K. Goto, R. Takayanagi, Y. Kashimura, M. Haji, H. Nawata, Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor, Endocrinology 142 (1) (2001) 437–445.
[23] A. Ai, Z. Tang, Y. Liu, S. Yu, B. Li, H. Huang, X. Wang, Y. Cao, W. Zhang, Characterization and identification of human immortalized granulosa cells derived from ovarian follicular fluid, Exp. Ther. Med. 18 (3) (2019) 2167–2177.
[24] M. Kano, A.E. Sosulski, L. Zhang, H.D. Saatcioglu, D. Wang, N. Nagykery, M.E. Sabatini, G. Gao, P.K. Donahoe, D. Pepin, AMH/MIS as a contraceptive that protects the ovarian reserve during chemotherapy, Proc. Natl. Acad. Sci. U. S. A. 114 (9) (2017) E1688–E1697.
[25] M. Kanatsu-Shinohara, J. Lee, T. Miyazaki, H. Morimoto, T. Shinohara, Adeno-associated-virus-mediated gene delivery to ovaries restores fertility in congenital infertile mice, Cell Rep Med 3 (5) (2022) 100606.
[26] H. Jang, O.H. Lee, Y. Lee, H. Yoon, E.M. Chang, M. Park, J.W. Lee, K. Hong, J.O. Kim, N.K. Kim, J.J. Ko, D.R. Lee, T.K. Yoon, W.S. Lee, Y. Choi, Melatonin prevents cisplatin-induced primordial follicle loss via suppression of PTEN/AKT/FOXO3a pathway activation in the mouse ovary, J. Pineal Res. 60 (3) (2016) 336–347.
[27] R. Wang, L. Wang, L. Wang, Z. Cui, F. Cheng, W. Wang, X. Yang, FGF2 is protective towards cisplatin-induced KGN cell toxicity by promoting FTO expression and autophagy, Front. Endocrinol. 13 (2022) 890623.
[28] D.C. Woods, C. Alvarez, A.L. Johnson, Cisplatin-mediated sensitivity to TRAIL-induced cell death in human granulosa tumor cells, Gynecol. Oncol. 108 (3) (2008) 632–640.
[29] Y. Wu, C. Ma, H. Zhao, Y. Zhou, Z. Chen, L. Wang, Alleviation of endoplasmic reticulum stress protects against cisplatin-induced ovarian damage, Reprod. Biol. Endocrinol. 16 (1) (2018) 85.
[30] E.M. Chang, E. Lim, S. Yoon, K. Jeong, S. Bae, D.R. Lee, T.K. Yoon, Y. Choi, W.S. Lee, Cisplatin induces overactivation of the dormant primordial follicle through PTEN/AKT/FOXO3a pathway which leads to loss of ovarian reserve in mice, PLoS One 10 (12) (2015) e0144245.
[31] D.C. Fingar, C.J. Richardson, A.R. Tee, L. Cheatham, C. Tsou, J. Blenis, mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E, Mol. Cell Biol. 24 (1) (2004) 200–216.
[32] J. Wang, J. Wu, Y. Zhang, J. Zhang, W. Xu, C. Wu, P. Zhou, Growth hormone protects against ovarian granulosa cell apoptosis: alleviation oxidative stress and enhancement mitochondrial function, Reprod. Biol. 21 (2) (2021) 100504.
[33] A. Al-Shahat, M.A.E. Hulail, N.M.M. Soliman, T. Khamis, L.M. Fericean, A.H. Arisha, R.S. Moawad, Melatonin mitigates cisplatin-induced ovarian dysfunction via altering steroidogenesis, inflammation, apoptosis, oxidative stress, and PTEN/PI3K/Akt/mTOR/AMPK signaling pathway in female rats, Pharmaceutics 14 (12) (2022).
[34] X. Zhang, L. Zhang, S. Huo, J. Wang, S. Cui, Neonatal superior ovarian nerve transection inhibits follicle development by enhancing follicular atresia and suppressing granulosa cell proliferation in rats, Reprod. Fertil. Dev. 22 (7) (2010) 1148–1158.
[35] K. Oktay, R.S. Schenken, J.F. Nelson, Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat, Biol. Reprod. 53 (2) (1995) 295–301.
[36] M. Pargianas, I. Kosmas, K. Papageorgiou, C. Kitsou, A. Papoudou-Bai, A. Batistatou, S. Markoula, S. Salta, A. Dalkalitsis, S. Kolibianakis, B.C. Tarlatzis, I. Georgiou, T.M. Michaelidis, Follicle inhibition at the primordial stage without increasing apoptosis, with a combination of everolimus, verapamil, Mol. Biol. Rep. 47 (11) (2020) 8711–8726.
[37] T. Ekiz Yilmaz, M. Tasdemir, M. Kaya, N. Arican, B. Ahishali, The effects of magnesium sulfate on cyclophosphamide-induced ovarian damage: folliculogenesis, Acta Histochem. 122 (2) (2020) 151470.
[38] J. Li, H. Long, Y. Cong, H. Gao, Q. Lyu, S. Yu, Y. Kuang, Quercetin prevents primordial follicle loss via suppression of PI3K/Akt/Foxo3a pathway activation in cyclophosphamide-treated mice, Reprod. Biol. Endocrinol. 19 (1) (2021) 63.
[39] H. Roness, I. Spector, Y. Leichtmann-Bardoogo, A.M. Savino, S. Dereh-Haim, D. Meirow, Pharmacological administration of recombinant human AMH rescues ovarian reserve and preserves fertility in a mouse model of chemotherapy, without interfering with anti-tumoural effects, J. Assist. Reprod. Genet. 36 (9) (2019) 1793–1803.
[40] K. Jankowska, Premature ovarian failure, Prz Menopauzalny 16 (2) (2017) 51–56.
[41] M.A. Fenwick, P.R. Hurst, Immunohistochemical localization of active caspase-3 in the mouse ovary: growth and atresia of small follicles, Reproduction 124 (5) (2002) 659–665.
[42] S.J. Tan, L.J. Lee, C.R. Tzeng, C.W. Wang, M.I. Hsu, C.H. Chen, Targeted anti-apoptosis activity for ovarian protection against chemotherapy-induced ovarian gonadotoxicity, Reprod. Biomed. Online 29 (5) (2014) 612–620.
[43] A. Akdemir, B. Zeybek, L. Akman, A.M. Ergenoglu, A.O. Yeniel, O. Erbas, A. Yavasoglu, M.C. Terek, D. Taskiran, Granulocyte-colony stimulating factor decreases the extent of ovarian damage caused by cisplatin in an experimental rat model, J Gynecol Oncol 25 (4) (2014) 328–333.
[44] A.K. Jensen, K.T. Macklon, J. Fedder, E. Ernst, P. Humaidan, C.Y. Andersen, 86 successful births and 9 ongoing pregnancies worldwide in women transplanted with frozen-thawed ovarian tissue: focus on birth and perinatal outcome in 40 of these children, J. Assist. Reprod. Genet. 34 (3) (2017) 325–336.
[45] H.C.F. Moore, J.M. Unger, K.A. Phillips, F. Boyle, E. Hitre, A. Moseley, D.J. Porter, P.A. Francis, L.J. Goldstein, H.L. Gomez, C.S. Vallejos, A.H. Partridge, S. R. Dakhil, A.A. Garcia, J.R. Gralow, J.M. Lombard, J.F. Forbes, S. Martino, W.E. Barlow, C.J. Fabian, L.M. Minasian, F.L. Meyskens, R.D. Gelber, G. N. Hortobagyi, K.S. Albain, Final analysis of the prevention of early menopause study (POEMS)/SWOG intergroup S0230, J. Natl. Cancer Inst. 111 (2) (2019) 210–213.
[46] Z. Blumenfeld, E. Dann, GnRH agonist for the prevention of chemotherapy-induced ovarian failure in lymphoma, J. Clin. Oncol. 31 (29) (2013) 3721.
[47] S.J. Lee, L.R. Schover, A.H. Partridge, P. Patrizio, W.H. Wallace, K. Hagerty, L.N. Beck, L.V. Brennan, K. Oktay, O. American Society of Clinical, American Society of Clinical Oncology recommendations on fertility preservation in cancer patients, J. Clin. Oncol. 24 (18) (2006) 2917–2931.
[48] Ethics Committee of American Society for Reproductive, M, Fertility preservation and reproduction in patients facing gonadotoxic therapies: a committee opinion, Fertil. Steril. 100 (5) (2013) 1224–1231.
[49] K. Oktay, B.E. Harvey, A.H. Partridge, G.P. Quinn, J. Reinecke, H.S. Taylor, W.H. Wallace, E.T. Wang, A.W. Loren, Fertility preservation in patients with cancer: ASCO clinical practice guideline update, J. Clin. Oncol. 36 (19) (2018) 1994–2001.
[50] K. Hancke, O. Strauch, C. Kissel, H. Gobel, W. Schafer, D. Denschlag, Sphingosine 1-phosphate protects ovaries from chemotherapy-induced damage in vivo, Fertil. Steril. 87 (1) (2007) 172–177.
[51] S.A. Mohamed, S.M. Shalaby, M. Abdelaziz, S. Brakta, W.D. Hill, N. Ismail, A. Al-Hendy, Human mesenchymal stem cells partially reverse infertility in chemotherapy-induced ovarian failure, Reprod. Sci. 25 (1) (2018) 51–63.
[52] Y. He, D. Chen, L. Yang, Q. Hou, H. Ma, X. Xu, The therapeutic potential of bone marrow mesenchymal stem cells in premature ovarian failure, Stem Cell Res. Ther. 9 (1) (2018) 263.
[53] Y.X. Fu, J. Ji, F. Shan, J. Li, R. Hu, Human mesenchymal stem cell treatment of premature ovarian failure: new challenges and opportunities, Stem Cell Res. Ther. 12 (1) (2021) 161.

[54] B.B. Ma, V.W. Lui, E.P. Hui, C.P. Lau, K. Ho, M.H. Ng, S.H. Cheng, S.W. Tsao, A.T. Chan, The activity of mTOR inhibitor RAD001 (everolimus) in nasopharyngeal carcinoma and cisplatin-resistant cell lines, Invest. N. Drugs 28 (4) (2010) 413–420.
[55] J. Sheng, L. Shen, L. Sun, X. Zhang, R. Cui, L. Wang, Inhibition of PI3K/mTOR increased the sensitivity of hepatocellular carcinoma cells to cisplatin via interference with mitochondrial-lysosomal crosstalk, Cell Prolif. 52 (3) (2019) e12609.
[56] X. Liang, C. Yu, Y. Tian, X. Xiang, Y. Luo, Inhibition of STX17-SNAP29-VAMP8 complex formation by costunolide sensitizes ovarian cancer cells to cisplatin via the AMPK/mTOR signaling pathway, Biochem. Pharmacol. 212 (2023) 115549.