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Chk1/2 inhibitor AZD7762 blocks the growth of preantral follicles by inducing apoptosis, suppressing proliferation, and interfering with the cell cycle in granulosa cells

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Summary. Background. Checkpoint kinases 1/2 (Chk1/2) have an important role in somatic cell development and oocyte meiotic maturation. However, the role of Chk1/2 in folliculogenesis has not been fully elucidated. The aim of this study was to assess the effects of Chk1/2 inhibition on ovarian folliculogenesis and granulosa cell development in mice.

Methods. Preantral follicles (100-120 μm) and granulosa cells from pre-ovulatory follicles (pre-GCs) of mice were isolated and cultured with or without Chk1/2 inhibitor AZD7762. Preantral follicles were cultured for 96h. Then, follicle morphology and follicular growth were assessed every 48h. Granulosa cells were cultured for 48h with or without AZD7762, after which cell apoptosis, cell proliferation, and cell cycle analysis were assessed; meanwhile, the mRNA expression of *PCNA* and *Bax* were measured by real-time RT-PCR, and PCNA and Bax protein were measured by Western blot.

Results. Compared with control follicles, AZD7762 inhibited growth of preantral follicles ($P<0.05$). Furthermore, inhibition of Chk1/2 significantly induced apoptosis ($P<0.05$) and inhibited the proliferation of granulosa cells ($P<0.01$), arrested cell cycle at S and G2/M phases, and decreased G1 phase fraction ($P<0.001$). Also, the expression of PCNA mRNA and protein were reduced ($P<0.01$), while Bax mRNA and protein were increased ($P<0.05$) post AZD7762 treatment in granulosa cells.

Conclusions. This study revealed that Chk1 and Chk2 have a crucial role during preantral follicular development by regulating the proliferation and apoptosis of granulosa cells.

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Introduction

In mammals, oocytes arrested in the diplotene stage of the first meiotic prophase are surrounded by a single, squamous layer of somatic cells to form a finite population of non-growing primordial follicles (Sen and Caiazza, 2013). Primary follicles are recruited from the primordial pool as oocytes grow. These cells continue to proliferate to form many layers surrounding the oocyte and eventually become granulosa cells (Candelaria et al., 2020). This transition is associated with participation in the subsequent phases of follicular growth, as the measured recruitment of primordial follicles from the resting pool of follicles is crucial for the development of folliculogenesis throughout the reproductive lifespan of mammals (Rimon-Dahari et al., 2016). However, apoptosis reduces this endowment by two-thirds before birth. In addition, granulosa cell apoptosis is the main cause of follicular atresia at different stages of their growth in the mammalian ovaries (Findlay et al., 2015; Zhou et al., 2019).

When atresia occurs, pyknotic nuclei are first observed in granulosa cells. Then a detachment of granulosa cell layer and fragmentation of basal membrane occurs, ultimately resulting in hypertrophied thecal cells and disruption of thecal integration and thecal vessels (Zhang et al., 2019). Granulosa cell apoptosis may occur much earlier than the morphological changes in follicular atresia, which can be observed only when granulosa cell apoptosis reaches a certain degree (Zhang et al., 2019). Generally,

Abbreviations. Chk1/2, checkpoint kinases 1/2; pre-GCs, granulosa cells from pre-ovulatory follicles.

proliferation and differentiation of granulosa cells lead to follicular maturation and ovulation, whereas apoptosis and degeneration of granulosa cells result in follicular atresia (da Silva Bitecourt et al., 2018). Many apoptosis-related factors have been implicated in follicular atresia, including death ligands and receptors, intracellular pro- and anti-apoptotic molecules, cytokines, growth factors, and several apoptosis-related genes (Khristi et al., 2018). Although new regulatory factors are continuously being identified, knowledge of the signaling networks that function during granulosa cell apoptosis remains limited.

Checkpoint kinases are threonine/serine that can be divided into two subtypes, Chk1 and Chk2, which have a critical role in DNA damage responses, cell cycle control, and cell survival (Chen and Poon, 2008). In response to DNA damage, Chk1 and Chk2 are activated by PI3 kinase-related kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), respectively, aiming at many downstream substrates that coordinate cell cycle checkpoint activation, DNA restitution, and apoptosis (Yang et al., 2017). Moreover, Chk1/2 have also been implicated in anaphase entry, chromosome condensation, and maintenance of genome integrity in somatic cells in the absence of DNA damage (Chaurasiya et al., 2020). Chk1 knockout mice are embryonically lethal, suggesting that Chk1 is an important molecule during early embryonic development (Schuler et al., 2019). Moreover, embryonic stem cells specific Chk1 knock-out mice display premature activation of Cdc2/cyclin B and mitotic catastrophe (Niida et al., 2005). At the same time, Chk2-deficient cells show significant defects in UV-induced apoptosis and G1/S arrest (Tanoue et al., 2018). Our previous study showed that the expression of Chk1 fluctuates during follicular development and plays important roles in mice oocyte meiosis (Liu et al., 2021); meanwhile, Chk2 is essential for oocyte meiosis under DNA damage or normal conditions (Bolcun-Filas et al., 2014; Dai et al., 2014), suggesting the importance of Chk1/2 in mammalian folliculogenesis; yet, the exact role in follicular development is not fully understood.

In the present study, Chk1/2 inhibitor (AZD7762) was used to further investigate the role of Chk1/2 during preantral follicular development and cellular proliferation and apoptosis.

Materials and methods

Animals

Female Kunming white mice of 12-14 (9-10g) or 21-23 (12-14g) days old were obtained from the Centre of Laboratory Animals of Hubei Province (Wuhan, PR China). Mice were housed in an environment with a temperature of 24±1°C, relative humidity of 50±1%, and a light/dark cycle of 12/12h, and given food and water ad libitum. All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of the Hubei Research Center of Exper-imental Animals and conducted according to the AAALAC and the IACUC guidelines (Approval ID: SCXK (Hubei) 2008-0005).

Isolation and culture of preantral follicles and granulosa cells

Preantral follicles (100-120 μm) obtained from the ovaries of 12-14 days old female mice were gently separated using the 1 ml syringe needle under a stereomicro-scope (CKX41SF; Olympus Optical Technology Philippines Inc., Lapu Lapu City, Philippines), and observed under an inverted microscope (TE2000 U; Nikon). Follicles with two or three layers of granulosa cells and a diameter between 100-120 μm were collected and cultured in follicle culture media, which consisted of 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Grand Island, NY, USA), 10 μg/mL of ITS (0.55 mg/mL human transferrin, 1.0 mg/mL recombinant human insulin, and 0.5 μg/mL sodium selenite; Sigma-Aldrich, St. Louis, MO, USA) and 100 mIU/mL of follicle-stimulating hormone (FSH, Sigma-Aldrich, St. Louis, MO, USA) in α-Minimum Essential Media (α-MEM, Gibco, Grand Island, NY, USA). Follicles were cultured in 96-well plates with one follicle per well in 100 μL follicle culture media, covered by mineral oil in a humidified atmosphere containing 5%CO_2/95% air at 37°C for 48h. After that, follicles were cul-tured in follicle culture media with or without 1 μM of AZD7762 (Axon Medchem BV, Groningen, Netherland; Cat. No. Axon 1399) for an additional 96h. Follicles were measured every other day under microscopy (TE2000 U; Nikon) for assessment of morphology and follicular growth, as indicated by follicular diameter (F.D). Every 2 days, 50 μL of follicle culture media was removed and replaced with fresh medium. The concentration of the Chk1/2 inhibitor used in our study was selected based on previous studies (Dai et al., 2014; Liu et al., 2021).

GCs from pre-ovulatory follicles (pre-GCs) were taken from ovaries of 21-23 days old mice injected with 10 IU pregnant-mare serum gonadotropin (PMSG, SanSheng, Ningbo, China) for 44-48h and cultured as previously described (Cao et al., 2018). Briefly, granulosa cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient F-12 (DMEM/F12; Gibco) medium with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). All cultures were maintained in DMEM/F12 medium in a humidified atmosphere containing 5%CO_2/95% air at 37°C.

Cell proliferation assay

Cell proliferation assay was measured using a WST-1 Cell Proliferation Assay kit (Beyotime, Wuhan, China). Briefly, granulosa cells were cultured in a 96-well culture plate (4×10^3 cells/well) for 24h. Cells were

then exposed to a gradually increased con-centration of AZD7762 (1, 5, 10, 20, and 50 μM) for 24h, 48h, and 72h. After each time point, 10 μL of freshly prepared WST 1 solution was added to each well, along with the culture medium. The absorbance of the samples was measured after 1h at 37°C using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm.

Cell cycle assay

For cell cycle analysis, pre-GCs were cultured in a 6-well culture plate with 0 μM or 1 μM AZD7762 for 48h. After being washed with PBS, the cells were digested and harvested using the Cell Cycle Detection kit (KeyGen Biotech Co., Ltd., Nanjing, Chi-na). Cells were then fixed in 70% ethanol at 4°C overnight, washed with PBS, and in-cubated with 100 μL RNase A at 37°C for 30 min. Then, the cells were stained with 400 μL PI in the dark for 30 min at 4°C and analyzed through flow cytometry using a BD FACS Calibur [excitation wavelength (Ex), 488 nm; emission wavelength (Em), 530 nm; Becton Dickinson, Mountain View, CA, USA].

Cell apoptosis assay

Pre-GCs were cultured in 6-well culture plate by adding 0 μM or 1 μM AZD7762 for 48h. After being washed in PBS, cells were digested and harvested. Annexin V-FITC/PI kit (AntGene, Wuhan, China) was used to detect the proportion of apoptotic cells according to the manufacturer's instructions. Cells were incubated in AnnexinV-FITC and PI solution at room temperature in the dark for 15 min, after which a 300 μL of 1×binding buffer was added to each sample. Flow cytometric analysis was planned through a BD FACS Calibur (Becton, Dickinson and Company, Franklin Lakes, NJ, USA; Ex, 488 nm and Em, 530 nm). Cells that stained positive for annexin V-FITC were calculated as apoptotic cells.

Real-time RT-PCR analysis

Real-time RT-PCR (RT-qPCR) analysis was performed to confirm that inhibition of Chk1/2 by AZD7762 could regulate genes expression, *PCNA*, and *Bax*. Pre-GCs were cultured with 0 μM or 1 μM AZD7762 for 48h, after which a total RNA was extracted using the RNAprep pure Cell/Bacteria Kit (TIANGEN, Beijing, China), and in vitro transcription was performed through RevertAid^{TM} First-strand cDNA Synthesis kit (Thermo, Waltham, MA, USA). RT-qPCR was quantified using special primer pairs (Table 1) and QuantiFast® SYBR® Green RT-PCR kit (QIAGEN, Duesseldorf, Germany) on the Roche LightCycler® 480 according to the manufacturer's instructions. The gene expression results were normalized to the basal level of β-actin. The $2^{-\Delta\Delta Ct}$ was used to calculate the relative fold change of each gene.

Western blot

After AZD7762 treatment for 48h, cells were harvested in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which contained 10 mg/mL protease in-hibitors cocktail (Santa Cruz Biotechnology) and 10 mM phenylmethylsulfonyl fluoride (PMSF; Ding-Guo, Beijing, China). The concentration of total protein was determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, USA), and 20 mg of total protein was subjected to gel electrophoresis as previously described (Cao et al., 2018). Monoclonal mouse anti-β-actin IgG (1:1000 dilution; Santa Cruz Biotechnology), monoclonal mouse anti-PCNA IgG (1:600 dilution; Santa Cruz Biotechnology), and monoclonal rabbit anti-Bax IgG (1:1000 dilution; Epigentek, Farmingdale, NY, USA) were used as the primary antibody, and HRP-conjugated anti-mouse or rabbit secondary antibodies (1:2000 dilution; Boster, Wuhan) were used. The images were measured with a Gel Pro analyzer 4.0 (Media Cybernetics, Silver Spring, MD, USA). The scanning intensities of the Western blots were analyzed using ImageJ software to quantify the target bands compared to the corresponding β-actin bands.

Statistical analysis

Experiments were independently performed at least three times, and data are presented as mean±SD. Differences between each group were analyzed by one-way ANOVA followed by Tukey's Honesty Significant Difference (HSD) test using SPSS (Version 17.0; SPSS, Chicago, IL, USA); $P<0.05$ was regarded as a statistically sig-nificant difference.

Results

Chk1/2 are essential for preantral follicular development

In order to assess the role of Chk1/2 during follicular development, we cultured preantral follicles with Chk1/2 broad-spectrum inhibitor (AZD7762) *in vitro*. In the control group, the gradual growth of follicles was observed, while follicles cultured with AZD7762

Table 1. RT-qPCR primer pairs.

Genes	Primer sequences	
PCNA	Forward	AGACAGTGGAGTGGCTTTT
	Reverse	CCGAGACCTTAGCCACATT
Bax	Forward	CCAGGATGCGTCCACCAA
	Reverse	CAAAGTAGAAGAGGGCAACCAC
β-actin	Forward	CCCATCTACGAGGGCTAT
	Reverse	TGTCACGCACGATTTCC

showed no growth or cell number reduction (Fig. 1A,B, $P<0.05$). Meanwhile, as shown in Fig. 1C, the granulosa cells of follicles treated with AZD7762 around the outer layer showed cell shrinkage and weak connection between cells compared with the control follicles. Thus, we predicted that the arrested development of preantral follicles and abnormalities of the morphology of follicles might be related to the granulosa cells status, including proliferation and apoptosis.

Inhibition of Chk1/2 reduces granulosa cell proliferation

In order to study whether Chk1/2 affect granulosa cells proliferation, cells were cultured with different concentrations of AZD7762 for 24, 48, or 72h, respectively. When cells were cultured for 24h, the percentage of proliferation was similar, and no significant difference between the control group and 1 μM AZD7762 was seen ($P>0.05$); yet, the percentage of proliferation was

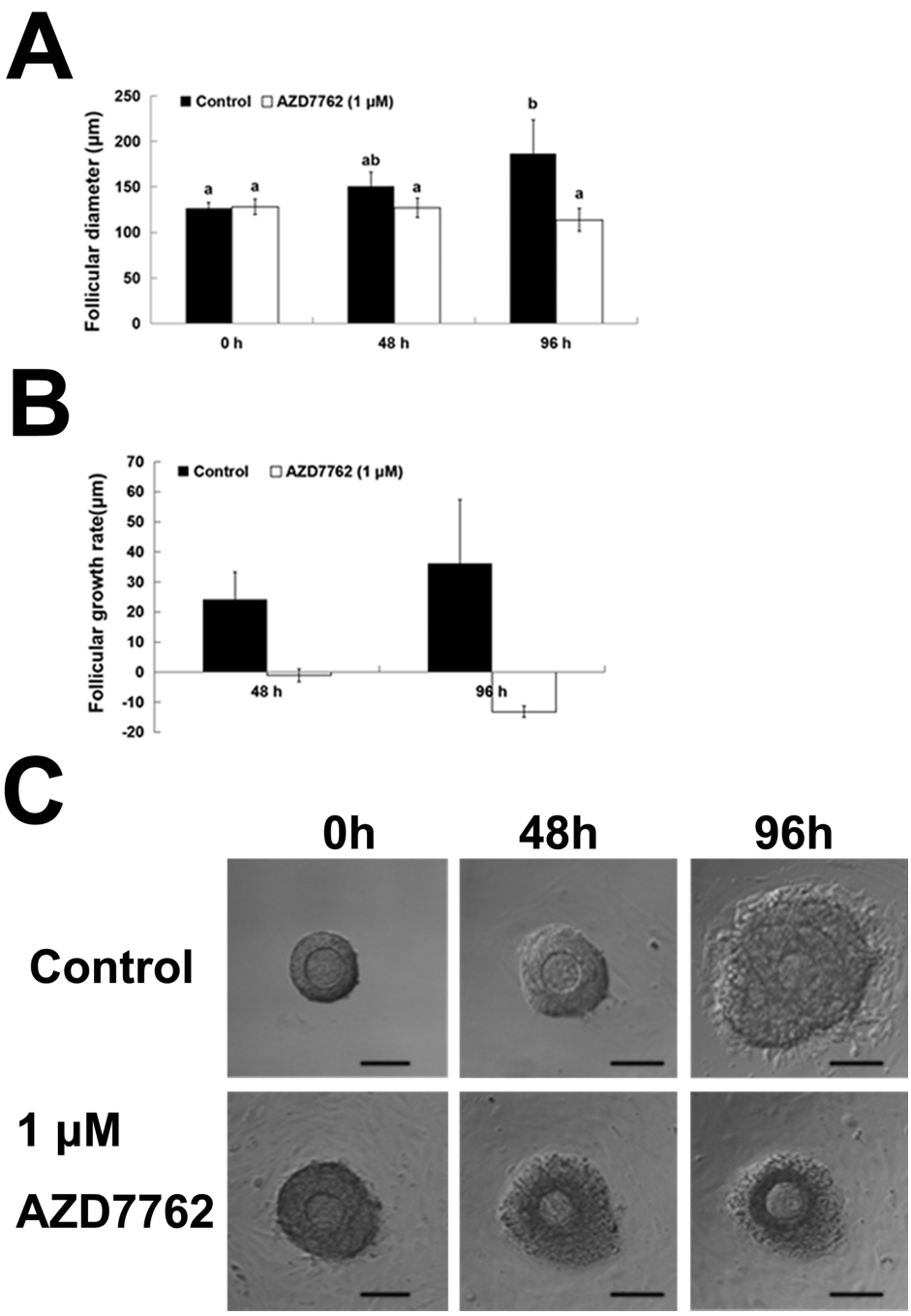

Fig. 1. Inhibition of Chk1/2 blocks the development of preantral follicles *in vitro*. **A.** Inhibition of Chk1/2 blocks follicular development. Follicles were cultured with 0 μM or 1 μM AZD7762 for 0h, 48h, and 96h. Fifty follicles per set and the value expressed by each bar represent mean± SD. a vs. b, $P<0.05$. **B.** The difference in follicular growth. The abscissa represents the time interval every 48h of culture. **C.** Abnormal morphology and structure of preantral follicles cultured with Chk1/2 inhibi-tor AZD7762. Scale bar: 100 μm.

significantly reduced when cells were treated with a higher concentration of AZD7762 (5, 10, 20, and 50 μM, $P<0.01$, Fig. 2A). However, after 48 or 72h, the percentage of proliferation was also significantly reduced in 1 μM AZD7762 (Fig. 2A, $P<0.01$), while no significant difference was found among a higher concentration of AZD7762 (5, 10, 20, and 50 μM, Fig. 2A). Therefore, granulosa cells cultured with 1 μM AZD7762 for 48h were selected for the following studies.

In addition, RT-qPCR was applied to detect the expression level of PCNA, which is a marker of proliferation. After culturing cells with AZD7762 for 48h, the expres-sion of PCNA mRNA was significantly decreased compared with control cells (Fig. 2B, $P<0.01$). Furthermore, Western blot results also showed decreased expression of PCNA protein in AZD7762 cultured cells (Fig. 2C, $P<0.01$).

Inhibition of Chk1/2 induces granulosa cell apoptosis

As shown in Fig. 3A, granulosa cells cultured with 1 μM AZD7762 for 48h showed abnormal cell morphology. As seen in Fig. 3B, the apoptosis rate in the AZD7762 group was significantly higher than in the control group ($P<0.05$). In addi-tion, the expression of Bax, a marker of apoptosis, was significantly up-regulated in both mRNA and protein levels (Fig. 3C,D, $P<0.001$ and $P<0.05$).

Inhibition of Chk1/2 arrests cell cycle at S and G2/M stages

Next, we explored the effect of Chk1/2 arrests of cell cycle at S and G2/M stages. As shown in Fig. 4, the percent of the G1 stage in the AZD7762 group was signifi-cantly lower than the control cells ($P<0.001$), while the percent of S and G2 stages were significantly increased in the AZD7762 group compared to control ($P<0.001$). Therefore, our results indicated that inhibition of Chk1/2 by AZD7762 suppressed the proliferation of cells and disturbed the normal cell cycle. Taken together, inhibition of Chk1/2 resulted in

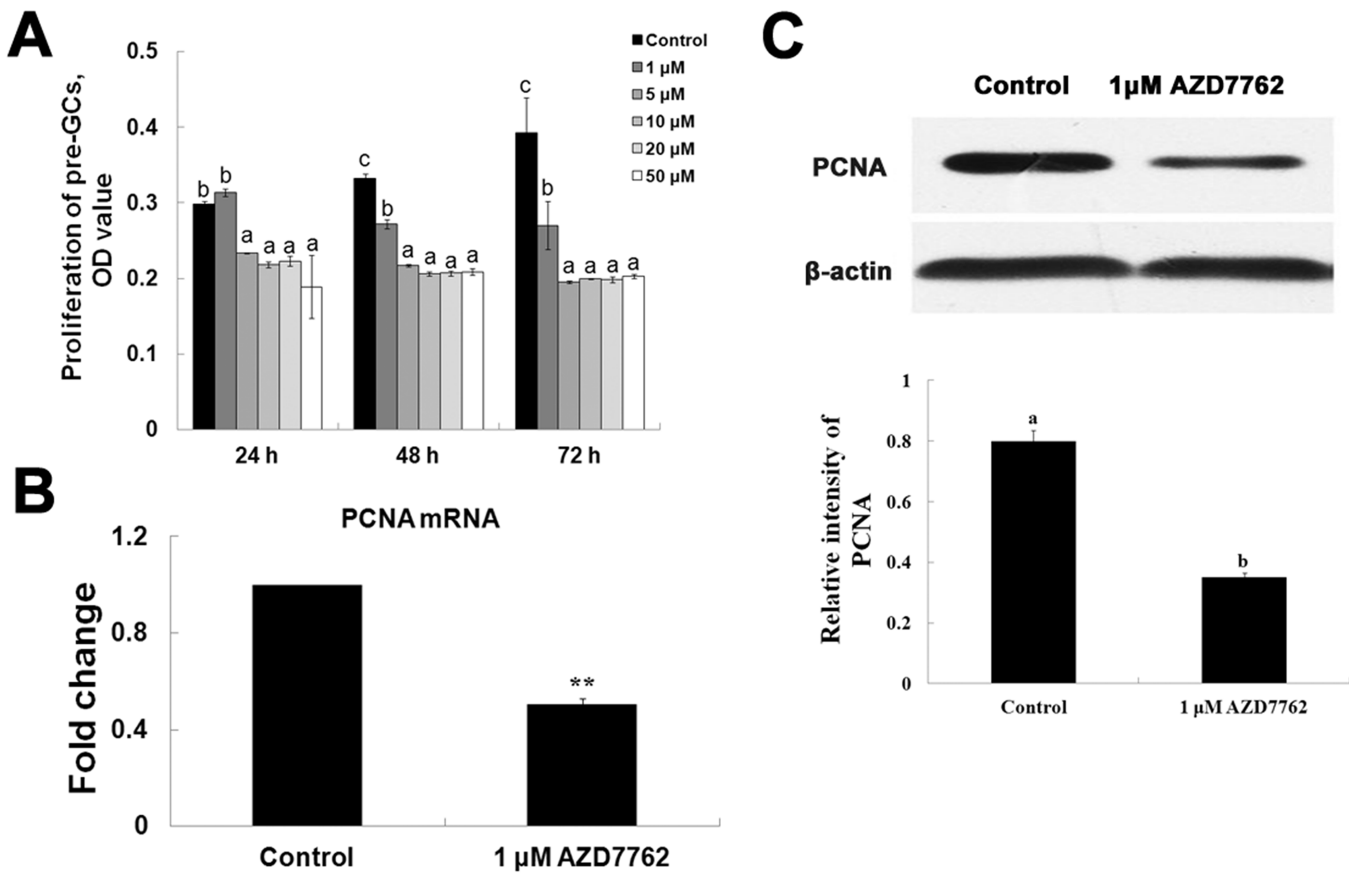

Fig. 2. Inhibition of Chk1/2 reduces granulosa cell proliferation. **A.** The proliferation of granulosa cells cultured with AZD7762. The value expressed by each bar represents mean± SD. 24h, a vs. b, $P<0.01$; 48h, a or b vs. c, $P<0.001$; 72h, a or b vs. c, $P<0.001$. **B.** Relative expression of *PCNA* mRNA in granulosa cells treated with AZD7762. Fold changes were calculated from β-actin normalized Ct values. The value expressed by each bar represents mean± SD. ** $P<0.01$. **C.** Relative expression of *PCNA* protein. The total amount of β-actin present in the lower set of lanes was used to standardize the amount of PCNA. The same batch of protein samples was used in Fig. 3D, so the lane of β-actin was the same. The value expressed by each bar represents the mean± SD. a vs. b, $P<0.01$.

inhibition of proliferation and promotion of apoptosis of granulosa cells.

Discussion

In this study, AZD7762 was used to inhibit the function of both Chk1 and Chk2. Preantral follicles treated with AZD7762 showed developmental abnormality. Moreover, granulosa cells treated with AZD7762 showed decreased cell growth, increased apoptosis, and abnormal cell cycle distributions. These results suggest that Chk1/2 have an important role in preantral follicular development and the growth of granulosa cells.

The development of preantral follicles includes oocyte growth, granulosa cell proliferation, differentiation, and apoptosis. However, more than 99% of follicles disappear, primarily due to the apoptosis of granulosa cells, and the majority of follicles become atretic during the early antral stage of development

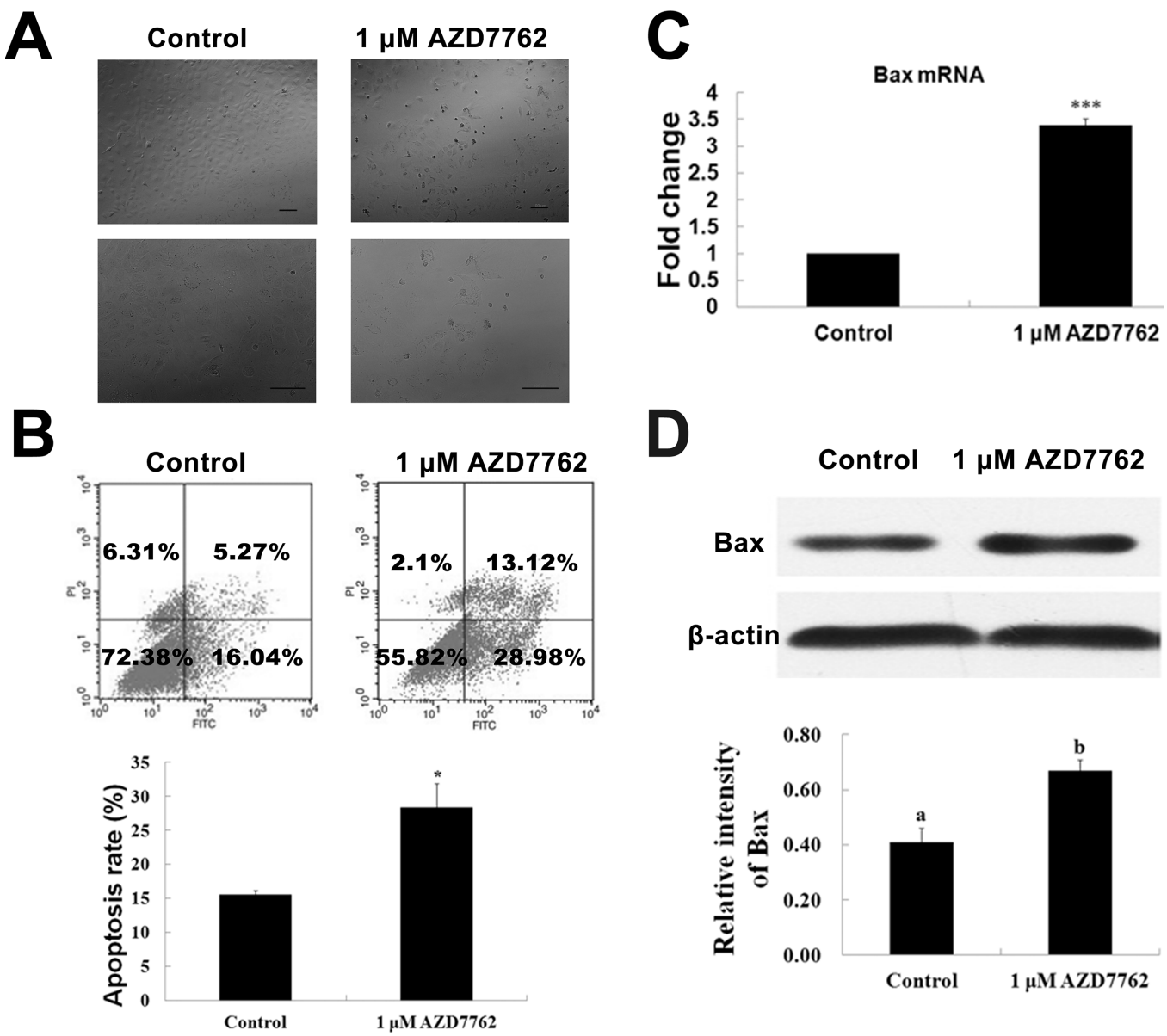

Fig. 3. Inhibition of Chk1/2 led to apoptosis of granulosa cells in vitro. **A.** Inhibition of Chk1/2 led to a contraction of the cytoplasm of cells during culture. Scale bar: 100 μm. **B.** The apoptosis rate of pre-GCs cultured with 0 μM or 1μM AZD7762 for 48h. The value expressed by each bar represents mean± SD. * P<0.05. **C.** Relative expression of *Bax* mRNA in granulosa cells treated with AZD7762. Fold changes were calculated from β-actin normalized Ct values. The value expressed by each bar repre-sents mean± SD. *** P<0.001. **D.** Relative expression of Bax protein. The total amount of β-actin present in the lower set of lanes was used to standardize the amount of Bax. The same batch of protein samples was used in Fig. 2C. The value expressed by each bar represents the mean± SD. a vs. b, indicate statistical difference (P<0.05).

(Hsueh et al., 2015). Thus, we selected the preantral follicles in this experiment, which were then treated with a Chk1/2 inhibitor to monitor the follicular development. Activated Chk1 and Chk2 have a full spectrum of substrates that are key cell cycle regulators. In the control group, the gradual growth of follicles was observed, while follicles cultured with AZD7762 showed no growth or even negative growth. These results suggested that Chk1/2 is essential for follicular development.

Gonadotropin can promote the differentiation of the granulosa cells, making them vulnerable to apoptosis. Thus, pre-GCs were selected to study the role of Chk1/2 in regulating the development of granulosa cells. The cells treated with AZD7762 showed decreased cell growth and increased cell apoptosis. Similarly, a previous study has sug-gested that Chk1 is required for mitotic progression and proliferation of Hela cells through negative regulation of polo-like kinase 1 Plk1 (Tang et al., 2006). Meanwhile, Chk1-depleted lobuloalveolar mammary epithelial cells do not proliferate and undergo apoptosis, suggesting that cell proliferation is important for apoptosis (Lam et al., 2004). Likewise, in our study, a proliferation of granulosa cells was significantly inhibited and showed an uncoordinated cell cycle. The link between apoptosis and proliferation suggests that death resulting from Chk1 depletion may involve mitotic alteration (Okada and Mak, 2004). However, in some circumstances, Chk2 appeared to be at least in part able to make up for the loss of Chk1 in some cells (Niida et al., 2010). Our preliminary studies of Chk1/2 inhibition in mouse oocytes supported this hypothesis (Liu et al., 2021). Moreover, the cell cycle of pre-GCs was disturbed by inhibition of Chk1/2 and showed increased G2 and S stages. As Chk1/2 are the key cell cycle checkpoint kinase, and the major function of Chk1 is to coordinate the cell cycle checkpoint response, including G1, S, G2/M, and M phase (Zhang and Hunter, 2014), Chk2 is needed for the optimal G2/M delay of G2 phase cells; Chk2- deficient cells show G1/S arrest (Nikitin et al., 2014). Our study showed that Chk1/2 might affect ovarian function by regulating the state (proliferation or apoptosis) of GCs

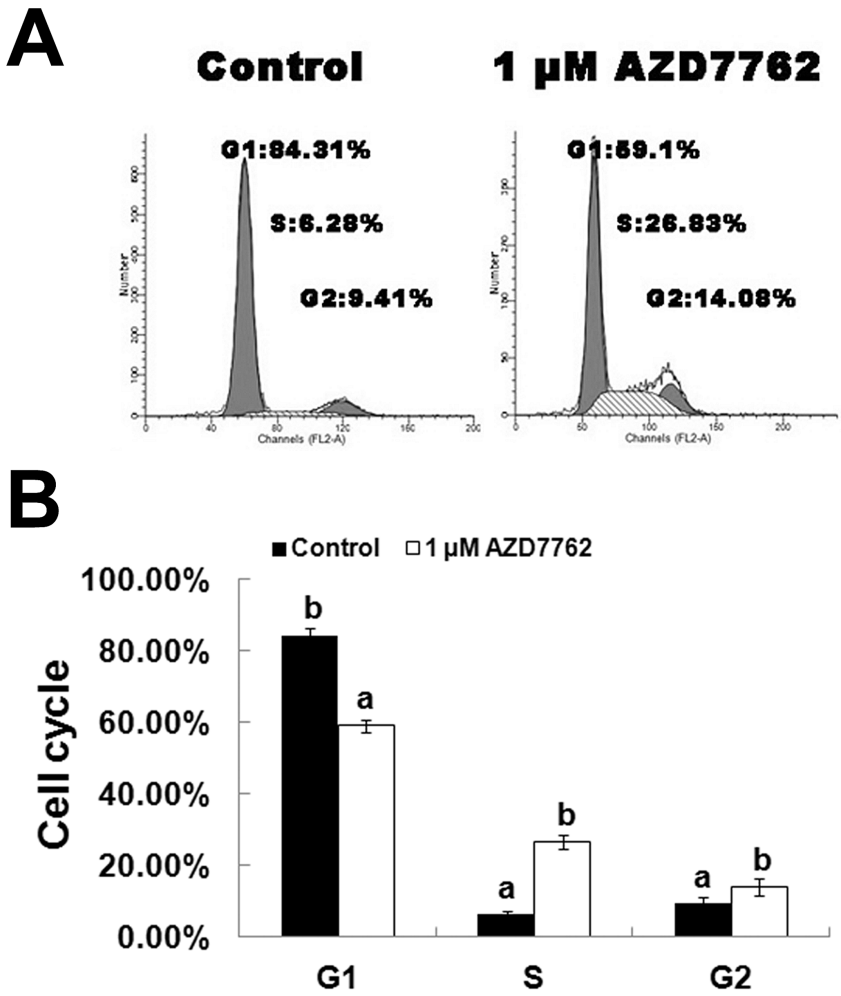

Fig. 4. Inhibition of Chk1/2 arrested cell cycle at G2 stage. **A.** The cell cycle of pre-GCs cultured with 0 μM and 1 μM AZD7762 for 48h. The data shown in this figure represent one of the repeats. **B.** The ratio of the value expressed by each bar represents mean± SD. a vs. b, P<0.001.

and the fate (growth or atresia) of follicular development.

Future studies should investigate the exact function of Chk1 and Chk2 in follicular development and the regulatory mechanism of the Chk1/2 network responsible for follicular development. Studies have shown that Chk1 is a potential target for treating cancer (Zhang and Hunter, 2014), so another important issue is evaluating the possibility of Chk1/2 in reducing follicular atresia. Therefore, we propose that Chk1/2 could represent an option for suppressing follicular atresia.

Conclusions

Our present results provide insight into the roles of Chk1/2 in mouse ovaries, including follicular development, granulosa cell proliferation, and apoptosis. These results suggest that Chk1/2 may have an important role in follicular development and ovarian functions. Furthermore, future research on the safe application of AZD7762 as drugs in clinical therapy of cancer (especially female patients) is warranted.

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Conflict of Interests. There is no conflict of interest for all authors.

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