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Crybb2 deficiency impairs fertility in female mice

Qian Gao [a,1], Li-Li Sun [b,a,1], Fen-Fen Xiang [c], Li Gao [d], Yin Jia [a], Jian-Rong Zhang [a], Hai-Bo Tao [a], Jun-Jie Zhang [e,*], Wen-Jie Li [a,*]

[a] Department of Laboratory Diagnosis, Changhai Hospital, Second Military Medical University, Shanghai 200433, PR China
[b] Aviation Medical Evaluation and Training Center of Airforce in Dalian, Dalian, Liaoning Province 116013, PR China
[c] Department of Laboratory Medicine, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200062, PR China
[d] Department of Pathology, Changhai Hospital, Second Military Medical University, Shanghai 200433, PR China
[e] Department of Obstetrics and Gynecology, Changhai Hospital, Second Military Medical University, Shanghai 200433, PR China

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ABSTRACT

Beta-B2-crystallin (CRYBB2), encoded by *Crybb2* gene, is a major protein in the mammalian eye lens that plays an important role in maintaining the transparency of the ocular lens. However, CRYBB2 also plays important roles in many extra-lenticular tissues and organs such as the retina, brain and testis. Our previous studies demonstrated that male *Crybb2* deficient (*Crybb2*$^{-/-}$) mice have reduced fertility compared with wild-type (WT) mice, while female *Crybb2*$^{-/-}$ mice exhibited reduced ovary weights and shorter estrous cycle percentages. Here we specifically investigated the role of CRYBB2 in the female reproductive system. Our studies revealed that ovaries from female *Crybb2*$^{-/-}$ mice exhibited significantly reduced numbers of primordial, secondary and pre-ovulatory follicles when compared with WT mice, while the rate of atretic follicles was also increased. Additionally, fewer eggs were collected from the oviduct of *Crybb2*$^{-/-}$ female mice after superovulation. Estrogen levels were higher in the metestrus and diestrus cycles of female *Crybb2*$^{-/-}$ mice, while progesterone levels were lower in diestrus cycles. Furthermore, the expression of survival and cell cycle genes, *Bcl-2*, *Cdk4* and *Ccnd2*, were significantly decreased in granulosa cells isolated from female *Crybb2*$^{-/-}$ mice, consistent with the predominant expression of CRYBB2 in ovarian granulosa cells. Our results reveal a critical role for CRYBB2 in female fertility and specific effects on the proliferation and survival status of ovarian granulosa cells.

1. Introduction

Beta-B2-crystallin (CRYBB2), encoded by *Crybb2* gene, is an important crystalline protein in the lens, which plays a critical role in maintaining lens transparency and refractive index. *Crybb2* expression is induced after birth [1], and also contributes to normal protein folding and stability as well as protein–protein interactions [2]. Our previous study generated a mouse model of age-related cataract by deleting *Crybb2* gene [3]. Ganguly et al. [4] reported that *Crybb2* is expressed in the cerebellum, olfactory bulb, cerebral cortex, and hippocampus. Liedtke et al. [2] also demonstrated that axonal regeneration is related to the movement of CRYBB2. In male mice, it has been reported that CRYBB2 is expressed as a microtubule-associated protein in interstitial cells and mature sperm in the testis, suggesting an important role in preventing germ cells from degeneration and maintaining their motility [5]. Moreover, we demonstrated a critical role for *Crybb2* in male fertility through the generation of *Crybb2*$^{-/-}$ mice [6]. We specifically observed that both proliferation and apoptosis were abnormal in testicular germ cells of *Crybb2*$^{-/-}$ mice, leading to a decrease in spermatogenesis and male reproductive function. In female mice, CRYBB2 is expressed in the ovary and we showed that female *Crybb2*$^{-/-}$ mice exhibited reduced ovary weights [7]. However, the mechanisms underlying a role for CRYBB2 function in female fertility are largely unknown.

Folliculogenesis is a complex process that is controlled by many factors. In every cycle, only a small portion of follicles are able to reach maturity for ovulation and most of the follicles will undergo atresia induced by apoptosis [8]. Granulosa cells play an important role in the process of follicular development mainly by interacting

* Corresponding authors at: Department of Laboratory Diagnosis, Changhai Hospital, Second Military Medical University, 168 Changhai Road, Shanghai 200433, PR China (W.-J. Li), Department of Obstetrics and Gynecology, Changhai Hospital, Second Military Medical University, 168 Changhai Road, Shanghai 200433, PR China (J.-J. Zhang).
E-mail addresses: zhangjj910@163.com (J.-J. Zhang), wenjieli@pku.org.cn (W.-J. Li).
[1] These authors contributed equally to this work.

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with the oocyte and producing sex steroids and a myriad of growth factors. As a result, these hormones and factors can directly regulate follicle development and ovulation [9,10].

In the present study, we investigated the effects of CRYBB2 on follicular development, proliferation and apoptosis of granulosa cells and the production of sex hormones, while exploring potential mechanisms.

2. Materials and methods

2.1. Animals

Wild type male and female C57BL/C mice were obtained from the Experimental Animal Center of the Second Military Medical University (Shanghai, China). *Crybb2* gene knockout mice were generated by the Genious Targeting Laboratory, Inc. (Stony Brook, NY) as previously described [11]. All mice were maintained on a 12 h light: 12 h darkness cycle in a pathogen-free mice room with free access to food and water *ad libitum*. Mice were sacrificed by cervical dislocation following anesthetization with ether. This study was conducted in accordance with institutional guidelines and approved by the Animal Care and Use Committee, Changhai hospital (permission number CH20110917-05).

2.2. Fertility assay

WT and *Crybb2*$^{-/-}$ female mice aged 11 weeks ($n = 8$) were caged with fertile wild-type C57BL/C male mice for 5 months. The number of litters and pups born from each mating cycle was recorded. The mean numbers of total pups and female pups per litters in the five-month period were also calculated.

2.3. Superovulation

WT and *Crybb2*$^{-/-}$ female mice (11 weeks, $n = 6$) underwent superovulation by intraperitoneal injection of 36 IU of pregnant mare's serum gonadotropin (PMSG; PROSPEC, Rehovot, Israel) followed by 5 IU of human chorionic gonadotropin (hCG; PROSPEC) 48 h later. Mice were sacrificed 18 h after the second injection. The eggs from the ampulla of the oviduct were isolated and counted, meantime the ovaries were collected from WT group and saline treated female WT mice to detect CRYBB2 expression using western blot. GAPDH was used as an internal control.

2.4. Granulosa cell culture

Six-week old WT and *Crybb2*$^{-/-}$ female mice ($n = 6$) in estrus were injected with 36 IU of PMSG to induce follicular development. Granulosa cells from small follicles (<5 mm) were collected by aspiration and filtered through a stainless steel filter (45 μm, Tokyo Screen Co., Ltd., Tokyo, Japan) to remove oocytes, then resuspended in 6-well plates in DMEM/F12 medium containing 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Life Technologies, Grand Island, NY) and 10% fetal bovine serum (GIBCO, Life Technologies). Granulosa cells were cultured at 37 °C in an incubator with 5% CO_2 and the medium was replaced 24 h after plating to remove any unattached cells [12].

2.5. Western blot analysis

Mouse granulosa cells were collected from six-week-old WT and *Crybb2*$^{-/-}$ female mice ($n = 6$) in the estrus. Equal amount of protein was loaded and resolved by 12% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE), and then transferred onto a PVDF membrane (Millipore Corporation, Bedford, MA). The membrane was blocked in 5% non-fat milk in TBST solution for 2 h at room temperature, followed by incubation with anti-beta-B2-crystallin (1:100 dilution, Santa Cruz Technology, Santa Cruz, CA) and anti-beta-actin antibodies (1:1000 dilution, Beyotime Institute of Biotechnology, Haimen, Jiangsu Province, China) at 4 °C overnight. The membrane was washed and incubated with HRP-conjugated anti-goat or anti-mouse secondary antibodies for 2 h. After several washes, the immunoblot was detected with enhanced chemiluminescence (Pierce Biotechnology) according to the manufacturer's instructions.

2.6. Immunofluorescence microscopy

Granulosa cells were collected from six-week-old WT and *Crybb2*$^{-/-}$ female mice ($n = 6$) in the estrus. Cells were fixed in 4% paraformaldehyde (37 °C) for 30 min, and permeabilized in 0.1% (v/v) Triton X-100 in PBS (pH 7.2) for 15 min. Cells were blocked with 5% (w/v) bovine serum albumin (BSA) for 30 min at 37 °C, and then incubated with CRYBB2 antibody (1:100 dilution) at 4 °C overnight. The coverslips were incubated with flouresceine-5-isothiocyanate (FITC)-conjugated anti-goat IgG (1:500, Jackson Immunoresearch Laboratories, Inc. West Grove, PA), followed by counterstaining with 4, 6-diamidino-2-phenylindole (DAPI) (Beyotime Institute of Biotechnology) for 2 min at room temperature and then photographed by using an OLYMPUS fluorescence microscope (Olympus Co., Tokyo, Japan).

2.7. Histological classification and quantification of ovarian follicles

Ovaries in estrus at postnatal 11 weeks ($n = 7$ for each genotype) were collected. Each ovary was serially sliced into 4-μm sections, and stained with hematoxylin and eosin (HE). In every fifth ovarian section, the numbers of primordial (a partial or complete layer of squamous granulosa cells), primary (a single layer of cuboidal granulosa cells), secondary (more than one layer of granulosa cells), preovulatory (a rim of cumulus cells surrounding the oocyte) follicles and atretic follicles (morphological signs of death such as pyknosis, cellular fragmentation and disintegration) were counted. To ensure that each follicle was counted only once, follicles were counted only when the nucleus of the oocyte was visible on the section. The number of follicles in the selected sections was then multiplied by 5 to get an estimate of the total number of follicles in each ovary [13,14]. The rate of follicular atresia (%) was calculated as follows: [(number of atretic follicles/total number of follicles) × 100] [15].

2.8. Measurement of serum estrogen and progesterone

Serum was collected from WT and *Crybb2*$^{-/-}$ mice at postnatal 11 weeks in different stages of the estrous cycle ($n = 5$ per stage). Serum estradiol levels were measured using an Estradiol Kit (No. 33540) (Beckman Coulter Inc., Fullerton, CA) on the Beckman Coulter Access 2 System [16]. Serum progesterone concentration was determined by the Progesterone Kit (No. 33550) (Beckman Coulter Inc.) on the Beckman Coulter Access 2 System. The intra-assay coefficients of variation were less than 10% for all hormone assays.

2.9. Flow cytometry analysis of cell cycle

The cellular DNA content in granulosa cells was determined by flow cytometric measurement of PI binding as previously described [17]. Cells ($1–2 \times 10^6$) were stained with a solution containing 5 μg/ml PI, 0.01% Triton X-100 and 30 μg/ml deoxyribonuclease-free ribonuclease A (Sigma, Poole, UK). Cells (10,000 per sample) were analyzed by flow cytometry. Cell cycle analysis of

G0/G1, S or G2/M phases was performed using the Modfit 5.2 multitrapezoid model (Verity Software House, Topsham, ME).

2.10. Apoptosis analysis by TUNEL and Annexin V assay

Cell apoptosis assays were carried out by using by using the DeadEnd™ Colorimetric TUNEL System kit (Promega, Madison, WI). The percentage of apoptotic granulosa cells were also quantified by using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences Pharmingen, San Diego, CA) according to manufacturer's instructions. Stained cells were analyzed by flow cytometry within 30 min.

2.11. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated from granulosa cells from ovaries of WT and *Crybb2*$^{-/-}$ mice by using the TRIzol reagent (Invitrogen, Life Technologies). Reverse transcription was performed with the PrimeScript RT Reagent Kit (Takara Biotechnology, Dalian, China) according to manufacturer's instructions. Quantitative real-time PCR-based gene expression analysis was performed using a Real-Time PCR machine (Applied Biosystems 7300, Life Technologies) and standard SYBR-Green PCR Kit. (Takara Biotechnology) Reactions were conducted at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 30 s. The relative expression of each target gene compared to beta-actin was calculated by using the 2-ΔΔct method. Specific primers for all genes were as follows: beta-actin: 5′-AGCCATGTACGTAGCCATCC-3′ (forward) and 5′-CTCTCAGCTGTGGTGGTGAA-3′ (reverse); *Cdk4*: 5′-GCAGTCTACATACGCAACACC-3′ (forward) and 5′-CAGAGATTCGCTTATGTGGGT-3′ (reverse); *Ccnd2*: 5′-CAAAAGGAGAAGCTGTCCCTG-3′ (forward) and 5′-GTTATGCTGCTCTTGACGGAA-3′ (reverse); *Bcl-2*: 5′-GGATAACGGAGGCTGGGATGCCT-3′ (forward) and 5′-CAGAGTGATGCAGGCCCCGAC-3′ (reverse); P21(*Cdkn1a*): 5′-TTAGGCAGCTCCAGTGGCAACC-3′ (forward) and 5′-ACCCCCACCACCACACACCATA-3′ (reverse); P27(*Cdkn1b*): 5′-TCGCAGAACTTCGAAGAGG-3′ (forward) and 5′-TGACTCGCTTCTTCCATATCC-3′ (reverse).

2.12. Data analysis

To ensure the creditability of results, all experiments were repeated at least three times. All data were presented as mean ± standard deviation (SD). All data were analyzed using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL). Statistical analysis was performed using an unpaired Student's *t*-test, or by one-way ANOVA followed by Tukey's test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Crybb2$^{-/-}$ female mice exhibit reduced fertility

To evaluate reproductive function of female *Crybb2*$^{-/-}$ mice, a continuous mating study was carried out for five months. Compared with WT female mice, *Crybb2*$^{-/-}$ female mice exhibited reduced fertility as represented by the significant decrease in pups per litter (4.00 ± 1.41 *vs* 6.50 ± 2.52; Fig. 1A) and in the number of litters per female (1.75 ± 0.71 *vs* 4.05 ± 1.49; Fig. 1B). To explore the effects of *Crybb2* deletion on folliculogenesis, the numbers of follicles at different stages were counted. The numbers of primordial, secondary and preovulatory follicles in *Crybb2*$^{-/-}$ ovaries were significantly less than WT ovaries (Fig. 1C). These data demonstrate that *Crybb2*$^{-/-}$ female mice have reduced fertility.

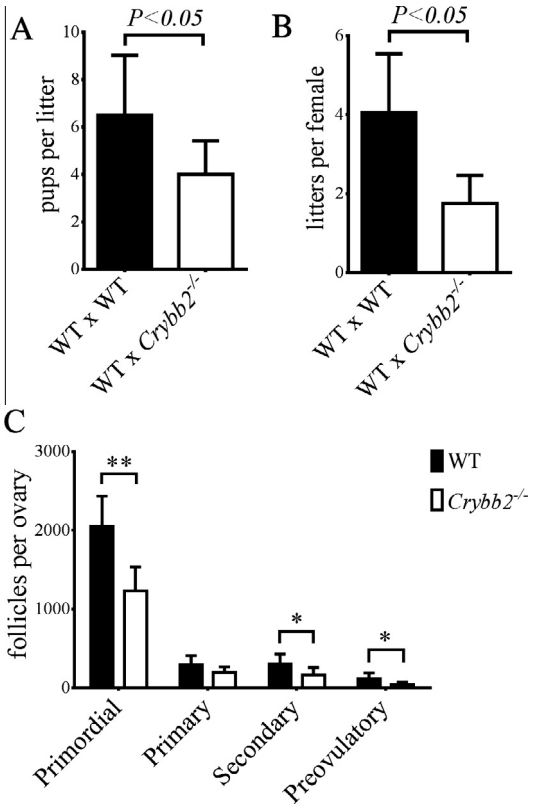

Fig. 1. Reduced fertility in *Crybb2*$^{-/-}$ female mice. (A) Pups per litter were reduced in *Crybb2*$^{-/-}$ female mice ($n = 8$). (B) Litters per female were reduced in *Crybb2*$^{-/-}$ female mice ($n = 8$). (C) The number of primordial, primary, secondary and preovulatory follicles in wild type (WT) and *Crybb2*$^{-/-}$ ovaries ($n = 7$). $^{*}P < 0.05$, $^{**}P < 0.01$.

3.2. Crybb2 deletion dysregulates sex hormone production and response

We also investigated the effect of *Crybb2* deletion on the production of sex hormones. The estrogen (E2) and progesterone (P) levels at different stages of the estrous cycle were examined in WT and *Crybb2*$^{-/-}$ mice. The E2 levels at proestrous and estrus were not significantly different between *Crybb2*$^{-/-}$ and WT mice, but the levels of E2 at metestrus and diestrous in *Crybb2*$^{-/-}$ mice were higher than those in WT mice (Fig. 2A). The level of P in the diestrus cycle but not other estrous cycle stages was significantly lower in *Crybb2*$^{-/-}$ mice compared to WT mice (Fig. 2B). To examine the response of *Crybb2* deletion on hormones, we performed a superovulation study. We found that egg numbers from the ampulla of the oviduct of *Crybb2*$^{-/-}$ mice were significantly less than that from WT mice (Fig. 2C). These data support that *Crybb2* deletion induces the dysfunction of sex hormones. Interestingly, the endogenous expression of CRYBB2 was increased after PMSG and hCG treatment compared with control (Suppl. Fig. 1), revealing that CRYBB2 may be regulated by sex hormones.

3.3. Crybb2 deletion decreases granulosa cell proliferation

Our previous studies showed that CRYBB2 was mainly expressed in granulosa cells, with weak expression in theca cells [7]. We validated the expression of CRYBB2 in granulosa cells using Western blot and immunofluorescence analyses. CRYBB2 was detected in granulosa cells derived from WT but not in *Crybb2*$^{-/-}$ mice (Fig. 3A). Similarly, immunofluorescence microscopy findings

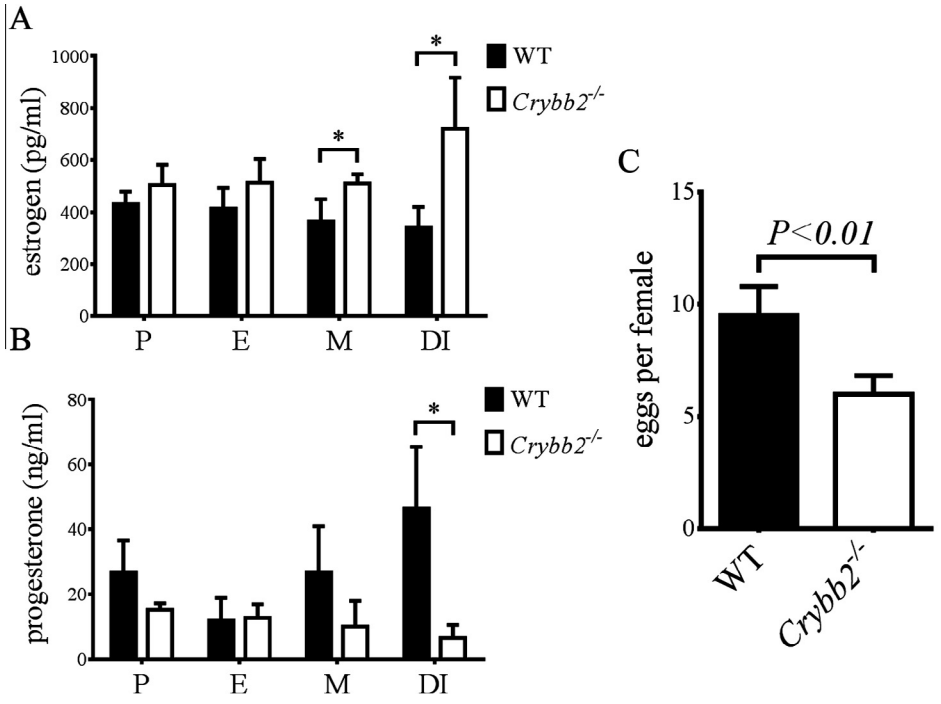

Fig. 2. *Crybb2* deletion affects the production of sex hormones and the response to reproductive hormones. (A) Quantification of estrogen levels in proestrous (P), estrus (E), metestrus (M) and diestrus (MI) cycles in WT and *Crybb2*$^{-/-}$ mice ($n = 5$). *$P < 0.05$. (B) Quantification of progesterone levels in proestrous (P), estrus (E), metestrus (M) and diestrus (MI) cycles in WT and *Crybb2*$^{-/-}$ mice ($n = 5$). *$P < 0.05$. (C) The number of eggs isolated from the ampulla of the oviduct after superovulation are measured ($n = 6$).

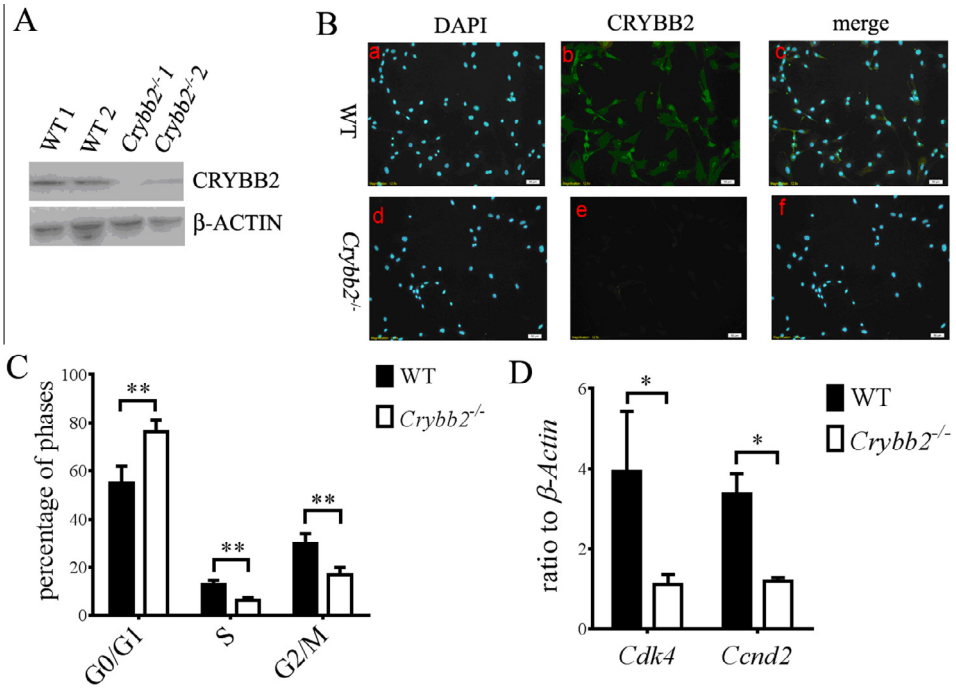

Fig. 3. *Crybb2* deletion decreases granulosa cell proliferation. (A) Detection of CRYBB2 expression in granulosa cells from WT and *Crybb2*$^{-/-}$ mice by Western blot methods. (B) Immunofluorescence analysis of CRYBB2 expression in granulosa cells from WT and *Crybb2*$^{-/-}$ mice. Bar = 50 μm. (C) Effect of *Crybb2* deletion on cell cycle progression in granulosa cells. **$P < 0.01$. (D) Expression of *Cdk4* and *Ccnd2* in granulosa cells from ovaries of WT and *Crybb2*$^{-/-}$ mice. *$P < 0.05$.

further confirmed that CRYBB2 was mainly expressed in the cytoplasm of granulosa cells from WT but not *Crybb2*$^{-/-}$ mice (Fig. 3B). We also examined cell cycle changes in the granulosa cells from WT and *Crybb2*$^{-/-}$ mice by flow cytometry. Our results showed that the percentage of G0/G1 phase in granulosa cells from *Crybb2*$^{-/-}$ mice was significantly increased, while the percentages of S and G2/M phases in granulosa cells from *Crybb2*$^{-/-}$ mice were significantly decreased compared with granulosa cells from WT mice (Fig. 3C). To determine whether loss of CRYBB2 affected the cell cycle, we examined the expression of cell cycle-related genes (*Cdk4* and *Ccnd2*) in granulosa cells from WT and *Crybb2*$^{-/-}$ mice using qRT-PCR. Our results showed that *Cdk4* and *Ccnd2* mRNA

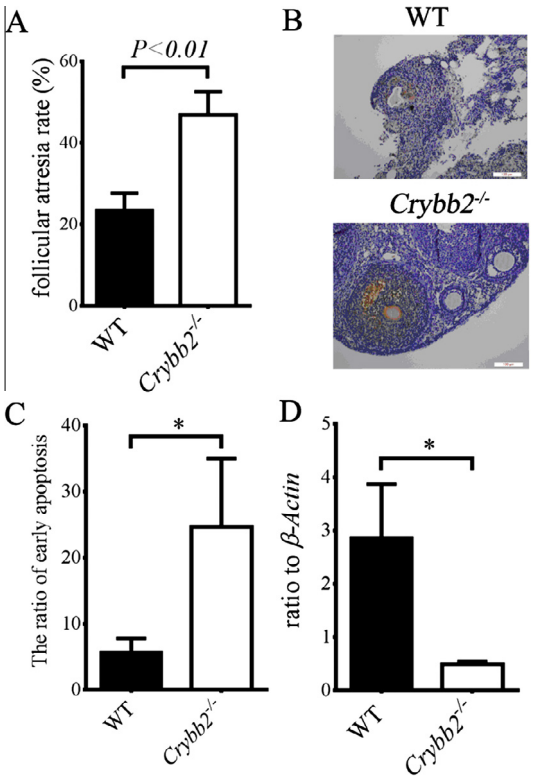

Fig. 4. Effects of *Crybb2* deletion on follicular atresia and apoptosis of granulosa cells. (A) The rate of atretic follicles in the ovaries from WT and *Crybb2*$^{-/-}$ mice. (B) Cell apoptosis was detected by TUNEL assay. Bar = 100 μm. (C) Detection of granulosa cell apoptosis by Annexin V assay. *$P < 0.05$. (D) qPCR analysis of *Bcl-2* mRNA expression in granulosa cells from WT and *Crybb2*$^{-/-}$ mice. *$P < 0.05$.

levels were significantly lower in granulosa cells from *Crybb2*$^{-/-}$ ovaries when compared to WT ovaries (Fig. 3D). Interestingly, *Cdkn1b* (*p27*) mRNA level was also increased in granulosa cells from *Crybb2*$^{-/-}$ ovaries (Suppl. Fig. 2).

3.4. Crybb2 deletion increases granulosa cell apoptosis

Since *Crybb2* deletion had striking effects on the inhibition of follicular development, we sought to examine the effect of *Crybb2* deletion on follicular atresia. We found that the rate of atretic follicles in *Crybb2*$^{-/-}$ ovaries was significantly higher than that in WT ovaries (Fig. 4A). TUNEL analysis revealed that *Crybb2*$^{-/-}$ ovaries harbored significantly more apoptotic granulosa cells than WT ovaries (Fig. 4B). Cell apoptosis in granulosa cells was further analyzed by flow cytometry. Granulosa cells isolated from *Crybb2*$^{-/-}$ antral follicles showed a significantly higher rate of apoptosis than compared to granulosa cells isolated from WT antral follicles (Fig. 4C). Moreover, we further examined the expression of the important anti-apoptotic gene, *Bcl-2*. We found that *Bcl-2* mRNA levels were significantly reduced in granulosa cells from *Crybb2*$^{-/-}$ ovaries when compared with WT ovaries (Fig. 4D).

4. Discussion

Previous studies have demonstrated that *Crybb2*-deficient mice are subfertile [5] and that *Crybb2* plays an important role in the testis [6]. However, the expression and function of *Crybb2* in the ovary has not been described. Here we investigated the *in vivo* function of *Crybb2* in the ovary using the *Crybb2*$^{-/-}$ mouse model. We demonstrated that (1) *Crybb2* deletion impaired female fertility; (2) *Crybb2* deletion dramatically affected the production of reproduction-related hormones and hormone response; (3) *Crybb2* deletion impaired follicular development and inhibited the proliferation of granulosa cells and (4) *Crybb2* deletion promoted follicular atresia and apoptosis in granulosa cells.

Follicular cells can secrete a variety of steroid hormones and biologically active peptides, including estrogen, progesterone, and a small amount of androgens. These hormones play important roles in ovarian development and fertility. Progesterone can directly affect the function of granulosa cells, thereby affecting ovulation, implantation and pregnancy. Estrogen is necessary for follicular development and promoting granulosa cell differentiation. In this study, we found that estradiol levels in female *Crybb2*$^{-/-}$ mice were significantly higher in metaestrus and diestrus cycles, while the level of progesterone in the diestrus cycle was lower in *Crybb2*$^{-/-}$ mice compared to WT mice. Interestingly, we found that CRYBB2 was increased after PMSG and hCG treatment. These data indicate that CRYBB2 may play a role in ovulation. *Crybb2* deletion disrupts the balance of sex hormones and the response to reproductive hormones, and thus, reduces female fertility.

Both estrogen and progesterone play critical roles in follicular development [18,19] and CRYBB2 is mainly expressed in the ovarian granulosa cells, thereby we hypothesized that *Crybb2* may affect the function of ovarian granulosa cells. Follicular development is a complex process, which requires vigorous proliferation of granulosa cells [20]. Here we found that the granulosa cell proliferation in ovaries from *Crybb2*$^{-/-}$ mice was significantly decreased because the cell cycle progression of granulosa cells was inhibited after *Crybb2* deletion. CDK4 and CCND2 are two important cell cycle regulators [21,22]. CCND2 acts as a positive regulator of cell cycle progression by binding with CDK4 and CDK6, thereby activating a cascade of events, which permits cell progression through the G1 phase of the cell cycle [23]. The mRNA expression of *Cdk4* and *Ccnd2* was also significantly reduced in ovarian granulosa cells from *Crybb2*$^{-/-}$ mice, which is consistent with slower cell cycle progression in granulosa cells from *Crybb2*$^{-/-}$ mice. The cyclin-dependent kinase inhibitor, p27, inhibits cell cycle transition from G1 to S phase, and the upregulation of p27 in *Crybb2*$^{-/-}$ mice further supports cell cycle arrest at G1/G0 phase in the granulosa cells from *Crybb2*$^{-/-}$ mice. These data suggest that reduced granulosa cell proliferation delays follicular development, and thus results in reduced number of primordial, secondary and preovulatory follicles in *Crybb2*$^{-/-}$ mice.

Interestingly, we also observed that the rate of follicular atresia in *Crybb2*$^{-/-}$ mice was significantly higher in WT mice. Follicular atresia is closely associated with the granulosa cell apoptosis [8]. Not surprisingly, the ratio of apoptotic granulosa cells in *Crybb2*$^{-/-}$ mice was significantly higher than in WT mice. The BCL-2 family plays a major anti-apoptotic role in many kinds of cells [24]. BCL-2 is expressed in developing follicles and plays an important role in maintaining the growth of follicular cells [25]. In *Crybb2*$^{-/-}$ mice, the mRNA expression of *Bcl-2* was significantly lower compared to WT mice, suggesting that *Crybb2* may affect female fertility by regulating the granulosa cell apoptosis and follicular atresia. It is reported that p27 plays an important role in ovarian development and follicle atresia [26]. CRYBB2 deficiency may result in p27 increase and cell cycle arrest in G1 phase, which may inhibit follicle growth and induce follicle atresia. However, the detailed mechanisms underlying these effects need further investigation.

In addition, CRYBB2 is a Ca^{2+}-binding protein [27]. The Ca^{2+} dynamics in the ER, cytoplasm and mitochondria are modulated by the apoptosis-regulating BCL-2 family proteins [28] and CDK4. CCND2 is also related to Ca^{2+} receptor calmodulin (CaM) [29]. Therefore, future studies will focus on whether *Crybb2* affects granulosa cell cycle progression and apoptosis via a Ca^{2+} signaling pathway as well as the mechanisms of how *Crybb2* manipulates the expression of *Bcl-2*, *Cdk4* and *Ccnd2*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.049.

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