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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