Decreased levels of Ca\(^{2+}\)-calmodulin-dependent protein kinase IV in the testis as a contributing factor to reduced fertility in male Crybb2\(^{-/-}\) mice

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Abstract. \(\beta\)B2-crystallin (Crybb2), a member of the \(\beta\gamma\)-crystallin superfamily, in conjunction with \(\alpha\)-crystallin, constitute the major proteins of the mammalian eye lens. Crybb2 is also expressed outside the lens, and certain related functions in these tissues have been reported. In the present study, in order to define the physiological role of Crybb2, we generated mice with a targeted deletion of the Crybb2 gene. Surprisingly, fertility was markedly reduced in male homozygous knockout mice compared to wild-type (WT) mice. Further experiments were performed to explore the underlying mechanism of subfertility in male Crybb2\(^{-/-}\) mice. Our results showed that Crybb2 was mainly expressed in the spermatogonia from the testes of mice with the WT C57BL/C genetic background. The testes of 4-week-old Crybb2\(^{-/-}\) mice were significantly hyperplastic, and no significant difference was found within 3 weeks postpartum. Additionally, there was a marked increase in the proliferation and apoptosis of germ cells, and the biological defects of these cells correlated with the decreased Bcl-2 levels, which correlated with the reduction of Ca\(^{2+}\)-calmodulin-dependent protein kinase IV (CaMKIV) in the testis. These results suggest that the reduced fertility of Crybb2\(^{-/-}\) male mice may result from the disordered proliferation and apoptosis of germ cells in the testis, possibly due to reduced CaMKIV from the loss of Crybb2.

Introduction

Crystallins are the major structural proteins of the vertebrate eye lens. There are 2 superfamilies: \(\alpha\)- and \(\beta\gamma\)-crystallins (1), which account for approximately 90% of total soluble proteins. \(\beta\)-crystallins have been reported to function as stress proteins, playing a crucial role in maintaining lens transparency, a high refractive index and solubility of the adult lens (2). \(\beta\)B2-crystallin (Crybb2 in mice) is the most abundant and the most thermally stable \(\beta\)-crystallin of the lens, and is resistant to modification (2-4).

The Crybb2 gene in mice is located on chromosome 5 within a cluster containing an additional 3 Cryb genes consisting of 4 Greek key motifs, which is the common character of all members of the \(\beta\)- and \(\gamma\)-crystallin superfamilies (2). Certain studies have reported that \(\alpha\)-crystallins are capable of functioning as molecular chaperones (5,6), while \(\beta\gamma\)-crystallins play a structural role in the mammalian eye lens. However, certain other studies have proposed that \(\beta\gamma\)-crystallins play unknown and unconceved non-crystallin roles (7), and that Crybb2 is expressed in some extralenticular tissues, such as the retina, brain and testis (4). Previous studies have shown that Crybb2 is localized in the retinal ganglion cells (RGCs) (8) and that the protein is secreted and is responsible for neurite outgrowth during retinal regeneration (9).

In our previous study, mice with a targeted deletion of the Crybb2 gene were used to investigate the role of Crybb2 in mice suffering from cataract (10). In our present study, we found that the fertility of male mice with Crybb2 deficiency was reduced. Crybb2 has also been implicated in the subfertility of mice exhibiting mutant Crybb2 (11); however, the actual mechanism remains elusive. In this study, we discovered that Crybb2 was mainly expressed in the spermatogonia from the testes of mice with normal fertility. The proliferation of Crybb2\(^{-/-}\) mouse germ cells was enhanced significantly, and apoptosis was also increased compared with the wild-type (WT) mice. In addition, Bcl-2 and Ca\(^{2+}\)-calmodulin-dependent protein kinase IV (CaMKIV) levels were decreased in Crybb2\(^{-/-}\) mouse testis. A previous study has shown that the function of CaMKIV is regulating Bcl-2 levels and rescuing proliferation defects (12). Our data reveal that the disordered
proliferation and apoptosis of Crybb2−/− germ cells may result from the decreased expression of Bcl-2, possibly due to reduced CaMKIV from the loss of Crybb2.

Materials and methods

Animals and mouse models. Using Crybb2 target vector construction, Crybb2−/− mice were generated by deleting the first and second exons of Crybb2 and the 2 known transcription initiation sites (13). Genotyping was performed as described previously (13). Both Crybb2−/− and WT mice were of C57BL/C genetic background, and were housed and maintained in the Laboratory Animal Center of the Second Military Medical University (Shanghai, China) under a 12-h light/dark cycle. Food and water were provided ad libitum, and complete care was given in compliance within the National Institutes of Health and the institutional guidelines on the use of laboratory and experimental animals.

Fertility test. Fertility was assessed by setting up natural matings between a single male and female. The number of litters and the number of pups/litter produced by each pair were calculated over a 3-month period. Fecundity was calculated as the total number of pups produced/mating/30-day period.

Histological and immunofluorescence analysis of testis. The testes isolated from 4-week-old mice were fixed in fresh 4% paraformaldehyde for 24 h, washed in 70% ethanol, and decalcified for 72 h. Glycol methacrylate infiltration and embedding were performed using a JB-4 embedding kit (Polysciences, Warrington, PA, USA). Sections of 4 µm were prepared and stained with hematoxylin and eosin (H&E).

For immunofluorescence analysis of Crybb2 expression in the mouse testis, paraffin sections were blocked with 2% BSA for 1 h, followed by incubation in anti-Crybb2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C overnight. The samples were then washed and incubated with FITC-conjugated anti-goat IgG (Jackson Immuno Research Laboratories, Inc.) and analyzed under a Nikon TE2000 microscope.

Quantitative real-time RT-PCR analysis. For qRT-PCR analysis, a 4-week-old male mouse testis was removed and the tissue was homogenized with an electric homogenizer. Total-RNA was isolated by using the TRIzol reagent kit, and was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc.), according to manufacturer’s directions. Quantitative real-time PCR-based gene expression analysis was performed on a Real-Time PCR machine (7300; Applied Biosystems, USA) using a standard SYBR-Green PCR reagent kit (Takara Bio, Inc.), according to manufacturer’s instructions.

Western blot analysis. For the whole protein extracts, the testicular tissue was homogenized in lysis buffer (Promega, Madison, WI, USA), then abraded with an electric homogenizer and centrifuged at 12,000 x g for 15 min. All buffers received a protease inhibitor cocktail (Konchem, China) prior to use. The protein concentration of each sample was determined. Equal amounts of protein were loaded and separated discontinuously on 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), and subsequently transferred onto a nitrocellulose membrane (Amershams Pharmacia, UK). The membrane was then incubated in TBST blocking solution (Tris-buffered saline including 0.1% Tween-20) containing 5% skim milk for 2 h at room temperature, followed by incubation with primary antibodies containing anti-CaMKIV (Abcam), anti-Crybb2 (Santa Cruz Biotechnology, Inc.), anti-Bax, anti-Bcl-2 (Cell Signaling), anti-β-actin (Beyotime, Jiangsu, China) at 4˚C overnight. After washing, the membrane was reacted with secondary antibodies, HRP-conjugated anti-mouse, anti-rabbit or anti-goat secondary antibodies for 2 h. After several washes, the immunoblot was detected with enhanced chemiluminescence (Pierce Biotechnology), which was performed according to the manufacturer’s instructions.

Proliferation analysis by bromodeoxyuridine (BrdU) assay. For in vivo BrdU labeling assays, 3 mice in each group were injected intraperitoneally with 100 µg/g BrdU (Sigma) 2 h before sacrifice. The testis samples were then excised quickly and fixed with 4% paraformaldehyde overnight at 4˚C. After fixation, 4-µm sections were prepared and washed in 0.1 M PBS containing 1% Triton X-100. The testis was then treated with 2 N HCl for 20 min at 37˚C. After neutralization in 0.1 M borate buffer, the testis was washed in PBST 3 times and blocked by PBST with 5% normal goat serum for 2 h and stained with anti-BrdU antibody.

Apoptosis analysis by TUNEL and Annexin V assay. Terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) assays were carried out using the DeadEnd™ Colorimetric TUNEL System kit (Promega) following the manufacturer’s instructions. The apoptotic index of TUNEL-positive cells was calculated using the total number of positive cells/field of sight at 5 random locations in each testis under light microscopy (x200).

The percentage of apoptotic cells in the mouse germ cells was also quantitated using the Annexin V fluorescein (FITC) kit (Bender MedSystem, Vienna, Austria) according to the manufacturer’s instructions. Stained cells were analyzed by flow cytometry within 30 min.

Statistical analysis. For all the analyses, measurements obtained from the groups were expressed as the means ± SD for all parameters determined. Statistical analysis was performed using an unpaired Student’s t-test followed by Tukey’s test. P-values <0.05 were considered to indicated statistically significant differences.
Table I. Number of progeny from breeding male Crybb2 knockout mice.

<table>
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<tr>
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<tr>
<td>+/+</td>
<td>+/+</td>
<td>13</td>
<td>114</td>
<td>9.1±1.4</td>
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<td>7</td>
<td>33</td>
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*aMeans ± SD. bP<0.05 when compared to +/- mice.

Results

Patterns of Crybb2 expression in Crybb2–/– and WT C57BL/C mouse testis. To investigate the role of Crybb2, Crybb2 knockout mice were produced with the assistance of the Ingenious Targeting Laboratory, Inc. (Stony Brook, NY, USA) (13). To investigate the Crybb2 expression in WT mouse testis and verify that Crybb2 was not produced by the knockout mice, RNA samples were first extracted from the Crybb2–/– and WT mouse testis, and the Crybb2 mRNA expression was analyzed by semi-quantitative RT-PCR using Crybb2 specific primers. The results indicated that the Crybb2 gene expression was not detected in the testis of the knockout mice (Fig. 1A). Crybb2 protein expression in the testis was then analyzed by western blot analysis. The Crybb2 protein was not produced in the Crybb2–/– testis while it was expressed in WT mouse testis (Fig. 1B). The results were further confirmed by immunofluorescence using a polyclonal antibody raised against Crybb2 (Fig. 1C), showing that Crybb2 was mainly expressed in the spermatogonia of WT mouse seminiferous tubules, which was consistent with the results of a previous study (11). Additionally, Crybb2 expression was not detected in the Crybb2–/– mouse testis. These data suggest that the intended alteration of Crybb2 was successful in eliminating Crybb2 from the testis.

Subfertility of male Crybb2–/– mice and abnormal development of testis. During the study, we intended to generate a large number of Crybb2–/– mice for various pathological and physiological studies by interbreeding between Crybb2–/– mice. Surprisingly, the reproductive performance of male Crybb2–/– mice was inferior to that of male WT mice. To further understand this unexpected observation, the reproductive performance of the male mice was closely observed (Table I). Five couples of WT mice gave birth to 13 litters (mean litter size, 9.1±1.4) in a 3-month period, only 7 litters (mean litter size, 4.8±1.5) were yielded from an equal number of Crybb2–/– male and normal female mice.

To further understand this unexpected observation, the testis morphology of the male mice was closely followed for 4 weeks postpartum. It was found that the testes of the 4-week-old Crybb2–/– mice were significantly larger compared to the age-matched WT mice, while no significant difference was observed between them within 3 weeks postpartum (Fig. 2A). Accordingly, the testis weight was increased in 4-week-old Crybb2–/– mice and the organ mass of testis was also higher, while there was no significant difference in body weight (Fig. 2B). We then performed histological analysis of the testis and found that the seminiferous tubules in Crybb2–/– mouse testis were thinner and scattered compared with those of the WT mouse testis (Fig. 2C).

Enhanced proliferation and apoptosis of germ cells in Crybb2–/– mouse testis. To investigate the reasons for the increased testis size and weight in Crybb2–/– mice, the testis sections were examined by BrdU assay. The results showed that the number of BrdU-positive cells increased significantly in Crybb2–/– mouse testis compared with those in WT mouse testis (Fig. 3A and B) (P<0.01), indicating that the proliferation of mutant cells was increased.

The testis sections were also examined by TUNEL assay based on the specific binding of TdT to 3'-OH ends of DNA. Compared with WT mice, apoptotic cells were increased in Crybb2–/– mouse testis (Fig. 3A and B) (P<0.05). In addition, the apoptotic rate of Crybb2–/– and WT mouse testis was analyzed by flow cytometry, while the Annexin V-positive: propidium iodide (PI)-negative population included cells that were in the early stages of apoptosis. The results also indicated that there were more dead cells in Crybb2–/– testis as compared with WT mouse testis (Fig. 3C).

Reduction of Bcl-2 and CaMKIV in the testis of Crybb2–/– mice. Knowing that Bcl-2 may inhibit both apoptosis and proliferation, and that the balance of Bcl-2 family and the pro-apoptotic Bax protein is important for normal spermatogenesis, the levels of Bcl-2 mRNA and protein were investigated in the mouse testis. The results indicated that the Bcl-2 mRNA level was higher in the Crybb2–/– testis compared to those in the WT mouse testis (P<0.05) (Fig. 4A). In addition, the protein level of Bcl-2 was also higher in Crybb2–/– testis (Fig. 4B). It was also found that Bax protein levels were increased in Crybb2–/– mouse testis compared with those in WT mouse testis (data not shown), while the Bax mRNA level was also higher, although there was no significant difference between them (P>0.05) (data not shown). The results indicated that the specific value of Bax/Bcl-2 in Crybb2–/– testis was abnormal, and that the lower level of Bcl-2 may be the reason for the excessive proliferation and apoptosis of Crybb2–/– germ cells.

CaMKIV is a multifunctional serine/threonine (Ser/Thr) protein kinase, expressed primarily in the brain, thymus, testis, ovary, bone marrow and adrenal glands (14). It has been reported that mice absent in CaMKIV are more prone to infertility (15), and that CaMKIV may regulate the normal proliferation of the cells, whose effect may be in part mediated via the regulation of Bcl-2 (12,16). Therefore, we analyzed the expression patterns of CaMKIV mRNA and protein in the mouse testis to further clarify the possible mechanism of the
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Figure 1. Evaluation of Crybb2 expression by qRT-PCR, western blot analysis and immunofluorescence. (A) qRT-PCR analysis of Crybb2 mRNA expression in WT mouse testis showed no Crybb2 mRNA expression in the Crybb2⁻/⁻ mouse testis. (B) Western blot analysis of Crybb2 expression was carried out in accordance with (A). (C) Immunofluorescence analysis showed that Crybb2 was mainly expressed in basal cells of certain WT mouse testis seminiferous tubules, but staining was not detected in Crybb2⁻/⁻ mice. Paraffin sections of the mouse testis were stained with anti-Crybb2 antibody (green) and nuclear DAPI (blue). (a-c) WT mice testes: (a) DAPI staining; (b) anti-Crybb2 antibody staining; (c) overlay of (a and b). (d-f) Crybb2⁻/⁻ mice testes: (d) DAPI staining; (e) anti-Crybb2 antibody staining; (f) overlay of (d and e).

Figure 2. Increased size and weight of testis in 4-week-old Crybb2⁻/⁻ mice. (A) Gross appearance of Crybb2⁻/⁻ and WT mouse testis. The testis was isolated from (a and d) 2-week-old, (b and e) 4-week-old and (c and f) 6-week-old mice. There was no difference between the 2 mouse testis in 2-week-old mice, while Crybb2⁻/⁻ testis was significantly larger in 4-week-old mice. (B) The testis weight, the value of testis/body and the body weight of Crybb2⁻/⁻ and WT mice. (C) H&E-stained paraffin sections of Crybb2⁻/⁻ and WT mice. Original magnification, x200. The Crybb2⁻/⁻ testis seminiferous tubules were thinner compared with WT mice.
poor reproductive performance of Crybb2−/− males. The results indicated that both CaMKIV mRNA (Fig. 4A) and protein levels were decreased compared to WT mouse testis (Fig. 4B).

**Discussion**

Since the discovery of Crybb2 in extralenticular tissues (4), a number of studies have reported on the function of Crybb2 in these tissues (8,9,11). It was discovered in our study that male mice lacking Crybb2 had reduced fertility, which was apparently associated with Crybb2 deficiency, as its expression was detected in WT mouse testis. Immunofluorescence assay showed that Crybb2 was mainly expressed in the spermatogonia of seminiferous tubules in testis but it was not detected in Crybb2−/− mouse testis by qRT-PCR, western blot analysis and immunofluorescence analysis, suggesting that the intended alteration of Crybb2 was successful in eliminating Crybb2 from the testis.

Crybb2 begins to be expressed after birth in rodents; therefore, it does not contribute to the development of the fetal
cells (11). These results indicate that the decreased level of CaMKIV of Crybb2⁺⁄⁻ mice, may affect the expression of Bcl-2, which further disturbs the proliferation and apoptosis of germ cells in Crybb2⁺⁄⁻ testis.

Collectively, our data indicate that a correlation exists between the presence of Crybb2, Ca²⁺, CaMKIV and Bcl-2 in testicular germ cells. Clarification of the potential role of Crybb2 in regulating the CaMKIV expression may provide new insights into the mechanism of male fertility.

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References