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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. βB2-crystallin (Crybb2), a member of the βγ-crystallin superfamily, in conjunction with α-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.

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Introduction

Crystallins are the major structural proteins of the vertebrate eye lens. There are 2 superfamilies: α- and βγ-crystallins (1), which account for approximately 90% of total soluble proteins. β-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). βB2-crystallin (*Crybb2* in mice) is the most abundant and the most thermally stable β-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 β- and γ-crystallin superfamilies (2). Certain studies have reported that α-crystallins are capable of functioning as molecular chaperones (5,6), while βγ-crystallins play a structural role in the mammalian eye lens. However, certain other studies have proposed that βγ-crystallins play unknown and unconceived 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 reverse transcription was performed 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 kit. Reactions were conducted at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 30 sec, The relative expression of each target gene compared with β-actin was calculated using the $2^{-\Delta\Delta ct}$ method. Primers used were as follows: mouse β-actin (5'-AGCCATGTACGTAGCCATCC-3' and 5'-CTCTCAGCTGTGGTGGTGAA-3'); Crybb2 (5'-CAGACACAGGCGGGCAAGCC-3' and 5'-CTCGTAGCCCACCCAGGGTCC-3'); CaMKIV (5'-TGGAGTCAGAGCTGGGACGGG-3' and 5'-TTCGGGTGTGAGAGACGCAGGAG-3'); Bcl-2 (5'-GGATAACGGAGGCTGGGATGCCT-3' and 5'-CAGAGTGATGCAGGCCCCGAC-3'); Bax (5'-CAGGATGCGTCCACCAAGAA-3' and 5'-GTTGAAGTTGCCATCAGCAAACA-3').

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 (Amersham 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 1 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.

Male	Female	No. of litters	Total no. of pups	Litter size[a]
+/+	+/+	13	114	9.1±1.4
-/-	+/+	7	33	4.8±1.5[b]

[a]Means ± SD. [b]P<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 were 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

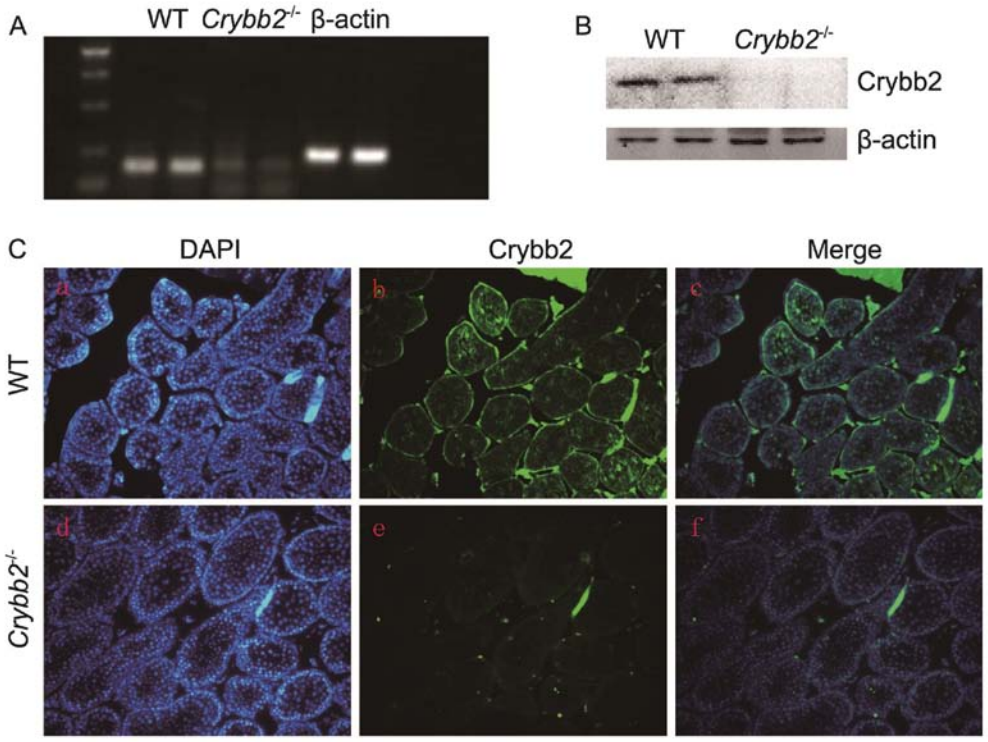

Figure 1. Evaluation of Crybb2 expression by qRT-PCR, western blot analysis and immunofluorescence. (A) qRT-PCR analysis of *Crybb2* mRNA expression in WT mouse testis showed no *Crybb2* mRNA expression in the *Crybb2*$^{-/-}$ mouse testis. (B) Western blot analysis of Crybb2 expression was carried out 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).

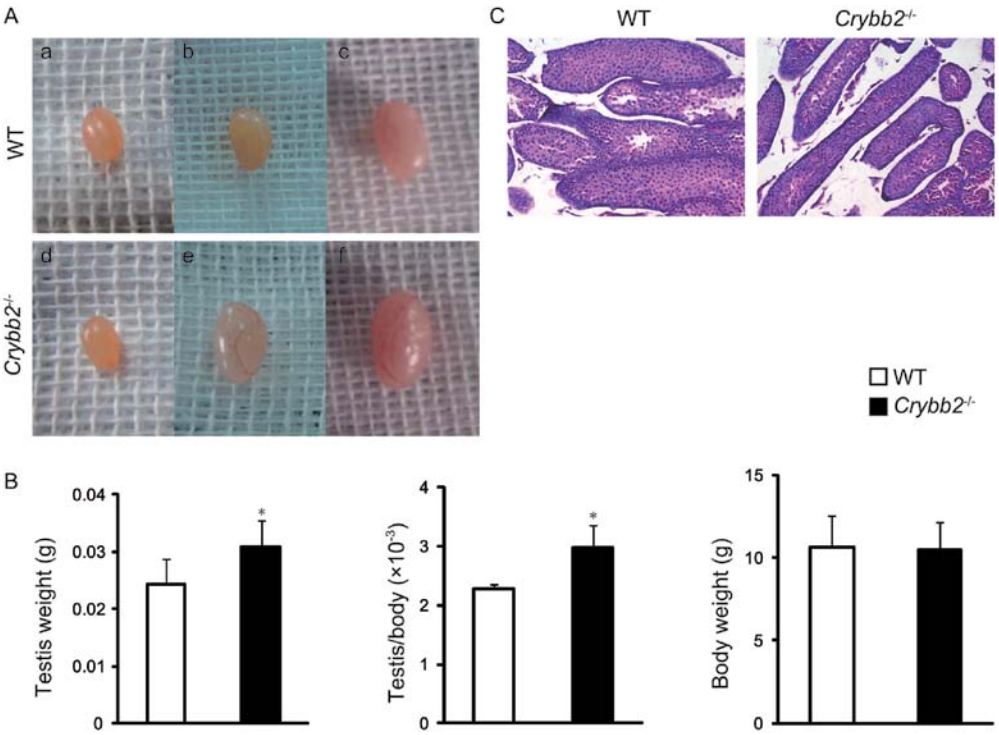

Figure 2. Increased size and weight of testis in 4-week-old *Crybb2*$^{-/-}$ mice. (A) Gross appearance of *Crybb2*$^{-/-}$ and WT mouse testis. The testis was isolated from (a and d) 2-week-old, (b and e) 4-week-old and (c and f) 6-week-old mice. There was no difference between the 2 mouse testes in 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.

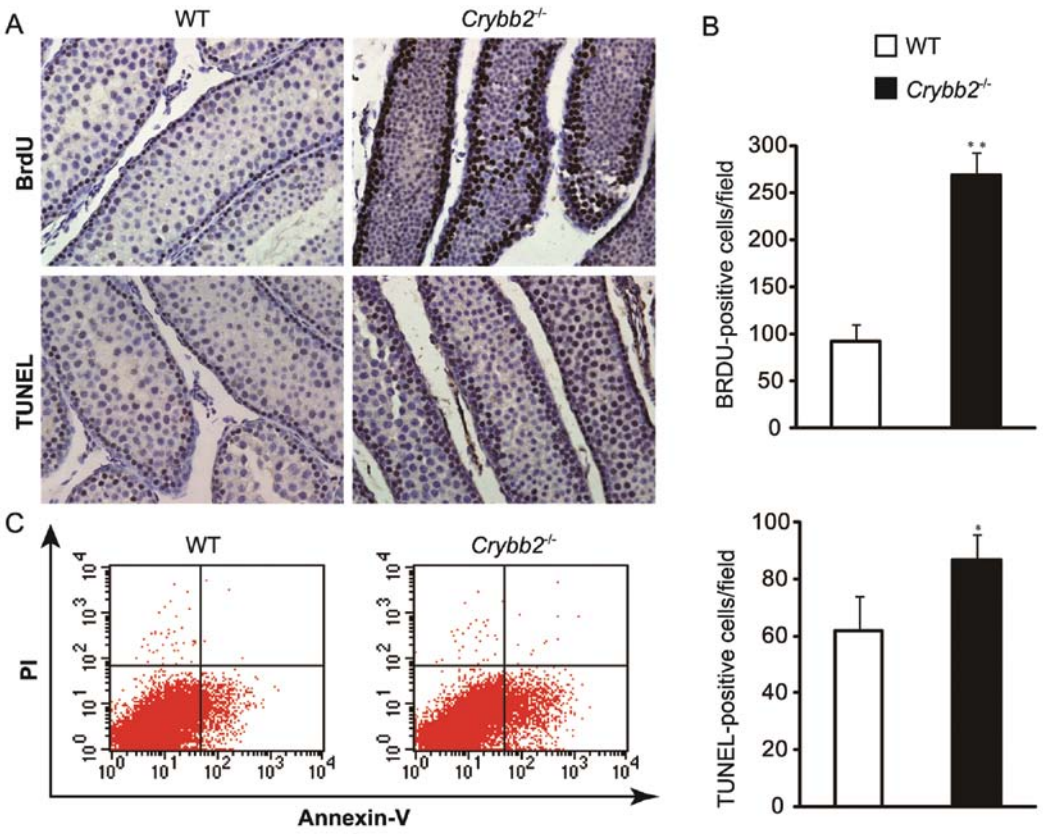

Figure 3. Enhanced proliferation and apoptosis of germ cells in *Crybb2*$^{-/-}$ mouse testis. (A) Testis sections of *Crybb2*$^{-/-}$ and WT mice stained with BrdU or TUNEL immunohistochemistry. Original magnification, x400. (B) The quantitative analysis of BrdU- and TUNEL-positive cells in *Crybb2*$^{-/-}$ and WT mouse testis. (C) Early apoptosis (Annexin V^{+}/PI^{-}) assayed by flow cytometry.

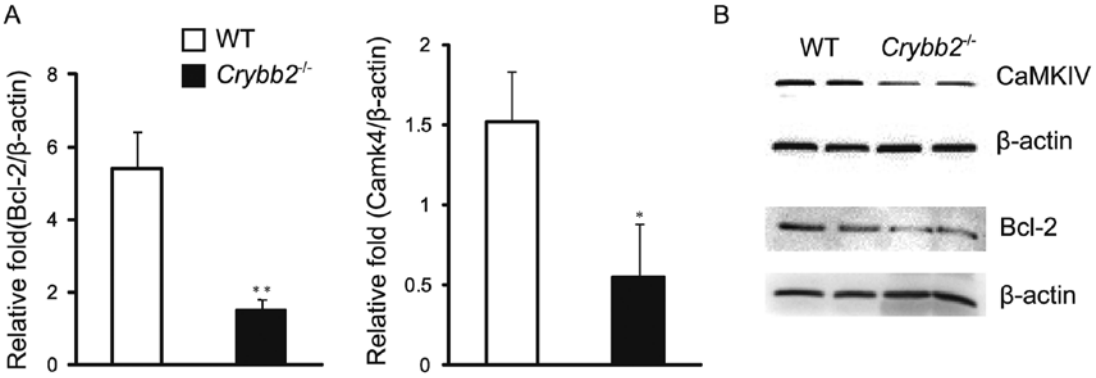

Figure 4. Decreased expression of Bcl-2 and CaMKIV in *Crybb2*$^{-/-}$ mouse testis. (A) Real-time PCR analysis of Bcl-2 and CaMKIV mRNA expression. The results were normalized to the amount of β-actin as the internal control. Each value represents the average from 3 independent experiments. (B) Western blot analysis of the Bcl-2 and CaMKIV protein expression in each group. β-actin expression served as the loading control.

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

testis. Our finding that *Crybb2*$^{-/-}$ testis developed normally for weeks after birth conforms to this later function of Crybb2. We found that the testis size and weight of 4-week-old *Crybb2*$^{-/-}$ mice were markedly increased, and the seminiferous tubules in *Crybb2*$^{-/-}$ mice testes were thinner and scattered compared with those of WT mice. However, these changes were not observed within 3 weeks postpartum.

To determine the cause of the change in *Crybb2*$^{-/-}$ testis morphology, the proliferation and apoptosis of testicular germ cells were detected by BrdU and TUNEL assays. It was found that the proliferation and apoptosis of germ cells were markedly increased in *Crybb2*$^{-/-}$ testes, compared with those in WT mice. The *Crybb2* gene was mainly expressed in the spermatogonia of the mouse testis, which perhaps reflected the early defect in spermatogonial proliferation and apoptosis, and finally resulted in the overall reduction in spermatogenesis, thus contributing to the subfertility of the male mice. Considering the restricted tissue distribution of Crybb2, a change in the expression level of Crybb2 may be a therapeutic option for defective spermatogenesis, during which germ cell proliferation and apoptosis play a significant role (17).

A balance of anti-apoptotic members of the Bcl-2 family and the pro-apoptotic Bax protein is extremely important for normal spermatogenesis in the regulation of germ cell survival (18,19). Any absence of Bcl-2, Bcl-x, Bcl6 and Bax may cause defective fertility as a result of subfertility or infertility (20-22). In addition to its role in cell survival, Bcl-2 has also been reported to play a role in maintaining cellular quiescence (23,24). However, our results indicated that both Bcl-2 mRNA and protein levels were decreased, which may be the reason for the hyperproliferative phenotype of the germ cells due to the reduced function of inhibiting proliferation and apoptosis of Bcl-2.

Calcium (Ca^{2+}) is a pervasive intracellular second messenger that initiates signaling cascades, leading to essential biological processes, such as secretion, cell proliferation, differentiation and migratory movement (25). However, many of the Ca^{2+} effects are mediated via Ca^{2+}-induced activation of the ubiquitous Ca^{2+} receptor calmodulin (CaM) (26). In turn, Ca^{2+}/CaM stimulates the increase of certain enzymes including those that comprise the family of multifunctional, Ser/Thr kinases (CaMKs), one of which is CaMKIV (27) and it is also expressed in the spermatogonia of the male mouse testis (28). In our study, we found that the level of CaMKIV was decreased in *Crybb2*$^{-/-}$ testis, which may be the reason for *Crybb2*$^{-/-}$ subfertility, as CaMKIV deficiency may result in the infertility of male mice as a profound impairment to spermiogenesis (14). It has been discussed that Crybb2 is a Ca^{2+}-binding protein (29), proposing the 4-Greek key crystallin fold as a Ca^{2+}-binding motif (30). We speculate that Crybb2 may not be regulated by Ca^{2+} due to the loss of the Crybb2 function of Ca^{2+} binding, resulting in the change of Ca^{2+} signaling, which further induces a decrease in the CaMKIV level.

However, several studies have shown that the transcription of the pro-survival Bcl-2 gene may be stimulated by Ca^{2+} (31,32). CaMKIV plays an important role in supporting the survival of dendritic cells by regulating the expression of Bcl-2 (15). In addition, re-expression of CaMKIV can restore the Bcl-2 levels and rescue both the hyperproliferation and the rapid exhaustion phenotypes characteristic of *Camk4*$^{-/-}$ KLS 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, Ca^{2+}, 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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