

Decreased levels of Ca²⁺-calmodulin-dependent protein kinase IV in the testis as a contributing factor to reduced fertility in male *Crybb2*^{-/-} mice

FENFEN XIANG^{1,4}, BEI CUI², QIAN GAO¹, JIANRONG ZHANG¹, JUNJIE ZHANG³ and WENJIE LI¹

Departments of ¹Laboratory Diagnosis, ²Ophthalmology and ³Obstetrics and Gynecology, Changhai Hospital, Second Military Medical University, Shanghai 200433; ⁴Department of Laboratory Medicine, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200062, P.R. China

Received April 27, 2012; Accepted July 6, 2012

DOI: 10.3892/ijmm.2012.1116

Abstract. β B2-crystallin (*Crybb2*), a member of the $\beta\gamma$ -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 Ca²⁺-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: α - and $\beta\gamma$ -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). β B2-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 $\beta\gamma$ -crystallins play a structural role in the mammalian eye lens. However, certain other studies have proposed that $\beta\gamma$ -crystallins play unknown and 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 Ca²⁺-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

Correspondence to: Dr Wenjie Li, Department of Laboratory Diagnosis, Changhai Hospital, Second Military Medical University, 168 Changhai Road, 200433 Shanghai, P.R. China
E-mail: wenjieli@pku.org.cn

Dr Junjie Zhang, Department of Obstetrics and Gynecology, Changhai Hospital, Second Military Medical University, 168 Changhai Road, 200433 Shanghai, P.R. China
E-mail: zhangjj910@tom.com

Key words: β B2-crystallin, proliferation, apoptosis, Bcl-2, calcium, Ca²⁺-calmodulin dependent protein kinase IV

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 μ m 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'-CTCGTAGCCACCCAGGGTCC-3'); CaMKIV (5'-TGGAGTCAGAGCTGGGACGGG-3' and 5'-TTCGGGTGTGAGAGACGCAGGAG-3');

Bcl-2 (5'-GGATAACGGAGGCTGGGATGCCT-3' and 5'-CAGAGTGATGCAGGCCCGAC-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 \times 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 μ g/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- μ m 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 \pm 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

^aMeans ± SD. ^bP<0.05 when compared to +/+ mice.

Results

Patterns of *Crybb2* expression in *Crybb2*^{-/-} and WT C57BL/6 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

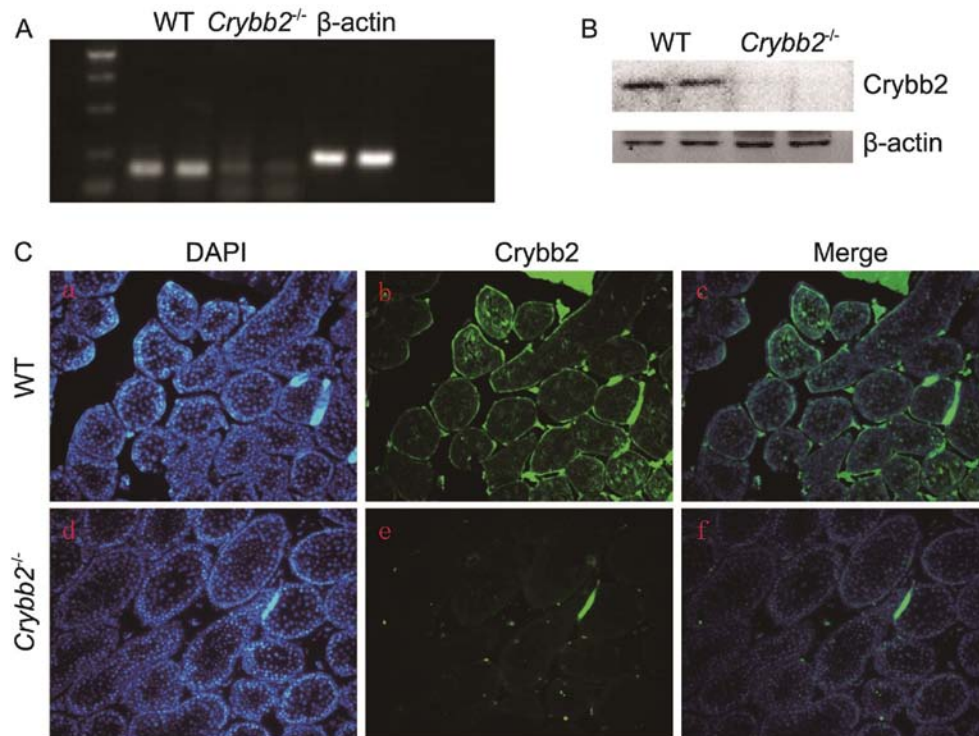


Figure 1. Evaluation of *Crybb2* expression by qRT-PCR, western blot analysis and immunofluorescence. (A) qRT-PCR analysis of *Crybb2* mRNA expression in WT mouse testis showed no *Crybb2* mRNA expression in the *Crybb2*^{-/-} mouse testis. (B) Western blot analysis of *Crybb2* expression was carried 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 mouse testes: (a) DAPI staining; (b) anti-*Crybb2* antibody staining; (c) overlay of (a) and (b). (d-f) *Crybb2*^{-/-} mouse testes: (d) DAPI staining; (e) anti-*Crybb2* antibody staining; (f) overlay of (d) and (e).

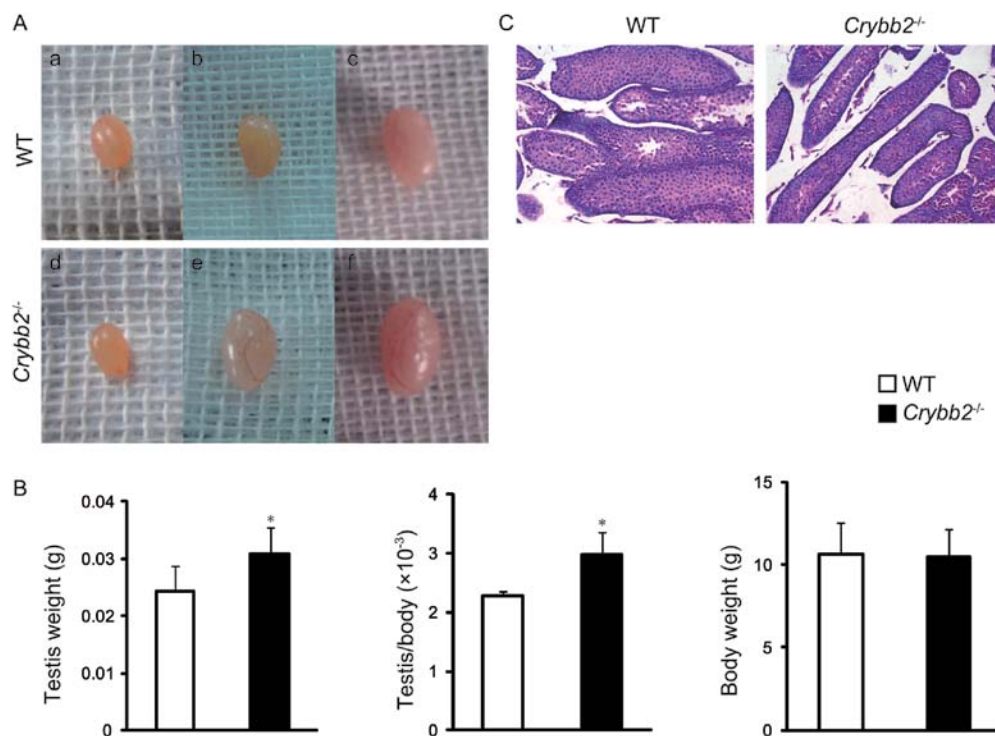


Figure 2. Increased size and weight of testis in 4-week-old *Crybb2*^{-/-} mice. (A) Gross appearance of *Crybb2*^{-/-} and WT mouse testis. The testis was isolated from (a and d) 2-week-old, (b and e) 4-week-old and (c and f) 6-week-old mice. There was no difference between the 2 mouse testes in 2-week-old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

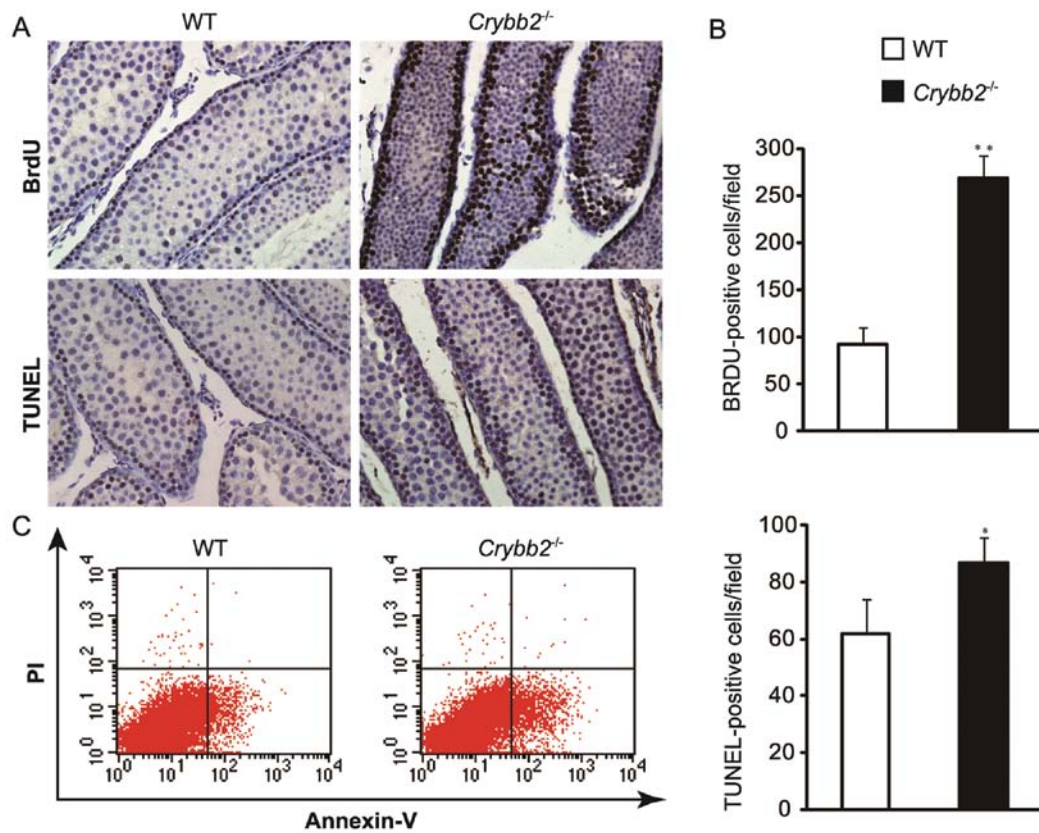


Figure 3. Enhanced proliferation and apoptosis of germ cells in *Crybb2*^{-/-} mouse testis. (A) Testis sections of *Crybb2*^{-/-} and WT mice stained with BrdU or TUNEL immunohistochemistry. Original magnification, x400. (B) The quantitative analysis of BrdU- and TUNEL-positive cells in *Crybb2*^{-/-}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

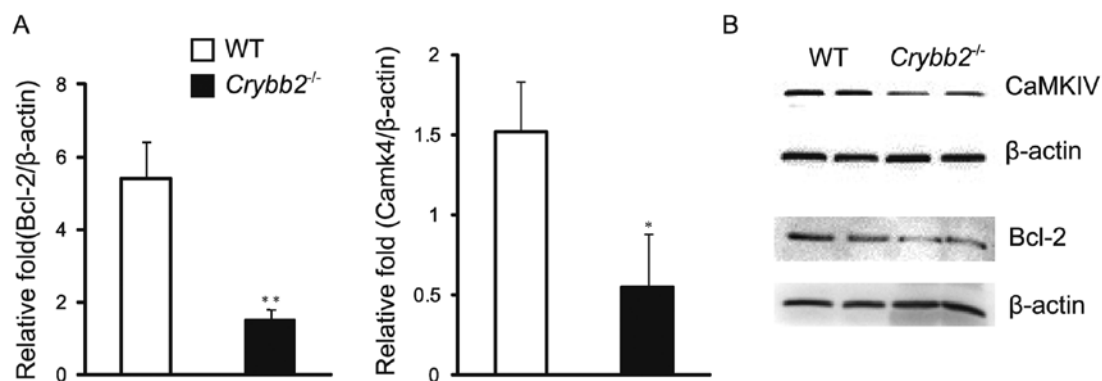


Figure 4. Decreased expression of Bcl-2 and CaMKIV in *Crybb2*^{-/-} mouse testis. (A) Real-time PCR analysis of Bcl-2 and CaMKIV mRNA expression. The results were normalized to the amount of β-actin as the internal control. Each value represents the average from 3 independent experiments. (B) Western 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 (Ca²⁺) 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 Ca²⁺ effects are mediated via Ca²⁺-induced activation of the ubiquitous Ca²⁺ receptor calmodulin (CaM) (26). In turn, Ca²⁺/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 Ca²⁺-binding protein (29), proposing the 4-Greek key crystallin fold as a Ca²⁺-binding motif (30). We speculate that *Crybb2* may not be regulated by Ca²⁺ due to the loss of the *Crybb2* function of Ca²⁺ binding, resulting in the change of Ca²⁺ 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 Ca²⁺ (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*, 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.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (no. 81170834) and the Shanghai Municipal Science and Technology Commission of China (no. 11ZR1447500).

References

1. Wistow GJ and Piatigorsky J: Lens crystallins: the evolution and expression of proteins for a highly specialized tissue. *Annu Rev Biochem* 57: 479-504, 1988.
2. Magabo KS, Horwitz J, Piatigorsky J and Kantorow M: Expression of betaB(2)-crystallin mRNA and protein in retina, brain, and testis. *Invest Ophthalmol Vis Sci* 41: 3056-3060, 2000.
3. Feng J, Smith DL and Smith JB: Human lens beta-crystallin solubility. *J Biol Chem* 275: 11585-11590, 2000.
4. Zhang Z, David LL, Smith DL and Smith JB: Resistance of human betaB2-crystallin to in vivo modification. *Exp Eye Res* 73: 203-211, 2001.
5. Peschek J, Braun N, Franzmann TM, *et al*: The eye lens chaperone alpha-crystallin forms defined globular assemblies. *Proc Natl Acad Sci USA* 106: 13272-13277, 2009.
6. Kumar PA, Kumar MS and Reddy GB: Effect of glycation on alpha-crystallin structure and chaperone-like function. *Biochem J* 408: 251-258, 2007.
7. Bhat SP: Transparency and non-refractive functions of crystallins - a proposal. *Exp Eye Res* 79: 809-816, 2004.
8. Piri N, Song M, Kwong JM and Caprioli J: Modulation of alpha and beta crystallin expression in rat retinas with ocular hypertension-induced ganglion cell degeneration. *Brain Res* 1141: 1-9, 2007.
9. Liedtke T, Schwamborn JC, Schroer U and Thanos S: Elongation of axons during regeneration involves retinal crystallin beta b2 (*crybb2*). *Mol Cell Proteomics* 6: 895-907, 2007.
10. Zhang J, Li J, Huang C, *et al*: Targeted knockout of the mouse betaB2-crystallin gene (*Crybb2*) induces age-related cataract. *Invest Ophthalmol Vis Sci* 49: 5476-5483, 2008.
11. Duprey KM, Robinson KM, Wang Y, Taube JR and Duncan MK: Subfertility in mice harboring a mutation in betaB2-crystallin. *Mol Vis* 13: 366-373, 2007.
12. Kitsos CM, Sankar U, Illario M, *et al*: Calmodulin-dependent protein kinase IV regulates hematopoietic stem cell maintenance. *J Biol Chem* 280: 33101-33108, 2005.
13. Zhang J, Huang CG, Li WJ, Weng W and Wang JQ: Establishment of a β B2 crystallin gene knockout mice model. *Acad J Sec Mil Med Univ* 27: 1246-1248, 2006.
14. Wang SL, Ribar TJ and Means AR: Expression of Ca(2+)/calmodulin-dependent protein kinase IV (CaMKIV) messenger RNA during murine embryogenesis. *Cell Growth Differ* 12: 351-361, 2001.
15. Wu JY, Ribar TJ, Cummings DE, Burton KA, McKnight GS and Means AR: Spermiogenesis and exchange of basic nuclear proteins are impaired in male germ cells lacking Camk4. *Nat Genet* 25: 448-452, 2000.
16. Illario M, Giardino-Torchia ML, Sankar U, *et al*: Calmodulin-dependent kinase IV links Toll-like receptor 4 signaling with survival pathway of activated dendritic cells. *Blood* 111: 723-731, 2008.
17. Corriero A, Medina A, Mylonas CC, *et al*: Proliferation and apoptosis of male germ cells in captive Atlantic bluefin tuna (*Thunnus thynnus* L.) treated with gonadotropin-releasing hormone agonist (GnRHa). *Anim Reprod Sci* 116: 346-357, 2009.

18. Aitken RJ, Findlay JK, Hutt KJ and Kerr JB: Apoptosis in the germ line. *Reproduction* 141: 139-150, 2011.
19. Shaha C, Tripathi R and Mishra DP: Male germ cell apoptosis: regulation and biology. *Philos Trans R Soc Lond B Biol Sci* 365: 1501-1515, 2010.
20. Matzuk MM and Lamb DJ: Genetic dissection of mammalian fertility pathways. *Nat Cell Biol* 4: S41-S49, 2002.
21. Hu MC, Hsu NC, El Hadj NB, *et al*: Steroid deficiency syndromes in mice with targeted disruption of *Cyp11a1*. *Mol Endocrinol* 16: 1943-1950, 2002.
22. Robertson KM, O'Donnell L, Jones ME, *et al*: Impairment of spermatogenesis in mice lacking a functional aromatase (*cyp 19*) gene. *Proc Natl Acad Sci USA* 96: 7986-7991, 1999.
23. Greider C, Chattopadhyay A, Parkhurst C and Yang E: BCL-x(L) and BCL2 delay Myc-induced cell cycle entry through elevation of p27 and inhibition of G1 cyclin-dependent kinases. *Oncogene* 21: 7765-7775, 2002.
24. Janumyan YM, Sansam CG, Chattopadhyay A, *et al*: Bcl-xL/Bcl-2 coordinately regulates apoptosis, cell cycle arrest and cell cycle entry. *EMBO J* 22: 5459-5470, 2003.
25. Berridge MJ, Bootman MD and Roderick HL: Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 4: 517-529, 2003.
26. Chin D and Means AR: Calmodulin: a prototypical calcium sensor. *Trends Cell Biol* 10: 322-328, 2000.
27. Hook SS and Means AR: Ca(2+)/CaM-dependent kinases: from activation to function. *Annu Rev Pharmacol Toxicol* 41: 471-505, 2001.
28. Wu JY and Means AR: Ca(2+)/calmodulin-dependent protein kinase IV is expressed in spermatids and targeted to chromatin and the nuclear matrix. *J Biol Chem* 275: 7994-7999, 2000.
29. Jobby MK and Sharma Y: Calcium-binding to lens betaB2- and betaA3-crystallins suggests that all beta-crystallins are calcium-binding proteins. *FEBS J* 274: 4135-4147, 2007.
30. Rajini B, Shridas P, Sundari CS, *et al*: Calcium binding properties of gamma-crystallin: calcium ion binds at the Greek key beta gamma-crystallin fold. *J Biol Chem* 276: 38464-38471, 2001.
31. Apati A, Janossy J, Brozik A, Bauer PI and Magocsi M: Calcium induces cell survival and proliferation through the activation of the MAPK pathway in a human hormone-dependent leukemia cell line, TF-1. *J Biol Chem* 278: 9235-9243, 2003.
32. Riccio A, Ahn S, Davenport CM, Blendy JA and Ginty DD: Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. *Science* 286: 2358-2361, 1999.