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Testicular germ cell apoptosis in Bcl6-deficient mice

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SUMMARY

Bcl6 protein has been detected in testicular germ cells, mainly spermatocytes, of normal mice, but its physiological role is largely unknown. The number of spermatozoa in the cauda epididymis of adult Bcl6-deficient (*Bcl6*$^{-/-}$) mice is lower than that of *Bcl6*$^{+/+}$ mice. We have found numerous apoptotic spermatocytes at the metaphase I stage with induction of Bax protein in adult *Bcl6*$^{-/-}$ testes. Developmentally, the incidence of germ cell apoptosis of *Bcl6*$^{-/-}$ mice was similar to that of *Bcl6*$^{+/+}$ mice until six weeks of age and increased after eight weeks of age. The incidence of apoptosis in heterozygous *Bcl6*$^{+/-}$ mice was also higher than that of *Bcl6*$^{+/+}$ mice. Since the activated form of p38 MAP kinase was detected in spermatocytes of adult *Bcl6*$^{-/-}$ mice, the germ cell apoptosis may be induced by stressors. Treatment of testes of adult *Bcl6*$^{+/+}$ mice with a mild hyperthermia resulted in germ cell apoptosis predominantly in metaphase I spermatocytes with induction of Bax protein and activation of p38 MAP kinase and this apoptosis mimics that in adult *Bcl6*$^{-/-}$ mice. Thus, Bcl6 may play a role as a stabilizer in protecting spermatocytes from apoptosis induced by stressors.

Key words: Bax, *Bcl6* knockout, Heat stress, p38 MAP kinase, Spermatogenesis, Mouse

INTRODUCTION

The *Bcl6* gene was first identified on the break-point of a chromosomal translocation involving 3q27 in human diffuse large B cell lymphomas (Kerckaert et al., 1993; Miki et al., 1994; Ye et al., 1993). Bcl6 protein contains a BTB/POZ domain in N-terminal region and six zinc-finger motifs in C-terminal region. It is located in the nucleus, where it functions as a sequence specific transcriptional repressor (Chang et al., 1996; Seyfert et al., 1996). Expression of *Bcl6* is ubiquitous, but is especially high in germinal center B cells (Cattoretti et al., 1995; Onizuka et al., 1995). The germinal center formation is defective in *Bcl6*-deficient (*Bcl6*$^{-/-}$) mice (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). *Bcl6*$^{-/-}$ mice also show growth retardation and eosinophilic inflammation including myocarditis. Since *Bcl6* is strongly expressed in mature cardiac myocytes, it may play a role in protecting myocytes from cell death (Yoshida et al., 1999). Gene transfection experiments demonstrate that overexpression of *Bcl6* enhances the viability of differentiating myocytes by preventing apoptosis (Kumagai et al., 1999); however, overexpression of *Bcl6* induces apoptosis in CV-1 and HeLa cells (Yamochi et al., 1999). The role of Bcl6 in apoptosis has therefore remained controversial.

Germ cell apoptosis naturally occurs in testis of rats (Billig et al., 1995) and mice (Rodriguez et al., 1997) at the prepubertal age. A peak of the physiological apoptosis, which is called the apoptotic wave, occurs around three to four weeks of age. The apoptotic cells are mainly spermatocytes and these cells express a large amount of Bax protein (Rodriguez et al., 1997). Overexpression of Bcl2 in germ cells inhibits the apoptotic wave followed by degeneration of germ cells in testes of transgenic adult mice (Furuchi et al., 1996; Rodriguez et al., 1997), indicating that the apoptotic wave is necessary for proper spermatogenesis. Many genes are expressed in germ cells during spermatogenesis (Eddy and O'Brien, 1998). Deficiency of genes such as *Bax* (Knudson et al., 1995), *Bcl-w* (*Bcl2l2* – Mouse Genome Informatics; Print et al., 1998; Ross et al., 1998), *Atm* (Xu et al., 1996), *Hsp70-2* (Dix et al., 1996), *cyclin A1* (Ccna1 – Mouse Genome Informatics; Liu et al., 1998), *Bsg* (Igakura et al., 1998; Toyama et al., 1999) and *Mlh1* (Edelmann et al., 1996) induces extensive germ cell apoptosis and arrest of spermatogenesis in mice. Thus, proper expression of these gene products is critical for spermatogenesis. Since *Bcl6* mRNA is present in normal mouse testis (Fukuda et al., 1995) and *Bcl6*$^{-/-}$ male mice have a very low fertility, Bcl6 may play a role in spermatogenesis. However, a role of Bcl6 in spermatogenesis has not been established.

Germ cell apoptosis is also induced by stress stimuli such as heat shock in adult rats (Lue et al., 1999). Stress stimuli activate mitogen-activated protein kinases (MAPKs) through phosphorylation. Several members of the MAPK family have

been identified, including extracellular signal-regulated kinase (ERK) (Pelech and Sanghera, 1992; Marshall, 1994), c-JUN-N-terminal protein kinase (SAPK/JNK) (Kyriakis and Avruch, 1990; Kyriakis et al., 1994; Verheij et al., 1996) and p38 MAP kinase (Freshney et al., 1994; Lee et al., 1994; Rouse et al., 1994). ERK is principally activated by stimulation of growth factors, while both SAPK/JNK and p38 MAP kinase are activated by stress stimuli such as severe heat shock (Nagata et al, 1999), strong oxidants and UV irradiation (Derijard et al., 1994; Kyriakis et al., 1994; Raingeaud et al., 1995). In the present study, we examined spermatogenesis in testes of *Bcl6*$^{-/-}$ mice. Although mature sperm developed in *Bcl6*$^{-/-}$ mice, the number of spermatozoa in the cauda epididymis was lower than that in *Bcl6*$^{+/+}$ mice. Numerous apoptotic spermatocytes, mainly at the metaphase I stage, were evident in adult *Bcl6*$^{-/-}$ mice. Since the activated form of p38 MAP kinase was detected in spermatocytes of adult *Bcl6*$^{-/-}$ mice, germ cell apoptosis may be induced by stressors. We discuss the role of Bcl6 in regulating stress-induced apoptosis during spermatogenesis.

MATERIALS AND METHODS

Animals

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). *Bcl6*$^{-/-}$ mice described elsewhere (Yoshida et al., 1999) were maintained under specific pathogen free conditions in the animal center of Chiba University School of Medicine.

Examination of reproductive performance

Reproductive performance of *Bcl6* $^{-/-}$ mice was examined after eight weeks of age. Briefly, a *Bcl6*$^{-/-}$ male mouse was mated with two wild-type female mice for 2 months. If no pregnancy was observed during the first month, these female mice were replaced with other females and pregnancy of the latter mice was monitored for the second one-month period. For short-term mating study, a *Bcl6*$^{-/-}$ male mouse at 12 weeks of age was mated with two six-week-old C57BL/6 female mice that had been superovulated by injection of 5 IU of pregnant mare serum gonadotropin (Sigma) and 5 IU of human chorionic gonadotropin (Sigma) for a 48-hour interval, and the presence of copulatory plugs was examined the next morning. For in vitro fertilization, spermatozoa from the cauda epididymis of *Bcl6* $^{-/-}$ mice at 12 weeks of age were prepared in 250 μl TYH (Toyoda, Yokohama and Hoshi) medium (Toyoda and Chang, 1974) containing bovine serum albumin (4 mg/ml), and sperm were allowed to disperse into the medium. Sperm were capacitated by incubation for 90 minutes at 37°C. Sperm counts and motility were determined visually by phase contrast microscopy. C57BL/6 female mice were superovulated and oocytes were isolated on the following morning. Twenty oocytes were mixed with 15,000 capacitated sperm in 100 μl TYH medium, incubated for 24 hours at 37°C, and examined for developmental progression to the two-cell stage by phase contrast microscopy.

Histology and electron microscopy

Testes were fixed by perfusion through the heart with either 4% paraformaldehyde or 3% glutaraldehyde in Hepes buffer (10 mM, pH 7.4) for 20 minutes. Then, the testes were removed, cut into small blocks, and immersed in the fixative for an additional 12 hours. Glutaraldehyde-fixed testes were postfixed with 1% OsO_4, dehydrated and embedded in Epon 812. Semi-thin sections (1 μm thick) were stained with 0.1% Toluidine Blue and analyzed histologically by light microscopy. Ultra-thin sections (80 nm thick) were stained with uranyl acetate followed by lead citrate and examined by electron microscopy (JEOl 1200 EX, Tokyo, Japan).

Immunohistochemistry

For immunohistochemistry, 4% paraformaldehyde-perfused testes were immersed in 20% sucrose and embedded in OTC compound (Miles, Elkhart, IN), and frozen 10 μm sections were cut. Perfused testes were dehydrated with ethanol, embedded in paraffin, and 5 μm thick paraffin sections were cut. We used rabbit polyclonal antibodies against Bax and Bcl6 (N-3) (Santa Cruz Biotechnology, Santa Cruz, CA) on paraffin sections, and against Bcl-x_L, p53, Cdc2, cyclin B1 (Santa Cruz Biotechnology) and phospho-p38 MAP kinase (Thr 180/Tyr 182) (New England Biolabs, Beverly, MA) on frozen sections. Sections were incubated overnight with the primary antibody. Normal rabbit serum was used for a control. The biotinylated goat anti-rabbit immunoglobulin antibody (Nichirei, Tokyo, Japan) and the Vectastain Elite ABC-kit (Vector Laboratories, Burlingame, CA) were used as the second- and the third-phase reagents, respectively. Bound peroxidase activity was visualized with the diaminobenzidine detection kit (DAB kit; Nichirei). Sections were counterstained with hematoxylin.

TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay

TUNEL assay was carried out as described (Gavrieli et al., 1992) with slight modification. Briefly, 4% paraformaldehyde-perfused testes were embedded in paraffin wax. Sections (5 μm thick) were deparaffinized in xylene and treated with the proteinase K solution for 30 minutes at 37°C. The tailing reaction was then carried out in TdT buffer in the presence of dUTP-biotin for 1 hour at 37°C (The Mebstain Apoptosis kit, Medical & Biological Laboratories, Nagoya, Japan). Signals were visualized by the Vector Elite ABC-kit and the DAB kit. Sections were counterstained with hematoxylin.

Quantitative analysis of TUNEL positive cells

Three to nine testes of each group were used for each experiment. The apoptotic cell number was calculated and averaged by counting apoptotic cells in 30-50 apoptotic tubule cross sections (TCS) within 400 TCS in each testis.

In vivo hyperthermia

Testes in the scrotum of adult mice were immersed in a thermostatically controlled water bath for 15 minutes at 42°C. Testes were removed 6 hours later, fixed with 4% paraformaldehyde, and apoptotic cells in the testes were detected by TUNEL assay.

Immunoblot analysis

Testicular cells (1×10^8) were suspended in 1 ml of IP (immunoprecipitation) buffer (Kobayashi et al, 1997) at 4°C and the clarified lysate was obtained by centrifugation. The amount of protein in cell lysates was determined using the BioRad protein assay (BioRad Laboratories). Cell lysates (80 μg) were resolved by SDS-PAGE and transferred to a polyvinyl difluoride membrane (Immobilon-P, Millipore, Bedford, MA). Blots were blocked in Blockace (Yukijirushi, Sapporo, Japan) for 1 hour, washed three times with Tris-buffered saline with 0.2% Tween 20 (TBST), and incubated with the antibody for phospho-p38 MAP kinase (Thr 180/Tyr 182) and p38 MAP kinase (New England Biolabs) in TBST for overnight at 4°C. The blots were then washed three times with TBST, incubated with affinity-purified donkey anti-rabbit antibody conjugated with horseradish peroxidase (HRP, Amersham) for 1 hour at room temperature, washed four times with TBST and developed with enhanced chemiluminescence reagents (Amersham).

Statistical analysis

Paired comparisons of testicular weight, epididymal spermatozoa numbers, in vitro fertilization rates of sperm, serum testosterone levels, and the apoptotic index in testes among *Bcl6*$^{+/+}$, *Bcl6*$^{+/-}$ and *Bcl6*$^{-/-}$ mice were performed for statistical significance by calculating means±s.e.m. and Student's *t* test.

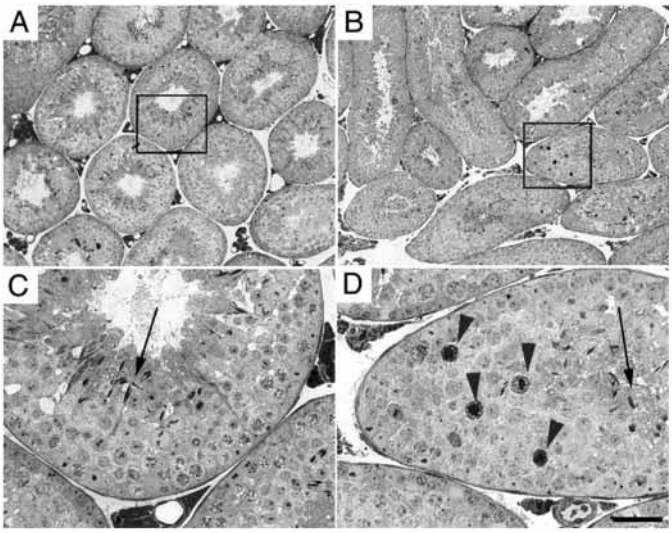

Fig. 1. Many germ cells are degenerated in testes of adult *Bcl6*$^{-/-}$ mice. Semi-thin sections of seminiferous tubules of *Bcl6*$^{+/+}$ (A,C) and *Bcl6*$^{-/-}$ (B,D) mice at 12 weeks of age. Sections were stained with Toluidine Blue. The seminiferous tubules of *Bcl6*$^{-/-}$ mice appear normal with mature spermatids (arrow), except for the presence of many degenerated germ cells (arrowheads). Scale bar: 100 μm in A,B; 25 μm in C,D.

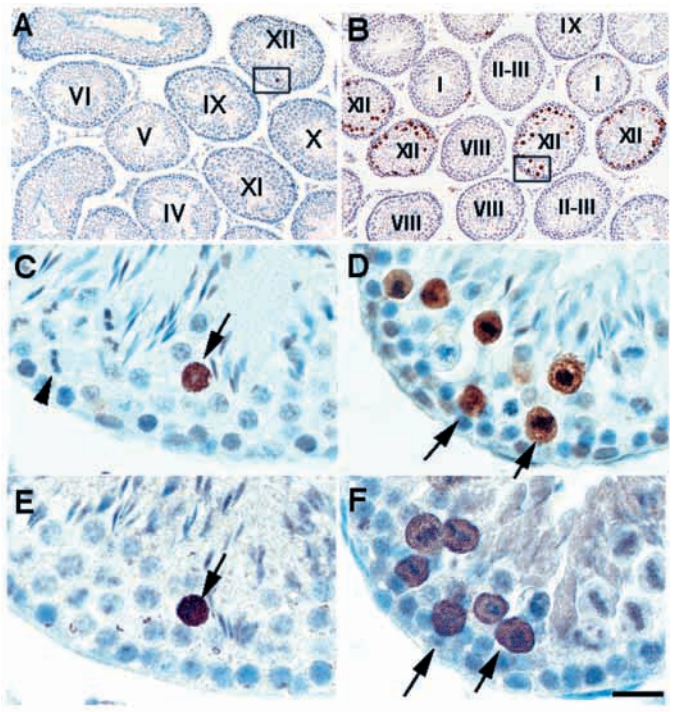

Fig. 2. Apoptotic cells with a large amount of Bax protein are frequently observed in metaphase I spermatocytes of adult *Bcl6*$^{-/-}$ mice. Sections of seminiferous tubules of *Bcl6*$^{+/+}$ (A,C,E) and *Bcl6*$^{-/-}$ (B,D,F) mice at 12 weeks of age. Parts of A and B are shown at a higher magnification in C and D, respectively. (A-D) Apoptotic cells were detected by TUNEL assay. TUNEL-positive cells (arrows) are rarely detectable in seminiferous tubules of *Bcl6*$^{+/+}$ mice (A,C) but frequently detectable in those of *Bcl6*$^{-/-}$ mice (B,D) at metaphase I spermatocytes of the stage XII seminiferous tubules. The arrowhead in C shows a TUNEL-negative metaphase I spermatocyte. (E,F) The adjoining sections were stained with an anti-Bax antibody. Apoptotic (TUNEL^{+}) cells express a detectable amount of Bax protein (arrows). Counter stained with hematoxylin. Scale bar: 100 μm in A,B; 25 μm in C-F.

RESULTS

The reproductive function of adult *Bcl6*$^{-/-}$ mice is lower than that of *Bcl6*$^{+/+}$ mice

The reproductive function of *Bcl6*$^{-/-}$ male mice was examined by mating them with wild-type female mice. Only 15% of *Bcl6*$^{-/-}$ mice (four out of 27 mice) at 8-12 weeks of age were able to make female mice pregnant whereas all 26 *Bcl6*$^{+/-}$ and *Bcl6*$^{+/+}$ mice examined were fertile. A short-term mating study was carried out to determine whether the absence of Bcl6 might affect the mating performance of *Bcl6*$^{-/-}$ mice or not. *Bcl6*$^{-/-}$ males produced copulatory plugs in three out of nine mates, the frequency of which is lower than that of *Bcl6*$^{+/-}$ (9 out of 9 mates) and *Bcl6*$^{+/+}$ males (9 out of 9 mates). Testes of *Bcl6*$^{-/-}$ mice weighed slightly less (79±12 mg) than those of *Bcl6*$^{+/+}$ mice (93±11 mg) but not significantly so (P=0.25). The weight of those from *Bcl6*$^{+/-}$ mice (102±19 mg) was similar to testes from *Bcl6*$^{+/+}$ mice (P=0.41). The number of spermatozoa in the cauda epididymis of the *Bcl6*$^{-/-}$ mice (2.2±0.4 ×10^6/ml) was less than the number in *Bcl6*$^{+/+}$ mice (9.8±2.0 ×10^6/ml; P=0.0031). The number of spermatozoa in the cauda epididymis of the *Bcl6*$^{+/-}$ mice (8.2±3.0 ×10^6/ml) was similar to that of the *Bcl6*$^{+/+}$ mice (P=0.75). In vitro fertilization of normal oocytes was carried out using equivalent numbers of motile sperm from *Bcl6*$^{-/-}$ and *Bcl6*$^{+/+}$ mice. The fertilization rate of *Bcl6*$^{-/-}$ sperm (27±6%) was less than that of *Bcl6*$^{+/+}$ sperm (80±5%; P<0.0001).

Germ cell apoptosis is frequently observed in metaphase I spermatocytes of adult *Bcl6*$^{-/-}$ mice

Testes of *Bcl6*$^{-/-}$ and *Bcl6*$^{+/+}$ mice at 12 weeks of age were histologically examined. Testes of *Bcl6*$^{+/+}$ mice revealed seminiferous tubules that contained normal spermatogenic cells at various stages of development and numerous numbers of mature spermatids (Fig. 1A,C). Although seminiferous tubules of *Bcl6*$^{-/-}$ mice contained spermatogenic cells at various stages, including mature spermatids, many degenerating germ cells were identified (Fig. 1B,D). We then carried out a TUNEL assay to detect apoptotic cells in testes of *Bcl6*$^{-/-}$ and *Bcl6*$^{+/+}$ mice at 12 weeks of age. Seminiferous tubules of *Bcl6*$^{-/-}$ mice (Fig. 2B,D) contained larger numbers of TUNEL-positive cells than did those of *Bcl6*$^{+/+}$ mice (Fig. 2A,C). The majority (>90%) of TUNEL-positive cells were metaphase I spermatocytes where the chromosomes were clearly visible as a cluster in stage XII seminiferous tubules (Russell et al., 1990) (Fig. 2D). Some of TUNEL-positive cells (<10%) were pachytene spermatocytes. To explore the relationship between these apoptotic cells and modified levels of protein involved in cell death, immunohistochemistry was performed on serial sections of the testes with antibodies specific for Bax, Bcl-x_L or p53. The apoptotic cells in seminiferous tubules of both *Bcl6*$^{+/+}$ and *Bcl6*$^{-/-}$ mice express a detectable amount of Bax protein (Fig. 2E,F) but not Bcl-x_L or p53 protein (data not shown).

Germ cell apoptosis in *Bcl6*$^{-/-}$ mice at 12 weeks of age was confirmed by electron microscope analysis. A normal

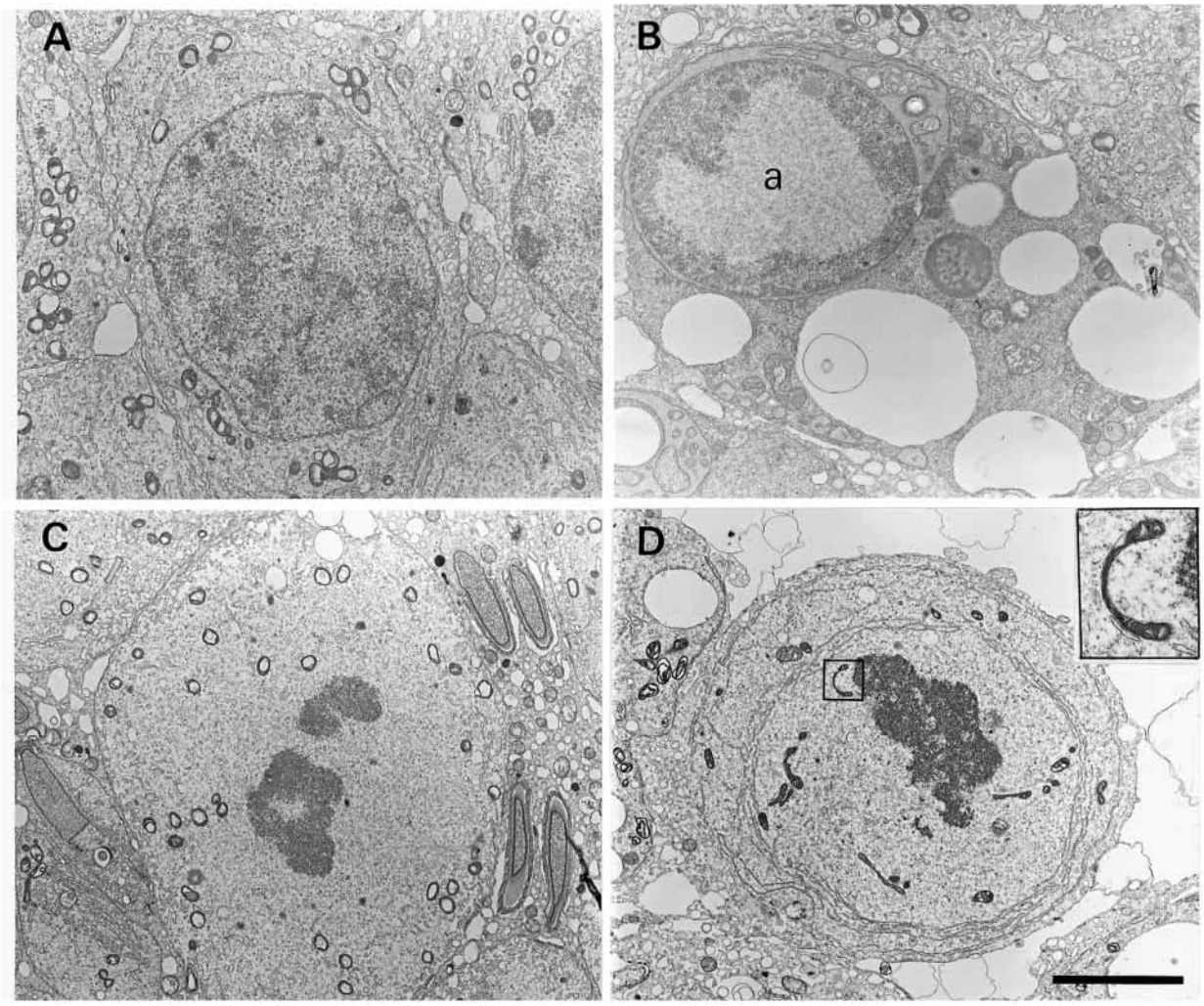

Fig. 3. Apoptotic spermatocytes are detected in testes of adult *Bcl6*$^{-/-}$ mice by an electron microscope. Ultra-thin sections of seminiferous tubules of *Bcl6*$^{+/+}$ (A,C) and *Bcl6*$^{-/-}$ (B,D) mice at 12 weeks of age. (A) A normal pachytene spermatocyte. (B) A spermatocyte contains a clear area in the nucleus (a), showing a typical appearance of apoptotic degeneration. (C) A normal metaphase I spermatocyte. Chromosome is condensed and locates in the center of the cell. The nuclear membrane disappears at this stage. (D) An apoptotic metaphase I spermatocyte. Note the deformed mitochondria (inset in D) and a peripherally located endoplasmic reticulum. Scale bar: 4.2 μm in A; 2.8 μm in B; 4.8 μm in C; 5.0 μm in D.

pachytene spermatocyte in *Bcl6*$^{+/+}$ mice had round nuclei with a regular contour (Fig. 3A). A spermatocyte in *Bcl6*$^{-/-}$ mice was degenerated with a clear area in the nucleus (Fig. 3B). A normal metaphase I spermatocyte in *Bcl6*$^{+/+}$ mice shows condensed chromosomes without a nuclear membrane (Fig. 3C). However, a metaphase I spermatocyte in *Bcl6*$^{-/-}$ mice had deformed mitochondria (Fig. 3D, inset) and a peripherally located endoplasmic reticulum. These results confirmed the presence of apoptotic cells in testes of adult *Bcl6*$^{-/-}$ mice. Spermatogonia, Sertoli and Leydig cells of the *Bcl6*$^{-/-}$ mice were normal in this examination (data not shown).

Bcl6 mRNA was detected in total RNA of normal mouse testis (Fukuda et al., 1995). To examine the relationship between loss of *Bcl6* expression and the apoptosis in adult *Bcl6*$^{-/-}$ mice, the presence of Bcl6 protein in spermatocytes was examined in testes of *Bcl6*$^{+/+}$ mice at 12 weeks of age by immunohistochemistry. Bcl6 was detectable in nuclei of primary spermatocytes at the pachytene, diplotene and metaphase I stage, secondary spermatocytes, and spermatids up to step 8 (Fig. 4A). Sertoli and Leydig cells did not express Bcl6 in the testes. As a control for specificity of the anti-Bcl6 antibody, testes of *Bcl6*$^{-/-}$ mice were stained. Bcl6 protein was not detected in testes of *Bcl6*$^{-/-}$ mice (Fig. 4B).

Development of testis of *Bcl6*$^{-/-}$ mice is similar to that of *Bcl6*$^{+/+}$ mice

In order to examine the onset of germ cell apoptosis in testes of *Bcl6*$^{-/-}$ mice, we examined testes histologically at the prepubertal age. Seminiferous tubular lumens of *Bcl6*$^{+/+}$ and *Bcl6*$^{-/-}$ mice were closed at two weeks of age (Fig. 5A,B) but were open at the age of four weeks (Fig. 5C,D). Mature spermatids were detectable in testes of *Bcl6*$^{+/+}$ and *Bcl6*$^{-/-}$ mice at eight weeks of age, and there was no significant histological difference in testes between them until eight weeks of age (Fig. 5E,F).

Germ cell apoptosis at prepubertal age occurred in

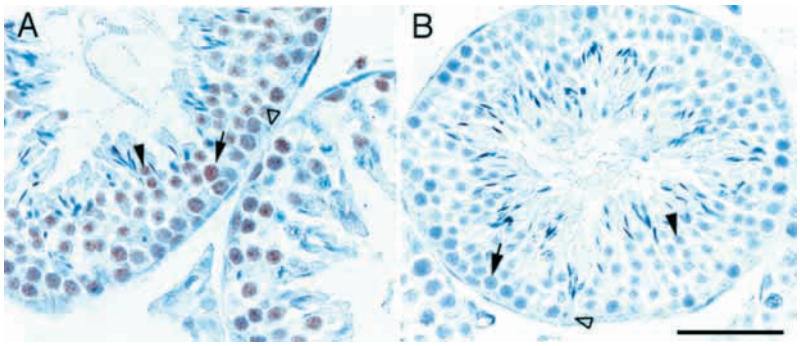

Fig. 4. Expression of Bcl6 protein in testes of adult *Bcl6*$^{+/+}$ mice. Testicular sections from *Bcl6*$^{+/+}$ (A) and *Bcl6*$^{-/-}$ (B) mice at 12 weeks of age were stained with an anti-Bcl6 antibody. Nuclei of pachytene spermatocytes (arrows) and step-7 spermatids (black arrowheads) are stained in testes from *Bcl6*$^{+/+}$ mice (A) but not from *Bcl6*$^{-/-}$ mice (B). Sertoli (white arrowheads) and Leydig cells are stained in neither testis. Counter stained with hematoxylin. Scale bar: 50 μm.

spermatocytes (Billing et al., 1995) mainly at the pachytene and the metaphase I stage of normal mice (data not shown). The number of apoptotic cells in a seminiferous tubule during development was calculated and statistically analyzed between *Bcl6*$^{+/+}$ and *Bcl6*$^{-/-}$ mice (Fig. 6). The apoptotic cell number of *Bcl6*$^{-/-}$ mice was similar to that of *Bcl6*$^{+/+}$ mice until four weeks of age, suggesting that the physiological apoptotic wave occurs normally in *Bcl6*$^{-/-}$ mice. Those apoptotic cells expressed a detectable amount of Bax protein (data not shown). The apoptotic cell number decreased in *Bcl6*$^{+/+}$ mice after six weeks of age. However, it continued at an increasingly high level in *Bcl6*$^{-/-}$ mice older than eight weeks.

Germ cell apoptosis in adult *Bcl6*$^{-/-}$ mice may be induced by stressors

Heat shock is a stress known to induce germ cell apoptosis in adult rats (Lue et al., 1999), so the pathology of germ cell apoptosis induced by heat stress in normal mice was examined. Testes of *Bcl6*$^{+/+}$ mice at eight weeks of age were subjected in vivo to hyperthermia (42°C) for 15 minutes, and apoptotic cells in the testes were detected by TUNEL assay 6 hours after the treatment. Testes of *Bcl6*$^{+/+}$ mice exhibited numerous apoptotic germ cells after heat treatment, with the majority (>90%) being metaphase I stage spermatocytes (Fig. 7A,B). These apoptotic cells expressed high levels of Bax protein (Fig. 7C). Since p38 MAP kinase is activated by stress stimuli (Nagata and Todokoro, 1999; Raingeaud et al., 1995), we examined expression of phospho-p38 MAP kinase in testes of *Bcl6*$^{+/+}$ mice at 12 weeks of age after heat treatment by immunoblotting. p38 MAP kinase (Fig. 8A) was activated in testes of *Bcl6*$^{+/+}$ mice 30 minutes to 1 hour after heat treatment. Phospho-p38 MAP kinase was also detected in leptotene, zygotene, pachytene spermatocytes 1 hour after heat treatment by immunohistochemistry (Fig. 8B). Interestingly phospho-p38 MAP kinase was obviously present in non-heat-treated testes of *Bcl6*$^{-/-}$ mice at 12 weeks of age by immunoblotting (Fig. 8C). Additionally, the nuclei of primary spermatocytes of the *Bcl6*$^{-/-}$ mice contained phospho-p38 MAP kinase by immunohistochemistry (Fig. 8D).

We next examined the relationship between the apoptotic cell number and the amount of Bcl6 in spermatocytes using testes of heterozygous *Bcl6*$^{+/-}$ mice, since *Bcl6*$^{+/-}$ mice are expected to express half the amount of *Bcl6* as *Bcl6*$^{+/+}$ mice (Yoshida et al., 1999). The apoptotic cell number in testes of *Bcl6*$^{+/-}$ mice at 12-16 weeks of age was examined by TUNEL assay and the data were compared with those of age-matched *Bcl6*$^{+/+}$ and *Bcl6*$^{-/-}$ mice (Fig. 9). Although the apoptotic cell number (17.3±5.4) of *Bcl6*$^{-/-}$ mice was the highest, the number in *Bcl6*$^{+/-}$ mice (6.8±3.7) was also higher than that in *Bcl6*$^{+/+}$ mice (2.5±0.4; $P<0.005$). Furthermore, heat sensitivity of spermatocytes of *Bcl6*$^{+/+}$, *Bcl6*$^{+/-}$ and *Bcl6*$^{-/-}$ mice at 12 weeks of age was examined by subjected testes in vivo to hyperthermia (42°C) for 15 minutes. Apoptotic cells in the testes were detected by TUNEL assay 6 hours after the treatment. The apoptotic cell numbers of *Bcl6*$^{+/+}$ (9.4±1.5) and *Bcl6*$^{+/-}$ (13.5±3.6) mice increased four and twofold after heat treatment, respectively. However, the number of apoptotic cells in *Bcl6*$^{-/-}$ mice (17.2±3.5) was not changed by heat treatment.

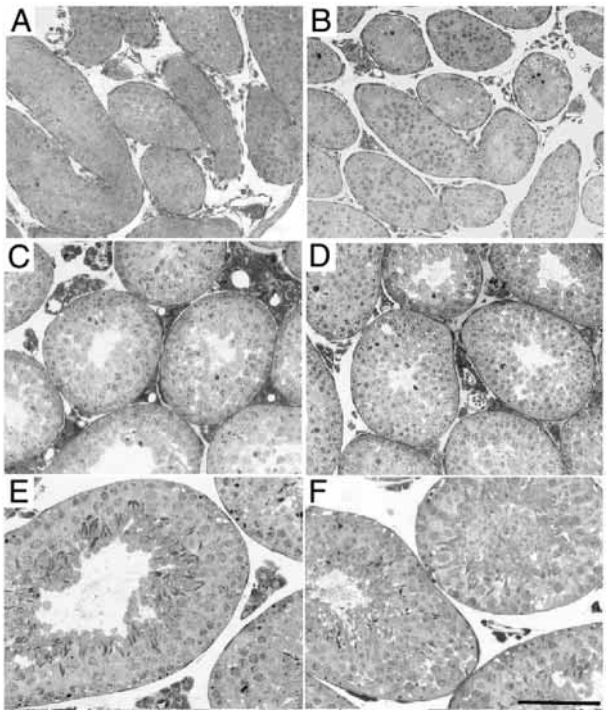

Fig. 5. Development of prepubertal testes in *Bcl6*$^{-/-}$ mice is similar to that in *Bcl6*$^{+/+}$ mice. Semi-thin sections of seminiferous tubules of *Bcl6*$^{+/+}$ (A,C,E) and *Bcl6*$^{-/-}$ (B,D,F) mice. Sections from mice at two weeks of age (A,B), four weeks (C,D) and eight weeks (E,F) were stained with Toluidine Blue. (A-D) Seminiferous tubular lumens of *Bcl6*$^{+/+}$ and *Bcl6*$^{-/-}$ mice are closed at two weeks of age (A,B) but are open at the age of four weeks (C,D). Mature spermatids develop in testes of *Bcl6*$^{-/-}$ mice at eight weeks of age, and there is no significant histological difference between testes from these mice and *Bcl6*$^{+/+}$ mice until eight weeks of age. Scale bar: 100 μm.

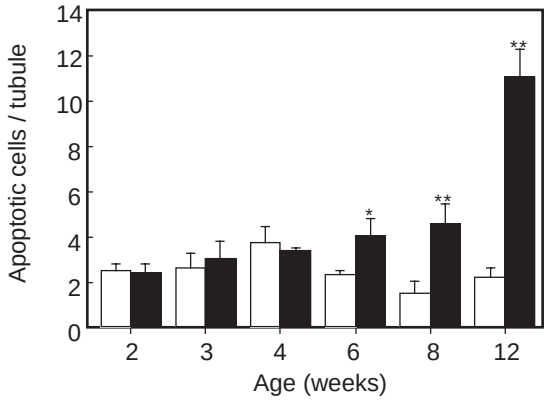

Fig. 6. Germ cell apoptosis continues in *Bcl6*$^{-/-}$ mice until adult age. Apoptotic germ cells in cross sections of the seminiferous tubules in *Bcl6*$^{+/+}$ (white) and *Bcl6*$^{-/-}$ (black) mice were detected by TUNEL assay. The number of TUNEL positive cells in 30-50 cross sections of the tubules with apoptotic cells was counted. The apoptotic cell number peaks between 3 and 4 weeks of age and decreases to lower level in *Bcl6*$^{+/+}$ mice after 6 weeks of age. In *Bcl6*$^{-/-}$ mice, the high apoptotic cell number continues to adult age. Results represent the means and variations (s.e.m.) of 3 mice. *Change compared with control is statistically significant, $P<0.05$, two-sample *t*-test for independent samples with unequal variances. **Change compared with control is statistically significant, $P<0.005$, two-sample *t*-test for independent samples with unequal variances.

DISCUSSION

Germ cell apoptosis in adult *Bcl6*$^{-/-}$ mice may be induced by stressors

Bcl6$^{-/-}$ male mice showed the lower reproductive function and only 15% of *Bcl6*$^{-/-}$ mice were fertile. The frequency of copulation (plugging ability) of *Bcl6*$^{-/-}$ mice was about one third that of *Bcl6*$^{+/+}$ mice, suggesting that the lower reproductive function is partly due to the abnormal reproductive behaviour of *Bcl6*$^{-/-}$ mice. Furthermore, the number of spermatozoa in the cauda epididymis of *Bcl6*$^{-/-}$ mice was about 20% of that of *Bcl6*$^{+/+}$ mice, and the in vitro fertilization rate of motile sperm from *Bcl6*$^{-/-}$ mice was lower than that from *Bcl6*$^{+/+}$ mice. Thus, the lower reproductive function of *Bcl6*$^{-/-}$ mice may be due to the abnormal reproductive behaviour, the lower number of spermatozoa and the abnormal fertilization function of spermatozoa. Since *Bcl6*$^{-/-}$ mice show growth retardation and eosinophilic inflammation (Yoshida et al., 1999), these abnormal physical conditions may be one of the reasons for the abnormalities.

The lower number of spermatozoa from *Bcl6*$^{-/-}$ mice may be due to germ cell apoptosis since numerous apoptotic spermatocytes mainly at the metaphase I stage were evident in adult *Bcl6*$^{-/-}$ testes. We suspected that the germ cell apoptosis in adult *Bcl6*$^{-/-}$ mice might be related with the abnormal physical conditions. However, apoptosis of metaphase I spermatocytes was also evident in heterozygous adult *Bcl6*$^{+/-}$ mice under conditions without growth retardation and eosinophilic inflammation, suggesting little relation between the germ cell apoptosis and the abnormal physical conditions. When levels of follicle stimulating hormone/luteinizing hormone decrease in normal rats at three to four weeks of age, testicular germ cell apoptosis is induced (Billig et al., 1995)

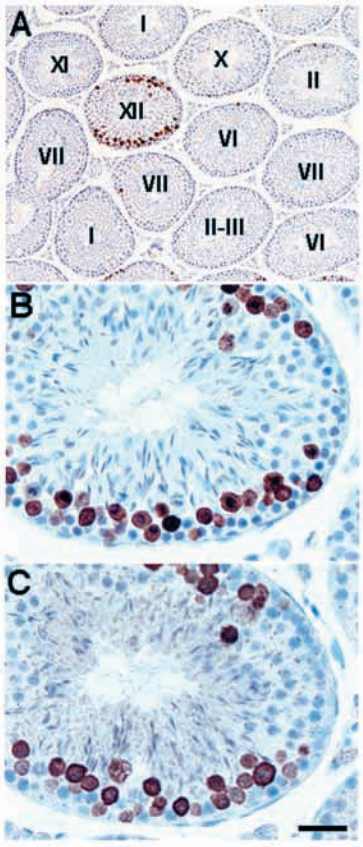

Fig. 7. A mild hyperthermia induces apoptosis in metaphase I spermatocytes with a detectable amount of Bax protein in testes of *Bcl6*$^{+/+}$ mice. Testes of *Bcl6*$^{+/+}$ mice at eight weeks of age were treated with a mild hyperthermia (42°C) for 15 minutes. Sections of seminiferous tubules of the mice were prepared 6 hours after treatment. (A,B) Apoptotic germ cells in the sections were detected by TUNEL assay. Part of A is shown at a higher magnification in B. Note that many spermatocytes are TUNEL positive after treatment and the majority of TUNEL-positive cells are metaphase I spermatocytes at the stage XII seminiferous tubules. (C) The adjoining section was stained with an anti-Bax antibody. Apoptotic (TUNEL^{+}) cells express a detectable amount of Bax protein. Counterstained with hematoxylin. Scale bar: 100 μm in A; 25 μm in B,C.

and the germ cell apoptosis is mostly detected in early primary spermatocytes and spermatids in rats (Hikim et al., 1995). The spermatogenic stages of the apoptotic cells are different from those in adult *Bcl6*$^{-/-}$ mice. Furthermore, the hormonal change has no effect on spermatogenesis in adult rats (Billig et al., 1995) and mice (Kumar et al., 1997), suggesting that germ cell apoptosis in adult *Bcl6*$^{-/-}$ mice is not due to decreased levels of the gonadotropins. Although Sertoli and Leydig cells are critical for spermatogenesis (Griswold, 1995; Lue et al., 1999; Ross et al., 1998), Sertoli and Leydig cells did not express Bcl6 in normal mice (Fig. 4A), and these cells of adult *Bcl6*$^{-/-}$ mice were histologically normal. These results suggest that germ cell apoptosis in adult *Bcl6*$^{+/-}$ and *Bcl6*$^{-/-}$ mice is induced under conditions where a number of cytological and hormonal networks appeared normal.

Testicular germ cell apoptosis occurs in rats (Billig et al., 1995) and mice (Rodriguez et al., 1997) at the prepubertal age. A large amount of Bax protein is detected in testicular

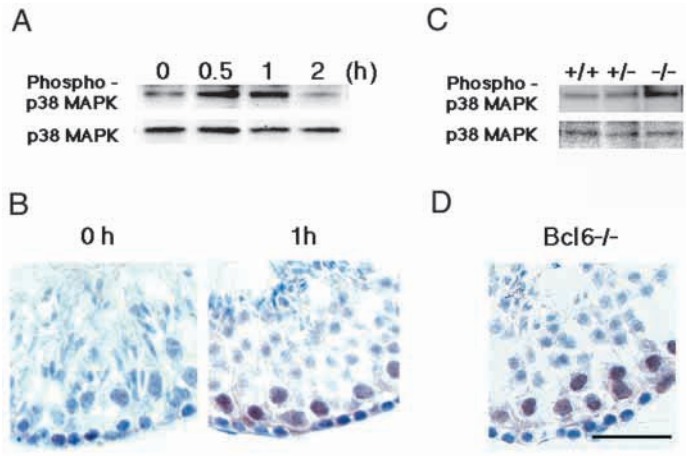

Fig. 8. A mild hyperthermia induces activation of p38 MAP kinase in spermatocytes of *Bcl6*$^{+/+}$ mice. (A) p38 MAP kinase in testicular cell lysates from *Bcl6*$^{+/+}$ mice at eight weeks of age treated with a mild hyperthermia (42°C) for 15 minutes was analyzed by western blot analysis using anti-phospho p38 MAP kinase and anti-p38 MAP kinase. p38 MAP kinase is activated 30 minutes to 1 hour after heat treatment. (B) Phospho-p38 MAP kinase is detected by immunohistochemistry in nuclei of pachytene spermatocytes of *Bcl6*$^{+/+}$ mice 1 hour after heat treatment. (C,D) Phospho-p38 MAP kinase is detected by western blot analysis (C) and immunohistochemistry (D) in testes of *Bcl6*$^{-/-}$ mice at 12 weeks of age without heat treatment. Scale bar: 25 μm in B,D.

apoptotic cells during this physiological apoptotic wave (Schwartz et al., 1993; data not shown) that is due to a decrease in testosterone level (Rodriguez et al., 1997). p53, which is highly expressed in pachytene spermatocytes during the prepubertal age (Schwartz et al., 1993), may function as a *Bax* promoter (Miyashita and Reed, 1995; Selvakumaran et al., 1994). The apoptotic cells in adult *Bcl6*$^{-/-}$ mice did express a detectable amount of Bax but not p53. Furthermore, adult *Bcl6*$^{-/-}$ mice had normal levels of testosterone in their sera (data not shown). Thus, the mechanism of germ cell apoptosis in adult *Bcl6*$^{-/-}$ mice appears different from the physiological apoptotic wave. Since the apoptotic cell number of *Bcl6*$^{-/-}$ mice was similar to that of *Bcl6*$^{+/+}$ mice at prepubertal age, but increased as animals became adults and the activated form of p38 MAP kinase was detected in primary spermatocytes of adult *Bcl6*$^{-/-}$ mice, the germ cell apoptosis may be stress induced.

Germ cell apoptosis induced by heat stress in normal mice may mimic that in adult *Bcl6*$^{-/-}$ mice

Sporadic apoptotic cell death occurs at every stage of the spermatogenic cycle in normal adult rats (Blanco-Rodriguez and Martinez-Garcia, 1996). However, in adult *Bcl6*$^{-/-}$ mice, germ cell apoptosis preferentially occurred in metaphase I spermatocytes, which suggests that a specific mechanism governs the apoptosis in spermatocytes. Spermatocytes at the metaphase I stage are most sensitive to heat stress in rats (Lue et al., 1999) and mice (Fig. 7), though the mechanism of germ cell apoptosis induced by testicular hyperthermia is unknown. We found a detectable amount of Bax protein and the activated form of p38 MAP kinase in spermatocytes of heat-treated *Bcl6*$^{+/+}$ mice. Thus, Bax and p38 MAP kinase are associated with heat-induced germ cell apoptosis, and both were also detected in spermatocytes of adult *Bcl6*$^{-/-}$ mice without heat treatment. These results suggest that the mechanism of germ cell apoptosis in adult *Bcl6*$^{-/-}$ mice is related to that in *Bcl6*$^{+/+}$ mice induced by heat stress. Since germ cell apoptosis is induced in rats even when testes are exposed to abdominal temperature (Yin et al., 1997), metaphase I spermatocytes without Bcl6 may die when subjected to mild heat stress.

A member of the heat shock protein gene family, the *Hsp70-2* gene, is expressed in primary spermatocytes (Allen et al., 1988; Zakeri et al., 1988) and spermatogenesis fails to proceed to meiosis in mice with null mutation of the gene (Dix et al., 1996; Dix et al., 1997; Zhu et al., 1997). Thus, dysregulated expression of the *Hsp70-2* gene by deficiency of Bcl6, a transcriptional repressor (Chang et al., 1996; Seyfert et al., 1996), might induce germ cell apoptosis in *Bcl6*$^{-/-}$ mice. Hsp70-2 is required for Cdc2/cyclin B complex formation (Zhu et al., 1997), and kinase activity of Cdc2/cyclin B complexes is critical for synaptonemal complex desynapsis (Chapman and Wolgemuth, 1994; Zhu et al., 1997). However, these proteins are detected in spermatocytes of both *Bcl6*$^{+/+}$ and *Bcl6*$^{-/-}$ mice without obvious differences in levels (data not shown), suggesting that the mechanism of germ cell apoptosis in adult

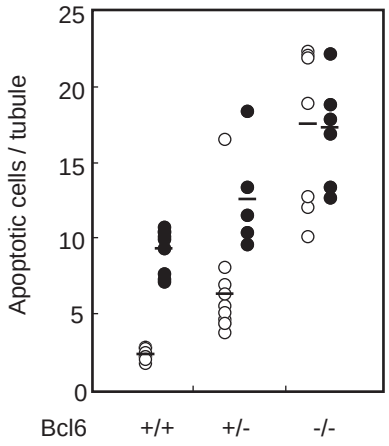

Fig. 9. The frequency of germ cell apoptosis increases in testes of heterozygous adult *Bcl6*$^{+/-}$ mice. Apoptotic germ cells in seminiferous tubule sections of *Bcl6*$^{+/+}$, *Bcl6*$^{+/-}$ and *Bcl6*$^{-/-}$ mice at 12-16 weeks of age were counted by TUNEL assay. Each circle indicates a result from a testis. The apoptotic cell numbers (white) in *Bcl6*$^{+/-}$ (6.8±3.7) and *Bcl6*$^{-/-}$ (17.3±5.4) mice are significantly higher than that of *Bcl6*$^{+/+}$ (2.5±0.4) mice. Testes of *Bcl6*$^{+/+}$, *Bcl6*$^{+/-}$ and *Bcl6*$^{-/-}$ mice at 12 weeks of age were treated with a mild hyperthermia (42°C) for 15 minutes. Sections of seminiferous tubules of the mice were prepared 6 hours after treatment. Apoptotic germ cells in the sections were detected by TUNEL assay. Each closed circle indicates a result from a testis. The apoptotic cell number (black) of *Bcl6*$^{+/+}$ (9.4±1.5) and *Bcl6*$^{+/-}$ (13.5±3.6) mice after heat treatment are significantly higher than those of *Bcl6*$^{+/+}$ and *Bcl6*$^{+/-}$ mice without treatment, respectively. However, the number of *Bcl6*$^{-/-}$ (17.2±3.5) mice after heat treatment is almost similar to that of *Bcl6*$^{-/-}$ mice without treatment.

Bcl6$^{-/-}$ mice is not related to the process of synaptonemal complex desynapsis.

Bcl6 may play a role in protecting spermatocytes at the meiosis metaphase I stage from stress-induced apoptosis

Apoptosis of metaphase I spermatocytes was induced in adult *Bcl6*$^{-/-}$ and *Bcl6*$^{+/-}$ mice without heat treatment and the incidence of germ cell apoptosis was roughly correlated with the *Bcl6* dose. After heat treatment, the number of apoptotic spermatocytes was not increased in adult *Bcl6*$^{-/-}$ mice. It is suggesting that the germ cells that are most sensitive to heat stress were dead in adult *Bcl6*$^{-/-}$ mice without heat treatment, although it is still possible that Bcl6 is somehow required for the spermatocytes to initiate heat-induced germ cell apoptosis. Furthermore, the spermatogenic stages of phospho-p38 MAP kinase expression in spermatocytes of *Bcl6*$^{-/-}$ mice were overlapped with those of *Bcl6* expression of *Bcl6*$^{+/+}$ mice. Thus, Bcl6 may play a role in protection of spermatocytes mainly at the metaphase I stage from apoptosis induced by stressors. Any relationship between Bcl6 function and p38 MAP kinase activation is presently unclear. Additional work is required to elucidate specific target genes for Bcl6 to protect apoptosis during spermatogenesis.

Many genes are expressed in germ cells during spermatogenesis (Eddy and O'Brien, 1998). Deficiency of the genes such as *Atm* (Xu et al., 1996), *Hsp70-2* (Dix et al., 1996), *cyclin A1* (Liu et al., 1998) and *Mlh1* (Edelmann et al., 1996) induces arrest of spermatogenesis at the pachytene stage, indicating that these gene products are essential for spermatogenesis. Abnormal expression of some apoptosis-related gene products in germ cells also perturbs spermatogenesis. *Bax*-deficient mice (Knudson et al., 1995) show arrest of spermatogenesis with apoptotic spermatocytes. Overexpression of *Bcl2* in spermatogonia (Furuchi et al., 1996) induces massive accumulation of spermatogonia leading to degeneration of seminiferous tubules. In *Bcl-w*-deficient mice (Print et al., 1998), the seminiferous tubules are disorganized and no mature sperm is produced. In contrast, *Bcl6*$^{-/-}$ mice showed normal development of testes and normal physiological apoptotic wave at the prepubertal age. Although many spermatocytes go to apoptosis in adult *Bcl6*$^{-/-}$ testes, mature sperm are developed in adult *Bcl6*$^{-/-}$ mice. These data indicate that Bcl6 is not essential for spermatogenesis but necessary for stabilization of spermatocytes to protect against the germ cell apoptosis induced by stressors.

This protective role of Bcl6 in stress-induced apoptosis has also been reported in cardiac myocytes (Yoshida et al., 1999) and in skeletal muscle cells (Kumagai et al., 1999), which may explain a function of Bcl6 as an oncogene to produce B cell lymphoma (Ye et al., 1993). Since the BTB/POZ domain of Bcl6 interacts with the silencing mediator of retinoid and thyroid receptor protein (SMRT) (Dhordain et al., 1997) and SMRT forms a repressive complex with the histone deacetylase 1 (Nagy et al., 1997), Bcl6 may repress transcription of target genes through mechanisms involving SMRT recruitment and histone deacetylation. Thus, *Bcl6*$^{-/-}$ mice can serve as a useful model for studying transcriptional regulatory mechanisms that allow for adaptive response to various kinds of stress.

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