Cd52, known as a major maturation-associated sperm membrane antigen secreted from the epididymis, is not required for fertilization in the mouse

Ryo Yamaguchi[1,2], Kazuo Yamagata[3], Hidetoshi Hasuwa[1], Emiko Inano[1], Masahito Ikawa[1] and Masaru Okabe[1,2,*]

[1]*Research Institute for Microbial Diseases, and*
[2]*Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan*
[3]*Laboratory for Genomic Reprogramming, RIKEN, Center for Developmental Biology, Kobe 650-0047, Japan*

CD52 is a glycosylphosphatidylinositol (GPI)-anchored antigen expressed on lymphocytes and in epididymal epithelial cells. CD52 is also known as "maturation-associated sperm antigen" but its function is unknown. We therefore generated *Cd52* disrupted mice. The resulting *Cd52* null mice were healthy, even though *Cd52* is expressed on cells of the immune system. We then examined a possible role for CD52 in reproduction. Sperm from *Cd52*-deficient males were investigated and the viability, motility, morphology, and incidence of spontaneous acrosome reactions were found to be all similar to values for wild-type sperm. In *in vitro* fertilization system, the sperm showed normal fertilizing ability. As CD52 was found to be transferred onto sperm only after they had migrated into the vas deferens, we examined the behavior of sperm from *Cd52*-deficient mice *in vivo*. The mice mated naturally and we observed that a normal number of sperm passed through the uterotubal junction, known to the crucial hurdle for various gene knockouts resulting in infertile sperm. As a consequence, there was no difference in the litter size from the wild-type and *Cd52*-null males. Our results therefore indicate CD52 is not required for fertilization in the mouse either *in vivo* or *in vitro*.

Introduction

Sperm are produced from spermatogonial cells as a highly differentiated cell to fertilize eggs but they have no fertilizing ability when they leave testis. Sperm require a maturation process during passage through epididymis. For example, swimming ability and egg interacting ability of sperm are reported to be acquired during the maturation step (Cooper 1995; Toshimori 2003). The ability of sperm to undergo the acrosome reaction, a prerequisite for sperm to fertilize eggs, is also known to increase during the epididymal maturation (Williams *et al.* 1991). This sperm maturation process is associated with reorganization of membrane structures such as processing of membrane proteins on sperm surface (Kim *et al.* 2006), changes in plasma membrane phospholipids composition (Sullivan *et al.* 2005), and the accumulation or modification of glycoproteins on sperm surface (Kirchhoff 1996; Tulsiani 2006). Various glycoproteins are known to be secreted from the epithelial cells of male reproductive tract and transferred on sperm during epididymal passage (Cornwall *et al.* 1990; Vreeburg *et al.* 1990; Sullivan *et al.* 2005; Busso *et al.* 2007). Thus these glycoproteins may function as a primary interface between sperm and female reproductive tract and eggs.

A highly sialylated glycosylphosphatidylinositol (GPI)-anchored protein CD52 initially found on lymphocytes is also known as SAGA-1, GP20, Cambridge pathology 1 antigen and epididymal secretory protein E5. It is one of the few well-defined antigens secreted from the epididymal cells, is transferred to the sperm plasma membrane during epididymal passage, and alters the characteristics of sperm surface. CD52 is exposed in the equatorial region of the sperm head at the end of the capacitation process in human (Yeung *et al.* 2001). Both CD52 on sperm and lymphocyte share the same peptide sequence but carbohydrate moiety is different in these two cases. Thus, CD52 on sperm could be antigenic to females. Actually, anti-CD52 mab (MAb H6-3C4) is generated from an infertile woman's peripheral

Communicated by: Takeo Kishimoto
**Correspondence*: okabe@gen-info.osaka-u.ac.jp

DOI: 10.1111/j.1365-2443.2008.01210.x

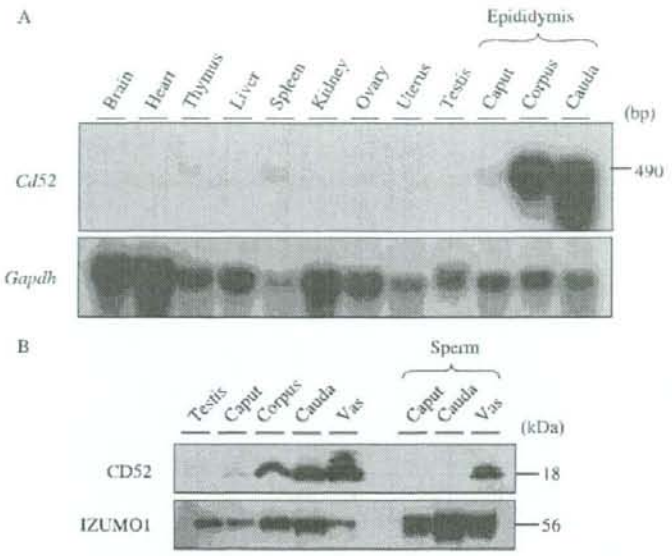

Figure 1 Tissue distribution of *Cd52* mRNA and protein. (A) Northern blot containing equal amounts of total RNA (10 μg) was hybridized with ^{32}P-dCTP labeled cDNA fragments of *Cd52* and *Gapdh*. (B) Testes, male reproductive ducts and sperm protein were extracted with lysis buffer containing Triton X-100 and subjected to immunoblot analysis. Western blots containing equal amounts of tissue proteins (30 μg) and sperm protein (10 μg) were hybridized with anti-CD52 polyclonal antibody. For control sperm protein, mouse IZUMO1 was detected with anti-IZUMO1 monoclonal antibody (#125).

blood lymphocytes. The antibody was shown to have a strong complement-dependent, sperm-immobilizing activity, which indicates the possibility of CD52 being a candidate contraceptive target molecule. Although a clear implication of CD52 in fertilization mechanism is not reported, the covering of sperm membrane with CD52 might have an effect on the sperm-egg, or sperm-female reproductive tract interactions (such as storage of spermatozoa in the caudal part of the isthmus, in tight contact with the epithelium cells lining the oviduct (Töpfer-Petersen 1999)).

In the study of mechanism of fertilization, many factors are designated as "important" factors from the experiments in which antibodies and ligands added to *in vitro* fertilization system showed inhibitory activities. However, many such discovered factors turned out to be "not essential" by gene disruption, suggesting that conclusive demonstrations of the protein functions become more reliable from the observation of the gene disrupted animals. Therefore, we tried to disrupt CD52 gene to examine its role in fertilization.

Results

Expression of Cd52 in various organs (Northern and Western blotting)

Cd52 is known to be expressed in thymus and in spleen (Kubota *et al.* 1990). The anti-CD52 monoclonal antibodies are used as one of the treatments to prevent the rejection of transplanted organs (Calne *et al.* 1999; Hale *et al.* 2000). However, the expression levels of *Cd52* were much higher in epididymal tissues than in those immunity-related organs (Fig. 1A). It has been reported that the human CD52 is secreted from the male reproductive tract and is bound to the sperm surface (Kirchhoff 1996). When we examined the presence of CD52 in the mouse using Western blotting, CD52 was not found in the caput epididymis but substantial amounts of CD52 were found starting from the corpus epididymal section to vas deferens. Interestingly, CD52-positive sperm (examined by Western blotting) were only found in the vas deferens (Fig. 1B).

Immunohistochemistry

The localization of CD52 was examined by immunostaining. We detected almost no staining in caput epididymal sections, but the epithelial cells of the cauda epididymal sections showed an intense staining (Fig. 2). In the vas deferens, an equally strong staining was observed in the epithelial cells, but different from epididymis, the staining was spread to the ductal area of the vas deferens. This staining was not a nonspecific binding, because no staining was observed in *Cd52*$^{-/-}$ mice.

While sperm from cauda epididymis showed no reactivity to anti-CD52 antibody, approximately 25% of sperm population from vas deferens was stained by anti-CD52 antibody (Fig. 3C). The staining pattern of CD52 was spotty and was basically localized in the midpiece area and

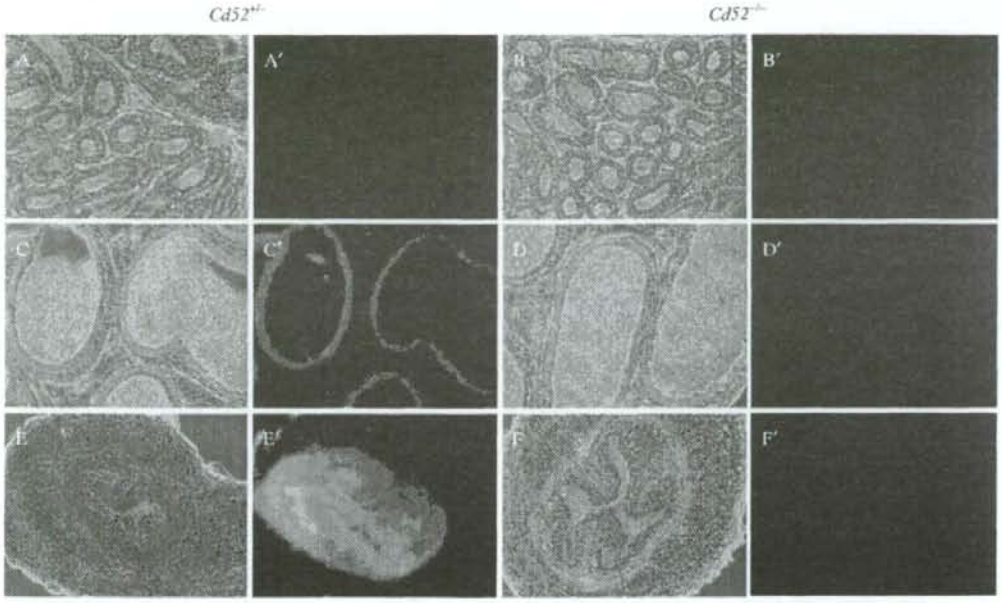

Figure 2 Expression of CD52 in epididymis and vas deferens. The sections of caput epididymis (A, B), cauda epididymis (C, D), and vas deferens (E, F) were subjected to indirect immunofluorescence employing anti-CD52 monoclonal antibody followed by Alexa Flour 488-conjugated anti-rat second antibody. Immunoreactivity was visualized with fluorescent microscopy. CD52 protein was only detected in the cauda epididymis and vas deferens from heterozygous mouse (C, E, and G). No fluorescence was detected in the tissues from *Cd52*-deficient mice (B, D, and F).

sometimes extended to the head regions. Ejaculated sperm recovered from the uterus within 1 h after plug formation showed a similar spotty staining pattern as sperm from vas deferens shown in Fig. 3E, indicating that the encounter of sperm with accessory gland secretions at ejaculation seems not to affect the localization of CD52 on sperm. Together with the result of Western and Northern blotting experiments, it was indicated that CD52 is secreted from the epithelial wall and binds to the sperm in the vas deferens in mouse.

Generation of Cd52-deficient mice

In order to examine the roles of CD52, we disrupted the *Cd52* gene by homologous recombination. The mouse *Cd52* gene consists of two exons and is mapped to chromosome 4 (Tone *et al.* 1999). The targeting vector was designed to remove both exons of *Cd52* (Fig. 4A) and was electroporated into D3 ES cells after linearization. Potentially targeted ES cell clones were separated by positive–negative selection with G418 and acyclovir. Correct targeting of the *Cd52* allele in ES cell clones was determined by PCR for homologous recombination on both ends (Fig. 4B). Mating between heterozygous mutant mice yielded the expected Mendelian ratios: $Cd52^{+/+}$, 26%; $Cd52^{+/-}$, 52%; $Cd52^{-/-}$, 22% of offspring ($n = 116$). Northern and Western blot analysis showed that *Cd52* mRNA expression was undetectable in the $Cd52^{-/-}$ epididymis (Fig. 4C). CD52 protein was also not detected in sperm from the vas deferens in $Cd52^{-/-}$ mice (Fig. 4D). $Cd52^{-/-}$ mice exhibited normal development and grew up as healthy adults with normal CD4/CD8 positive cell ratios (Fig. S1 in Supplementary Material). We do not exclude the possibility that we overlooked the phenotype, but the chance is high that the role of CD52 in immune system is masked by some compensating factors or the role is not essential in lymphocytes and in splenocytes.

Fertility of sperm in CD52 null epididymis

CD52 expressed in epididymis may contribute to supporting epididymal function *per se* in nursing sperm. We collected and observed the motility of epididymal sperm from $Cd52^{-/-}$ male mice using automated sperm analyzer

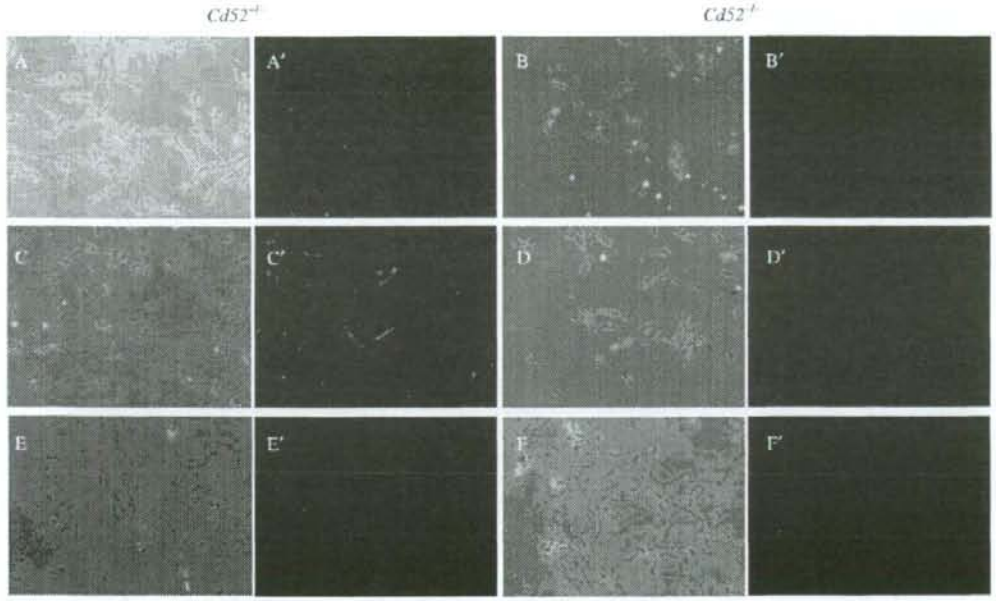

Figure 3 Immunolocalization of CD52 protein on mouse sperm. Sperm from cauda epididymis (A, B), vas deferens (C, D) and recovered from uterus (E, F) were immunostained with anti-CD52 monoclonal antibody combined with Alexa Flour 488-conjugated second antibody. Immunoreactivity was visualized with fluorescent microscopy (×400). No fluorescence was detected on sperm from the cauda epididymis (A) and on *Cd52*-deficient sperm (B, D, and F).

SMAS. No difference in the motility or the swimming pattern was found compared to sperm from wild-type mice (Table 1 & Fig. 5). Using the double transgenic mouse line, CD52 KO bearing *Acr-EGFP* transgene, we examined the effect of CD52 disruption on the spontaneous acrosome reaction of sperm from cauda epididymis and from vas deferens by a flow cytometer. Sperm from $Cd52^{-/-}$ male showed no significant difference from those from control male, even sperm from the cauda epididymis and vas deferens (Fig. 6). The ionophore induced acrosome reaction which was caused by the addition of A23187 was also found not to be affected by the CD52 disruption (data not shown). To confirm the fact that sperm maturation in epididymis was normal in CD52 null mice, *in vitro* fertilization assay was performed using sperm from CD52 −/−, +/−, and +/+ mice and it resulted in a similar fertilization ratio as expected (Table 2).

Fertility of ejaculated sperm in CD52 null mice

Usually, cauda epididymal sperm are used for *in vitro* fertilization experiments. However, this sperm population is not yet covered with CD52 (Fig. 3), indicating that mouse epididymal sperm are competent to bind and penetrate eggs without having CD52 on their surface. Therefore, if CD52 functions in fertilization, it might be through a characteristic step involved in *in vivo* fertilization processes, such as ejaculated sperm moving up the female reproductive tract to where fertilization takes place.

To clarify the role of CD52 in fertilization *in vivo*, we mated $Cd52^{-/-}$ male mice with superovulated wild-type female mice. Two hours after coitus, whole mount sections of oviducts were made and serial sections were observed by bright field microscopy. Close examination of sections of the uterotubal junction revealed that sperm derived from both CD52 +/+ and −/− sperm had migrated through the ostium of the colliculus tubarius, and were found inside the oviduct (Fig. 7).

There was no difference in the mating ratio and fertility compared to wild-type mice (Table 3). In natural mating, the number of pups sired by wild-type and $Cd52^{-/-}$ male mice were similar (control, 9.55 ± 1.70, $n = 18$; homozygous, 9.14 ± 1.02, $n = 14$), coinciding with the result of migrating equal number of sperm into the oviduct. Thus the disruption experiment revealed that CD52 is "dispensable" in fertilization.

Table 1 Comparison of sperm motility prepared from CD52 +/+, +/−, and −/− mice

Parameters	Incubation time (min)	+/+	+/−	−/−
Percentage of motile spermatozoa (%)	15	76.1 ± 10.8	82.7 ± 8.4	76.3 ± 2.6
	60	72.7 ± 11.1	73.1 ± 7.1	67.2 ± 4.8
	120	72.4 ± 6.9	70.4 ± 16.8	64.2 ± 3.9
Straight line velocity (μm/s)	15	108.6 ± 16.9	99.7 ± 8.0	104.4 ± 15.7
	60	74.3 ± 18.7	90.5 ± 16.2	97.3 ± 6.1
	120	73.8 ± 17.7	72.6 ± 10.6	83.4 ± 19.4
Curvilinear (μm/s)	15	306.1 ± 17.2	291.0 ± 17.1	279.4 ± 31.0
	60	289.9 ± 42.9	305.8 ± 28.7	312.2 ± 20.8
	120	273.4 ± 63.5	276.2 ± 31.1	306.9 ± 47.5
Linearity	15	0.37 ± 0.039	0.35 ± 0.029	0.38 ± 0.026
	60	0.27 ± 0.077	0.31 ± 0.027	0.32 ± 0.014
	120	0.28 ± 0.013	0.27 ± 0.019	0.27 ± 0.025
Amplitude of lateral head displacement (μm)	15	9.0 ± 0.7	8.2 ± 0.23	7.9 ± 1.06
	60	8.5 ± 1.0	8.5 ± 0.39	9.0 ± 1.01
	120	7.9 ± 0.7	7.3 ± 0.80	8.9 ± 1.18

The data represent the means ± SD of three independent experiments.

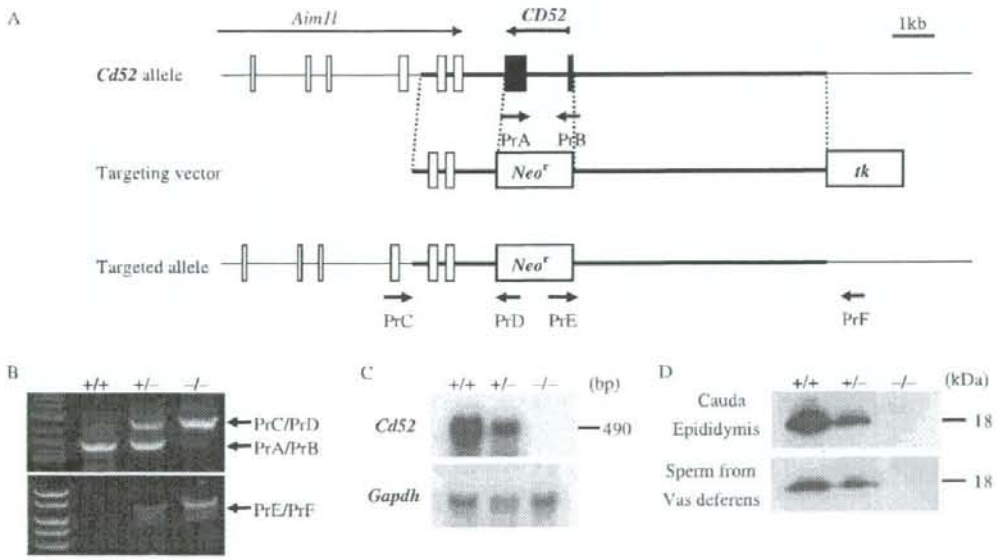

Figure 4 Production of *Cd52*-deficient mice. For the targeted disruption of mouse Cd52 allele, all two exons encoding mouse CD52 protein (closed boxes) was replaced by the neomycin-resistant-cassette (*Neo*^{r}). A herpes simplex virus thymidine kinase gene (*tk*) was introduced into the targeting construct for negative selection. (B) Genotyping of tail tip DNA by PCR amplification with primers indicated in the figure. (C) Northern blot analysis of total RNA from *Cd52*$^{+/+}$ (+/+), *Cd52*$^{+/-}$ (+/−), and *Cd52*$^{-/-}$ (−/−) cauda epididymis. Blots were hybridized with ^{32}P-dCTP labeled cDNA fragments of *Cd52* and *Gapdh*. (D) Western blot analysis of cauda epididymal lysate and sperm lysate in vas deferens from *Cd52*$^{+/+}$ (+/+), *Cd52*$^{+/-}$ (+/−), and *Cd52*$^{-/-}$ (−/−) mice.

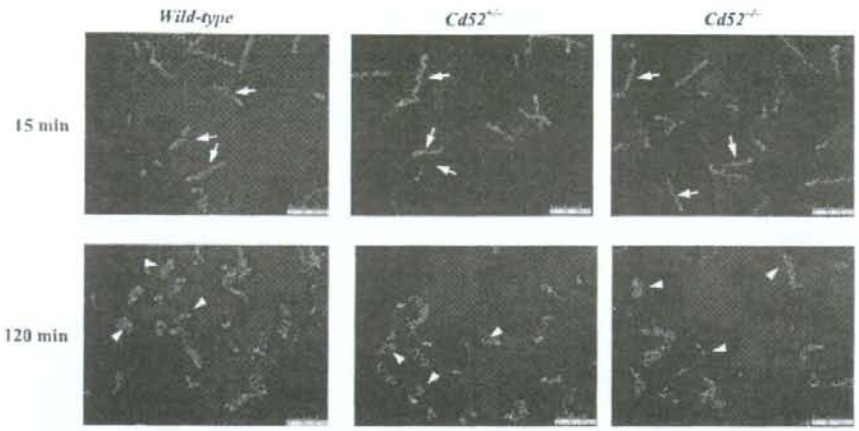

Figure 5 A change in swimming patterns of sperm during incubation in TYH medium. Sperm from CD52 +/− and −/− mice were incubated in TYH medium and analyzed by the computer-aided Sperm Motility Analysis System (SMAS). Almost all the sperm showed straightforward movement at 15 min of incubation (arrows), but a comparable number of characteristic hyperactivated movements (arrowheads) appeared in the sperm population after 120 min of incubation both in sperm from CD52 +/− and −/− mouse lines.

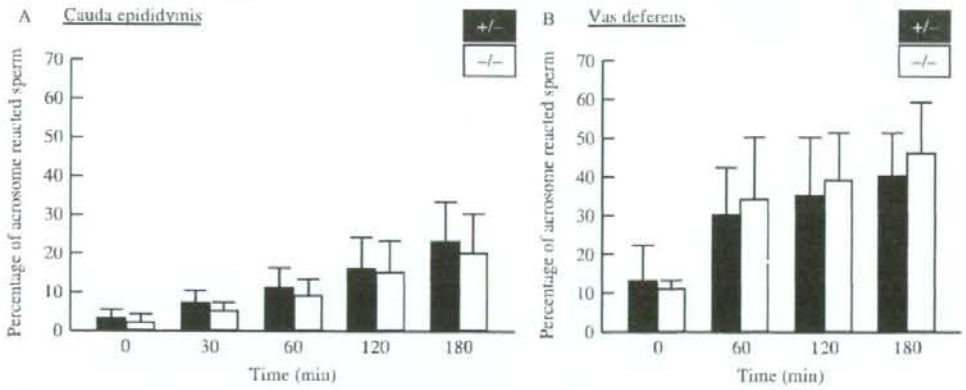

Figure 6 Time course of the acrosome reaction in *Cd52*$^{+/-}$ mice. Mice with the *Cd52*$^{+/-}$ allele were bred with a green sperm transgenic mouse line and a double-transgenic mouse line, *Cd52*$^{-/-}$ mice with green sperm was obtained. Sperm from cauda epididymis (A) and vas deferens (B) from this double-transgenic mouse line were incubated in TYH medium and analyzed for their acrosomal integrity by flow cytometry. Sperm from *Cd52*$^{+/-}$ (closed column) and *Cd52*$^{-/-}$ (open column) littermate mice were compared. Error bars represent mean ± SDs from seven (cauda epididymis) or four (vas deferens) independent experiments.

Discussion

In the process of studying fertilization, various antibodies to sperm were produced in the past. The clarification of the antigens and subsequent disruptions of the corresponding genes indicated various antigens to be essential (ADAM2, ADAM1a, ADAM3, ACE, and IZUMO1). On the other hand, many factors turned out to be "not essential" (acrosin, CD46, PH20, ADAM1b, GalTase etc.) for fertilization (see review, Okabe & Cummins (2007)). These data suggest that we have to be careful when we examine the roles of a certain factor in fertilization using antibodies and/or ligands. The gene disruption experiment is a time-consuming effort but could often provide clear understanding which is not possible to obtain with normal biochemical and/or physiological analysis.

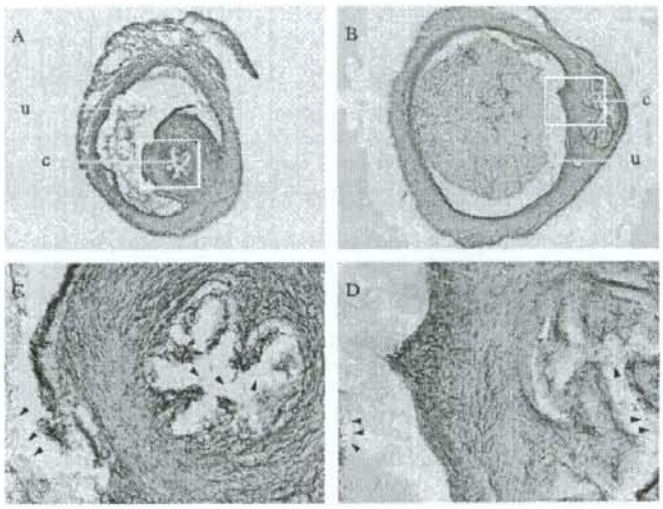

Figure 7 Migration of *Cd52*$^{-/-}$ sperm into oviduct. Sperm transits from uterus to oviduct were observed by removing the uterotubal junction 2 h after coitus and making frozen sections stained with hematoxylin. (A, C) heterozygous type: (B, D) *Cd52*$^{-/-}$. Boxed areas in (A) and (B) were magnified in (C) and (D). Arrow heads indicate sperm present in the uterus (u) and colliculus (c).

Table 2 Comparison of fertilizing ability of sperm prepared from CD52 +/+, +/− and −/− mice *in vitro*

CD52	Total number of eggs examined	8 h after insemination: Number of pronuclear eggs ($n = 3$)*	24 h after insemination: Number of 2-cell eggs (%) ($n = 3$)
+/+	195	188 (96.4 ± 2.6)	180 (92.3 ± 2.5)
+/−	273	262 (96.0 ± 2.6)	252 (92.3 ± 5.2)
−/−	229	218 (95.2 ± 2.6)	216 (94.3 ± 1.2)

*Polyspermy eggs with more than three pronuclei were eliminated from the count.
Mean ± SD of three independent IVF results using three different males.

Table 3 Fertilized eggs recovered from oviducts from copulated females

Male genotype	Number of females used	Total number of eggs obtained	Fertilized eggs*	Fertilization (%) mean ± SD
+/+ or +/−	7	277	256	94.9 ± 2.8
−/−	7	235	222	95.2 ± 5.4

*Assessed by observing the pronuclear formation.

CD52 is a GPI-anchored membrane protein found in mouse, rat, monkey, dog and human (Hale 2001). Although the lengths of the mature peptides are different among species, all of them have a single potential site for the N-linked glycosylation. The rat CD52 antigen has been characterized for many years as the "major maturation-associated antigen" of sperm (Kirchhoff 1996; Yeung *et al.* 2001). It is the most abundant antigen among the sperm glycoproteins and its acquisition during epididymal transit explains much of the remarkable change in surface charge and lectin-binding characteristics which occurs during sperm maturation (Kirchhoff & Schroter 2001). In view of the accumulation of CD52 in rat and human sperm, it is natural to expect an important role of CD52 in sperm maturation in various species. Therefore, we chose CD52 as a candidate gene to disrupt to elucidate the mechanism of epididymal maturation process toward fertilization.

Although CD52 was highly expressed in the epididymal tissues, the disruption of CD52 caused no apparent effect on the epididymal functions *per se*. As an alternative role, we could speculate that CD52, as one of the membrane proteins after transition to sperm, is functioning to interact with the female reproductive tract. However, the sperm from *Cd52*-deficient mice could successfully migrate into the oviduct and fertilize the eggs and *Cd52*-deficient males sired similar numbers of pups. The fertilizing ability of *Cd52*-deficient males remained normal even at 50 weeks of age (data not shown), indicating that no immunological disorders in the reproductive tract took place, differing from the case of CD59-disrupted mouse line, in which a progressive loss of fertility associated with immobile dysmorphic and fewer sperm cells after 5 months of age was observed (Qin *et al.* 2003; Qin *et al.* 2005). Thus with respect to all these aspects, CD52 turned out to be not essential in the fertilization system. Approximately 25% of the sperm population had detectable CD52. If CD52 has an inhibitory or stimulatory activity on sperm function when attached to the sperm surface, the remaining CD52-free population might mask the effect of CD52 in our experimental system. Although, no apparent phenotype of CD52 disruption was observed in the present experiment, the fact that the CD52 has been retained as an active gene in epididymis and is found on mouse, rat, monkey and human sperm suggests that CD52 has unknown but important roles in fertilization.

In human, all sperm are reported to possess CD52 on their surface, while in mouse, approximately a quarter of the population was found to react to anti-CD52 antibody (Fig. 3). The localization of CD52 on mouse sperm turned out to be different from human (midpiece in mouse versus whole sperm in human) and the site of CD52 transition from epididymal tissue to sperm seemed to be different between mouse and human (vas deferens in mouse versus in epididymis in human). It is not clear how CD52 is transferred from epididymal epithelium to sperm membrane. As a similar mechanism of transfer of membrane proteins to sperm, prostasomes are known in prostate and are speculated to transport membrane proteins to the sperm membrane (Ronquist & Brody 1985). Recently, exosome (Leblanc *et al.* 2006) in the epididymis, termed epididymosome, was reported to serve in epididymal maturation of sperm (Rejraji *et al.* 2006). CD52 might be transported through this secretion system and epididymosomes might be the origin of the speckled stainings observed in immunofluorescent analysis. Further investigation is awaited to learn if CD52 is transferred from epididymal epithelium to sperm plasma membrane or CD52-containing exosomes are only attaching on sperm membrane.

Although CD52 was found to be dispensable in fertilization, it does not mean that CD52 has no function in the system in wild-type mice. In the present paper, we could not find any apparent phenotype derived from the disruption of CD52 in immune system or in fertilization in mice kept under normal conditions. However, with combination to other gene disrupted mice, a severe phenotype might be expressed as in the case shown in Hox gene disruptions (Davis *et al.* 1995). In other words, various factors are known to be compensated by other factors, but their role becomes evident if the disruption is overlaid on another genetic background in which some other gene was disrupted (Nef *et al.* 2003). The CD52-disrupted mouse line may have such a characteristic. In order to make it possible to pursue further analysis of the role of CD52 *in vivo*, the CD52 disrupted mouse line was submitted to Riken BioResource center and is available to the scientific community.

Experimental procedures

Northern blot analysis

Northern hybridization was performed using 10 μg of total RNA extracted from various tissues of adult ICR mice. RNAs were separated by electrophoresis on agarose gels, transferred to Hybond-N$^+$ membranes (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), and hybridized to ^{32}P-labeled probes at 60 °C overnight. Mouse *Cd52* and glyceraldehydes-3-phosphatate dehydrogenase (*Gapdh*) cDNAs were used as probes. The *Cd52* probe consisted of a cDNA fragment amplified from mouse epididymal total RNA by RT-PCR using 5′-TGAATTCTTCAAAGTGGCCTGCA GACTGTC-3′ and 5′-TGAATTCGCCATTGGCTGTCAAC TTTAGCC-3′ as primers.

Antibodies

Rabbit anti-mouse CD52 polyclonal antiserum was produced by immunization with mouse CD52 polypeptide (AASGTNKNST-STKKTPLKSG). Rat monoclonal antibody against mouse CD52 was a kind gift from Dr Nagahiro Minato (Kyoto University, Kyoto, Japan) (Kubota *et al.* 1990). A new monoclonal antibody against mouse IZUMO1 (Inoue *et al.* 2005) was produced by screening after immunization of whole mouse sperm to rat and termed Mab #125. Monoclonal antibodies against mouse ADAM2 (fertilin β 9D2.2) were purchased from Chemicon International, Inc. (Temecula, CA).

Immunohistochemistry

Epididymis and vas deferens were collected from adult *Cd52*$^{+/-}$ and *Cd52*$^{-/-}$ mice and embedded in a TissueTek O.C.T. compound (Sakura Finetechnical Co., Tokyo, Japan). Frozen sections (16 μm) prepared from these tissues were mounted on APS (aminosilane)

coated glass slides. Sperm from cauda epididymis or vas deferens were swum up in TYH medium and resuspended in PBS (Toyoda *et al.* 1971). Ejaculated sperm were collected from the uterus just after mating and resuspended in PBS. Sperm suspensions were mounted on glass slides and dried up. All samples were fixed in 4% paraformaldehyde/PBS for 30 min. After washing with PBS, slides were blocked with 10% New Born Calf Serum (NBCS)/PBS for 1 h and incubated with rat anti-mouse CD52 monoclonal antibody in 10% NBCS/PBS at 4 °C overnight. After washing with 10% NBCS/PBS containing 0.05% Tween-20, the slides were incubated with anti-rat IgG labeled with Alexa Fluor 488 (Invitrogen) in 10% NBCS/PBS for 1 h. After washing with PBS containing 0.05% Tween-20, the slides were observed under an Olympus IX-70 fluorescence microscope.

Immunoblot analysis

Immunoblot analysis was performed as described previously (Yamaguchi *et al.* 2006). Briefly, sperm from the epididymis and vas deferens were collected and incubated in lysis buffer containing 1% TritonX-100 for 20 min on ice. The testis, epididymis, and vas deferens were excised, minced, and homogenized in lysis buffer, and then placed on ice for 1 h. The sperm and tissue extracts were centrifuged, and the supernatants were collected. Proteins were separated by SDS-PAGE under reducing conditions and transferred electrophoretically to PVDF membranes. After blocking, blots were incubated with primary antibody overnight at 4 °C, and then incubated with horseradish-peroxidase conjugated goat anti-rabbit IgG, goat anti-mouse IgG and goat anti-rat IgG (GE Healthcare Bio-Sciences Corp.). The detection was performed using an ECL Western blotting detection kit (GE Healthcare).

Construction of the Cd52 gene disruption vector

A targeting vector was constructed using pPNT containing the Neo-resistance gene (*Neo*r) as a positive selection marker and a herpes simplex virus thymidine kinase (*tk*) as a negative selection marker (Tybulewicz *et al.* 1991). A 2.1-kb *Not*I-*Sal*I fragment as a short arm and a 6.0-kb *Spe*I-*Kpn*I fragment as a long arm were obtained by PCR using genomic DNA in D3 embryonic stem (ES) cells as a template. The PCR primers used were as follows: 5′-GCGGCCGCAGTTAAAAGCACTTGTTGCAAGCCGGGC AG-3′ and 5′-TTTGTCGACGTGCGGCAGTATTAGGAGT GAACCCAGTAC-3′ for the short arm, 5′-GGACTAGT GGCCACTTTGAACCTGGCTGCTTTTTCTGC-3′ and 5′-TGGTACCAGAGGTCTCAACCTGTGGCTTGTGACCC AG-3′ for the long arm.

These two fragments were inserted into a pPNT vector and the targeting construct was linearized with *Not*I digestion. ES cells were electroporated and colonies were screened.

Generation of Cd52 mutant mice

G418-resistant colonies were amplified, and genomic DNA was prepared from them and screened by PCR analysis. Several of the recombinant ES cell lines carrying the disrupted *Cd52* allele were identified and subsequently used to generate chimeras by injection into blastocysts from C57BL/6 Cr mice (> 2 months old; Japan SLC, Inc., Shizuoka, Japan). Injected blastocysts were transferred to ICR pseudopregnant foster mothers, resulting in the birth of male chimeric mice. These mice were crossed with C57BL/6 to obtain F1 heterozygous offspring. *Cd52*-deficient mice were generated by the intercrossing F1 offspring mice. Mice used in this study were of B6; 129 mixed background.

All experiments were performed with the consent of the Animal Care and Use Committee of Osaka University.

Analysis of acrosome reaction and sperm motility

To investigate the influence of *Cd52* disruption on the sperm acrosome reaction, females from a transgenic mouse line which have enhanced green fluorescent protein in sperm acrosome (*Acr-EGFP*) were crossed with $Cd52^{-/-}$ males (Nakanishi *et al.* 1999). Double transgenic F1 offspring were intercrossed to generate $Cd52^{-/-}$ and $Acr\text{-}EGFP^{+/-}$. Sperm from double-transgenic mice were assayed as described in our previous paper (Inoue *et al.* 2003). Briefly, sperm were squeezed out from the incisions made in cauda epididymis or vas deferens and were suspended and incubated in TYH medium to induce a spontaneous acrosome reaction. Acrosomal statuses were analyzed from the acrosomal fluorescence by flow cytometer at 0, 30, 60, 120 and 180 min after insemination. Sperm motility was measured using epididymal sperm and automated Sperm Motility Analysis System (SMAS, Kaga Electronics Co. Ltd, Tokyo, Japan).

Sperm migration analysis

B6D2F1 females were superovulated by intraperitoneal injection of 5 units of equine chorionic gonadotropin followed 48 h later by 5 units of human chorionic gonadotropin (HCG). Superovulated females were caged together with test males 12 h after HCG injection, and the formation of vaginal plug was observed every 30 min. About 2 h after copulation, oviducts were excised together with the connective part of the uterus. To detect sperm in the uterotubal junction, the oviducts with attached uterus were fixed in 4% paraformaldehyde–PBS for 6 h, followed by washing with PBS, and were then prepared for frozen sections. Total of three females were examined using three males of each genotype.

Assessment of the fertilizing ability of Cd52-deficient mice

Female B6D2F1 mice (older than 8 weeks; Clea Japan Inc., Tokyo, Japan) were superovulated following intraperitoneal injections of eCG (Teikoku Zoki, Co. Ltd, Kanagawa, Japan) and hCG (Teikoku Zoki) at 48 h intervals. $Cd52^{+/-}$ or $Cd52^{-/-}$ males were mated with superovulated females 7 h after hCG injection. Eggs were recovered from females at their pronuclear stage and placed in a KSOM medium (Ho *et al.* 1995). Fertilization rates were assessed by pronuclear formation and subsequent 2-cell formation, observed by Olympus IX-70 microscope. *In vitro* fertilization was performed as previously described (Ikawa *et al.* 1997).

Acknowledgements

Authors thank Dr Nagahiro Minato (Kyoto University) for providing the antibody against CD52. Authors also thank Y. Maruyama, A. Kawai and Y. Koreeda for technical assistance with gene disruption. This work was supported in part by grants from the Ministry of Education, Science, Sports, Culture, and Technology, and the 21st Century 200 COE program from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Received: 2 February 2008
Accepted: 13 May 2008

Supplementary material

The following supplementary materials are available for this article online:

Figure S1 Cell populations of thymocytes and splenocytes in terms of expression of CD antigens.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2443.2008.01210.x
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Am J Physiol Endocrinol Metab 295: E1349–E1357, 2008.
First published October 7, 2008; doi:10.1152/ajpendo.90704.2008.

Bis deficiency results in early lethality with metabolic deterioration and involution of spleen and thymus

Dong-Ye Youn,[1]* Dong-Hyoung Lee,[1]* Mi-Hyun Lim,[1] Jung-Sook Yoon,[2] Ji Hee Lim,[2] Seung Eun Jung,[1] Chung Eun Yeum,[3] Cheol Whee Park,[2] Ho-Joong Youn,[2] Jae-Seon Lee,[4] Seong-Beom Lee,[3] Masahito Ikawa,[5] Masaru Okabe,[5] Yoshihide Tsujimoto,[6,7] and Jeong-Hwa Lee[1]

[1]Department of Biochemistry, [2]Department of Internal Medicine, and [3]Department of Pathology, College of Medicine, Catholic University of Korea and [4]Division of Radiation Cancer Research, Korea Institute of Radiological and Medical Science, Seoul, Korea; and [5]Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, [6]Department of Medical Genetics, Laboratory of Molecular Genetics, Osaka University Medical School, and [7]Solution-Oriented Research for Science and Technology, Japan Science and Technology Agency, Osaka, Japan

Submitted 18 August 2008; accepted in final form 25 September 2008

Youn DY, Lee DH, Lim MH, Yoon JS, Lim JH, Jung SE, Yeum CE, Park CW, Youn HJ, Lee JS, Lee SB, Ikawa M, Okabe M, Tsujimoto Y, Lee JH. Bis deficiency results in early lethality with metabolic deterioration and involution of spleen and thymus. *Am J Physiol Endocrinol Metab* 295: E1349–E1357, 2008. First published October 7, 2008; doi:10.1152/ajpendo.90704.2008.— Bcl-2 interacting cell death suppressor (Bis), also known as Bag3 or CAIR-1, is involved in antistress and antiapoptotic pathways. In addition to Bcl-2, Bis binds to several proteins, suggesting it has diverse functions in normal and pathological conditions. To better define the physiological function of Bis in vivo, we developed *bis*-deficient mice with a *cre-loxP* system. Targeted disruption of exon 4 of the *bis* gene was demonstrated by Southern blotting and PCR, and Western blotting showed that no intact or truncated Bis protein was synthesized in $bis^{-/-}$ mice. While heterozygotes were fertile and appeared normal, Bis-deficient mice showed growth retardation and died by 3 wk after birth. The relative weight of the thymus and spleen was reduced and the total numbers of white blood cells, splenocytes, and thymocytes were significantly reduced compared with wild-type littermates. Serum profiles indicated significant hypoglycemia as well as decrease in triglyceride and cholesterol levels. Expression profiles of metabolic genes indicated that gluconeogenesis and β-oxidation are activated in the liver of $bis^{-/-}$ mice. This activation, as well as a decrease in peripheral fat and an induction of fatty liver, appears to be an adaptive response to hypoglycemia. Our study reveals that the absence of Bis has considerable influences on postnatal growth and survival, possibly due to a nutritional impairment.

bis; knockout; hypoglycemia

THE BCL-2 INTERACTING DEATH SUPPRESSOR (*bis*) gene has been identified as encoding a Bcl-2 binding protein in protein interaction techniques (18). Bis has also been reported as Bag3 and CAIR-1, which bind to heat shock protein (HSP)70 and PLC-γ, respectively (7, 33). The ability of Bis to bind to several proteins suggests that it has distinct functions depending on its cellular environment. A possible role for Bis in modulating cell death was revealed in in vitro DNA transfection experiments in which Bis was shown to significantly enhance the antiapoptotic function of Bcl-2 (18). Supporting this, Bis has also been shown to be specifically expressed or overexpressed in several cancers, including pancreatic cancer, thyroid carcinoma, and some leukemia (1, 5, 21, 27, 28). Furthermore, the downmodulation of Bis results in an increased susceptibility for the induction of apoptosis in cancer cells (1, 5, 26). Bis has been also proposed as an antistress protein, based on the upregulation of its expression, concomitant with HSP70, in cells exposed to stressful stimuli such as heat shock or heavy metals (21, 23). In addition to the stressful conditions given for cellular levels, the expression of Bis is significantly upregulated in several in vivo disease models such as stroke and seizure models (19, 20, 31). Moreover, Bis is robustly expressed in reactive astrocytes in areas of gliosis in the brain of human immunodeficiency virus (HIV) encephalopathy patients (29). Light damage also increases the expression of Bis in the mouse retina (4). These results suggest that the expression of Bis may be induced to protect cells from stressful conditions, but the persistent and/or uncontrolled expression of Bis may contribute to the progression of cancer.

In addition to its possible role as a stress- or survival-related protein, Bis has been implicated to have other cellular functions. Overexpression of Bis promotes the differentiation of human promyelocytic cells and cell cycle arrest (32). Roles for Bis in cell adhesion and migration have been recently reported by separate groups, although their results differ: in one study overexpression of Bis is shown to inhibit the migration and adhesion of breast cancer cell lines, whereas in the other study *bis*-deficient fibroblasts have reduced motility and delayed formation of focal adhesion complex (12, 14). These results suggest that complex mechanisms are involved in the regulation of cellular motility by Bis. Furthermore, cytoplasmic Bis protein modulates the transcription of the HIV-1 gene and the replication of the varicella-zoster virus (15, 29). Therefore, it appears that Bis exerts diverse functions in pathophysiological conditions in vivo, which may be partly ascribed to its ability to interact with several known and yet to be identified proteins.

To better define the function of Bis in vivo, we developed *bis*-deficient mice with a *cre-loxP* system targeting exon 4. Here we show that disruption of exon 4 of the *bis* gene by homologous recombination led to a complete inhibition of Bis protein synthesis, which resulted in serious hypoglycemia, a fatty liver, and 100% lethality before 3 wk of age. Bis-deficient mice also exhibited a significant involution of the spleen and thymus. Our results are inconsistent with a previous study in

* D.-Y. Youn and D.-H. Lee contributed equally to this work.
Address for reprint requests and other correspondence: J.-H. Lee, 505 Banpo-Dong, Seocho-gu, Seoul 137-701, Korea (e-mail: leejh@catholic.ac.kr).

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which retrovirus-targeted deletion of the *bis* gene resulted in massive degeneration of myofibrils with apoptotic features in heart and skeletal muscles and no abnormalities in other organs (10). Possible explanations for the differences observed in *bis*-deficient mice in the previously published study and the present study are discussed below.

METHODS

Construction of targeting vector and generation of bis-mutant mice. A 6,018-bp genomic clone that includes coding exons 3 and 4 of the *bis* gene (nucleotides 17173-23356 from the start codon) was cloned from D3 mouse embryonic stem (ES) cells as the long arm and introduced into a pMulti-ND 1.0 vector with *PmeI* and *PacI* sites (11). The *loxP* sequences were inserted into an *Eco*RV site located between exon 3 and exon 4. For homologous recombination, the downstream short arm spanning nucleotides 23357-27370 was also cloned and introduced into a *NotI* site of a pMulti vector. The resulting *PmeI*-digested targeting vector was electroporated into D3 ES cells derived from 129Sv and screened for neomycin resistance. Of 98 neomycin-resistant clones, four clones were shown to have the desired homologous recombination as determined by Southern blotting with two different probes for the 5′ and 3′ regions external to the targeting vector and one probe for the neomycin sequences. Four homologous recombinant ES clones were independently injected into C57B6 blastocysts to generate chimeric mice. Male chimera derived from one ES clone transmitted the recombinant allele to the next generation. To generate heterozygous mutants with deletion of exon 4 of the *bis* gene on one chromosome, the germ line-transmitted male mice were mated with *CAG-cre* C57B6 females.

All research procedures involving animals were performed in accordance with the Laboratory Animals Welfare Act, the *Guide for the Care and Use of Laboratory Animals*, and the Guidelines and Policies for Rodent Experiments provided by the Institutional Animal Care and Use Committee (IACUC) at the College of Medicine, Catholic University of Korea and were reviewed and approved by the IACUC.

Southern blotting and allele-specific genomic PCR. Genomic DNA extracted from wild-type or *bis*-mutant mice livers was digested with *Bam*HI enzyme and electrophoresed through 0.8% agarose. After transfer onto nylon membrane by capillary blotting, the membrane was hybridized with a digoxigenin (DIG)-labeled specific DNA probe and then immunodetected with alkaline phosphatase-conjugated anti-DIG antibody and a chemiluminescent substrate (Roche Applied Science, Mannheim, Germany) as described previously (17). The following primers were used to incorporate DIG-11 dUTP for the DNA probes: 5′-TGA GGT AAG AAG AGA CCC AGA GAC (forward primer) and 5′-TAC AGA CGT AGG AAA CAC ATC TCC (reverse primer).

PCR reactions were also performed to detect the truncated *bis* allele in genomic DNA with two sets of primers, 5′-TGA GAG CCA GCA TGC TGT TTC ATT and 5′-TGG CCC TCA GGG GAC AAC CTG CAG designed to amplify a region of 500 bp in the wild-type allele and 5′-CTT TCA AGG ATT TAA CTT ATC TGA CCA and 5′-ACA GCA AGC ATA TTC CTC TAC CTA AG to amplify a 3,003-bp product in the wild-type allele and a 1,043-bp product in the post-*cre* allele. PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide staining.

Western blotting. Proteins from various tissues of wild-type or *bis*-mutant mice were prepared and Western blotting was performed as described previously (17). To analyze Bis expression, the blotted membranes were incubated with polyclonal antibodies against the COOH-terminal half of human Bis (306-575 aa) (18) or against whole human Bis (Abnova, Taiwan, Taipei). Polyclonal antibodies raised in rabbit against the NH_2-terminal of human Bis (48-63 aa) (Peptron, Daejeon, Korea) were also used to detect smaller truncated Bis proteins. Antibodies for HSP70 and Bcl-2 were purchased from BD Biosciences (San Jose, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Complete blood count and assay of metabolites in blood and liver. The complete blood count was determined with a Hemavet 850 automated hematologic analyzer (CDC Technologies, Oxford, CT). The concentration of glucose in the blood was determined by Hemocue Glucose 201+ (Hemocue, Angelholm, Sweden). Plasma concentration of insulin was measured with a mouse insulin enzyme-linked immunoabsorbent assay kit (Linco Research, Erie, PA). Measurements of triglyceride, free fatty acid, and cholesterol in the serum and in the liver were performed with the Triglyceride E-test, NEFA-HR (2), and Labassay Cholesterol, respectively (Wako Pure Chemical Industries, Osaka, Japan).

Histological analysis. Paraffin sections (10 μm) from various organs were processed for hematoxylin and eosin (H & E) staining. Frozen liver sections (6 μm) were fixed with 10% formalin, stained with 0.5% Oil Red O, and counterstained with Mayer's hematoxylin. To examine the state of apoptosis in situ in muscles, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was also performed with the ApopTag Peroxidase In Situ Apoptosis Detection Kit S7100 (Chemicon, Temecula, CA). Specimens were examined under a light microscope (Axioskop40, Carl Zeiss, Gottingen, Germany). For electron microscopy, the tissue samples were fixed with 2.5% glutaraldehyde for 1 h. After fixation, the samples were postfixed in 1% O_5O_4, dehydrated in ethanol, and embedded in Epon 812 (Polysciences, Warrington, PA). Ultrathin sections were contrasted with uranyl acetate and lead citrate. Sections were examined in a JEM 1010 CX transmission electron microscope (JEOL, Akishima, Japan).

RNA extraction and quantitative real-time PCR. Total RNA from liver was isolated with RNA-Bee (Tel-Test, Friendswood, TX). cDNA was synthesized from 2 μg of total RNA with AccuPower Cycle Script (dN6) (Bioneer, Daejeon, Korea). mRNA levels of genes involved in glucose and lipid metabolism were measured by quantitative real-time PCR using a cDNA template and appropriate primers as previously described (Refs. 9, 25, 34; Supplemental Table S1).[1] Quantitative real-time PCR was performed with the IQ5 Real Time PCR detection System (Bio-Rad Laboratories, Hercules, CA) and iQ TM SYBR Green Supermix (Bio-Rad Laboratories). Relative levels of PCR products were determined after normalizing to an endogenous cyclophilin control.

Statistical analysis. The number of mice in each experimental group is indicated in Figs. 2 and 3. A two-tailed Student's *t*-test was used to calculate *P* values. All values are presented as means ± SE. Differences were considered significant if $P < 0.05$.

RESULTS

Targeting the bis gene and generation of bis-mutant mice. The coding region of mouse *bis* consists of four exons. The 315-amino acid peptide encoded by exon 4 includes the bag domain and a proline-rich region, which are required for the regulation of HSP70 chaperone activity and cellular motility, respectively (14, 33). To disrupt exon 4, we generated a targeting vector in which exon 4 was bracketed by *loxP* sites as shown in Fig. 1*A*. The germ line-transmitted male mice were obtained and mated with *CAG-cre* C57B6 females as described in METHODS. The resulting heterozygous male *bis* mutants were backcrossed into C57B6 females for more than eight generations to minimize the contribution of the 129Sv genetic background of ES cells on the phenotype of *bis* mutants. Male and female *bis* heterozygotes were interbred to generate homozygous mice. In $bis^{-/-}$ mice, the *loxP* sites and the intervening

[1] The online version of this article contains supplemental material.

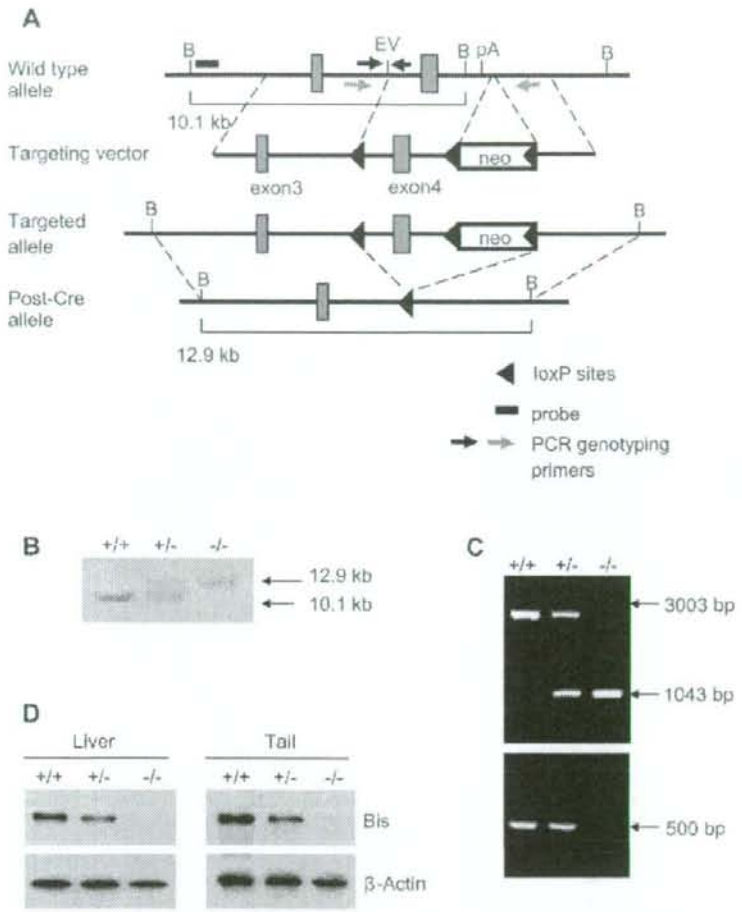

Fig. 1. Targeted disruption of the *bis* gene. *A*: schematic representation of a part of the *bis* genomic locus, targeting vector, and mutant allele. The targeting vector includes the 5′ long arm, the neomycin-resistant gene (*neo*), and the 3′ short arm for homologous recombination. Exon 4, as well as *neo*, was flanked by *loxP* sequences, shown as arrowheads. The sizes of *Bam*HI DNA fragments are indicated beneath the wild-type allele and post-*cre* allele. The 5′ external probe used for Southern blotting is shown as a black square. The small black and gray arrows indicate the locations of the primers used for genotyping. B, *Bam*HI; EV, *Eco*RV; pA, poly A. *B*: Southern blot analysis. Genomic DNA (10 μg) was extracted from liver of mice of the indicated *bis* genotypes. Hybridization of genomic DNA with the external probe, shown in *A*, revealed a 10.1-kb *Bam*HI fragment for wild-type allele and a 12.9-kb *Bam*HI fragment for knockout allele, corresponding to the deletion of a *Bam*HI site and exon 4 by *Cre* excision. *C*: PCR analysis. Genomic DNA was isolated from mouse tails, and PCR screening was performed with 2 pairs of primers, indicated in *A*. A pair of primers (gray arrows in *A*) were designed to produce a 3,003-bp product from the wild-type allele and a 1,043-bp product from the post-*cre* allele. Another pair of primers (black arrows in *A*) failed to amplify a 500-bp product in homozygous $bis^{-/-}$ mice because of the deletion of a section of DNA that contained the reverse primer site. *D*: Western blotting using whole protein extracts from liver and tail revealed that there is no intact Bis protein in $bis^{-/-}$ mice.

DNA, including a *Bam*HI site, were deleted, generating a 12.9-kb fragment of *Bam*HI compared with a 10.1-kb fragment in $bis^{+/+}$ mice, as shown in a Southern blot using genomic DNA extracted from the tail (Fig. 1*B*). PCR analysis using two pairs of primers, upstream and downstream of either the first *loxP* site or all three *loxP* sites, also confirmed the elimination of the DNA fragment flanked by the *loxP* sites (Fig. 1*C*). Expression of the 80-kDa Bis protein was reduced in $bis^{+/-}$ heterozygous and undetectable in $bis^{-/-}$ homozygous mouse liver tissues in a Western blot with Bis-specific antibody against the COOH terminus of Bis (Fig. 1*D*). Neither anti-Bis antibodies raised against whole peptides of Bis nor anti-Bis antibodies specific for its NH_2 terminus showed any smaller size of Bis protein products in heterozygous and homozygous tissues, excluding the possibility of the presence of truncated Bis protein composed of exon 1 from exon 3 (Supplemental Fig. S1). Therefore, disruption of exon 4 of the *bis* gene resulted in the complete inhibition of synthesis of both intact Bis protein and aberrant forms of Bis.

General characteristics of $bis^{-/-}$ mice. The $bis^{-/-}$ offspring were born roughly in a Mendelian ratio: 67 $bis^{-/-}$ homozygous, 135 $bis^{+/-}$ heterozygous, and 75 $bis^{+/+}$ wild type. While $bis^{+/-}$ heterozygous mice appeared normal and were fertile, all $bis^{-/-}$ homozygous mice died before 3 wk of age. As shown in Fig. 2*A*, the difference in body weight between homozygous $bis^{-/-}$ and both heterozygous and wild-type mice was imperceptible at birth but became noticeable within 1 wk after birth and obvious until 12–13 days after birth. Thereafter, the $bis^{-/-}$ mice failed to gain weight and began to gradually lose body weight before they died. Apparently, the thymus and spleen of $bis^{-/-}$ mice shrank dramatically to 51% and 36% of wild type, respectively, in terms of weight per total body weight at 16 days after birth (Fig. 2, *C* and *D*). The involution of spleen in *bis*-deficient mice appeared before that of thymus, showing a reduction of relative weight to 50% of wild type at 14 days after birth but no reduction of thymus (Fig. 2*E*). In addition, the external surface of livers from $bis^{-/-}$ mice, which were 80% of the relative weight of wild-type livers, appeared pale (Fig. 2, *C*

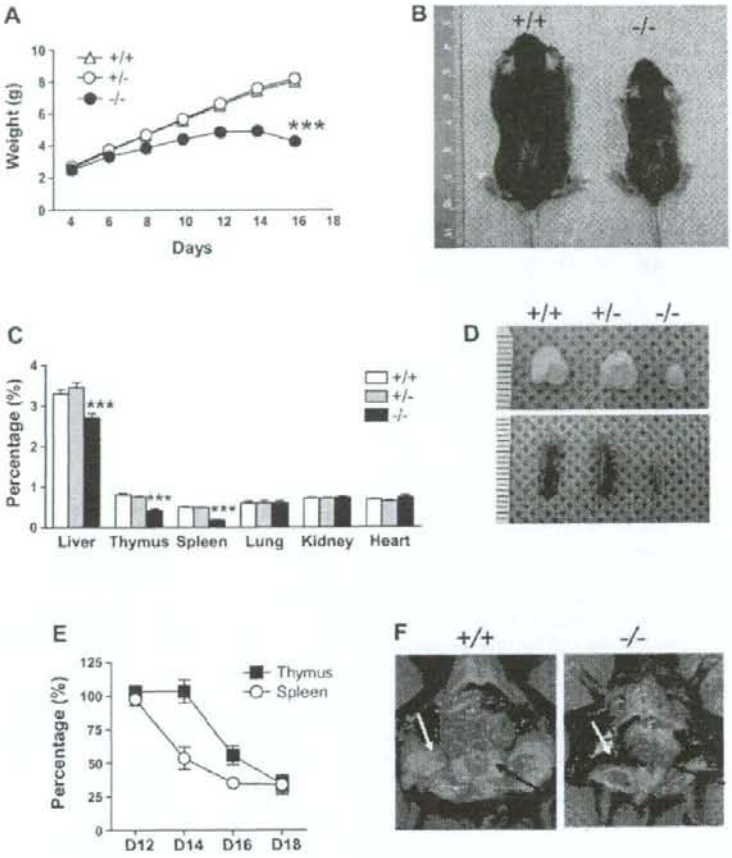

Fig. 2. Characterization of *bis*-deficient mice. *A*: growth of wild-type and $bis^{-/-}$ mice. Offspring generated from heterozygous intercrosses of $bis^{+/-}$ mice were weighed at 2-day intervals from 4 days until 16 days after birth. [n = 46 for wild-type (+/+), 104 for $bis^{+/-}$, 36 for $bis^{-/-}$]. ***$P < 0.001$, compared with wild-type littermates. *B*: representative picture showing significant growth retardation of a $bis^{-/-}$ mouse compared with a wild-type littermate at 16 days of age. *C*: relative weight of each organ to total body weight as shown as %. The ratios of thymus and spleen weight to total body weight in homozygous $bis^{-/-}$ mice were significantly decreased compared with those in wild-type and heterozygous mice older than 16 days of age (n = 15 for $^{+/+}$, 12 for $bis^{+/-}$, 16 for $bis^{-/-}$). ***$P < 0.001$, compared with wild-type littermates. *D*: representative morphology of thymus (*top*) and spleen (*bottom*) at 16 days of age showing notable reduction in size in *bis*-deficient mice. *E*: the decreased size of the thymus and spleen in *bis*-deficient mice was not obvious until age 12 days; thereafter, shrinkage of the spleen occurred before that of the thymus. The relative weight of the thymus and the spleen in $bis^{-/-}$ mice was compared with that of wild-type littermates, and the ratio is shown as %. The data are means ± SE. The number of animals measured each day is 4, 6, 8, and 5 for days (D)12, 14, 16, and 18, respectively. *F*: reduction of subcutaneous fat (white arrow) and perigonadal fat (black arrow) in a male *bis*-deficient mouse at 16 days of age compared with a wild-type male littermate.

and *F*). Notably, the subcutaneous fat and the perigonadal fat were severely reduced in $bis^{-/-}$ mice compared with wild-type mice at day 16 (Fig. 2*F*).

Decreased number of thymocytes, splenocytes, and leukocytes in peripheral blood of bis-deficient mice. As predicted from the reduced size of the thymus and spleen of *bis*-deficient mice, the number of splenocytes and thymocytes was significantly decreased, about one-tenth and one-fifth compared with wild type in the spleen and thymus, respectively, at ≥16 days of age (Table 1). The *bis*-deficient mice also had a >50% decrease in the number of total peripheral leukocytes, but the proportion of neutrophils and lymphocytes was not significantly different from that in wild-type littermates (Table 1). The difference in the number of red blood cells and platelets in *bis*-deficient and wild-type mice was insignificant.

Bis deficiency caused hypoglycemia and hepatic steatosis. The reduction in perigonadal and subcutaneous fat in $bis^{-/-}$ mice suggested that the mice suffered from malnutrition and led us to inspect the metabolic parameters in the serum. As shown in Table 2, serum glucose levels were decreased to one-third the levels of wild type in $bis^{-/-}$ mice. Insulin levels were also lower in *bis*-deficient mice than in $bis^{+/+}$ mice, showing that the hypoglycemia observed in the $bis^{-/-}$ mice was not due to high levels of insulin. Total cholesterol and triglyceride levels were also significantly decreased in *bis*-deficient mice, 60% and 26% of those in wild-type littermates, respectively. The levels of β-hydroxybutyrate, a product of

Table 1. *Comparison of cellularity in spleen and thymus and total blood cell counts in wild-type and bis-deficient mice*

	$bis^{+/+}$	$bis^{-/-}$
Splenocytes, $\times 10^6$	46.2 ± 11.4 (7)	4.67 ± 1.12 (12)*
Thymocytes, $\times 10^7$	16.7 ± 2.79 (7)	2.97 ± 0.87 (12)†
RBC, $\times 10^{12}$/l	6.18 ± 0.18 (9)	6.77 ± 0.16 (13)*
Platelets, $\times 10^9$/l	333 ± 47.2 (9)	329 ± 48.2 (13)
WBC, $\times 10^9$/l	6.63 ± 0.63 (9)	2.76 ± 0.34 (13)‡
Neutrophils	1.72 ± 0.25 (9)	0.71 ± 0.13 (13)†
Lymphocytes	3.98 ± 0.32 (9)	1.63 ± 0.17 (13)‡
Others	0.84 ± 0.18 (9)	0.42 ± 0.07 (13)*

Values are means ± SE for numbers of animals in parentheses. RBC, red blood cells; WBC, white blood cells; Others, monocytes, eosinophils, and basophils. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared with $bis^{+/+}$ littermates.

Table 2. *Profile of serum metabolites of wild-type and bis-deficient mice*

Parameter	$bis^{+/+}$	$bis^{-/-}$
Glucose, mg/dl	212.9 ± 11.9 (13)	71.46 ± 4.03 (17)‡
Insulin, pg/ml	768.5 ± 119.7 (7)	282.9 ± 77.5 (11)†
TAG, mg/dl	158.6 ± 21.3 (7)	41.7 ± 11.7 (7)‡
FFA, meq/l	1.2 ± 0.2 (7)	1.0 ± 0.2 (7)
Cholesterol, mg/dl	137.7 ± 11.6 (7)	80.4 ± 5.3 (7)‡
β-Hydroxybutyrate, mmol/l	2.80 ± 0.56 (3)	6.44 ± 1.30 (3)*

Results were obtained from mice at age 16 days and expressed as means ± SE for numbers of animals indicated in parentheses. TAG, triglyceride; FFA, free fatty acids. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared with wild-type littermates.

ketogenesis, were increased in $bis^{-/-}$ mice to ~2.5-fold above wild-type levels.

Although no obvious changes were observed by H & E staining (data not shown), Oil Red O staining revealed marked accumulation of lipids throughout the $bis^{-/-}$ liver tissues (Fig. 3*A*). Ultrastructural analysis of the hepatocytes of $bis^{-/-}$ mice revealed the presence of enlarged lipid particles and an increased number of lipid particles (Fig. 3*B*). The lipid contents of the $bis^{-/-}$ livers were analyzed to identify the type of accumulated lipids. In contrast to the serum profile of free fatty acids (FFA), which showed no difference between $bis^{-/-}$ and $bis^{+/+}$ mice, hepatic FFA levels in $bis^{-/-}$ livers were increased to twofold compared with wild-type littermates. $bis^{-/-}$ mice also had 2.8-fold and 3.4-fold increases in hepatic triglyceride and cholesterol levels, respectively, compared with control mice (Fig. 3*C*).

Quantitative RT-PCR revealed increased hepatic expression of mRNAs involved in gluconeogenesis in $bis^{-/-}$ mice, including glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (Fig. 3*D*). The expression of several lipogenic genes, including fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1), was markedly diminished, suggesting that de novo synthesis of fatty acids is inhibited in $bis^{-/-}$ mice (Fig. 3*D*). In addition, several hepatic genes involved in β-oxidation, such as carnitine palmitoyltransferase I (CPT-1) and medium-chain acyl-CoA dehydrogenase (MCAD), were induced in $bis^{-/-}$ mice (Fig. 3*D*). Thus hepatic steatosis in $bis^{-/-}$ mice is likely due to fatty acid delivery that exceeds the capacity for hepatic fatty acid oxidation to generate energy for gluconeogenesis, which are the typical metabolic changes in response to fasting (3, 8).

Bis deficiency caused no prominent apoptosis in diaphragm and cardiomyocytes. Bis is highly expressed in skeletal muscles (18), and a previous study with mice in which the *bis* gene had been disrupted by retroviral insertion described that, as the only abnormal finding, *bis*-deficient mice developed a fulminant myopathy characterized by noninflammatory myofibrillar degeneration with apoptotic features (10). However, in our model, no significant differences in H & E staining were found between the skeletal muscles from wild-type and *bis*-deficient mice (Fig. 4*A*), and the ventricular cardiomyocytes revealed similar frequencies in cells positive for TUNEL staining in wild-type and $bis^{-/-}$ mice (Fig. 4*B*). The diaphragm of $bis^{-/-}$ mice revealed a slight increase in TUNEL-positive apoptotic cells (Fig. 4*C*) but not as prominent as previously described by Homma et al. (10). When wild-type mice with body weight similar to $bis^{-/-}$ mice at day 12 after birth were fasted for 48 h, TUNEL-positive cells were increased in the diaphragm compared with feeding control (data not shown). Thus the increase of apoptotic cells in the diaphragm of $bis^{-/-}$ mice might represent a nutritionally insufficient status rather than acceleration of apoptosis due to the absence of Bis. Although no considerable abnormalities were noted in H & E staining, ultrastructures of muscles from *bis*-deficient mice exhibited discontinuous arrangement of myofibrils with thick and short Z bands but nuclei preserved normal morphology (Fig. 4*D*). Previous reports showed the colocalization of Bis with Z-disk proteins such as α-actinin and desmin (10). Thus Bis protein may contribute to preservation of the architecture of myofibrils, especially the integrity of Z bands, rather than the viability of myocytes.

DISCUSSION

Bis is expressed in various tissues, including skeletal muscle, heart, and kidney, and known to bind with several proteins, suggesting that it has diverse physiological functions. Using a *cre-loxP* system, we generated *bis* knockout mice and showed that these mice died within 3 wk after birth with metabolic derangements such as hypoglycemia and hepatic steatosis and significant reduction in the cellularity of the thymus and spleen. A previous study with mice in which the *bis* gene had been disrupted by retroviral insertion also reported premature death before weaning, although these mice died ~1 wk later than the time of death we observed (10). Furthermore, the previous study described severe degeneration and apoptosis in skeletal muscles and myocardium and no evidence of abnormality in other organs (10). In the present study, we found that the skeletal muscle fibers from $bis^{-/-}$ mice were irregular and smaller than those of wild-type littermates but found no evidence for massive apoptosis in the diaphragm, quadriceps, and cardiac muscles (Fig. 4 and data not shown). In addition, several of the phenotypes we report here, such as shrinkage of lymphoid organs and perturbations in metabolic homeostasis, were not observed in the previous report.

At present, the precise reasons for the differences in the phenotypes of our model and the previous model are not entirely clear. The method used for gene targeting may contribute to the different phenotypes observed. The previous *bis*-deficient model was developed with ES clones that had been mutagenized by retroviral insertion (10); our *bis*-deficient mice model was developed by precise deletion of exon 4 of the *bis* gene with a *Cre-loxP* system. Although the previous report does not describe which part of the *bis* gene was disrupted by retroviral insertion, partial disruption of the *bis* gene may have resulted in the expression of truncated Bis protein products, and these may have retained some function. In our system, we did not detect any full-length or truncated Bis protein by Western blotting using three kinds of antibodies raised against whole, COOH-terminal, and NH_2-terminal Bis peptides (Fig. 1 and Supplemental Fig. S1). However, the possibility that the sensitivity of immunoblotting was not high enough to detect a tiny amount of truncated Bis protein in our assay, as well as in the previous model, cannot be excluded. Another possible explanation for the discrepancy in the reported phenotypes of *bis*-deficient mice may be the extent of homogeneity in the genetic background. Diverse genetic backgrounds in hybrid

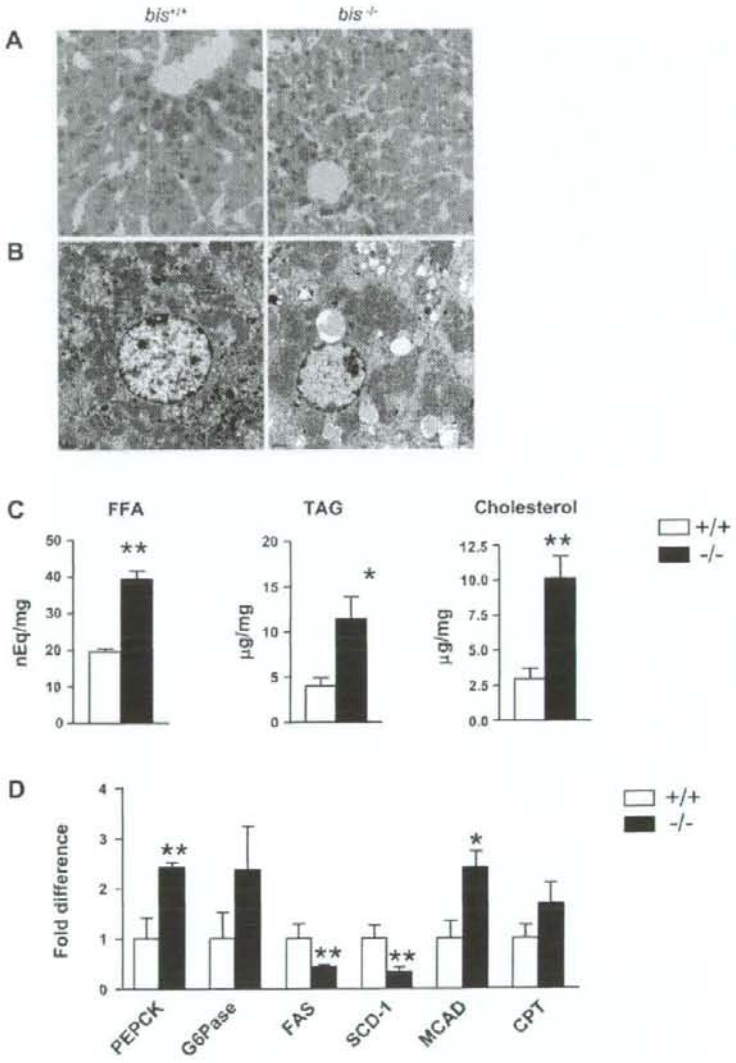

Fig. 3. Lipid accumulation in the liver of *bis*-deficient mice. *A*: Oil Red O staining of histological sections of liver from $bis^{+/+}$ and $bis^{-/-}$ mice. Red staining indicates neutral lipid accumulation. *B*: representative electron micrographs of $bis^{+/+}$ and $bis^{-/-}$ mice livers. Scale bars, 1 μm. *C*: increased levels of free fatty acid (FFA), triglyceride (TAG), and cholesterol in livers of $bis^{-/-}$ mice compared with livers of wild-type littermates. Results are expressed as means ± SE for 5 animals at 16 days of age. $*P < 0.05$, $**P < 0.01$, compared with wild-type littermates. *D*: alteration in mRNA levels of several genes involved in glucose or lipid metabolism in *bis*-deficient mice: quantitative RT-PCR of selected genes from livers of wild-type and *bis*-deficient mice. Data are means ± SE of 3 animals in each group, older than 15 days of age. Data are normalized relative to cyclophilin mRNA in the same samples, and wild-type values were arbitrarily set as 1.0. $*P < 0.05$, $**P < 0.01$, compared with wild-type littermates.

strains result in different degrees of compensatory responses, especially in response to metabolic challenges (2). For the generation of homozygous $bis^{-/-}$ mice we used heterozygous mice that were backcrossed with C57BL6 more than eight generations. Thus the effect of the Sv129 genetic background on the phenotypes of our study appeared insignificant. It is also possible that the metabolic disturbances observed in this study using biochemical and ultrastructure assays were not noticeable in the histological examinations performed by the previous research group.

The cause of death of the $bis^{-/-}$ mice was previously suggested to be respiratory failure, based on the marked degeneration of the diaphragm and intercostal muscle (10). It was also postulated that the decreased cardiac performance and subsequent pulmonary edema may have played a role in the death of the $bis^{-/-}$ mice (10). In the present study, massive apoptosis and degeneration of skeletal muscles were not observed in $bis^{-/-}$ mice (Fig. 4), suggesting that the loss of antiapoptotic activity in muscles is not the primary cause of death in these mice. Instead, the serious metabolic deterioration, such as sustained hypoglycemia and lipid accumulation in the liver, observed in our $bis^{-/-}$ mice model, may be ultimately responsible for the death of the animals.

What causes the perturbations in glucose and lipid metabolism in $bis^{-/-}$ mice? Analysis of the hepatic expression of key enzymes in the pathways of glucose and lipid metabolism

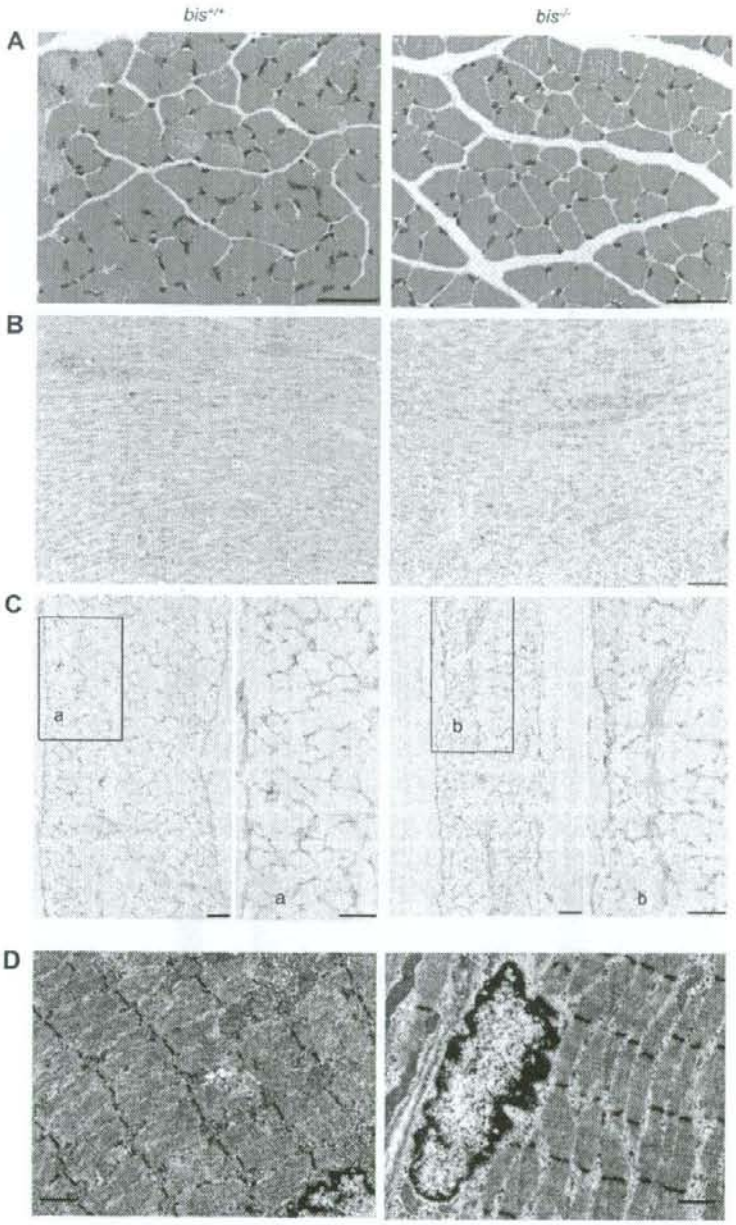

Fig. 4. No massive apoptotic features in myocytes of *bis*-deficient mice. *A*: hematoxylin and eosin staining of quadriceps femoris muscles of wild-type and *bis*-deficient mice. Scale bars, 30 μm. *B* and *C*: TUNEL staining of ventricle (*B*) and thin sections of diaphragm (*C*) of wild-type and $bis^{-/-}$ mice. *C*, *a* and *b*: Higher magnifications of boxed areas. Scale bars, 50 μm. *D*: transelectron microscopy of quadriceps femoris muscles of wild-type and *bis*-deficient mice. Scale bars, 1 μm.

revealed an increase in gluconeogenesis and lipolysis and a decrease in lipogenesis in $bis^{-/-}$ mice (Fig. 3*D*). These changes, which were also accompanied by a decrease in peripheral fat and serum triglyceride levels (Table 2), are typical of the adaptive response to a scarcity of glucose in serum that supplies the energy for gluconeogenesis in the liver, which is observed after fasting (35). Since we frequently observed that, even throughout their weight loss, $bis^{-/-}$ mice were trying to suckle, it is unlikely that isolation from the feeding mother or loss of appetite was the cause of their hypoglycemia. An impediment in the uptake or absorption of milk possibly caused delayed growth, due to an insufficiency

of nutrients for normal growth, and substantially metabolic deterioration, the same results of fasting. Since the amounts of milk in the stomachs of *bis*$^{-/-}$ mice were low at ≥16 days of age and no obvious histological abnormalities were found in the intestines of *bis*$^{-/-}$ mice (Supplemental Fig. S2), the ingestion of milk, rather than the process of absorption, appears to be impaired in *bis*$^{-/-}$ mice. The hypothesis that hypophagia or dysphagia is linked to nutritional problems and growth retardation in *bis*$^{-/-}$ mice is supported by a previous mutation study of *starvin* (*stv*), a *Drosophila* gene encoding a Bag-domain protein (6). The Bag domain is located in the COOH terminal of Bis, shared with several proteins comprising the Bag family (33). Coulson et al. (6) showed that mutation of *stv* results in a failure of larvae to grow after hatching and a severely impaired ability to take up food. The expression of STV was shown to be highly specific in embryonic somatic muscle and tendon cells, suggesting a role in muscle development or function. However, the gross morphology and function of somatic muscles including mouth-hook movement is predominantly normal in *stv* mutants, indicating that the feeding disability of *stv* mutants is not linked to dysfunction of skeletal muscles. Thus, in light of the study of *stv* mutants of *Drosophila*, the malnutrition status observed in *bis* deficiency is associated with impairment in uptake of milk, which is probably not caused by dysfunction of skeletal muscles. However, although obvious apoptotic changes were not found in the skeletal muscles in *bis*$^{-/-}$ mice, it is possible that Bis deletion caused functional weakness of muscles involved in suckling or swallowing or abnormal esophageal motor function shown in achalasia, a esophageal motility disorder in humans (16). Therefore, the role of Bis in the physiological regulation of swallowing remains to be elucidated.

We also observed a dramatic involution of the thymus and spleen in mice with a homozygous *bis* gene deletion (Fig. 2, *C* and *D*). At present, the direct link between the two representative phenotypes of *bis*$^{-/-}$ mice, metabolic deterioration and involution of the thymus and spleen, remains unclear. The thymus has been shown to be significantly affected in malnutrition, undergoing a severe atrophy due to apoptosis-induced thymocyte depletion (22, 24, 30). We showed that the reduction in the relative weight of the thymus and spleen was not obvious until 12 days after birth (Fig. 2*E*), at a time when body weight was still increasing and the serum glucose level was within the normal range (Fig. 2*A* and data not shown). Thus the involution of the thymus and spleen appears to be directly or indirectly linked to the nutritional status of *bis*$^{-/-}$ mice. Shrinkage of the thymus and spleen has also been described in *bcl-2*-deficient mice (13, 36). Since Bis binds Bcl-2 (18), interaction between Bis and Bcl-2 may be required for normal physiology of these lymphoid organs. However, *bcl-2*$^{-/-}$ mice have selective lymphopenia, but *bis*$^{-/-}$ mice have an overall decrease in white blood cells (Table 1). Furthermore, thymic and hepatic levels of Bcl-2 and HSP70, another Bis binding partner (33), were not decreased in protein extracts from *bis*-deficient mice in a Western blot analysis (data not shown). Thus the phenotypes observed in *bis*$^{-/-}$ mice are not mainly due to the disruption of the interaction between Bis and Bcl-2, or HSP70, but due to the specific effect of ablation of *bis* gene.

Bis has been shown to be highly expressed in lymphocytic leukemia cells, and downmodulation of Bis increases susceptibility to apoptosis in normal and neoplastic leukocytes (26–28). Therefore, our results showing significant decrease in leukocytes in peripheral blood cells from *bis*$^{-/-}$ mice support the previous reports for survival-sustaining roles of Bis in leukocytes. However, it is not certain whether the absence of Bis affects the viability of peripheral leukocytes or the function of progenitor cells in bone marrow. Thus, with the shrinkage of lymphoid organs, the decreases in the leukocyte numbers in *bis*$^{-/-}$ mice suggest the expanded roles of Bis in the physiology of hematopoietic cells and in the development of lymphoid organs, not confined to prosurvival activity of lymphocytes.

In this study, we generated *bis*-deficient mice and demonstrated that *bis* ablation resulted in growth retardation and early lethality with serious metabolic deterioration and involution of the thymus and spleen. Our results suggest that Bis is critical for postnatal growth and survival. However, the critical role for Bis in the regulation of feeding and the physiology of the thymus and spleen, which may or may not be linked, remains to be fully defined.

ACKNOWLEDGMENTS

We thank Y. Maruyama and A. Kawai for technical assistances and I. H. Oh and J. Kim for helpful discussions.

GRANTS

This work was partly supported by a Korea Research Foundation Grant funded by the Korean government (MOEHRD) (KRF-2004-041-E00043), a Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean government (MOST) (R01-2006-000-10208-0), grants for Scientific Research from the Ministry of Education, Science, Sports and Culture, and a grant for Research on Dementia and Bone Fracture from the Ministry of Health, Labor and Welfare, Japan.

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