

Title: Analysis of factors decreasing testis weight in MRL mice

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Abstract

MRL/MpJ (MRL) mouse testes have several unique characteristics, including the appearance of oocytes, the occurrence of metaphase-specific apoptosis of meiotic spermatocytes, and the presence of heat-shock resistant spermatocytes. In the present study, we used chromosomal mapping to determine the genomic background associated with small testis size in MRL mice. We prepared and analyzed C57BL/6-based congenic mice carrying MRL mouse loci. Quantitative trait loci (QTL) analysis revealed susceptibility loci for small testis size at 100 cM on chromosome (Chr) 1 and at around 80 cM on Chr 2. Analysis with B6.MRLc1 and B6.MRLc2 congenic mice, and double congenic mice, confirmed the QTL data and showed that low testis weight in MRL mice was caused by germ cell apoptosis. Through histological examinations, we found that B6.MRLc1 and B6.MRLc2 mice showed stage-specific apoptosis in their testes, the former at metaphase stage XII and the later at pachytene stage IV. Metaphase-specific apoptosis of spermatocytes occurs due to mutation of the exonuclease 1 (*Exo1*) gene located at 100 cM on Chr 1. Thus the mutation of the *Exo1* gene is also responsible for low testis weight caused by metaphase-specific apoptosis. In conclusion, testis weight is reduced in MRL mice due to apoptosis of germ cells caused by mutations in loci on Chrs 1 and 2.

Introduction

Germ cells are highly specialized cells that transmit genomic information to the next generation. Male germ cells, sperm, are produced in a cyclic and complicated process called spermatogenesis, which occurs in the seminiferous tubules in the testes. Spermatogenic cells
55 differentiating in the mouse seminiferous tubules can be divided into 12 discrete histological stages (I-XII) (Leblond and Clermont 1951; Oakberg 1956).

Spermatogenesis consists of mitotic and meiotic processes. In mitosis, stem cells divide in order to replace themselves and to supply germ cells destined to develop into mature sperm. In contrast, meiosis is composed of a series of two cell divisions, the segregation of
60 homologous chromosomes at the first meiotic division and separation of sister chromatids at the second meiotic division to produce haploid cells from diploid cells. Abnormalities in meiosis cause aneuploidy, and intracellular surveillance systems called checkpoints detect meiotic errors (Roeder and Bailis 2000; Cohen et al., 2006; Wang and Sun 2006). At least two meiotic checkpoints, one at pachytene stage IV and the other at metaphase stage XII, have been reported
65 during spermatogenesis. Abnormal germ cells detected by these checkpoints are eliminated via apoptosis.

The MRL/MpJ (MRL) inbred mouse strain originated from a series of crosses with strains of C57BL/6 (B6) (0.3%), C3H/He (12.1%), AKR (12.6%) and LG/J (75%) mice, followed by inbreeding (Murphy 1981). MRL mice, including their mutant strain *lpr/lpr*, have several
70 unique characteristics in regenerative wound healing such as the closure of ear punches and cardiomyocyte regeneration and are used as models for several autoimmune diseases such as systemic lupus erythematosus, polyarteritis nodosa, rheumatoid arthritis, and systemic sclerosis (Clark et al., 1998; Leferovich et al., 2001). These phenotypes depend on the MRL genetic background, but not on the *lpr* allele (Theofilopoulos and Dixon 1985). In addition, a study on
75 autoimmune glomerulonephritis in MRL mice and recently clarified that the telomeric region of

chromosome (Chr) 1 contained a susceptibility locus called MRL autoimmune glomerulonephritis (*Mag*) (Ichii et al., 2008)

The testes of MRL mice possess many unique characteristics, including oocytes (Otsuka et al., 2008a), metaphase-specific apoptosis of meiotic spermatocytes, heat-shock resistant spermatocytes, and smaller testis size than other inbred strains (Kon et al., 1999; Kon and Endoh 2000 and 2001; Namiki et al., 2004 and 2005; Kon, 2005). We have reported that the appearance of testicular oocytes in MRL mice correlates with the polymorphism of CAG repeats within Sex-determining region Y (*Sry*) on the Y chromosome (Otsuka et al., 2008b). Additionally, metaphase-specific apoptosis and heat-shock resistant spermatogenesis are closely related to exonuclease 1 (*Exo1*) mutation, with *Exo1* mutation playing an important role in DNA repair (Namiki et al., 2003 and 2005). The MRL *Exo1* gene mutation is located at the branchpoint sequence in intron 8, which causes alternative splicing of the 9th exon, and the truncated protein acts as a dominant negative product (Namiki et al., 2005). On the basis of reported research it may be concluded that mutated germ cells that are not repaired by *Exo1* proceed in their differentiation until they are arrested at the metaphase stage XII checkpoint. Hence, the unique characteristics of MRL mouse testes suggest that MRL mice may serve as a useful model for investigations regarding the mechanisms of sex differentiation and spermatogenesis.

In the present study, we used chromosomal mapping to determine the genomic background associated with small testis size in MRL mice. To this end, we created and analyzed B6-based congenic mice carrying loci derived from MRL mice. Additionally, as low testis weight is often caused by germ cell loss, we used MRL and congenic mice to verify the relationship between low testis weight and germ cell apoptosis.

Mice

Test subjects came from inbred B6 and MRL mouse strains. Eight-week-old male and female mice purchased from Japan SLC (Shizuoka, Japan) were maintained in our facility with free access to food and water. In the handling of experimental animals, the investigators
105 adhered to the “Guide for the Care and Use of Laboratory Animals, Hokkaido University, Graduate School of Veterinary Medicine.”

Genotyping and quantitative trait loci (QTL) analysis

To examine the genes inducing low testis weight in MRL mice, QTL analysis was
110 performed using 69 male backcross progeny (N2) that were created by mating female B6MRLF1 (F1 between female B6 and male MRL) with male MRL mice. The standard protocol was used to prepare genomic DNA from the tail, with a length of approximately 5 mm, for each of the N2 progeny. We used ninety-eight microsatellite markers identified in the Mouse Genome Database of the Jackson Laboratory (www.informatics.jax.org) for a wide scan
115 with a mean intermarker distance of 10-20 cM to genotype the B6 and MRL alleles. Genotypes and the ratio of the testis weight to the body weight (hereafter referred to as testis weight ratio) were entered into the Map Manager QTX program (Manly et al., 2001). The degree of linkage of genotype to the testis weight ratio was reported using the likelihood ratio statistic (LRS) portion of the Map Manager program. Because two causative loci were
120 detected on Chr 1 (*D1Mit403*) and Chr 2 (*D2mi452*) by QTL analysis, we created and genotyped an additional 552 male N2 progeny with *D1Mit403* and *D2mit452* loci.

Creation of nine congenic strains

To produce congenic mouse strains, the female backcrossed mice genotyped using
125 microsatellite markers on Chr 1 (*D1Mit202–403*) or Chr 2 (*D2Mit340-456*) were mated with

male B6 mice for more than 16 generations (Fig. 1). Polymorphisms between the segregated strains and the B6 strain were detected by a PCR-based method as previously described (Namiki et al., 2003). MRL heterozygotes carrying the telomeric regions of Chr 1(82-100 cM) and Chr 2(69-86 cM) were bred to each sex, and fundamental homozygous strains designated
130 B6.MRLc1(82-100) and B6.MRLc2(69-86) were made. We created mice designated B6.MRLc1(82-100, 82-86, 93-100, and 100) and B6.MRLc2(69-86, 69, 69-78, 78, and 78-86) by mating MRL mice with B6 mice and segregating into a short distance of the MRL-derived genome. These hybrid mice were maintained for 5-10 generations by further inbreeding and then used in the experiments.

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Tissue preparation and histological analysis

At the age of 9 or 10 weeks, each animal was weighed, and then euthanized by cervical dislocation. The removed testes were immediately weighed, fixed with Bouin's solution for 24 hr, cut into 5 μ m-thick paraffin sections, and stained with hematoxylin-eosin (HE) or
140 periodic acid Schiff (PAS). We used the sections stained with PAS to determine the seminiferous epithelial stage based on the definition described by Russell et al. (1990).

We performed immunohistochemistry for ssDNA to detect apoptotic cells. Briefly, the deparaffinized sections were autoclaved with 10 mM citrate for 5 min at 105°C. The samples were then incubated in a 3% hydrogen peroxide/methanol solution for 10 min to block
145 endogenous peroxidase activity. Sections blocked in a 1% blocking reagent (Roche Diagnostics, Basel, Switzerland) were incubated with a goat anti-ssDNA antibody (1:1000; Dako Cytomation, Tokyo, Japan), at 4°C overnight. Negative controls were left in the normal goat serum blocks, and were not incubated with the primary antibody. The sections were next treated with a secondary biotinylated anti-rabbit IgG antibody (Chemicon, Tokyo, Japan) for 30
150 min, then in an avidin-biotin-HRP complex (Vectastain ABC kit®; Vector Laboratories,

Burlingame, USA) for 30 min, and finally in a 3,3'-diaminobenzidine tetrahydrochloride-H₂O₂ solution. The sections were observed under a differential interference microscope (BX50F4, Olympus, Tokyo, Japan).

To analyze the frequency of apoptotic cells in testes, we counted the number of apoptotic
155 germ cells located in the seminiferous tubules and then determined the frequency of
seminiferous tubules with apoptotic germ cells. To evaluate these values, we randomly
selected 40 seminiferous tubules from 5 mice each of the B6.MRLc1(82–100),
B6.MRLc2(69–86), double congenic, B6, and MRL groups (a total of 200 seminiferous tubules
per strain were examined). Then the numbers of apoptotic germ cells within these
160 seminiferous tubules were counted. The seminiferous tubules were then categorized into 6
groups according to the numbers of apoptotic germ cells they contained and the relative
percentages of each group of tubules were calculated.

To quantify sperm production in each strain, we estimated the Sertoli cell index (the
number of sperm per Sertoli cell) in the 10 cross sections of stage VII or VIII seminiferous
165 tubules from the MRL, B6, B6.MRLc1(82–100), and B6.MRLc2(69–86) mice testes.

Electron microscopic analysis

To confirm the epithelial stage of the seminiferous tubules, the testes were fixed with 5%
glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C for 6 hr immediately after collection.
170 The testes were then post-fixed with 1% osmium tetroxide in a 0.1 M cacodylate buffer at room
temperature for 2 hr, and dehydrated with graded alcohol, and embedded in epoxy resin.
Ultrathin sections were stained with uranyl acetate and lead citrate, and were observed with an
electron microscope (JEM-1210, JOEL, Japan).

Statistical Analysis

The body weight and testis weight values, testis weight ratios, and numbers of apoptotic cells within the seminiferous tubules were expressed as means \pm SEM. We used the Kruskal-Wallis test to compare the testis weight ratios and Sertoli cell indexes among strains, and the Scheffé's method for multiple comparisons when a significant difference was observed (P < 0.05).

Results

QTL interval mapping

To study the genetic mechanism of small testis in MRL mice, QTL analysis was carried out using N2 backcross progeny. During the interval mapping of the testis weight ratios using 69 N2 males, we detected two significant peaks, around 100cM (*D1Mit403*) on Chr 1 (LRS = 74.6) and around 80cM (*D2Mit452*) on Chr 2 (LRS = 25.6) (Fig. 2). The LRS for suggestive, significant, and highly significant levels were 6.8, 12.7, and 18.2, respectively. The peaks for Chrs 1 and 2 were above the highly significant level, and no other peaks were observed in any chromosomal position.

Comparison of testis weight ratios among four genotypes in male backcross progeny

To determine the influence of Chr 1 or 2 on the testis weight ratios, four types of male backcross progeny were created: BM:BM (heterozygous Chrs 1 and 2), BM:MM (heterozygous Chr 1 and MRL homozygous Chr 2), MM:BM (MRL homozygous Chr 1 and heterozygous Chr 2), and MM:MM (MRL homozygous Chrs 1 and 2). All 552 mice were examined and the genotypes of these mice were checked by PCR with *D1Mit403* for 100cM on Chr1 and *D2Mit452* for 80cM on Chr2. Comparison of the testis weight ratios among these four kinds of backcross progeny revealed that MM:MM mice had the smallest testis weight ratios and that

MM:BM mice had the next smallest testis weight ratios. The testis weight ratios of MM:MB mice were close to those of the MRL mice, and no significant difference was seen between the testis weight ratios of these two groups (Table 1). In contrast, BM:BM mice exhibited the largest testis weight ratio values, and these ratios were almost the same as those seen in B6 mice. When BM:MM mice and MM:BM mice were compared, MM:BM mice had smaller testis weight ratios than BM:MM mice. Compared to MRL mice, BM:BM mice, BM:MM mice, and B6 mice showed significantly larger testis weight ratios.

Comparison of testis weight ratios among congenic and original strains

We created congenic strains consisting of nine MRL-type homozygous mouse strains designated B6.MRLc1(82-100, 82-86, 93-100, and 100), B6.MRLc2(69-86, 69, 69-78, 78, and 78-86), and B6.MRLc1(82-100)c2(69-86) (double congenic), as shown in Figure 1 (MRL-type homozygous loci are indicated with gray boxes). Then the testis weight ratios of these congenic strains were examined and compared to confirm the effects of the loci that showed significant values in QTL analysis (Table 2). When the four B6.MRLc1 strains were compared with the B6 strain, all strains except B6.MRLc1(82-86) had significantly smaller testis weight ratios than did the B6 strain. Additionally, only B6.MRLc1(82-86) mice showed significantly larger testis weight ratios than did MRL mice. In contrast, a significant decrease in testis weight ratios was detected between B6 and B6.MRLc2 (69-86, 69-78, and 78-86) mice. MRL and double congenic strain mice also had significantly smaller testis weights than did B6 mice.

Detection of apoptotic germ cells within congenic and original strain mice

Since we speculated that testis weight reduction was caused by germ cell loss, we examined the number of apoptotic germ cells located in one seminiferous tubule and the frequency of

seminiferous tubules containing apoptotic germ cells by immunohistochemistry with an anti-ssDNA antibody. Additionally, we compared these values among B6, MRL, B6.MRLc1(82-100), B6.MRLc2(69-86), and double congenic strains. We found that the occurrence of apoptotic germ cells and seminiferous tubules with apoptotic germ cells was remarkably increased in the B6.MRLc1(82-100) and double congenic mice, and that these 2 types of mice contained as many apoptotic germ cells as did MRL mice (Fig. 3 A and B). In contrast, B6.MRLc2(69-86) strain mice had fewer apoptotic germ cells than did B6.MRLc1(82-100) strain mice and double congenic strain mice. To identify and compare the apoptotic germ cells and seminiferous stages, B6.MRLc1(82-100) and B6.MRLc2(69-86) strain mice were examined and their spermatogenesis stages were categorized using PAS stain for serial sections. In B6.MRLc1(82-100) strain mice, apoptotic germ cells were mostly found at stage XII, as metaphase spermatocytes (Fig. 4A-C); however, in B6.MRLc2(69-86) strain mice, apoptotic germ cells were mostly found at stage IV, as pachytene spermatocytes (Fig. 4D-F). To confirm the apoptosis in pachytene spermatocytes at stage IV, testes were observed by electron microscopy. Round spermatids containing acrosomal vesicles attached to the nuclear surface with an angle subtended up to 40°, which are known as cells specific to stage IV, were observed in the same seminiferous tubules as apoptotic spermatocytes (Fig. 5A and B).

Comparison of sperm production among congenic and original strains

To investigate the effects of germ cell apoptosis on sperm production, the Sertoli cell index at stage VII or VIII was calculated and compared among strains (Table 3). As a result, MRL, B6.MRLc1(82-100) and double congenic mice showed significantly lower numbers of sperm than did B6 mice. In particular, sperm production in double congenic mice was dramatically low.

Discussion

In the present study, we performed genetic analysis of the low testis weight trait in MRL mice. Our results showed that this phenotype is controlled by multiple genetic loci; major QTL were observed in the 100 cM region on Chr 1 and at 78 cM on Chr 2. The QTL peak found on Chr 1 was sharp and a decrease in testis weight was only found in B6.MRLc1 strain mice that contained the 100 cM MRL genotype, this suggests that one of the factors associated with low testis weight in MRL mice is located at 100 cM on Chr 1. In comparison, the QTL peak detected on Chr 2 was gentle, and examination of several B6.MRLc2 mice revealed the existence of multiple factors. Since testis weights were not decreased in the B6.MRLc2(78) strain, our results suggest that there were causative factors located at 69-78 cM and 78-86 cM. Based on the information from the Mouse Genome Database, about 20 genes involved in spermatogenesis, cellular signaling, and development have been known to exist within this telomeric region of Chr 1. In addition, 15 genes related to cell cycle and cell proliferation have been reported between the 69 and 86 cM of Chr 2.

Histological and immunohistochemical analyses of B6.MRLc1 and B6.MRLc2 congenic mice revealed that low testis weight is strongly related to germ cell apoptosis and low sperm production. Although B6.MRLc1 and B6.MRLc2 mice both possessed apoptotic germ cells and reduced testis weights, MRL type Chr 1 was assumed to be the primary cause because data showed lower testis weight in B6.MRLc1 mice than in B6.MRLc2 mice. Interestingly, both the B6.MRLc1 and the B6.MRLc2 strains showed stage-specific germ cell apoptosis, the former at metaphase stage XII and the later at pachytene stage IV. These findings indicate that MRL type Chrs 1 and 2 influence this phenotype independently. Additionally, the stages in which apoptosis appeared had checkpoint mechanisms (Roeder and Bailis 2000; Cohen et al., 2006; Wang and Sun 2006). Taken together, the causative genes on Chrs 1 and 2 in MRL mice produced abnormal germ cells that were later detected and eliminated by stage-specific

checkpoints via apoptosis.

During our years of research on MRL mouse testes we have clarified that metaphase-specific apoptosis of meiotic spermatocytes is associated with *Exo1*, which is located at 100cM on Chr 1 (Namiki et al., 2003; Kon 2005). These previous reports are confirmed by our QTL analysis findings regarding testis weight and by the metaphase-specific analysis observed in immunohistochemistry for ssDNA. Additionally, the *Exo1* gene contains just one nucleotide substitution in the branchpoint sequence of the intron 8, and this mutation causes incomplete alternative splicing in MRL mice (Namiki et al., 2004). Thus, the *Exo1* gene mutation also plays a role in the germ cell apoptosis and small testis size in the MRL mouse. Metaphase germ cell death has also been observed in some other animals, such as *Mlh1* (mutL homolog 1)-deficient mice, *Exo1* deficient mice, and Robertsonian heterozygous mice (Eaker et al., 2001 and 2002; Wei et al., 2003; Merico et al., 2008). In these mice, as in MRL mice, the checkpoint mechanism in metaphase spermatocytes at stage XII actively monitors meiotic defects, and in the spermatocytes there is failure of DNA repair and/or spindle assembly. Interestingly, *Exo1* has been shown to interact with the other mismatch repair genes *Mlh1* and *Msh2* (Schmutte et al., 1998 and 2001). Mutations of *Mlh1* and *Msh2* not only cause cancer but also affect meiosis (Hoffmann and Borts, 2004). In particular, *Msh2* mutation has been reported to increase the frequency of aneuploidy in human sperm (Martin et al., 2000). These facts suggest that the MRL mouse is a useful model for investigating the interactions among mismatch repair genes during spermatogenesis.

Unlike *Exo1* on Chr 1, the causative genes that exist on Chr 2 are unknown. However, studies on pachytene-specific apoptosis provide clues that allow us to hypothesize which roles they play during meiosis. During the pachytene stage, recombination between homologous chromosomes takes place. This process consists of complicated steps such as a double-strand break, exonucleolytic digestion, strand invasion, and heteroduplex DNA formation.

Pachytene-specific apoptosis (or arrest) has been observed in mouse strains that lack genes involved in DNA repair and recombination. These strains include ataxia telangiectasia mutated homolog (*Atm*), disrupted meiotic cDNA 1 homolog (*Dmc1*), mutS homolog 5 (*Msh5*), early growth response homolog 4 (*Egr4*), and sporulation protein, meiosis-specific, SPO11 homolog (*Spo11*) (Xu et al., 1996; Yoshida et al., 1998; Edelman et al., 1999; Tourettelotte et al., 1999; Baidat et al., 2000; Romanienko et al., 2000; Mahadevaiah et al., 2008). Additionally, the disruption of genes such as synaptonemal complex protein 1 (*Sycp1*), synaptonemal complex protein 3 (*Sycp3*), and structural maintenance of chromosomes 1B (*Smc1 β*), involved in both sister chromatid cohesion and pairing, and in synapsis of homologous meiotic chromosomes, cause apoptosis at this stage (Hamer et al., 2008). Although none of the genes listed above are located at 69–78 cM or 78-86 cM on Chr 2, these reports suggest that the causative factors on Chr 2 might be involved in some crossing over processes, and that abnormalities in these genes cause pachytene-specific apoptosis at stage IV.

It is interesting to note that mutation of *Exo1* causes metaphase-specific apoptosis, whereas defects of other genes involved in DNA repair, such as *Atm*, *Dmc1*, and *Msh5*, are associated with pachytene-specific apoptosis. It has been speculated that *Exo1* may not play a direct role in crossing over but may be required for the DNA transactions associated with double strand break repair that are prerequisites for crossover resolution (Hoffmann and Borts, 2004). Unlike *Exo1*, *Atm*, *Dmc1*, and *Msh5* play roles not only in DNA repair, but also in DNA recombination and pairing. This indicates that the checkpoint mechanisms affected by these genes depends on how each gene contributes to spermatogenesis (Yoshida et al., 1998; Edelman et al., 1999; Barchi et al., 2008). The following two possibilities have been suggested for this phenomenon: (i) the pachytene stage checkpoint may detect more defects in crossing over than in DNA repair, and (ii) the pachytene checkpoint ignores DNA damage until the metaphase checkpoint detects it.

The spontaneous apoptosis of male germ cells is necessary in order to eliminate abnormal cells and to balance the Sertoli cell/germ cell ratio. It is well known that abnormalities in spermatogenesis enhance germ cell apoptosis, which results in spermatogenetic arrest and male infertility. The various factors causing this pathological change include genetic mutation and hormone deficiency (Sairam and Krishnamurthy, 2001; Dohle et al., 2003; Toshimori et al., 2004; Carrell et al., 2006). As some sex hormone defects cause stage-specific germ cell loss (Selva et al., 2000), we could not eliminate the hormone influence on germ cell apoptosis in MRL mice. However, the results of our QTL analysis on congenic strains and our histological data showed no testicular cancer in subjects of either the previous or the present studies. Therefore, the increased number of apoptotic germ cells in MRL mice was considered the result of purely genetic factors. The sterility model is used to investigate genetic influences on spermatogenesis; however, difficulties are often encountered in breeding. Although slightly lower sperm production was observed in MRL mice in this study, the mice's fertility levels were normal. This suggests that the MRL mouse is useful as model for investigating both the pachytene and metaphase checkpoints during spermatogenesis and the mechanisms underlying germ cell apoptosis.

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455 **Figure legends**

Fig. 1. Representation of MRL/MpJ-derived congenic B6.MRLc1 and B6.MRLc2 mice. The MRL/MpJ-type congenic intervals are represented by gray boxes. The microsatellite markers used for genotyping and their positions (cM) are indicated at the left.

460 Fig. 2. Quantitative trait loci linkage analysis with testis weight ratio parameters. The three vertical lines on the right indicate suggestive, significant, and highly significant levels. The number at the peak indicates the likelihood ratio statistic (LRS) score. Chr, chromosome.

Fig. 3. Comparison of appearance of apoptotic cells among congenic mice. (A) Numbers of
465 apoptotic cells located in one seminiferous tubule and (B) frequencies of seminiferous tubules containing apoptotic cells. Double congenic, B6.MRLc1(82–100)c2(69–86); B6, C57BL/6; MRL, MRL/MpJ. Values = mean \pm SEM.

Fig. 4. Comparison between apoptotic germ cells in B6.MRLc1 and B6.MRLc2 strains.
470 (A–C) B6.MRLc1(82–100); (D–F) B6.MRLc2(69–86); (A and D) HE stain; (B and E) PAS stain; (C and F) ssDNA immunostaining. Scale bar = 50 μ m, and all images are the same magnification.

Fig. 5. Ultrastructure of stage IV seminiferous tubules. (A) Lower magnification and (B)
475 higher magnification of area indicated by square in A. Arrows, apoptotic germ cells;

arrowhead, acrosomes of spermatids. Scale bars = 1 μm .