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Title: Genomic analysis of the appearance of testicular oocytes in MRL/MpJ mice

Running head: Autosomal effects on testicular oocytes in MRL mice

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Abstract

Mammals produce sperm or oocytes depending on their sex; however, newborn MRL/MpJ (MRL) male mice produce oocytes within their testes. We previously reported that one of the genes responsible for this phenotype is present on the MRL-type Y chromosome (Y^{MRL}), and that multiple genes, probably autosomal, are also required for the development of this phenotype. In this study, we focused on the autosomal genes and examined their relationship with this phenotype by analyzing the progeny from crosses between MRL mice and other strains. We first observed the male F1 progeny from the crosses between female A/J, C57BL/6 (B6), BALB/c, C3H/He, or DBA/2 mice and male MRL mice, and 2 consomic strains, male B6- Y^{MRL} and MRL- Y^{B6} . Testicular oocytes that were morphologically similar to those of MRL mice were detected in all mouse strains except BALBMRLF1; however, the incidence of testicular oocytes was significantly lower than that in MRL mice. The appearance of testicular oocytes in MRL- Y^{B6} mice indicates that this phenotype is strongly affected by genomic factors present on autosomes, and that there is at least 1 other causative gene on the MRL-type autosomes (MRL testicular oocyte production; *mtop*) other than that on Y^{MRL} . Furthermore, a quantitative trait locus (QTL) analysis using N2 backcross progeny from crosses between female MRLB6F1 and male MRL mice revealed the presence of susceptibility loci for the appearance of testicular oocytes at 8–17cM on Chr 15. These findings demonstrate that the appearance of testicular oocytes is regulated by the genetic factors on Chr 15 and on Y^{MRL} .

Introduction

Gonads undergo a unique developmental process because of their bipotential nature, the ability to differentiate into a testis or an ovary. In most mammals, sex determination is genetically controlled by the presence or absence of the Y chromosome (Chr). The male pathway is initiated by the gonadal expression of the Y-linked gene, sex-determining region of Chr Y (*Sry*), which induces the differentiation of Sertoli cells (Koopman et al., 1991). Germ cells also have the plasticity to develop into either oogonia or prospermatogonia; however, this sex differentiation is directed by signals from somatic cells in the gonads, and not by the germ cells themselves (McLaren 1995). The initiation of meiosis at different time points between males and females is the key factor influencing the sex differentiation of primordial germ cells in mammals. In mice, this phenomenon occurs at embryonic day (E) 13.5, when ovarian germ cells enter meiosis and testicular germ cells are arrested at the G0/G1 phase of the mitotic cell cycle (McLaren 1984). These sex differentiation mechanisms “are the reasons why, universally, mammalian males produce only sperms in their testes and females produce only oocytes in their ovaries”.

However, there are many cases in which this universal rule does not apply because of genetic abnormalities. For example, the mutations or functional loss of genes involved in sex differentiation such as *Sry* and *Sox9* result in male-to-female sex reversal, i.e., the development of the primordial gonad into an ovary or ovotestes, in XY humans and mice (Berta et al., 1990; Barrionuevo et al., 2006; Sim et al., 2008). In particular, nearly all human patients carrying SRY mutations show complete gonadal dysgenesis with internal and external female genitalia and are sterile.

As a new model of sex reversal in mice, we previously reported the existence of testicular oocytes in the newborns of MRL/MpJ mouse strains, MRL/MpJ-+/+ (MRL) and MRL/MpJ-*lpr/lpr*, on the basis of morphological characteristics and the expression of

oocyte-specific genes such as zona pellucida glycoproteins 1-3 (*Zp1*, *Zp2*, and *Zp3*), and oocyte maturation, alpha (*Omt2a*) (Otsuka et al., 2008a). As described in previous studies, testicular oocytes appeared as early as at birth, formed *Zp3*-positive zona pellucidae, and developed until the early secondary follicular stage. Their appearance peaked at day 14, and they disappeared after day 30. In the MRL fetal testis, some germ cells entered meiosis prematurely, (as early as E13.5) these meiotic germ cells are thought to develop into testicular oocytes. In spite of this unique phenotype, adult MRL mice are fertile. We also examined the F1 generation from a cross between MRL and C57BL/6 (B6) mice, B6MRLF1 mice (F1 generation from a cross between female B6 and male MRL mice), and MRLB6F1 mice (F1 generation from a cross between female MRL and male B6 mice) but only detected testicular oocytes in B6MRLF1 mice. Therefore, one of the causative genes must be located on the MRL-type Y chromosome (Y^{MRL}). We also reported that the MRL-type *Sry* contains shortened CAG repeat regions that are associated with the appearance of testicular oocytes (Otsuka et al., 2008b). However, B6MRLF1 mice showed a much lower incidence of testicular oocytes than MRL mice, which indicates that the development of this phenotype is associated with multiple autosomal genes (Otsuka et al., 2008a and b). In the present study, we focused on these autosomal genes and examined their relationship with the phenotype by analyzing the progeny arising from crosses between MRL and various other strains.

Methods

Mice

Inbred mouse strains AJ, B6, BALB/c (BALB), C3H/He (C3H), DBA/2 (DBA), and MRL were used. Eight-week-old male and female mice purchased from Japan SLC were maintained in our specific pathogen-free facility with free access to food and water. The investigators adhered to the “Guide for the Care and Use of Laboratory Animals, Hokkaido University,

Graduate School of Veterinary Medicine” for the handling of experimental animals. The mice were housed in an animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care, and the project was approved by the Animal Experiment Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval no. 11-0033).

Generation of F1 and backcross mouse strains

Female AJ, B6, BALB, C3H, and DBA mice were mated with male MRL mice to produce F1 progeny, AJMRLF1, B6MRLF1, BALBMRLF1, C3HMRLF1, and DBAMRLF1, respectively. To produce the consomic mouse strains B6-Y^{MRL} and MRL-Y^{B6}, the male backcrossed mice were mated with female B6 and MRL mice, respectively, for more than 10 generations. Genotyping was performed only for the N10 generation because the Y chromosome is only derived from male mice (Fig. 1). To examine the derivation of Chr Y, 5 mice were randomly chosen as samples for each consomic strain, and genomic DNA was extracted from the spleens of MRL, B6, B6-Y^{MRL}, and MRL-Y^{B6} mice by using a standard protocol. Polymorphism on Chr Y was detected by polymerase chain reaction (PCR) for zinc finger protein 2, Y linked (*Zfy2*) with primer pairs (forward primer 5'-GTGAGAGGCACAAGTTGGC - 3' and reverse primer 5' – GTGCTCCTGGTATGGTGTAT - 3') for distinguishing Chr Y between *Mus musculus musculus* and *Mus musculus domesticus* (Prager et al., 1997). PCR was performed with ExTaq (Takara) under the following PCR conditions: 5 min at 95 °C; 40 cycles of 30 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C; and finally, 5 min at 72 °C. B6 and AKR mice carry *M. m. musculus*-type Chr Y and *M. m. domesticus*-type Chr Y, respectively. As Y^{MRL} is derived from AKR mice, the polymorphism on *Zfy2* can be used to confirm the derivation of Chr Y in consomic mouse strains (Otsuka et al., 2008b). Furthermore, 266 male backcross progeny (MBM) were created for QTL analysis by mating female MRLB6F1 mice with male MRL mice.

Detection of testicular oocytes

To detect testicular oocytes, fresh whole-mount preparations of testes were used as described in previous studies (Otsuka et al., 2008a and b). Briefly, the testes of 14-day-old F1 (AJMRLF1, B6MRLF1, BALBMRLF1, C3HMRLF1, and DBAMRLF1), B6-Y^{MRL}, MRL-Y^{B6}, and MBM mice, and backcross progeny containing Y^{MRL} were removed and immediately mounted on glass-slides with cover slips. The testes were then crushed to generate whole-mounted single tubular sheets and observed by differential interference microscope (BX50F4, Olympus). The numbers of oocytes contained in the left and right testes were added to calculate the number of testicular oocytes in 1 mouse each of the MRL, B6MRLF1, and MBM strains. The average number of oocytes per testis was calculated as the oocyte score for each mouse strain.

Genotyping and QTL analysis

To examine the genes involved in testicular oocyte appearance in newborn MRL mice, QTL analysis was performed using 266 MBM mice. Genomic DNA was prepared from the spleen of each MBM mouse using a standard protocol. In total 113 microsatellite markers identified in the Mouse Genome Informatics (MGI) of the Jackson Laboratory (www.informatics.jax.org) were used for a genome-wide scan with a mean intermarker distance of 10–20 cM to genotype the B6 or MRL allele. PCR was performed with GoTaq (Promega) under the following PCR conditions: 2 min at 94 °C; 40 cycles of 40 s at 94 °C, 30 s at 58 °C, and 20 min at 72 °C; and finally, 5 min at 72 °C. Two phenotypic categories were used: mice with testicular oocytes (value = 1) and mice without testicular oocytes (value = 0). Genotyping data and phenotype values were entered into the Map Manager QTXb20 program and analyzed (Manly et al., 2001).

Statistical analysis

The oocyte scores for 2 groups of mouse strains were statistically compared by the non-parametric Mann–Whitney *U*-test ($P < 0.05$) using PASW version 18 (IBM).

Results

Morphology of testicular oocytes in F1 and backcross progeny carrying Y^{MRL} or Y^{B6}

Testicular oocytes were observed in all F1 progeny except for BALBMRLF1, and they appeared in every generation of backcross progeny carrying Y^{MRL} (Fig. 2A-K). Surprisingly, testicular oocytes also appeared in the MRL- Y^{B6} mice carrying Y^{B6} (Fig. 2L). The testicular oocytes in these mice had morphological characteristics similar to those of MRL mice; they coexisted with spermatogenic cells in the seminiferous tubules, mostly in the vicinity of the rete testis and efferent ductule (Fig. 2A). Each testicular oocyte had a diameter of 50–70 μ m, abundant cytoplasm, a large nucleus with 1 or 2 distinct nucleoli, and zona pellucida. The development of follicular epithelial-like cells in some of the testicular oocytes in the backcross progeny was relatively poor compared with that of the MRL mice (Fig. 2B, G-K).

Oocyte scores in F1 and backcross progeny carrying Y^{MRL} or Y^{B6}

The oocyte scores were different among F1 progeny the testicular oocytes in AJMRLF1, DBAMRLF1, and B6MRLF1 mice had significantly lower oocyte scores than those in MRL mice, whereas C3HMRLF1 mice had oocyte scores similar to those of MRL mice (Table 1). Although testicular oocytes appeared in every generation of backcross progeny carrying Y^{MRL} , the incidence of testicular oocytes gradually decreased as the number of generations increased (Table 1). Significant differences in oocyte scores were found between MRL mice and every backcross progeny. In addition, the oocyte score of MRL- Y^{B6} mice was significantly lower than that of the MRL and B6- Y^{MRL} mice.

QTL analysis of testicular oocytes in MBM mice

MBM mice were analyzed to study the genetic mechanism underlying the appearance of testicular oocytes in MRL mice. The testicular oocytes in MBM mice showed morphological characteristics similar to those of MRL mice, such as the existence of the zona pellucida and follicular epithelial-like cells (Fig. 3A and B). Approximately a quarter of MBM mice (74 of 266) had testicular oocytes, and the oocyte score was 0.517, which was significantly lower than that of MRL mice (Fig. 3C). The maximum number of testicular oocytes in 1 testis and 1 mouse was 16 and 20, respectively. The distribution of the number of testicular oocytes per mouse followed a gradient curve (Fig. 3D).

During interval mapping of the appearance of testicular oocytes in 266 MBM males, 2 significant peaks, 8.23–11.93cM (*D15Mit130–D15Mit18*) and 15.18–16.73cM (*D15Mit1007–D15Mit24*), on Chr 15 were detected with likelihood-ratio statistic (LRS) scores of 13.2 and 12.9, respectively (Fig. 4 and 5). The LRS scores for suggestive, significant, and highly significant levels were 6.4, 12.6, and 21.3, respectively. Peaks above the suggestive level were found on Chrs 1, 3, 4, 5, and 17. No peak was above the highly significant level.

Genotype–phenotype correlations

Genotype and phenotype data were examined to evaluate the allelic contribution to the effect of Chr 15 on the oocyte score (Table 2). According to the QTL results, the 8.23–16.73 cM region of Chr 15 was divided into 3 regions as follows: the region containing both peaks, *D15Mit130–D15Mit87* (8.23–16.73 cM), and regions containing either 1 of the 2 peaks, *D15Mit130–D15Mit18* (8.23–11.93 cM) and *D15Mit1007–D15Mit87* (15.18–16.73 cM). MBM mice were categorized according to genotype, and the oocyte score for each group was calculated. Mice with the homozygous MRL genotype had significantly higher oocyte scores than

heterozygous mice for all 3 regions. Interestingly, even MBM mice homozygous for the 8.23–16.73 cM region of Chr 15 had a significantly lower oocyte score than MRL mice. In addition, to examine which peak has a stronger influence on the appearance of testicular oocytes, the oocyte scores of 2 groups, 1 homozygous for *D15Mit130–D15Mit18* (8.23–11.93 cM) and heterozygous for *D15Mit1007–D15Mit87* (15.18–16.73 cM), and the other heterozygous for *D15Mit130–D15Mit18* (8.23–11.93 cM) and homozygous for *D15Mit1007–D15Mit87* (15.18–16.73 cM), were compared. There was, however, no significant difference between the 2 groups.

Discussion

Difference in the phenotypic expression of Y^{MRL} among inbred strains

Genetic background is known to play an important role in the process of gonadal sex determination and differentiation in mice. For example, B6 mice, but not other inbred strains, undergo sex reversal when Chr Y of the *M. m. domesticus* variant is introduced. These mice are named B6- Y^{DOM} as a group or B6- Y^{POS} , B6- Y^{TIR} , etc., for specific *M. m. domesticus* variants, and they develop ovaries or ovotestes (Eicher et al., 1982). Three interesting points arose during the observation of F1 progeny, when the phenotypic expression of Y^{MRL} was analyzed and compared with that of Y^{POS} among inbred strains. First, testicular oocytes were never observed in BALBMRLF1 mice. These data reflect the resistance of this strain against Y^{POS} and the stability of sex determination in the BALB background (Eicher and Washburn 1986). Second, Y^{DOM} did not cause sex reversal in the DBA background, and the sex reversal in B6- Y^{DOM} was suppressed by outcrossing to DBA or C3H (Eicher and Washburn 1986). These findings indicate that DBA and C3H mice are resistant to Y^{DOM} . In contrast, we discovered that the AJ, C3H, and DBA strains are sensitive to Y^{MRL} in addition to B6 (Otsuka et al., 2008a and b). It is therefore likely that the autosomal genes responsible for sex differentiation under Chr Y in these strains behave

differently between Y^{DOM} and Y^{MRL} . Finally, the oocyte score in C3HMRLF1 mice was much higher than that in AJMRLF1, B6MRLF1, and DBAMRLF1 mice. As one of the ancestor strains of the MRL strain, the C3H strain shares about 12% of its genetic background with the MRL strain (Murphy 1981). This similarity in genetic background between the MRL and C3H strains might be responsible for the propensity of C3H to produce testicular oocytes. Hence, the MRL and C3H genetic composition involves certain genes that play a role in oocyte production or survival in the testicular environment and thus result in an increase in number of oocytes, not observed in other inbred strains.

Existence of autosomal causative and modifier genes and their effect on the development of testicular oocytes

A series of studies revealed that sex reversal in B6- Y^{DOM} mice is associated with the interaction between Y^{DOM} and B6-type autosomal genes involved in testis determination (McElreavey et al., 1995; Eicher et al., 1996; Albrecht et al., 2003; Bullejos and Koopman 2005). The present study also provides evidence for the importance of the interaction between Y^{MRL} and autosomal genes in the sex differentiation of germ cells. Although the morphological characteristics of testicular oocytes were maintained in the F1 and backcross progeny carrying Y^{MRL} , the oocyte scores decreased dramatically when compared with the MRL mice. This result indicates the importance of MRL-type autosomal genes in the quantitative appearance of testicular oocytes. The MRL-type autosomes had a greater impact on this phenotype than we first expected. Surprisingly, not only B6- Y^{MRL} mice, but also the other consomic mouse strain, MRL- Y^{B6} produced testicular oocytes. This finding indicates the existence of at least 1 other responsible gene on the autosomes that, functions independently of Y^{MRL} during the all-or-none induction of testicular oocytes. Because MRLB6F1 mice did not contain testicular oocytes in previous studies (Otsuka et al., 2008a), the autosomal causative genes are suggested to be

homozygous recessive. We have named these autosomal responsible genes as MRL testicular oocyte production (*mtop*).

Furthermore, QTL analysis revealed that the appearance of testicular oocytes was significantly correlated with the 8.23–11.93 cM and 15.18–16.73 cM regions on Chr 15. This result may indicate the existence of autosomal causative genes, i.e., *mtop*, on 1 (or both) of these regions of Chr 15. An insufficient number of MBM mice with recombination within these regions made it difficult to determine which of the 2 loci on Chr 15 contains *mtop*; therefore, further analyses such as the generation and examination of congenic mice are necessary to identify the functions of these loci. The genotype–phenotype correlation analysis revealed that MBM mice with MRL-type Chr 15 had significantly higher oocyte scores than those with MRL/B6 heterozygous Chr 15. However, the former group still had a significantly lower oocyte score than the original MRL mice. In addition, the distribution of the number of testicular oocytes per MBM mouse followed a gradient curve, and there was a large variation in the number of oocytes among MBM mice. These results indicate that this phenotype is regulated by polygenetic inheritance, and the modifier genes that strongly affect the oocyte score exist on autosomes. Some of these modifier genes might be located on the loci of Chrs. 1, 3, 4, 5, and 17, which showed a suggestive correlation with the appearance of testicular oocytes on QTL analysis. In addition, although MRL-Y^{B6} mice contain MRL-type autosomes, their oocyte score was significantly lower than that of B6-Y^{MRL} mice. These data suggest a difference in potency between *mtop* and Y^{MRL} or the possibility that some of the autosomal modifier genes are functional only in the presence of Y^{MRL}.

Possible causative genes and their roles in testicular oocyte production

The appearance of a testicular oocyte in an MRL mouse is considered to represent sex reversal at the cellular level but not at the tissue or organ level. As reported previously, fetal

MRL testes contain meiotic germ cells within the testis cord as early as E13.5 [Otsuka et al., 2008a]. This premature initiation of meiosis in the fetal testis is the key phenomenon in the production of testicular oocytes. Another important aspect of this phenotype is that although MRL mice contain testicular oocytes within their seminiferous tubules, their testes have a normal structure and are capable of spermatogenesis throughout their life. On the basis of these characteristics, we consider that *mtop* (probably on Chr 15) and Chr Y induce the premature initiation of meiosis in specific regions of the fetal testis, but the resulting alternations may be so minor that they do not impede the male pathway in other regions of the fetal and postnatal testes.

In a previous study, we reported that MRL-type *Sry* contains shortened CAG repeat regions that are associated with the appearance of testicular oocytes [Otsuka et al., 2008b]. Because of its importance in sex differentiation and its strong correlation with this phenotype, *Sry* is thought to be the causative gene on Y^{MRL}. Although, the candidate genes for *mtop* on Chr 15 have not been identified yet, the ability of these genes to induce the development of testicular oocytes independently of MRL-type *Sry* and to increase the number of testicular oocytes in the presence of MRL-type *Sry* suggest that they have an important role and interact with *Sry* during sex determination. According to MGI, several genes thought to be involved in gonadal development are located at 8–17 cM on Chr 15, such as zinc finger protein, multitype 2 (*Zfp2*) and R-spondin 2 homolog (*Rspo2*) (Tevosian et al., 2002; Choi et al., 2007; Kocer et al., 2008). In particular, *Zfp2* has a great impact on the sex differentiation of gonadal somatic cells (Tevosian et al., 2002), as it interacts with and regulates the transcriptional activity of GATA binding protein 4 (GATA4), which is a member of the GATA family of transcription factors. In mice, *Zfp2* and *Gata4* induce the male pathway by regulating *Sry* expression in somatic cells starting at E10.5. Because *Zfp2* directly affects *Sry* expression, it is suggested to be a strong candidate gene for *mtop*. However, further analyses are necessary to identify the *mtop*, because other candidate genes may exist.

In conclusion, the testicular oocyte-producing pathway has at least 2 starting points: Chr Y and *mtop* on MRL-type Chr 15 and. Although they can independently induce the development of testicular oocytes, their interaction and cooperation with modifier genes are necessary for the manifestation of this phenotype. Identification of the causative and modifier genes, as well as the molecular pathway underlying the appearance of testicular oocytes, will help to elucidate the mechanism underlying sex differentiation and sex reversal in mammals.

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Figure legends

Figure. 1 *Zfy2* PCR genotype analysis for Y chromosome origin in the consomic strains MRL-Y^{B6} and B6-Y^{MRL}. MRL generates a 202-bp fragment; B6 generates 184-bp and 202-bp fragments (very weak signals).

Figure. 2 Testicular oocytes in whole-mount testes of MRL mice and F1 and backcross progeny aged 14 days. The testicular oocytes are observed in MRL (A and B), AJMRLF1 (C), B6MRLF1 (D), C3HMRLF1 (E), DBAMRLF1 (F), B6MRLN2 (G), B6MRLN4 (H), B6MRLN6 (I), B6MRLN8 (J), MRL-Y^{B6} (K), and B6-Y^{MRL} mice (L). Scale bars = 50 μ m. ED, efferent ductule; FE, follicular epithelial-like cell; N, nucleus; O, oocyte; ZP; zona pellucida.

Figure. 3 Testicular oocytes in MBM mice aged 14 days. Testicular oocytes in whole-mount testes of MBM mice (A and B). Distributions of the number of testicular oocytes in 1 mouse each of the MRL, B6MRLF1, and MBM strains (C). Distribution of the number of testicular oocytes per MBM mouse (D). Scale bars = 50 μ m. FE, follicular epithelial-like cell; N, nucleus; O, oocyte; ZP; zona pellucida.

Figure 4 Quantitative trait loci linkage analysis with the appearance of testicular oocytes. Peaks above the significant level are observed on Chr 15, and peakds above the suggestive level are found on Chrs 1, 3, 4, 5, and 17. From the top down, the 3 horizontal dotted lines indicate highly significant, significant, and suggestive levels. The LRS scores for each level were 6.4, 12.6, and 21.3, respectively.

Figure 5. Quantitative trait loci linkage analysis of Chr 15 with the appearance of testicular oocytes. Two significant peaks on Chr 15 were detected in the regions of 8.23–11.93cM (*D15Mit130–D15Mit18*) and 15.18–16.73cM (*D15Mit1007–D15Mit24*). From the top down, t 3 horizontal dotted lines indicate highly significant, significant, and suggestive levels in the stated order from the top. The LRS scores for each level were 6.4, 12.6, and 21.3, respectively.

Table 1. Appearance of testicular oocytes in F1 and backcross progenies

Strain	No. of testes	No. of oocytes	Oocyte score
MRL/MpJ	140	161	1.150
AJMRLF1	80	29	0.363 *
B6MRLF1	68	20	0.294 *
BALBMRLF1	56	0	0 *
C3HMRLF1	26	38	1.461
DBAMRLF1	34	5	0.147 *
<hr/>			
B6MRLN2	184	75	0.408 *
B6MRLN4	68	14	0.206 *
B6MRLN6	352	36	0.102 *
B6MRLN8	456	30	0.092 *
B6-Y ^{MRL}	330	22	0.067 *†
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MRL-Y ^{B6}	292	6	0.021 *†
<hr/>			
C57BL/6	56	0	0 *
MRLB6F1	82	0	0 *

Oocyte Score; No. of oocytes/No of testes; *, significant differences between MRL mice (Mann-Whitney *U*-test, $P < 0.05$); †, significant differences between MRL-Y^{B6} and B6-Y^{MRL} mice (Mann-Whitney *U*-test, $P < 0.05$).

Table 2. Genotype-phenotype correlations for the oocyte score in MBM mice

Region	Genotype	Oocyte score
<i>D15Mit130-D15Mit87</i>	M/M	0.820 [*] †
	M/B	0.323 [*]
<i>D15Mit130-D15Mit18</i>	M/M	0.770 [*] †
	M/B	0.320 [*]
<i>D15Mit1007-D15Mit87</i>	M/M	0.744 [*] †
	M/B	0.312 [*]

Oocyte Score; No. of oocytes/No of testes; *, significant differences between MRL mice (Mann-Whitney *U*-test, $P < 0.05$); †, significant differences between M/M homozygous and M/B heterozygous genotypes (Mann-Whitney *U*-test, $P < 0.05$); M/M, MRL homozygous; M/B, heterozygous.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5