Reproductive Isolation in Hybrid Mice Due to Spermatogenesis Defects at Three Meiotic Stages

Ayako Oka*, Akihiko Mita§, Yuki Takada**, Haruhiko Koseki** and Toshihiko Shiroishi§

*Transdisciplinary Research Integration Center, Research Organization of Information and Systems, Toranomon, Tokyo, Japan 105-0001
§Mammalian Genetics Laboratory, National Institute of Genetics, Mishima, Shizuoka, Japan 411-8540
**RIKEN Research Center for Allergy and Immunology, Yokohama, Kanagawa, Japan 230-0045

Short running title (>35 characters): meiotic disruptions in hybrid males
Key words: mouse, male sterility, speciation, reproductive isolation, meiosis

Corresponding author:
Name: Toshihiko Shiroishi,
Mailing address: Mammalian Genetics Laboratory, National Institute of Genetics, Yata 1111, Mishima, Shizuoka, Japan 411-8540
Tel: +81-55-981-6818, Fax: +81-55-981-6817
Email: tshirois@lab.nig.ac.jp
ABSTRACT

Early in the process of speciation, reproductive failures occur in hybrid animals between genetically diverged populations. The sterile hybrid animals are often males in mammals and they exhibit spermatogenic disruptions, resulting in decreased number and/or malformation of mature sperms. Despite the generality of this phenomenon, comparative study of phenotypes in hybrid males from various crosses have not been done, and thereby comprehensive genetic basis of the disruption is still elusive. In this study, we characterized the spermatogenic phenotype especially during meiosis in four different cases of reproductive isolation: B6-ChrX\textsuperscript{MSM}, PGN-ChrX\textsuperscript{MSM}, (B6 x \textit{Mus musculus musculus}-NJL/Ms) F\textsubscript{1} and (B6 x \textit{Mus spretus}) F\textsubscript{1}. The first two are consomic strains, both bearing the X chromosome of \textit{M. m. molossinus}; in B6-ChrX\textsuperscript{MSM}, the genetic background is the laboratory strain C57BL/6J (predominantly \textit{M. m. domesticus}), while in PGN-ChrX\textsuperscript{MSM} the background is the PGN2/Ms strain purely derived from wild \textit{M. m. domesticus}. The last two cases are F\textsubscript{1} hybrids between mouse subspecies or species. Each of the hybrid males exhibited cell-cycle arrest and/or apoptosis at either one or two of three distinct meiotic stages: prophase I and metaphase I. This study shows that the sterility in hybrid males is caused by spermatogenic disruptions at multiple stages, suggesting that the responsible genes function in different cellular processes. Furthermore, the stages with disruptions are not correlated with the genetic distance between the respective parental strains.
INTRODUCTION

When animals from genetically diverged populations hybridize, complete or partial sterility is often observed in the F₁ hybrids or in their descendants. This phenomenon belonging to postzygotic reproductive isolation accelerates irreversible genetic divergence by preventing free gene flow across the two diverging populations, and thereby plays a pivotal role in speciation. Sexual dimorphism is a general feature of reproductive isolation (Wu and Davis 1993; Laurie 1997; Orr 1997; Kulathinal and Singh 2008). In mammals, impairment is much more severe in males than in females, and in general the heterogametic sex is more sensitive to interspecific and intersubspecific genetic incompatibility. This phenomenon is well known as Haldane’s rule (Haldane 1922; Laurie 1997; Orr 1997).

In many animals, the reproductive isolation is caused by spermatogenic disruptions characterized by reduced number of germ cells and small testis size. These animals include Drosophila (Joly et al. 1997), stickleback fish Pungitius (Takahashi et al. 2005), caviomorph rodent Thrichomys (Borodin et al. 2006), house musk shrew Suncus (Borodin et al. 1998), wallaby Petrogale (Close et al. 1996) and genus Mus (Forejt and Iványi 1974; Matsuda et al. 1992; Yoshiki et al. 1993; Hale et al. 1993; Kaku et al. 1995; Gregorová and Forejt 2000; Elliott et al. 2001; Elliott et al. 2004; Good et al. 2008). Although reproductive isolation by spermatogenic impairment is a well-known phenomenon, its underlying genetic mechanism and molecular basis have remained elusive. Dobzhansky-Muller model, which infers that hybrid sterility or inviability is caused by deleterious epistatic interactions between nuclear genes derived
from their respective parent species or subspecies (DOBZHANSKY 1936; MULLER 1942), is widely accepted in animals and plants, and is also applicable to the sterility of hybrid animals in F₂ or backcross generations, so-called hybrid breakdown, in which the genes causing postzygotic reproductive isolation are partially recessive (ORR 2005).

The genetic incompatibility between house mouse subspecies is an ideal animal model for studying the early stage of speciation. Two subspecies of mouse, *Mus musculus domesticus* and *M. m. musculus*, diverged from their common ancestor 0.3-1.0 million years ago (YONEKAWA *et al.* 1980; MORIWAKI 1994; BONHOMME and GUÉNET 1996; BOURSOT *et al.* 1996; DIN *et al.* 1996). *M. m. domesticus* ranges across western Europe and the Middle East, whereas *M. m. musculus* ranges throughout eastern Europe and northern Asia (BONHOMME and GUÉNET 1996). The two subspecies meet in a narrow hybrid zone, which is most likely maintained by a balance between dispersal and selection against hybrids (HUNT and SELANDER 1973; BONHOMME and GUÉNET 1996; PAYSEUR *et al.* 2004). *M. m. domesticus* also displays reproductive isolation from the Japanese wild mouse, *M. m. molossinus*, which originated from hybridization of *M. m. castaneus* and *M. m. musculus*, and its nuclear genome is predominantly derived from *M. m. musculus* (YONEKAWA *et al.* 1980; YONEKAWA *et al.* 1988; MORIWAKI 1994; SAKAI *et al.* 2005). To investigate the reproductive isolation between *M. m. domesticus* and *M. m. molossinus*, we previously constructed a consomic strain B6-ChrX<sup>MSM</sup> (OKA *et al.* 2004). This strain has the X chromosome from the MSM/Ms strain, which is derived from *M. m. molossinus*, in the genetic background of the laboratory strain C57BL/6J (B6), which is predominantly derived from *M. m. domesticus* (MORIWAKI 1994). F₁ hybrid animals between B6 and MSM/Ms strains are fully fertile. On the
contrary, B6-ChrX^{MSM} shows male-specific sterility characterized by a reduced sperm number and dysfunction of the sperm, including abnormal morphology and low motility, indicating that B6-ChrX^{MSM} is a model of hybrid breakdown in animals (oka et al. 2004; Oka et al. 2007). Our previous study indicated that the abnormal morphology of the sperm head results from the genetic incompatibility between MSM/Ms-derived X-linked genes and B6 genes on autosomes including chromosomes 1 and 11 (Oka et al. 2007).

In this study, to understand the genetic mechanism of reproductive isolation in mice, we first undertook in-depth characterization of phenotype for each B6-ChrX^{MSM} male especially during meiosis. Meiosis is a special cell division that produces four haploid cells after one round of chromosome replication and two rounds of chromosome segregation. During meiosis, homologous chromosomes pair, synapse, undergo crossing-over, and achieve bipolar attachment to the spindle to segregate one set of chromosomes to each daughter cell. Homologous recombination is initiated during the leptotene stage of meiotic prophase I with the formation of DNA double-strand breaks (DSBs), which are repaired immediately during the zygotene stage or after crossing-over of homologous chromosomes during the pachytene stage (Roeder 1997; Tarsounas and Moens 2001).

During the first wave of spermatogenesis, most mitotic spermatogonia in the B6-ChrX^{MSM} testes fail to initiate meiotic DNA replication. Some proportion of those spermatogonia that enter into meiosis are again arrested and eliminated by apoptosis at the pachytene stage, resulting in the production of small number of sperms. We extended the same analysis to three other cases of reproductive isolation. Another
consomic strain PGN-ChrX^{MSM} has an MSM/Ms-derived X chromosome in the genetic background of the PGN2/Ms strain derived from wild mice (*M. m. domesticus*). PGN-ChrX^{MSM} males produce a small number of dysfunctional sperms as was the case with B6-ChrX^{MSM} males, but the former males show apoptosis mainly at metaphase of meiosis I. Furthermore, we examined F$_1$ hybrid males from intersubspecific cross of (B6 x *M. m. musculus*-NJL/Ms) and interspecific cross of (B6 x *M. spretus*). These F$_1$ hybrid males exhibited apoptosis at metaphase I and at the zygotene-to-pachytene stage of prophase I. As a whole, the postzygotic reproductive isolation in mice is caused by disruptions at a minimum of three different spermatogenic stages.
**MATERIALS AND METHODS**

**Mouse strains:** Construction of the consomic strain B6-ChrX^{MSM} was described in our previous report (OKA et al. 2004). The strain is maintained in the animal facility of the National Institute of Genetics (NIG). For maintenance of B6-ChrX^{MSM}, females that have the MSM-derived X chromosome heterozygously are selected by genotyping with twelve X-linked microsatellite markers and backcrossed to B6 males at each backcross generation. From these backcrosses, males with the MSM-derived X chromosome and males with the B6-derived X chromosome are obtained at a frequency of 12.5% each. Because the reproductive phenotype of males with the B6-derived X chromosome (B6-Chr^{B6}) is almost identical to that of B6 males (OKA et al. 2004), we used the B6-Chr^{B6} males as controls in this study.

The proximal and distal microsatellite markers used for the maintenance are DXMit89 at 10.0 Mb and DXMi160 at 162.5 Mb, respectively. Thus, B6-ChrX^{MSM} strain possibly has B6 genome in the proximal side beyond DXMit89 and the distal side beyond DXMi160 (Figure S1). To clarify the origin of the pseudoautosomal region, which starts from the middle of Mid1 gene at 165.2 Mb toward telomere, in B6-ChrX^{MSM} strain, we designed several primer sets for PCR, which can distinguish between B6 and MSM/Ms alleles. The primers are: X163466600 Forward (F) primer 5’-CAAGCTGTGCTGAATATCTGGTG-3’ and reverse (R) primer 5’-GCAGCCAGGCTTAAGGTAGGACC-3’; X164174029 F-primer 5’-CGTCATCTCTCAAAAGCTCTTCAC-3’ and R-primer 5’-GAAGATCTGTAATGTTTTACTGGG-3’; X164783477 F-primer 5’-
CACTTGAGGGAGAAATGTATTGTC-3’ and R-primer 5’-
CCCTAGAGGTCTATATGAAGCC-3’); X165318225 F-primer 5’-
GTCCTGTAATCCACCTCCTGGG-3’ and R-primer 5’-
CATTCAACAAATATCTAGTGCTCC-3’); X165319677 F-primer 5’-
AAATGAGTATGGAATGACCCAGC-3’ and R-primer 5’-
CACAGCCTAGGAACCATGCTGGCC-3’. Genotyping of B6-ChrX\textsuperscript{MM}M showed that the MSM genome remained only at marker X163466600, and the other regions were completely substituted by B6 genome, indicating that the pseudoautosomal region of B6-ChrX\textsuperscript{MM}M strain is derived from the B6 strain (Figure S1).

\textit{M. spretus} strain (BRC No. RBRC00208) was provided by RIKEN BRC, which is supported by the National Bio-Resource Project of MEXT, Japan. The NJL/Ms inbred strain was derived from wild \textit{M. m. musculus} mice trapped in Northern Jutland, Denmark and maintained by NIG. PGN-ChrX\textsuperscript{MM}M strain was constructed by the same strategy used in the construction of B6-ChrX\textsuperscript{MM}M. The inbred strain PGN2/Ms was derived from \textit{M. m. domesticus} mice trapped in Pigeon, Canada and maintained by NIG.

\textbf{Histology and detection of apoptosis:} Testes were placed in Bouin’s fixative and then embedded in paraffin. Sections (6 μm) were stained with hematoxylin and eosin (HE). Apoptotic cells were visualized by TUNEL assay (In Situ Cell Death Detection Kit, AP; Roche) following the manufacturer’s protocol. To examine the progression of the first-wave spermatogenesis, 50 tubules were randomly chosen from a section of each male, and were classified based on the cell types in the innermost layer of epithelium. Germ cells in the epithelium were classified according to the book by RUSSELL et al.
(RUSSELL et al. 1990). Three individuals from each strain were used for the evaluation.

Immunohistochemistry: For immunohistochemistry, testes were fixed in Mildform 20NM (Wako) and embedded in paraffin. After deparaffinization, antigen retrieval was performed by boiling the sections in 0.01 M citrate buffer, pH 6.0, in a microwave for 5 min. After blocking with 10% fetal calf serum (FCS) in phosphate-buffered saline (PBS), the sections were incubated with primary antibody for 1 h at room temperature (RT). After washing, the sections were incubated with Alexa-Fluor-488 conjugated secondary antibody at RT for 30 min. Then, after washing, the sections were counterstained with Hoechst 33258 in PBS. Primary antibodies used were rabbit polyclonal anti-γH2AX (1:50; Upstate), rabbit polyclonal anti-SYCP3 (1:50; Novus Biologicals), mouse monoclonal anti-PLZF (1:50, Santa Cruz), and rat monoclonal anti-CBX1/HP1β (1:50, Abcam).

For bromodeoxyuridine (BrdU) staining, we injected mice intraperitoneally with 40 mg of BrdU (Sigma) per kilogram body weight. After 1 h, testes were fixed in Mildform 20NM and processed in paraffin. We detected BrdU incorporation with monoclonal antibody to BrdU (Sigma), incubated samples with biotinylated secondary antibody and streptavidin-HRP and then detected with diaminobenzidine.

Testicular cell spread preparations: For spermatocyte preparations, 18- or 19-day-old mice were used. After removal of tunica albuginea, testes were minced in 10% FCS/PBS and large tissues were removed with 70-μm nylon mesh. Collected cells were
washed twice with 10% FCS/PBS and then fixed for 1 min in a solution of 1.85% formaldehyde and 0.05 M sucrose in PBS. After washing with 10% FCS/PBS, cells were pelleted and resuspended in a small volume of 10% FCS/PBS (<20 μl/testis). Cells were then smeared on APS-coated glass slides (Matsunami) and air-dried. Specimens were used for immunostaining immediately after the preparation.

For immunocytochemistry, slides were immersed in 0.5% Triton-X 100/PBS for 10 min, then washed with PBS and incubated with 10% FCS/PBS for 1 h at RT to block non-specific binding. Primary antibodies were applied and incubated either overnight at 4°C or 1 h at RT. After washing with 0.1% Triton-X 100 in PBS (PBST), slides were incubated with Alexa-Fluor-conjugated secondary antibodies for 30 min at RT. Following PBST washes, slides were mounted in Vectashield medium (Vector Laboratories). Staging of prophase spermatocytes was based on SYCP3 staining on autosomes and the sex chromosomes (ASHLEY et al. 2004; TURNER et al. 2004). The substaging of pachytene spermatocytes was based on H1t staining (YANG et al. 2008). Fluorescent signals were detected under a Carl Zeiss laser scanning confocal microscope (LSM510). Images of 50-80 cells for each male were captured for quantitative analysis. Primary antibodies used were rabbit polyclonal anti-SYCP3 (1:200; Novus Biologicals), goat polyclonal anti-ATR (1:20; Santa Cruz), rabbit polyclonal anti-γH2AX (1:200; Upstate), mouse monoclonal anti-RAD51 (1:20, Abcam), mouse monoclonal anti-MLH1 (1:20; BD Pharmingen), guinea pig polyclonal anti-H1t (1:1000), which was generously provided by Dr. Handel (INSELMAN et al. 2003), and mouse monoclonal anti-XLR (1:300), which was generously provided by Dr. Garchon (ESCALIER and GARCHON 2000). For the immunocytochemistry of γH2AX, we
used anti-SYCP3 antibody labeled by Zenon rabbit IgG labeling kits (Molecular Probes).
RESULTS

Failure to initiate meiotic S phase in B6-ChrX\textsuperscript{MSM} testes: We previously reported variation in the degree of testicular defects across seminiferous tubules within individual adult B6-ChrX\textsuperscript{MSM} males, ranging from relatively normal spermatogenesis to loss of all germ cells except spermatogonia (OKA \textit{et al.} 2004). To learn when the disruption of spermatogenesis starts, we examined testicular histology of B6-ChrX\textsuperscript{MSM} males at various stages in the first wave of spermatogenesis, which progresses synchronously in mice. After the several rounds of mitosis, type B spermatogonia enter the meiotic preleptotene stage, during which meiotic DNA replication occurs, at around 10 days post partum (dpp) (ELLIS \textit{et al.} 2004). Then, meiotic spermatocytes enter the prolonged prophase, which takes 10-12 days (ELLIS \textit{et al.} 2004). The first meiotic prophase is divided into five stages: leptotene, zygotene, pachytene, diplotene and diakinesis stages. Pachytene stage is the longest one, continuing for 5 to 7 days, and the first pachytene spermatocytes are observed at around 14 dpp (ELLIS \textit{et al.} 2004; COHEN \textit{et al.} 2006).

Histological examination of B6-ChrX\textsuperscript{MSM} testes by light microscopy revealed no detectable difference from those of control littermates at 8 dpp (data not shown). Perceptible changes in the constitution of germ cells within the tubules in B6-ChrX\textsuperscript{MSM} testes became visible at 10 dpp (Figure 1A). At 14 dpp, a clear difference in histology was noted between B6-ChrX\textsuperscript{MSM} and control testes: meiotic spermatocytes in the seminiferous tubules were abundant in control animals, but rarely seen in the B6-ChrX\textsuperscript{MSM} animals (Figure 1A). At 19 dpp, many tubules in the B6-ChrX\textsuperscript{MSM} testes still
contained no cells engaged in meiosis (Figure 1A). The disruption of spermatogenesis became more severe in the B6-ChrX<sup>MSM</sup> testes at 32 dpp. Some tubules contained surviving spermatocytes and round spermatids, but none of the mature sperm cells that were observed in most tubules of the control testes (Figure 1A). These results show that spermatogenesis of B6-ChrX<sup>MSM</sup> mice is affected in the meiotic phase. We quantified the progression of spermatogenic development by counting tubules containing germ cells of the various developmental stages in the innermost layer of seminiferous epithelium. As summarized in Figure 1B, delayed timing of emergence of developing germ cells was detected in the B6-ChrX<sup>MSM</sup> testes at all ages tested.

To examine whether mitotic spermatogonia are affected in B6-ChrX<sup>MSM</sup>, we performed immunohistochemical analysis of the testicular sections with antibodies against PLZF and CBX1/HP1β, which are localized to nuclei of undifferentiated type A and type B spermatogonia, respectively (Hoyer-Fender et al. 2000; Bauas et al. 2004; Costoya et al. 2004). The antibodies used for immunostaining in this study are listed in Table 1. Control and B6-ChrX<sup>MSM</sup> testes showed no obvious differences in the number of undifferentiated type A and type B spermatogonia at 14 dpp (Figure S2).

We further investigated proliferation of spermatogonia and spermatocytes in the testes at 19 dpp by analyzing the incorporation of BrdU. We distinguished proliferative spermatogonia from spermatocytes based on size and number of BrdU-positive nuclei in the seminiferous tubules: tubules containing preleptotene spermatocytes were recognized as those with a larger number of small positive nuclei. In the control testes, BrdU incorporation was detected in both spermatogonia and preleptotene spermatocytes (Figure 2A). In contrast, in the B6-ChrX<sup>MSM</sup> testes, BrdU
signals were detected mainly in spermatogonia, and rarely in the preleptotene spermatocytes (Figure 2B). Although most of tubules in the B6-ChrX\textsuperscript{MSM} testes were aberrant, the frequency of tubules containing BrdU-positive germ cells was not significantly different between control and the B6-ChrX\textsuperscript{MSM} testes (31.8 and 27.0% for the two control males; 36.1 and 28.9% for the two B6-ChrX\textsuperscript{MSM} males), suggesting relatively normal proliferation of spermatogonia in B6-ChrX\textsuperscript{MSM}.

We next investigated whether B6-ChrX\textsuperscript{MSM} germ cells enter meiosis. Immunohistochemistry was performed on testis sections at 14 dpp with antibody against the phosphorylated form of histone H2AX, $\gamma$H2AX. Since $\gamma$H2AX is observed in chromatin immediately after the induction of DSBs at the leptotene and zygotene stages and on the sex chromosomes at the pachytene stage (MAHADEVAIAH et al. 2001), we used $\gamma$H2AX as a marker of early spermatocytes. Almost all tubules of the control testes contained spermatocytes at the leptotene-to-zygotene stage or the pachytene stage (Figure 2C). By contrast, in the B6-ChrX\textsuperscript{MSM} testes, a small proportion of tubules showed $\gamma$H2AX-positive spermatocytes at the leptotene-to-zygotene stage but very few tubules showed spermatocytes at the pachytene stage (Figure 2D). We then quantified frequency of early spermatocytes by detecting SYCP3-positive cells. SYCP3 is a component of the synaptonemal complex that physically tethers homologous chromosomes, and is localized in different manners from leptotene stage to metaphase I. SYCP3 therefore serves as a marker of early spermatocytes. We performed immunocytochemical analysis with SYCP3 antibody for cell spreads from B6-ChrX\textsuperscript{MSM} and control testes at 18 or 19 dpp, and quantified the ratio of SYCP3-positive early spermatocytes to all testicular cells stained by Hoechst 33258. As shown in Figure 2E,
almost half (46.9%) of cells in the control testes were SYCP3-positive, whereas only a small proportion (6.4%) of cells in the B6-ChrX^{MSM} testes were SYCP3-positive. All these data indicate that in the B6-ChrX^{MSM} testes, proliferation and differentiation are normal prior to type B spermatogonia, but initiation of meiotic DNA replication is prevented in the majority of type B spermatogonia, allowing only a small proportion of the spermatogonia to enter into meiosis.

**Synaptic errors and apoptosis at pachytene stage in the B6-ChrX^{MSM} spermatocytes:** We next characterized the early meiotic spermatocytes found in the B6-ChrX^{MSM} testes. During prophase I, synapsis of the homologous chromosomes can be monitored by localization of SYCP3 (COHEN et al. 2006; HAMER et al. 2006). The lateral axes of synaptonemal complexes are formed during the leptotene stage, and synapsis between homologous chromosomes occurs during the zygotene stage and becomes complete prior to the pachytene stage (COHEN et al. 2006). Unsynapsed and synapsed chromosomes can be distinguished by fine vs. thick fibers of SYCP3. We observed that the pattern of SYCP3 signals in B6-ChrX^{MSM} was similar to that in the control spermatocytes during leptotene to zygotene (Figure S3A). Because pachynema is prolonged stage, we divided it into two substages (early and mid-to-late) based on the testis-specific H1 variant H1t, which replaces somatic H1 on chromatin at mid-pachytene and thus serves as a marker of mid-to-late pachytene spermatocytes (MOENS 1995; COBB et al. 1999). In most of early pachytene spermatocytes in B6-ChrX^{MSM} males, SYCP3 signals were observed as thick fibers as observed in control males, but B6-ChrX^{MSM} spermatocytes also frequently exhibited aggregation of SYCP3 (Figure
This SYCP3 aggregation was also found in mid-to-late pachytene B6-ChrX\textsuperscript{MSM} spermatocytes, though less frequently (Figure 3C). We then immunostained the pachytene spermatocytes with antibody against the kinase ATR, which during pachytene stage localizes on the sex chromosomes, but it also persists on unrepaird DSBs specifically on asynaptic autosomes (Keegan et al. 1996; Moens et al. 1999). Control pachytene spermatocytes showed ATR only on the sex chromosomes, whereas some proportion of B6-ChrX\textsuperscript{MSM} pachytene spermatocytes showed ATR on the asynaptic autosomes (Figure 3B). Such spermatocytes with asynapsis included both spermatocytes with and without signals on the sex chromosomes. Frequencies of spermatocytes with SYCP3 aggregation and asynapsis were significantly higher in B6-ChrX\textsuperscript{MSM} than in the control, though the variance between individuals was relatively large (Figure 3C). Those aberrant chromosomes were prominent during early pachytene substage. We then estimated the proportion of spermatocytes in the two pachytene substages. Compared with the control B6-ChrX\textsuperscript{B6}, B6-ChrX\textsuperscript{MSM} had a significantly reduced proportion of the mid-to-late pachytene spermatocytes (Figure 3D). This suggests that cell-cycle arrest occurred during the early pachytene substage in the B6-ChrX\textsuperscript{MSM} males.

To examine whether DSBs and recombination nodules are properly formed in B6-ChrX\textsuperscript{MSM} males, we immunostained the early spermatocytes with antibodies against \(\gamma\text{H2AX}\) and RAD51 as markers of DSBs and early recombination nodules, respectively. We found no obvious differences in the patterns of \(\gamma\text{H2AX}\) and RAD51 signals between control and the B6-ChrX\textsuperscript{MSM} testes in leptotene, zygotene and pachytene spermatocytes (Figure S3A and S3B). We next immunostained the pachytene spermatocytes with
antibody against MLH1, a protein that functions in meiotic crossing-over and DNA mismatch repair in the late recombination nodules from the mid-pachytene substage (Ashley et al. 2004). Again, we found no significant difference in the pattern of MLH1 foci between control males and B6-ChrX<sup>MSM</sup> (Figure S3A and S3B).

During pachytene stage, a specialized meiotic chromatin domain named the XY (sex) body is formed, in which X and Y chromosomes are transcriptionally silenced. To evaluate the proper development of pachytene spermatocytes, we examined the XY body formation with antibodies against three marker proteins, ATR, γH2AX and XLR, which during pachytene stage all accumulate exclusively in the XY body (Calenda et al. 1994; Escalier and Garchon 2000; Reynard et al. 2007). Most of the spermatocytes of the B6-ChrX<sup>MSM</sup> males properly formed an XY body, although the marker proteins were also frequently detected in autosomal regions (Figure S4A and S4B).

Next, we examined whether the spermatogonia arrested prior to the meiotic entry and spermatocytes arrested at pachytene stage are subject to apoptosis by using the TUNEL assay on testicular sections at 15 and 19 dpp. At 15 dpp, occasional TUNEL-positive cells were present in some tubules of both control and B6-ChrX<sup>MSM</sup> testes (Figure 4A). This suggests that most of the arrested B6-ChrX<sup>MSM</sup> spermatogonia at the premeiotic stage did not induce apoptosis. At 19 dpp, numerous apoptotic cells were detected in the tubules in the B6-ChrX<sup>MSM</sup> testes, but not in control testes (Figure 4A). Apoptosis was rarely observed in severely defective tubules lacking meiotic spermatocytes (Figure S5A). Most of the apoptotic spermatocytes were found in tubules at epithelial stage IV, as judged by the presence of mitotic intermediate spermatogonia.
and early type B spermatogonia (Figure S5B). We compared numbers of tubules containing apoptotic cells in sections of testes at various ages stained with hematoxylin and eosin (HE). The frequency of tubules containing apoptotic cells became higher in the B6-ChrX<sup>MSM</sup> testes than in the control testes starting at 19 dpp during first-wave spermatogenesis (Figure 4B).

**Meiotic cell cycle arrest in other cases of reproductive isolation:** We further investigated the meiotic phenotypes in three other cases of reproductive isolation. The first case is males of a second X chromosomal consomic strain, PGN-ChrX<sup>MSM</sup>. Because the classical laboratory inbred strains, including B6, have contributions of multiple subspecies to their genome, it was uncertain whether meiotic defects of the B6-ChrX<sup>MSM</sup> males indeed reflect the reproductive isolation between *M. m. domesticus* and *M. m. molossinus*. Thus, we constructed the consomic stain PGN-ChrX<sup>MSM</sup> with the X chromosome from the MSM/Ms strain in the genetic background of the PGN2/Ms strain, which is derived purely from wild mice (*M. m. domesticus*). PGN-ChrX<sup>MSM</sup> males showed phenotypes similar to those of B6-ChrX<sup>MSM</sup> males: sterility with reduced testis weight and abnormal morphology of mature sperm (Table S1, S2, Figure S6). This result indicated that PGN-ChrX<sup>MSM</sup> males show reproductive isolation like that of B6-ChrX<sup>MSM</sup> males. Immunohistochemical analysis with anti-SYCP3 antibody revealed that the number of SYCP3-positive early spermatocytes was almost identical in PGN-ChrX<sup>MSM</sup> testes and in control PGN-ChrX<sup>PGN</sup> and B6-ChrX<sup>B6</sup> testes (Figure 5A). This suggests that early spermatocytes in PGN-ChrX<sup>MSM</sup>, unlike those in B6-ChrX<sup>MSM</sup>, are not arrested or eliminated. On the other hand, histological analysis of HE- and TUNEL-
stained testes showed that the PGN-ChrX\textsuperscript{MSM} testes have increased apoptosis in spermatocytes at epithelial stage XII, which is equivalent to metaphase I, rather than stage IV (Figure 5B).

The second case of reproductive isolation is F\textsubscript{1} hybrid males from an intersubspecific cross of B6 and the NJL/Ms strain derived from east European wild mice (\textit{M. m. musculus}). These hybrids show male sterility with massive loss of germ cells during meiosis (KAKU \textit{et al.} 1995). Their testicular histology revealed extensive apoptosis in spermatocytes at the zygotene and pachytene stages (Figure 6A). Immunohistochemical analysis showed that the (B6 x NJL/Ms) F\textsubscript{1} hybrid males had a significantly decreased proportion of SYCP3-positive early spermatocytes (Figure 6B). We observed a smaller proportion of pachytene spermatocytes relative to zygotene spermatocytes in the (B6 x NJL/Ms) F\textsubscript{1} testes compared to control testes (data not shown). Since these testes contained only a small number of H1t-positive mid-to-late pachytene spermatocytes, we examined the marker proteins only at the early pachytene substage. Immunostaining with anti-ATR and anti-\(\gamma\)H2AX antibodies showed that these markers of XY bodies rarely accumulated in the F\textsubscript{1} hybrid spermatocytes (Figure 6C). Furthermore, their pachytene spermatocytes showed a significantly elevated frequency of persistent RAD51 foci and a lack of MLH1 foci on asynaptic axes (Figure 6C). These results suggest that DSBs were not repaired and homologous crossing-over was not completed in nearly all zygotene-to-pachytene spermatocytes in (B6 x NJL/Ms) F\textsubscript{1} hybrid males.

The last case of reproductive isolation is F\textsubscript{1} hybrid males from the interspecific cross of B6 and \textit{M. spretus}. It was reported that (B6 x \textit{M. spretus}) F\textsubscript{1} hybrid
males are sterile, showing spermatogenic breakdown at the first meiotic metaphase and only a small number of sperm, which are dysfunctional (Matsuda et al. 1991; Matsuda et al. 1992). Histological analysis of the F₁ hybrid testes revealed a significant increase of apoptosis in spermatocytes, mainly at epithelial stage XII, equivalent to metaphase I (Figure 6A). We further carried out immunohistochemical analysis of (B6 x M. spretus) F₁ hybrid males with antibodies directed against several meiotic marker proteins. The proportion of SYCP3-positive early spermatocytes in (B6 x M. spretus) F₁ hybrid males was not significantly different from that of control B6-ChrX<sup>B6</sup> males (Figure 6B). Immunostaining with ATR and γH2AX antibodies on cell spreads from the F₁ hybrid testes showed a slight but significant decrease in the frequency of XY body formation, especially at the early pachytene substage (Figure 6C). Furthermore, the axes of (B6 x M. spretus) F₁ hybrid pachytene spermatocytes displayed persistent RAD51 foci and lack of MLH1 foci (Figure 6C). This indicates that DSBs were not repaired and homologous crossing-over was not completed in the small population of spermatocytes in the F₁ hybrid, though the spermatocytes were mainly impaired at metaphase I.
DISCUSSION

Figure 7 summarizes the spermatogenic stages at which four different cases of reproductive isolation undergo cell-cycle arrest and apoptosis.

For B6-ChrX^{MSM}, we showed that the number of type B spermatogonia is unchanged, but most of them fail to initiate meiotic DNA replication, suggesting that cell-cycle arrest occurs at the stage prior to the meiotic entry. Premature cell-cycle arrest in spermatogonia has seldom been reported. One report showed that the knockout mutant of Cdk inhibitor p27, which negatively regulates the G1/S transition, has a small proportion of spermatogonia that fail to undergo meiotic entry (BEUMER et al. 1999). Thus, B6-ChrX^{MSM} is the first example in which spermatogonia are predominantly subject to cell-cycle arrest at the premeiotic stage. In this study, during the first wave of spermatogenesis, the number of meiotic spermatocytes in B6-ChrX^{MSM} males was reduced to less than one-sevenths of those in control males at 18 and 19 dpp. However, we previously observed that the number of mature sperms in adult B6-ChrX^{MSM} males was approximately one-thirds of control males (OKA et al. 2004). Thus, the premeiotic cell-cycle arrest in adult B6-ChrX^{MSM} males may be recovered partially.

Three of the types of hybrid males, B6-ChrX^{MSM}, (B6 x NJL/Ms) F₁ and (B6 x M. spretus) F₁, exhibited arrest at the zygotene-to-pachytene stage of meiosis I. A most prominent cell-cycle arrest at this stage occurs in the testes of intersubspecific (B6 x NJL/Ms) F₁ hybrids. They show extensive arrest and apoptosis at the late zygotene and early pachytene stages. Early studies of budding yeast, Saccharomyces cerevisiae, revealed that persistent unrepaired DSBs or asynapsis triggers meiotic arrest at the
pachytene stage (Roeder and Bailis 2000). This response to the meiotic defects, the pachytene checkpoint, is known to function in many animals including mice. In various mouse meiotic mutants, cell-cycle arrest and apoptosis at zygotene-to-pachytene are associated with unrepaired DSBs, asynapsis and possibly lack of the XY body, suggesting that the pachytene checkpoint monitors these defects (Pittman et al. 1998; de Vries et al. 1999; Yuan et al. 2000; Turner et al. 2004; de Vries et al. 2005). Thus, it is likely that zygotene and pachytene spermatocytes in (B6 x NJL/Ms) F1 males are subject to the pachytene checkpoint-dependent apoptosis, because the spermatocytes showed severe asynapsis and unrepaired DSBs, which were represented by the extensive RAD51 foci that persisted throughout the pachytene stage. On the other hand, pachytene spermatocytes in B6-ChrX^{MSM} males showed relatively mild synaptic defect and no persistence of unrepaired DSBs. Thus, cell-cycle arrest and apoptosis at pachytene stage in this strain less likely resulted from pachytene checkpoint response, and are rather attributable to the incompetence of meiosis; the early pachytene spermatocytes cannot complete cellular events due to the genetic incompatibility and may be hard to reach the mid-to-late pachytene stage.

A recent study demonstrated that one of the genes responsible for the reproductive isolation between the *M. m. musculus*-derived PWD strain and B6 is the histone H3 lysine 4 trimethyltransferase Prdm9 (Mihola et al. 2009). Both (PWD x B6) F1 spermatocytes and Prdm9 knockout spermatocytes fail to form XY bodies and undergo apoptosis at pachytene stage (Mihola et al. 2009), similar to the phenotype we observed in the (B6 x NJL/Ms) F1 hybrids. Epigenetic regulation induced by the genetic incompatibility was shown to cause the meiotic defects. It will be interesting to see
whether checkpoint responses are involved in these meiotic defects.

The third stage at which spermatocytes of the hybrid males are arrested is the first meiotic metaphase. Apoptosis at this stage occurs in spermatocytes in PGN-ChrX\textsuperscript{MSM} and, to a much greater extent, in interspecific (B6 x \textit{M. spretus}) F\textsubscript{1} hybrids. During both mitotic and meiotic metaphase, the faithful separation of chromosomes requires accurate connections between chromosomes and microtubules, with kinetochores playing the most significant roles. Some of the spindle checkpoint proteins, which monitor the attachment of microtubules to kinetochores in mitosis, are known to function in meiosis (\textit{Yin et al.} 2008). Moreover, cell-cycle arrest and apoptosis at metaphase of meiosis I have been observed in mutant mice having meiotic impairments such as lack of chiasmata and dissociation of homologous autosomes and XY chromosomes (\textit{Baker et al.} 1996; \textit{Lipkin et al.} 2002; \textit{Mark et al.} 2008). Previous studies revealed that genetic divergence between the parental species in the pseudoautosomal region of the XY chromosomes causes dissociation of these chromosomes in (B6 x \textit{M. spretus}) F\textsubscript{1} spermatocytes (\textit{Matsuda et al.} 1991; \textit{Matsuda et al.} 1992; \textit{Hale et al.} 1993). This dissociation is caused by asynapsis between sex chromosomes during pachytene (although X and Y chromosomes are still close to each other), and continues through metaphase I (\textit{Matsuda et al.} 1991; \textit{Matsuda et al.} 1992; \textit{Hale et al.} 1993). In this study, we observed that (B6 x \textit{M. spretus}) F\textsubscript{1} pachytene spermatocytes successfully form XY bodies that appear to contain normal amounts of ATR and \textgamma\textsubscript{H2AX}. This suggests that the dissociated XY chromosomes can be packaged into XY bodies, and are protected from pachytene checkpoint monitoring. Thus, we infer that the XY chromosome dissociation finally triggers the spindle checkpoint, and
the spermatocytes are eventually eliminated by apoptosis.

This study showed that male sterility in hybrid mice from various crosses is caused by disruptions at a minimum of three stages during meiosis. In addition to meiotic disruptions, consomic strains such as B6-ChrX^{MSM} and PGN-ChrX^{MSM} show impairments during spermiogenesis, terminal differentiation stage during spermatogenesis, resulting in the malformation of sperm heads. These results indicate that genes responsible for reproductive isolation do not function in a particular specific process, rather in different multiple processes. In many cases of mouse reproductive isolation, meiotic defects are predominantly observed (Matsuda et al. 1991; Kaku et al. 1995; Forejt 1996). This is possibly due to the high intricacy of meiosis, which includes many cellular events such as the induction of DSBs, homologous recombination, repair of DSBs and segregation of homologous chromosomes.

Notably, the genetic distance between the two parental strains was not explicitly correlated with the stages at which cell-cycle arrest and apoptosis occur. For instance, intersubspecific incompatibility in B6-ChrX^{MSM} causes arrest both at the premeiotic stage and the pachytene stage, but similar intersubspecific incompatibility in PGN-ChrX^{MSM} causes extensive apoptosis at metaphase I. In the case of another consomic stain B6-X^{PWD}, which has an X chromosome of wild M. m. musculus-derived PWD strain in the genetic background of B6 strain, it was reported that meiotic disruption occurs mainly at the pachytene stage (Storchová 2004). Interspecific incompatibility in the (B6 x M. spretus) F₁ hybrid causes partial arrest and apoptosis at the pachytene stage, but extensive apoptosis was observed at metaphase I as well. In a substantially long period of time for speciation, it is conceivable that mutations
 responsible for the reproductive isolation have continuously accumulated in the diverging populations, which may stochastically increase the chance that mutations responsible for reproductive defects at early meiotic stages, i.e. the premeiotic stage, occurs. Thus, the absence of obvious correlation between the genetic distance and the stages of the meiotic defects, which was observed in this study, may imply that these house mice are in early stage of speciation.
ACKNOWLEDGMENTS

We thank M. A. Handel for kindly providing H1t antibody, and H. J. Garchon for XLR antibody. We thank J. Turner for providing useful advice on experimental design and methodology, and D. G. de Rooij for helpful comments on histology. We thank members of the Shiroishi lab for providing critical comments on early versions of the manuscript. We are grateful to A. Yamakage, F. Kobayashi, H. Nakazawa, I. Okagaki and the staff of the animal facility of National Institute of Genetics for assistance with mouse husbandry. This study was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (‘‘Genome Science’’) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This study was also supported in part by the Transdisciplinary Research Integration Center, Research Organization of Information and Systems. This article is contribution no. 2514 from the National Institute of Genetics, Mishima, Japan.
FIGURE LEGENDS

FIGURE 1
Testicular histology and spermatogenic development in B6-ChrX<sup>MSM</sup> testes during first-wave spermatogenesis. (A) Hematoxylin and eosin (HE) staining of cross sections of testes from 10, 14, 19, and 32 dpp from control B6-ChrX<sup>B6</sup> males (left panels) and B6-ChrX<sup>MSM</sup> males (right panels). The B6-ChrX<sup>MSM</sup> testes showed few cells engaged in meiosis and vacuolation in the seminiferous tubules at ages after 14 dpp. Scale bar: 100 μm. (B) The proportion of developing germ cells in the innermost layer of seminiferous tubules in control (left) and B6-ChrX<sup>MSM</sup> (right) males during first-wave spermatogenesis. Leptotene and zygotene spermatocytes were grouped together as pre-pachytene spermatocytes.

FIGURE 2
Failure in initiation of meiotic S-phase and reduced proportion of meiotic spermatocytes in B6-ChrX<sup>MSM</sup> testes. (A, B) BrdU incorporation into control (A) and B6-ChrX<sup>MSM</sup> (B) testes at 19 dpp was detected by anti-BrdU antibody. In control testis, BrdU signals were detected in type A spermatogonia, intermediate spermatogonia (In), type B spermatogonia (SB) and preleptotene spermatocytes (preL). In B6-ChrX<sup>MSM</sup> testis, anti-BrdU antibody detected type A and B spermatogonia but not preleptotene spermatocytes. (C, D) Meiotic spermatocytes were indicated by γH2AX signals in testes at 14 dpp. In control testis (C), leptotene to zygotene spermatocytes (LZ) and pachytene spermatocytes with XY bodies (P) were detected. In contrast, a few leptotene to
zygotene spermatocytes were detected in B6-ChrX\textsuperscript{MSM} testis (D). Signals are $\gamma$H2AX (green) and Hoechst (gray). Scale bar: 200 $\mu$m. (E) Cell spreads from control (upper panel) and B6-ChrX\textsuperscript{MSM} (bottom panel) testis at 19 dpp. Anti-SYCP3 antibody (red) stained spermatocytes at meiotic prophase I. All nuclei were stained by Hoechst (blue). Only a few cells were SYCP3-positive in B6-ChrX\textsuperscript{MSM} testis. Frequency of spermatocytes at prophase I in control and B6-ChrX\textsuperscript{MSM} testes at 18-19 dpp (right graph). We counted 1731 and 1473 cells from four control and B6-ChrX\textsuperscript{MSM} males, respectively. The difference in frequency was significant by $t$-test.

FIGURE 3
Synaptic errors and cell-cycle arrest at early pachytene stage in B6-ChrX\textsuperscript{MSM} spermatocytes. (A) SYCP3 and H1t double immunostaining of control and B6-ChrX\textsuperscript{MSM} spermatocytes at early and late pachytene substages. The early pachytene spermatocyte from B6-ChrX\textsuperscript{MSM} showed aggregation of SYCP3 (arrows). (B) SYCP3 and ATR double immunostaining of control and B6-ChrX\textsuperscript{MSM} early pachytene spermatocytes. ATR localized to the sex chromosomes in the control spermatocyte. Note the dotted ATR signals along asynaptic regions on autosomes in the B6-ChrX\textsuperscript{MSM} spermatocyte. (C) Frequencies of aggregation and asynapsis at each substage. We counted 1035 pachytene spermatocytes from nine control testes and 1008 from seven B6-ChrX\textsuperscript{MSM} testes. (D) Proportion of early pachytene and mid-to-late pachytene spermatocytes, which were subtyped by staining with anti-H1t antibody. We counted 1463 pachytene spermatocytes from seven control males and 1334 from five B6-ChrX\textsuperscript{MSM} males. All p-values were evaluated by $t$-test.
FIGURE 4
Increased apoptosis at the pachytene stage in B6-ChrX\textsuperscript{MSM} testes. (A) Incidence of apoptosis in testes at 15 dpp and 19 dpp. Occasional apoptosis was observed in both control and B6-ChrX\textsuperscript{MSM} testes. In B6-ChrX\textsuperscript{MSM} testis at 19 dpp, extensive apoptosis was observed in the tubules with meiotic spermatocytes. Scale bars: 100 μm. (B) The frequency of seminiferous tubules with apoptotic cells during first-wave spermatogenesis. Fifty tubules for each of three males were counted at each age tested. Apoptotic cells were judged by the condensed staining of nuclei by HE staining.

FIGURE 5
Increased apoptotic spermatocytes at metaphase I in PGN-ChrX\textsuperscript{MSM} testis. (A, left panel) Cell spreads from PGN-ChrX\textsuperscript{MSM} testis at 19 dpp were immunostained with anti-SYCP3 antibody (red) and counterstained by Hoechst (blue). (A, right graph) Frequency of SYCP3-positive spermatocytes in control and PGN-ChrX\textsuperscript{MSM} testes at 18-19 dpp. The difference was not significant by \textit{t}-test. (B) Testicular histology of control PGN-ChrX\textsuperscript{PGN} (upper left panel) and PGN-ChrX\textsuperscript{MSM} (upper right panel) males at 24 dpp. TUNEL staining of adult control PGN-ChrX\textsuperscript{PGN} (bottom left panel) and PGN-ChrX\textsuperscript{MSM} (bottom right panel) testes. Increased apoptosis of metaphase I spermatocytes was observed at epithelial stage XII in PGN-ChrX\textsuperscript{MSM} testis (arrow heads in upper right panel and positive nuclei in bottom right panel). Scale bars: 200 μm.

FIGURE 6
Meiotic phenotypes of inter-subspecific and inter-specific F_1 hybrid males. (A) HE-stained testicular histology of (B6 x NJL/Ms) F_1 (upper left panel) and (B6 x M. spretus) F_1 (upper right panel) at 19 dpp. (B6 x NJL/Ms) F_1 testis contained tubules with apoptotic zygotene spermatocytes (ap) and tubules lacking spermatocytes (*). Increased apoptosis of metaphase I was observed at epithelial stage XII in (B6 x M. spretus) F_1 testis. TUNEL stained testicular histology of (B6 x NJL/Ms) F_1 (bottom left panel) and (B6 x M. spretus) F_1 (bottom right panel) at 24 dpp. Scale bars: 50 μm. (B) Ratio of SYCP3-positive meiotic spermatocytes to all testicular cells for each male at 18-19 dpp. Reduction of SYCP3-positive spermatocytes in (B6 x NJL/Ms) F_1 testes was significant by t-test. (C) Immunostaining of control, (B6 x M. spretus) F_1, and (B6 x NJL/Ms) F_1 pachytene spermatocytes with anti-ATR, γH2AX, RAD51 and MLH1 antibodies (indicated in green). All spermatocytes were double-stained with anti-SYCP3 antibody (red). Graphs at the side indicate the quantification of signal-positive spermatocytes at early and mid-to-late pachytene stages. * indicates significant difference by t-test (P<0.05).

FIGURE 7
Summary of meiotic arrest in four different cases of reproductive isolation. Horizontal arrows indicate the progression of spermatogenic development. Downward arrows (blue) indicate points of disruptions (arrest and/or apoptosis). Orange horizontal lines indicate defective spermiogenesis. Thickness of blue and orange lines represents the proportion of spermatocytes subjected to arrests and/or apoptosis.
# TABLE 1

MARKER PROTEINS USED FOR IMMUNOASSAYS

<table>
<thead>
<tr>
<th>Protein</th>
<th>Feature Marked</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLZF</td>
<td>Undifferentiated type A spermatogonia</td>
</tr>
<tr>
<td>CBX/HP1β</td>
<td>Type B spermatogonia</td>
</tr>
<tr>
<td>γH2AX</td>
<td>DSBs (leptotene-zygotene); XY body (pachytene)</td>
</tr>
<tr>
<td>SYCP3</td>
<td>Synaptonemal complex (leptotene-metaphase I)</td>
</tr>
<tr>
<td>ATR</td>
<td>XY body (pachytene); unrepaired DSBs on unsynapsed autosomes (pachytene)</td>
</tr>
<tr>
<td>XLR</td>
<td>XY body (pachytene)</td>
</tr>
<tr>
<td>H1t</td>
<td>Mid-to-late pachytene</td>
</tr>
<tr>
<td>RAD51</td>
<td>Early recombination nodules on DSBs (leptotene-pachytene)</td>
</tr>
<tr>
<td>MLH1</td>
<td>Late recombination nodules on DSBs (mid-to-late pachytene)</td>
</tr>
</tbody>
</table>
REFERENCES


BOURSOT, P., W. DIN, R. ANAND, D. DARVICHE, B. DOD et al., 1996 Origin and radiation


HALE, D. W., L. L. WASHBURN and E. M. EICHER, 1993 Meiotic abnormalities in hybrid mice of the C57BL/6J x Mus spretus cross suggest a cytogenetic basis for Haldane's rule of hybrid sterility. Cytogenet Cell Genet 63: 221-234.


JOLY, D., C. BAZIN, L. W. ZENG and R. S. SINGH, 1997 Genetic basis of sperm and testis
length differences and epistatic effect on hybrid inviability and sperm motility between *Drosophila simulans* and *D. sechellia*. Heredity **78** (Pt 4): 354-362.


Laurie, C. C., 1997 The weaker sex is heterogametic: 75 years of Haldane's rule. Genetics **147**: 937-951.


MATSUDA, Y., P. B. MOENS and V. M. CHAPMAN, 1992 Deficiency of X and Y chromosomal pairing at meiotic prophase in spermatocytes of sterile interspecific hybrids between laboratory mice (Mus domesticus) and Mus spretus. Chromosoma 101: 483-492.


OKA, A., T. AOTO, Y. TOTSUKA, R. TAKAHASHI, M.UEDA et al., 2007 Disruption of genetic interaction between two autosomal regions and the X chromosome causes reproductive isolation between mouse strains derived from different subspecies. Genetics 175: 185-197.

OKA, A., A. MITA, N. SAKURAI-YAMATANI, H. YAMAMOTO, N. TAKAGI et al., 2004 Hybrid breakdown caused by substitution of the X chromosome between two
mouse subspecies. Genetics 166: 913-924.


YONEKAWA, H., K. MORIWAKI, O. GOTOH, J. WATANABE, J. I. HAYASHI et al., 1980
Relationship between laboratory mice and the subspecies Mus musculus
domesticus based on restriction endonuclease cleavage sites. Jpn. J. Genet. 55:
289-296.

YOSHIKI, A., K. MORIWAKI, T. SAKAKURA and M. KUSAKABE, 1993 Histological studies
on male sterility of hybrids between laboratory and wild mouse strains. Develop

YUAN, L., J. G. LIU, J. ZHAO, E. BRUNDELL, B. DANEHOLT et al., 2000 The murine
SCP3 gene is required for synaptonemal complex assembly, chromosome
synapsis, and male fertility. Mol Cell. 5: 73-83.
Figure 1. Testicular histology and spermatogenic development in B6-ChrX<sup>MSM</sup> testes during first-wave spermatogenesis. (A) Hematoxylin and eosin (HE) staining of cross sections of testes from 10, 14, 19, and 32 dpp from control B6-ChrX<sup>B6</sup> males (left panels) and B6-ChrX<sup>MSM</sup> males (right panels). The B6-ChrX<sup>MSM</sup> testes showed few cells engaged in meiosis and vacuolation in the seminiferous tubules at ages after 14 dpp. Scale bar: 100 μm. (B) The proportion of developing germ cells in the innermost layer of seminiferous tubules in control (left) and B6-ChrX<sup>MSM</sup> (right) males during first-wave spermatogenesis. Leptotene and zygotene spermatocytes were grouped together as pre-pachytene spermatocytes.
Figure 2. Failure in initiation of meiotic S-phase and reduced proportion of meiotic spermatocytes in B6-ChrX^{MSM} testes. (A, B) BrdU incorporation into control (A) and B6-ChrX^{MSM} (B) testes at 19 dpp was detected by anti-BrdU antibody. In control testis, BrdU signals were detected in type A spermatogonia, intermediate spermatogonia (In), type B spermatogonia (SB) and preleptotene spermatocytes (preL). In B6-ChrX^{MSM} testis, anti-BrdU antibody detected type A and B spermatogonia but not preleptotene spermatocytes. (C, D) Meiotic spermatocytes were indicated by γH2AX signals in testes at 14 dpp. In control testis (C), leptotene to zygotene spermatocytes (LZ) and pachytene spermatocytes with XY bodies (P) were detected. In contrast, a few leptotene to zygotene spermatocytes were detected in B6-ChrX^{MSM} testis (D). Signals are γH2AX (green) and Hoechst (gray). Scale bar: 200 μm. (E) Cell spreads from control (upper panel) and B6-ChrX^{MSM} (bottom panel) testis at 19 dpp. Anti-SYCP3 antibody (red) stained spermatocytes at meiotic prophase I. All nuclei were stained by Hoechst (blue). Only a few cells were SYCP3-positive in B6-ChrX^{MSM} testis. Frequency of spermatocytes at prophase I in control and B6-ChrX^{MSM} testes at 18-19 dpp (right graph). We counted 1731 and 1473 cells from four control and B6-ChrX^{MSM} males, respectively. The difference in frequency was significant by t-test.
**Figure 3. Synaptic errors and cell-cycle arrest at early pachytene stage in B6-ChrX^{MSM} spermatocytes.** (A) SYCP3 and H1t double immunostaining of control and B6-ChrX^{MSM} spermatocytes at early and late pachytene substages. The early pachytene spermatocyte from B6-ChrX^{MSM} showed aggregation of SYCP3 (arrows). (B) SYCP3 and ATR double immunostaining of control and B6-ChrX^{MSM} early pachytene spermatocytes. ATR localized to the sex chromosomes in the control spermatocyte. Note the dotted ATR signals along asynaptic regions on autosomes in the B6-ChrX^{MSM} spermatocyte. (C) Frequencies of aggregation and asynapsis at each substage. We counted 1035 pachytene spermatocytes from nine control testes and 1008 from seven B6-ChrX^{MSM} testes. (D) Proportion of early pachytene and mid-to-late pachytene spermatocytes, which were subtyped by staining with anti-H1t antibody. We counted 1463 pachytene spermatocytes from seven control males and 1334 from five B6-ChrX^{MSM} males. All p-values were evaluated by $t$-test.
Figure 4. Increased apoptosis at the pachytene stage in B6-ChrX<sup>MSM</sup> testes. (A) Incidence of apoptosis in testes at 15 dpp and 19 dpp. Occasional apoptosis was observed in both control and B6-ChrX<sup>MSM</sup> testes. In B6-ChrX<sup>MSM</sup> testis at 19 dpp, extensive apoptosis was observed in the tubules with meiotic spermatocytes. Scale bars: 100 μm. (B) The frequency of seminiferous tubules with apoptotic cells during first-wave spermatogenesis. Fifty tubules for each of three males were counted at each age tested. Apoptotic cells were judged by the condensed staining of nuclei by HE staining.
Figure 5. Increased apoptotic spermatocytes at metaphase I in PGN-ChrX^{MSM} testis. (A, left panel) Cell spreads from PGN-ChrX^{MSM} testis at 19 dpp were immunostained with anti-SYCP3 antibody (red) and counterstained by Hoechst (blue). (A, right graph) Frequency of SYCP3-positive spermatocytes in control and PGN-ChrX^{MSM} testes at 18-19 dpp. The difference was not significant by $t$-test. (B) Testicular histology of control PGN-ChrX^{PGN} (upper left panel) and PGN-ChrX^{MSM} (upper right panel) males at 24 dpp. TUNEL staining of adult control PGN-ChrX^{PGN} (bottom left panel) and PGN-ChrX^{MSM} (bottom right panel) testes. Increased apoptosis of metaphase I spermatocytes was observed at epithelial stage XII in PGN-ChrX^{MSM} testis (arrow heads in upper right panel and positive nuclei in bottom right panel). Scale bars: 200 μm.
A

<table>
<thead>
<tr>
<th></th>
<th>(B6 x NJL)F₁</th>
<th>(B6 x M. spretus)F₁</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HE</strong></td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td><strong>TUNEL</strong></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
</tbody>
</table>

B

![Bar graph showing proportion of meiotic spermatocytes](image5)

C

<table>
<thead>
<tr>
<th></th>
<th>B6-ChrX₈⁶</th>
<th>(B6 x NJL)F₁</th>
<th>(B6 x M. spretus)F₁</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ATR</strong></td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td><strong>γH2AX</strong></td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
</tr>
<tr>
<td><strong>RAD51</strong></td>
<td><img src="image12" alt="Image" /></td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
</tr>
<tr>
<td><strong>MLH1</strong></td>
<td><img src="image15" alt="Image" /></td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
</tr>
</tbody>
</table>

**Proportion of meiotic spermatocytes**

-B6-X₈⁶ (control)
-NJL (control)
-(B6xNJL)F₁
-(B6xSpr)F₁

% of cells with ATR-positive XY body
% of cells with γH2AX-positive XY body
% of cells with >20 RAD51 foci
% of cells with MLH1 foci
Figure 6. Meiotic phenotypes of inter-subspecific and inter-specific F₁ hybrid males. (A) HE-stained testicular histology of (B6 x NJL/Ms) F₁ (upper left panel) and (B6 x M. spretus) F₁ (upper right panel) at 19 dpp. (B6 x NJL/Ms) F₁ testis contained tubules with apoptotic zygotene spermatocytes (ap) and tubules lacking spermatocytes (*). Increased apoptosis of metaphase I was observed at epithelial stage XII in (B6 x M. spretus) F₁ testis. TUNEL stained testicular histology of (B6 x NJL/Ms) F₁ (bottom left panel) and (B6 x M. spretus) F₁ (bottom right panel) at 24 dpp. Scale bars: 50 μm. (B) Ratio of SYCP3-positive meiotic spermatocytes to all testicular cells for each male at 18-19 dpp. Reduction of SYCP3-positive spermatocytes in (B6 x NJL/Ms) F₁ testes was significant by t-test. (C) Immunostaining of control, (B6 x M. spretus) F₁ and (B6 x NJL/Ms) F₁ pachytene spermatocytes with anti-ATR, γH2AX, RAD51 and MLH1 antibodies (indicated in green). All spermatocytes were double-stained with anti-SYCP3 antibody (red). Graphs at the side indicate the quantification of signal-positive spermatocytes at early and mid-to-late pachytene stages. * indicates significant difference by t-test (P<0.05).
Figure 7. Summary of meiotic arrest in four different cases of reproductive isolation. Horizontal arrows indicate the progression of spermatogenic development. Downward arrows (blue) indicate points of disruptions (arrest and/or apoptosis). Orange horizontal lines indicate defective spermiogenesis. Thickness of blue and orange lines represents the proportion of spermatocytes subjected to arrests and/or apoptosis.