An extreme bias in the germ line of XY C57BL/6 $\leftrightarrow$ XY FVB/N chimaeric mice

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Chimaeric analysis is a powerful method to address questions about the cell-autonomous nature of defects in spermatogenesis. *Symplastic spermatids* (sys) mice have a recessive mutation that causes male sterility due to an arrest in germ-cell development during spermiogenesis. Chimaeric mice were generated by aggregation of eight-cell embryos from sys (FVB/N genetic background) and wild-type C57BL/6 (B6) mice to determine whether the male germ-cell defect is cell-autonomous. The resulting FVB/N $\leftrightarrow$ B6 chimaeras ($\leftrightarrow$ denotes fusion of embryos) were mated with FVB/N mice and coat colour of offspring was used to identify transmission of FVB/N or B6 gametes. Regardless of the relative contribution of B6 to somatic tissues of the chimaeras, almost all (282 of 284; 99.3%) offspring of B6 XY $\leftrightarrow$ XY FVB/N (+/+ or sys/+) males ($n = 9$) received a FVB/N-derived paternal gamete. After mating of female B6 $\leftrightarrow$ FVB/N chimaeras, 51 of 73 (69.9%) offspring received an FVB-derived maternal gamete. Southern blot analysis of different tissues from

chimaeric males indicated that, despite the presence of balanced chimaerism in somatic tissues, the germ line in B6 XY $\leftrightarrow$ XY FVB/N mice was essentially FVB/N in composition. Thus there is a strong selective advantage for FVB/N male germ cells over B6 male germ cells in B6 $\leftrightarrow$ FVB/N-aggregation chimaeras at some stage during development of the male germ line. Each of three male chimaeras that were either B6 XY $\leftrightarrow$ XY FVB/N (sys/sys) or B6 XX $\leftrightarrow$ XY FVB/N (sys/sys) in composition was sterile, and testis histology was essentially sys mutant. This finding indicates that the function of the gene(s) affected in the sys mutation may be required in the testis, although whether expression is required in germ cells, somatic cells or both remains unknown. The extreme bias in transmission of male gametes has implications for experimental design in studies that use chimaeric analysis to address questions regarding the cell-autonomous nature of germ-cell defects in mice.

Introduction

Mammalian spermatogenesis involves the proliferation and differentiation of stem-cell spermatogonia that progress through meiosis as spermatocytes before undergoing terminal differentiation as haploid spermatids. During this process, germ-cell development is supported through intimate contact with the Sertoli cell, the somatic component of the seminiferous epithelium. Genetic defects can cause abnormalities in development of male germ cells. In such cases it can be difficult to determine whether the type of cell that is primarily affected by the mutation is the germ cell or a somatic cell. Chimaeric analysis is a powerful experimental method that can be used to determine whether a developmental defect is due to loss of gene function within the cell displaying the defect (that is, a cell-autonomous defect), or is the result of an indirect effect of

loss of the gene function in another type of cell (that is, a non-cell-autonomous defect) (Rossant and Spence, 1998). Historically, chimaeric studies in mice were first performed by aggregation of pairs of pre-compacted, preimplantation-stage embryos (Tarkowski, 1961). This method has been used to investigate the male germ cell-autonomous nature of several factors, including influences on sperm morphology and fertilizing capacity (Burgoyne, 1975; Krzanowska *et al.*, 1991) as well as genetic defects in spermatogenesis (Handel *et al.*, 1987). Studies involving a chimaeric analysis of testis function usually require production of a relatively large number of animals because of the 25% probability that any aggregated embryo will have been derived from a fusion of two XY embryos. In such cases, cells from either embryo can, in theory, contribute to development of the testis, including the male germ line. In contrast, although XY $\leftrightarrow$ XX chimaeras ($\leftrightarrow$ denotes fusion of embryos; Mintz, 1964) usually develop as phenotypic males, the postnatal germ line is formed exclusively from the XY component and Sertoli cells are almost always XY-derived. Thus, study of XY $\leftrightarrow$ XX chimaeras is uninformative when attempting to discriminate between a germ cell or Sertoli cell defect. An important premise of the experimental use of aggregation

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chimaeras is that a mixture of cells from each XY embryo will be found within each tissue in the adult animal.

Symplastic spermatids (sys) mice have a recessive mutation that results in male sterility (MacGregor *et al.*, 1990). During the first wave of spermatogenesis in sys mutants there is a developmental arrest between steps 6 and 8 of spermiogenesis, when clones of developing germ cells lose contact with the supporting Sertoli cells and the intercellular bridges linking the spermatids open prematurely (MacGregor *et al.*, 1990; Russell *et al.*, 1991). The gene defect responsible for this mutant phenotype has not yet been identified. It is unclear whether the function of the gene(s) affected by the sys mutation is required in the testis, and if so, in which type of testicular cell expression is required for normal spermatogenesis. Chimaeric mice were generated by aggregation of sys homozygous or control (+/+, sys/+) embryos on an FVB/N strain background and wild-type embryos on a C57BL/6 (B6) strain background to determine whether the germ-cell defect in sys homozygous males is germ cell-autonomous. The resulting chimaeric males were mated to determine whether homozygous sys germ cells undergo normal development in a testicular environment containing wild-type somatic cells.

Materials and Methods

Mice and superovulation

Inbred male and female C57BL/6 (*a/a*, *+^B/+^B*, *+^{Tyr-c}/+^{Tyr-c}*) mice were purchased from Harlan (Indianapolis, IN). Inbred FVB/N (*+^A/+^A*, *+^B/+^B*, *Tyr-c/Tyr-c*) mice and ICR female mice were purchased from the National Cancer Institute. Mice were maintained on a 14 h light cycle (on 06:00 h, off 20:00 h). For superovulation, 4–6-week-old mice were injected i.p. with 5 iu equine chorionic gonadotrophin (Calbiochem, La Jolla, CA) at 14:00 h on day 0. Mice were subsequently administered i.p. with 5 iu hCG (Sigma, St Louis, MO) at 12:00 h on day 2, after which females were caged with stud males. Pseudopregnant female recipients were generated by mating ICR females that were in oestrus with vasectomized B6D2F1 stud males. All studies were conducted in accordance with guidelines provided by the AMVA and a protocol approved by an Institutional Animal Care and Use Committee.

Production of chimaeric mice

Chimaeric mice were generated using the method described by Hogan *et al.* (1994). In brief, uteri were excised from superovulated and mated females at day 2.5 after mating (12:00 h of the day on which a copulation plug was observed was designated as day 0.5 after mating). Eight-cell embryos were flushed from the proximal end of uteri using a 1 ml syringe filled with BMOC-3 media (Invitrogen Corp., Carlsbad, CA) attached to a 33 g needle. Embryos were collected immediately and incubated in 50 µl pre-equilibrated BMOC-3, under paraffin oil, in 5% CO₂ in air at 37°C. Zonae pellucidae were removed using acidified Tyrode's solution, followed by four washes in pre-

equilibrated BMOC-3. Pairs of embryos were aggregated by making small depressions in a bacterial-grade plastic Petri dish (Sterilin, Stone) using the end of a pair of watchmaker forceps (Dumont, No. 5). Ten microlitres of BMOC-3 was added to each depression; the dish was covered with paraffin oil and pre-equilibrated at 37°C, in 5% CO₂ in air. One healthy embryo from each strain was placed in each depression and the embryos were pushed carefully together. After incubation for 1 h, the dish was removed gently from the incubator and examined. Any embryos that had separated were pushed together and the dish was incubated overnight at 37°C, in 5% CO₂ in air in a humidified incubator. It was important to minimize the time that the embryos were out of the incubator. The next morning, aggregated embryos, which had usually developed a small blastocoel, were transferred surgically to the uteri of pseudopregnant females that had been anaesthetized (2,2,2-tribromoethanol (Avertin); Aldrich, Milwaukee, WI) as described by Hogan *et al.* (1994) at day 2.5 after mating. Eight embryos were transferred per uterine horn.

Mating

Chimaeric animals were mated beginning at 8 weeks of age. Each chimaeric male was mated in a trio, with two FVB/N females, and each chimaeric female was mated with a single FVB/N male.

Southern blot analysis

Routine DNA sub-cloning was performed as described by Ausubel *et al.* (1994). DNA was extracted from tail, spleen or testis and Southern blot analysis was carried out as described by MacGregor *et al.* (1990). Paired testes from fully masculinized male chimaeras have almost identical contribution of tissue from each parental type (Gearhart and Oster-Granite, 1981; Handel *et al.*, 1987), so it was assumed that histology and DNA composition would be similar between paired testes. The plasmid pZap1 contains a piece of mouse-genomic DNA from the sys locus (MacGregor *et al.*, 1990). Plasmid pDP1171 (Mardon *et al.*, 1989), which contains a region of the mouse *Zfy1* locus, was the kind gift of D. Page (Whitehead Institute, Boston, MA). Southern blot hybridization was conducted as described by MacGregor *et al.* (1990). All hybridization filters were washed to a final stringency of $\times 0.1$ standard saline citrate, 1% (w/v) sodium dodecyl sulphate at 65°C before being exposed to Kodak XAR-5 film with an intensifying screen at -80°C.

Histology

Testes were excised and measured (pole–pole) using a Vernier calliper gauge before fixing by immersion in 15 ml of Bouin's fluid (15 parts saturated picric acid:five parts 37% formaldehyde:one part glacial acetic acid) for 2 h at 4°C. Testes were then removed, and the poles (approximately 10% at each end) were removed using a microtome

blade and re-immersed in Bouin's fluid for a further 16–24 h at 4°C. After dehydration in ethanols, clearing in xylenes and infiltration with paraffin wax (Paraplast Plus; Oxford Labware, St Louis, MO), testes were embedded in paraffin wax and sectioned at 5 µm. Sections were stained with periodic acid–Schiff–haematoxylin, mounted with a coverslip and viewed using a compound microscope equipped with a digital camera (SPOT; Diagnostic Instruments Inc., Sterling Heights, MI).

Results

Experimental strategy

The aim of the present study was to generate XY<->XY male chimaeras that were derived from fusion of a sys homozygous FVB/N and a wild-type B6 eight-cell embryo. Sys heterozygous mutant females were superovulated and mated with sys heterozygous males to generate the sys embryos. Similarly, B6 females were superovulated and mated with B6 stud males. One in four embryos from the sys mating were expected to be sys homozygous and half of the embryos from both matings were expected to be male. Therefore, the incidence of sys homozygous B6 XY<->XY FVB/N animals should be one in 16. Consequently, a target was set to produce at least 25 chimaeras for analysis, at least one of which was expected to consist of the desired genetic composition.

Production of chimaeric mice

Twenty-one male and six female chimaeric mice were generated. Dorsal views of each male and one female are shown (Fig. 1). FVB/N animals are albino and have white fur, whereas B6 mice have black coats. The fur of the chimaeras is composed of black, agouti and white hairs. Agouti pigmentation occurs in hair where B6 and FVB/N melanocytes are found in the same hair follicle (Mintz and Palm, 1969). The chimaeras displayed contributions from both parental strains. Approximately half of the chimaeras displayed balanced somatic chimaerism, as evidenced by their coat colour. After a breeding analysis, chimaeras were killed for molecular and histological analysis at 5 months of age.

Molecular analysis of chimaeric mice

Identification of XY<->XY chimaeras. In theory, the testes of XY<->XY male chimaeric mice are composed of a mixture of somatic and germ cells derived from each of the two strains used to generate the chimaeras. XY<->XX chimaeras normally develop as phenotypic male mice and can display balanced chimaerism in somatic tissues (Mystkowska and Tarkowski, 1968; McLaren, 1975; Gearhart and Oster-Granite, 1981; Krzanowska *et al.*, 1991). However, XX spermatogonia fail to proliferate after birth owing to the absence of genes on the Y chromosome, and the testis is composed exclusively of XY germ cells (for a review, see McLaren, 1983). The present study required the

production of male chimaeras with a mixture of male germ cells within the testis, so it was important to discriminate male XY<->XY chimaeras from male XY<->XX chimaeras. The Y chromosome of FVB/N mice is derived from *Mus musculus domesticus*, whereas that of B6 mice comes from *M. musculus musculus* (Mardon *et al.*, 1989). A *Taq I* restriction fragment length polymorphism (RFLP) at the *Zfy1* locus can be used to discriminate these chromosomes (Mardon *et al.*, 1989). Genomic DNA was purified from tail biopsies of chimaeric and control (non-chimaeric) mice and these were analysed by Southern blot hybridization to identify XY<->XY chimaeric males. DNAs were digested with *Taq I*, and the fragments were subjected to agarose gel electrophoresis and, after Southern blotting, were hybridized with a 1.6 kb *Taq I*–*EcoRI* fragment isolated from pDP1171 (Mardon *et al.*, 1989). This probe detects bands of 9.0 and 4.5 kb specific for the *M. m. musculus* (B6) Y chromosome and 7.0 and 5.5 kb for the *M. m. domesticus* (FVB/N) Y chromosome. The results indicate that of the 21 male chimaeras analysed, 12 were XY<->XY, four were XX(B6)<->XY(FVB/N) and five were XY(B6)<->XX(FVB/N) (Fig. 2a; Table 1).

Genotyping at the sys locus. The Southern blot (Fig. 2a) was stripped and re-probed with a portion of genomic DNA flanking the transgene complex integration site in the sys mice to determine the genotype of the FVB/N (sys) component of these chimaeras (MacGregor *et al.*, 1990). In conjunction with *Taq I* digestion, this probe permits discrimination of wild-type (B6), wild-type (FVB/N) and sys (FVB/N) alleles of chromosome 14. The results of the analysis are shown (Fig. 2b) and summarized (Table 1). Of the 21 male chimaeras analysed, four were wild type, 12 heterozygous mutant sys and four homozygous mutant sys. On the basis of the Southern blot hybridization (Fig. 2b), and subsequent testis histology, one animal (1207) appeared to have been derived from more than two embryos, one of which was sys/sys. Consequently, chimaera 1207 was excluded from further analysis.

Mating of chimaeras

Chimaeric males and females were mated with FVB/N mice and the coat colour of the progeny was examined to determine the gametic contribution from the chimaeric parent. FVB/N inbred mice are albino (Taketo *et al.*, 1991). Consequently, a zygote derived from fusion of two FVB/N gametes will produce albino progeny, whereas fusion of FVB/N and B6 gametes will produce offspring with agouti fur. All nine (B6) XY<->XY (FVB/N; +/- or +/-sys) males sired progeny (Table 1). Neither of the two XY<->XY animals that had a sys/sys component, nor the single B6 XX<->XY FVB (sys/sys) male produced progeny, despite mating with several different females over 4 months. In contrast to a previous report (Patek *et al.*, 1991), the fecundity of XY<->XX sex-chimaeric males was not significantly different from that of XY<->XY chimaeras (Table 2).

The coat colour of progeny from matings of XY<->XY

Fig. 1. Chimaeric mice used in the present study. Dorsal views of 21 male and one female (1143, upper right) chimaeric mice. Animals were anaesthetized before photography. On the basis of coat colour, approximately half of the animals display balanced somatic chimaerism.

male chimaeras, containing either a $+/+$ or $+/sys$ FVB/N component, to FVB/N females was anticipated to be either albino or agouti, indicating transmission of FVB/N or B6

gametes, respectively, from the chimaera. Surprisingly, of 284 offspring, only two had agouti coat colour, indicating an extremely low (approximately 0.7%) rate of transmission

Fig. 2. Molecular genetic analysis of FVB/N<->B6 chimaeric mice. (a) Southern blot analysis of Y-chromosome content of tail DNA from chimaeras. The blot was hybridized with a probe specific for the Y-linked *Zfy1* locus. The graphic on the right-hand side illustrates the hybridization patterns indicative of the presence of a *Mus musculus domesticus* (FVB/N) Y chromosome or a *M. m. musculus* (B6) Y chromosome. Lanes with numbers represent individual chimaeras. Control samples are shown on the right. (b) Southern blot analysis of tail DNA to determine genotype at *sys* locus of FVB/N component of chimaeras. The blot in (a) was stripped and re-hybridized with a probe from pZap1 (MacGregor *et al.*, 1990), which identifies one flank of the transgene integration site in the *sys* allele on chromosome 14 (MacGregor *et al.*, 1990). This probe detects a 2.3 kb band associated with the wild-type FVB allele (FVB (+)), a 0.9 kb band associated with the *sys* mutant FVB allele (FVB (*sys*)) and a 14 kb band associated with the C57BL/6 wild type allele (B6). The 0.9 kb band is relatively weak owing to deletion of much of the probe target sequences in the *sys* allele. Lanes with numbers represent individual chimaeras. Control samples are shown on the right. In chimaera 1145, the 0.9 kb (*sys* allele) band is extremely faint. However, confirmation of this genotype was made (Fig. 4). In chimaera 1207, the relative intensity of the FVB specific alleles (2.3 kb and 0.9 kb) is inverted when compared with chimaeras with a *sys*/+ component, indicating that the animal may have arisen from aggregation of more than two embryos, two of which were B6 XY and FVB (*sys/sys*) XY. Consequently, this animal was not considered further.

of B6 gametes from these males. In contrast, breeding analysis of five chimaeric females that were presumed to be XX<->XX produced progeny with a less biased contribution of B6 (30.1%) and FVB/N (69.9%) gametes. As expected, the offspring of male XY<->XX chimaeras inherited a gametic contribution exclusively from the XY component of the chimaera.

Testis histology of chimaeras

After completion of the mating analysis, chimaeric males were killed. Genomic DNA was prepared from the spleen

as a source of somatic tissue and one testis as a source of (predominantly) germ line tissue. The contralateral testis was fixed in Bouin's fluid for histological examination. Testes from XY<->XY chimaeras were mostly of a similar size, equivalent to those from non-chimaeric mice (Table 1). The exceptions were the two chimaeras that had an XY (*sys/sys*) component (chimaeras 1210 and 1294), the reduced testes sizes of which were similar to those in *sys/sys* (non-chimaeric) animals. Testes from XY<->XX chimaeras were either slightly smaller or similar to those in wild-type mice, with two exceptions. These were chimaera 1206, which contained an XY FVB (*sys/sys*) component, and

Table 1. Breeding analysis of FVB/N (sys) $\leftrightarrow$ C57BL/6 mice

ID number	B6 Y	FVB Y	+/+	+ / sys	sys / sys	Testicular size ^a	Percentage of aspermatogenic tubules (n)	Expected coat colour of progeny	Litters	Total progeny	Number of albino	Number of agouti
Phenotypic males												
1146	•	•	•			11.5	0.0 (128)	Albino and agouti	4	33	33	0
1209	•	•	•			11.0	0.0 (173)	Albino and agouti	5	31	31	0
1211	•	•	•			10.5	0.0 (138)	Albino and agouti	5	34	34	0
1147	•	•		•		12.0	0.0 (190)	Albino and agouti	6	50	49	1
1148	•	•		•		11.0	0.0 (162)	Albino and agouti	5	40	40	0
1149	•	•		•		11.5	0.0 (163)	Albino and agouti	4	24	24	0
1202	•	•		•		10.5	0.0 (179)	Albino and agouti	5	33	32	1
1203	•	•		•		10.5	0.0 (88)	Albino and agouti	1	8	8	0
1207	•	•	• +	• +	•	ND	ND	ND	ND	ND	ND	ND
1291	•	•		•		10.5	2.6 (114)	Albino and agouti	4	31	31	0
1210	•	•			•	6.0	ND	?	0	0	0	0
1294	•	•			•	6.5	ND	?	0	0	0	0
1144		•	•			10.0	0.0 (153)	Albino only	3	34	34	0
1208		•		•		9.5	0.0 (175)	Albino only	4	26	26	0
1293		•		•		10.0	0.0 (188)	Albino only	5	38	38	0
1206		•			•	6.0	ND	?	0	0	0	0
1145	•			•		9.0	34.4 (128)	Agouti only	3	15	0	15
1204	•			•		9.0	73.4 (192)	Agouti only	2	17	0	17
1205	•			•		9.5	1.9 (154)	Agouti only	3	25	0	25
1212	•			•		10.0	2.4 (210)	Agouti only	4	34	0	34
1292	•				•	5.5	ND	Agouti only	4	21	0	21
Phenotypic females												
1143			•			NA	NA	Albino and agouti	2	19	12	7
1230			ND			NA	NA	Albino and agouti	2	19	6	13
1231			ND			NA	NA	Albino and agouti	1	9	8	1
1232			ND			NA	NA	Albino and agouti	1	10	10	0
1233			ND			NA	NA	Albino and agouti	1	10	9	1
1234			ND			NA	NA	Albino and agouti	1	6	6	0

B6 Y: B6 male embryo; FVB Y: FVB/N male embryo; +/+, +/sys, sys/sys: genotype of FVB/N embryo at sys locus; expected coat colour of progeny: based upon transmission of FVB/N (albino)- or B6 (agouti)-derived gametes; litters: number of litters generated by chimaera; ND: not determined; NA: not applicable. ^aTesticular size: testes length (pole–pole, mm); ?: experimental outcome is dependent on whether sys mutant gem cells have cell-autonomous defect; (•): indicates the presence of a Y chromosome from either B6 and/or FVB/N, as well as the genotype of the FVB/N component at the sys locus. (• +): based on the Southern blot analysis in Fig. 2b, chimaera No. 1207 appeared to have been derived from more than two embryos, which included B6 XY and FVB XY (sys/sys), with the identity of the other uncertain; consequently, this chimaera was not considered for subsequent analysis.

Table 2. Comparison of fecundity of FVB/N (sys) $\leftrightarrow$ C57BL/6 mice

Category	Chimaera composition	Number of animals	Number of litters	Number of progeny per litter (mean $\pm$ SEM)
A	B6 XY $\leftrightarrow$ XY FVB	9	39	7.26 $\pm$ 0.34
B	B6 XX $\leftrightarrow$ XY FVB	3	12	8.17 $\pm$ 0.75
C	B6 XY $\leftrightarrow$ XX FVB	5	16	7.00 $\pm$ 0.71

Comparison of mean number of progeny per litter: categories A and B, $t(10) = 0.39$, $P > 0.05$; categories B and C, $t(6) = 0.38$, $P > 0.05$; categories A and C, $t(12) = 0.07$, $P > 0.05$.

Data in this table were derived from chimaeras with XY FVB/N components that were either +/+ or sys/+, that is, functionally wild type for spermatogenesis.

chimaera 1292 (B6 XY $\leftrightarrow$ XX FVB sys/sys), the testes of which were approximately half the normal size. Chimaera 1292 was of interest because despite a relatively small proportion of XY cells, the animal was male and fertile. In

no case was evidence for hermaphroditism observed in the adult animals.

Chimaeras 1210 and 1294 were both XY(B6) $\leftrightarrow$ XY(FVB/N sys/sys). Such animals were of particular interest, as their

Fig. 3. Testis histology from FVB/N (sys) $\leftrightarrow$ B6 chimaeric mice. (a) Testis histology of chimaera 1294 (B6 XY $\leftrightarrow$ XY FVB/N (sys/sys)). Note the presence of symplasts of spermatids (arrow indicates an example), indicative of a sys mutant phenotype, in most tubules. Staining was with periodic acid–Schiff–haematoxylin. (b) Testis histology of chimaera 1210 (B6 XY $\leftrightarrow$ XY FVB/N (sys/sys)). As in panel (a), symplasts of spermatids are observed in most tubules. A tubule containing a mixture of symplasts and normally developing elongate germ cells is boxed. (c) Higher magnification of box in (b). Symplasts of spermatids (arrowheads) and normally developing elongate spermatids (arrow) are present in the same tubule. (d) Testis histology of chimaera 1208 (B6 XX $\leftrightarrow$ XY FVB/N (sys/+)), showing normal spermatogenesis in all tubules. (e) Testis histology of chimaera 1145 (B6 XY $\leftrightarrow$ XX FVB/N (sys/+)), showing both aspermatogenic (arrowheads) and tubules with normal seminiferous epithelium (arrows). (f) Testis histology of chimaera 1204 (B6 XY $\leftrightarrow$ XX FVB/N (sys/+)). The majority of tubules are aspermatogenic (arrowheads) with relatively few tubules with normal seminiferous epithelium (arrows). Inset: Similar morphology of Sertoli cells (arrowheads) in tubules containing (upper), or lacking (lower) germ cells. Scale bars represent (a,b) 200 μ m, (c) 50 μ m, (d–f) 400 μ m and (inset in (f)) 30 μ m.

testes were predicted to contain a mixture of B6 and FVB/N (sys/sys) germ cells. Despite this prediction, examination of testis histology from these animals revealed an almost exclusive presence of developing germ cells with a sys mutant phenotype (Fig. 3a,b). However, analysis of multiple

sections of testis from chimaera 1210 revealed a small number of tubules that contained a mixture of germ cells with either a normal or sys mutant phenotype (Fig. 3c). All of the remaining XY $\leftrightarrow$ XY animals exhibited normal gross testis histology. Examination of testis histology from

Fig. 4. Molecular genetic analysis of cellular composition of testis and spleen in male FVB/N $\leftrightarrow$ B6 chimaeric mice. Southern blot analysis of genomic DNA extracted from testis (T) or spleen (S) from the individual (numbered) chimaeras, digested with *Taq*I and hybridized with the insert from pZap1, which identifies RFLPs that discriminate between B6 (14 kb; B6), the wild-type allele of FVB/N (2.3 kb; FVB (+)), and the *sys* allele of FVB/N (0.9 kb; FVB (*sys*)). Control DNA samples are shown on the right side of the lower blot. Photographs of the ethidium bromide-stained gels are shown on the left of each blot to indicate loading of DNA. In some lanes, an expected signal for the B6 allele (14 kb) cannot be clearly observed (for example, testis lane for chimaeras 1205 and 1292).

chimaera 1206 (B6 XX $\leftrightarrow$ XY FVB *sys/sys*) revealed that the seminiferous epithelium was exclusively *sys* mutant in nature (data not shown).

The number of aspermatogenic seminiferous tubules (that is, lacking germ cells) was quantified for each chimaera, excluding those with a *sys/sys* component (Table 1). Aspermatogenic tubules were found in one of nine XY $\leftrightarrow$ XY chimaeras (2.6%, in chimaera 1291) but in none of three FVB/N XY $\leftrightarrow$ XX B6 chimaeras (for an example, see Fig. 3d). In contrast, analysis of histology from all four B6 XY $\leftrightarrow$ XX FVB chimaeras revealed aspermatogenic tubules in each animal, with two chimaeras having a large proportion of these (34.4%, chimaera 1145, Fig. 3e; 73.4%, chimaera 1204, Fig. 3f). Sertoli cells with a normal appearance were present within the aspermatogenic seminiferous tubules (Fig. 3f, inset). Aspermatogenic tubules occasionally contained elongate spermatozoa, which had probably originated within adjacent regions of the tubule that had normal spermatogenesis.

Molecular analysis of somatic and germline tissues from chimaeras

The relative contribution of each donor embryo to cells within the testis of chimaeras was determined by Southern

blot analysis with the same DNA probe used to genotype at the *sys* locus (Fig. 4). As a control for a somatic tissue, DNA was also extracted from the spleen and the results were compared with those obtained from analysis of the tail DNA. Good direct correlation was found between the relative proportion of B6 and FVB/N DNA in tail and spleen. These results also correlated with the estimate for somatic chimaerism deduced from coat colour. For example, on the basis of coat colour, chimaera 1202 (XY $\leftrightarrow$ XY) had predominantly B6 contribution to somatic tissues (Fig. 1), which was reflected by the hybridization signals for the B6-derived alleles at the *sys* and *Zfy1* loci in the Southern blot analyses (Fig. 2a,b).

Conversely, on the basis of its mainly albino coat (Fig. 1), chimaera 1210 (XY $\leftrightarrow$ XY) had a large proportion of the FVB/N component, and this conclusion was supported by the Southern blot analysis of DNA from somatic tissues (Fig. 2a,b).

In contrast, Southern blot analysis of testicular DNA from the XY $\leftrightarrow$ XY chimaeras revealed a marked bias towards the FVB/N component (Fig. 4). In each case, a hybridization signal for the B6 allele was observed in the spleen DNA. However, only the FVB/N-derived hybridization signal was observed in the testis sample from the same chimaera. Thus,

irrespective of whether balanced somatic chimaerism was evident, there appeared to be an extreme bias in favour of FVB/N cells in the testes of these animals.

As expected, in XX<->XY male chimaeras, the Southern blot hybridization pattern was biased towards the XY component. For example, chimaeras 1144, 1206, 1208 and 1293, in which the XY embryo was FVB/N, displayed a FVB/N pattern only within the testis sample. Similarly, in chimaeras 1145, 1204 and 1212, which each had an XY B6 component, there was a B6 hybridization pattern only in the testis DNA sample.

Discussion

These results reveal a striking bias in the germ line of B6 XY<->XY FVB-aggregation chimaeric mice. Bias in gametic contribution from XY<->XY mice of other strain combinations has been reported by Mintz (1968) and Krzanowska *et al.* (1991). However, the bias observed in the present study is the most extreme example reported to date. Competition among mature spermatozoa can influence gametic contribution in mice (Krzanowska *et al.*, 1991). This mechanism is unlikely to be involved in the present study, as Southern blot analyses indicated that the genetic composition of the testis in B6 XY<->XY FVB mice is predominantly FVB in origin. A more plausible mechanism would involve a proliferative advantage of FVB germ cells at some stage during germ-line development.

Germ-line development in mice commences with specification of primordial germ cells (PGCs) from the proximal epiblast just before the onset of gastrulation (Lawson and Hage, 1994; Tam and Zhou, 1996). Subsequently the PGCs translocate to the developing hindgut endoderm. Between day 9.0 and day 9.5 of embryo development they leave the gut and migrate into the developing genital ridge between day 10.0 and day 10.5 of embryo development (Molyneaux *et al.*, 2001). In males, gonadal differentiation commences after expression of *Sry* in the pre-Sertoli cell lineage beginning at day 10.5, and the earliest morphological signs of testicular development are observed by day 12.5 (Capel, 2000). The PGCs differentiate into prospermatogonia, which briefly undergo mitosis before becoming arrested by day 13.5. Prospermatogonial mitosis resumes by day 3 after birth, with formation of spermatogonia by day 5 and commencement of meiosis between day 8 and day 10 after birth (Nebel *et al.*, 1961).

Analysis of testis histology in male B6 XY<->XX FVB chimaeras indicates that competition in germ-cell development may occur during embryogenesis. A significant number of aspermatogenic seminiferous tubules was not observed in B6 XX<->XY FVB or XY<->XY chimaeric mice. In contrast, two independent B6 XY<->XX FVB males displayed a high incidence of seminiferous tubules that lacked germ cells. Two X chromosomes are incompatible with male germ cell development, and XX prospermatogonia die shortly after birth (for a review, see McLaren, 1983). Thus, the large number of aspermatogenic tubules in

B6 XY<->XX FVB males may have arisen during embryogenesis, because of a proliferative advantage of FVB XX PGCs or prospermatogonia that subsequently died after birth. Differences in the rate of proliferation of PGCs during embryogenesis have been observed in KE and CBA/Kw mice, which also displayed bias of the germ line as XY<->XY chimaeras (Krzanowska *et al.*, 1991; Wabik-Sliz *et al.*, 1998). Aspermatogenic tubules might also have arisen as a consequence of excessive numbers of XX Leydig cells or Sertoli cells. However, XX cells in XX<->XY-aggregation chimaeras can form Leydig cells with a normal appearance that are capable of supporting spermatogenesis in adjacent tubules (Burgoyne *et al.*, 1988). In addition, genetic analysis (Mazeyrat *et al.*, 2001) has indicated that adult mouse spermatogenesis can be supported (at least up to the first meiotic metaphase) by XX Leydig cells that express the Y-linked gene *Eif2s3y*. Thus, the presence of two X chromosomes is not incompatible with Leydig-cell function and *Eif2s3y* is formally the only Y-linked gene the expression of which in Leydig cells might be required for adult spermatogenesis (at least until the first meiotic metaphase). Sertoli cells in adult XY<->XX chimaeric mice of different strain combinations are almost exclusively (> 90%) XY in origin (Palmer and Burgoyne, 1991; Patek *et al.*, 1991). On the basis of these findings, the aspermatogenic tubules are unlikely to arise as a consequence of a large number of XX Sertoli cells.

Ultimately, further studies are required to identify the precise stage(s) in development at which the bias in germline development occurs in B6<->FVB chimaeras and the basis for the advantage of FVB male germ cells compared with B6 male germ cells. Development of female germ cells in these chimaeras, which although less biased, was still evident, must also be examined. Such studies could be facilitated by use of ROSA26.B6 mice (Friedrich and Soriano, 1991), the cells of which are marked by β -galactosidase expression, enabling single-cell resolution of the parental origin of both somatic and germ cells in the developing gonad.

Testes from B6 XX or XY<->XY FVB *sys/sys* chimaeras contained essentially only germ cells with a *sys*-mutant phenotype, indicating that the wild-type B6 component of somatic tissues was unable to rescue the germ-cell defect. This finding indicates that if the defect in *sys* mice is germ-cell non-autonomous, it is likely to affect a type of somatic cell within the testis. The fact that the germ line in XY<->XY chimaeras was almost completely derived from the FVB component, and that the testis histology of B6 XY<->XY FVB (*sys/sys*) was almost exclusively *sys* mutant, is consistent with the contention that the *sys* defect is germ-cell autonomous. However, if the germ-cell defect is an indirect effect of defective Sertoli (or other somatic) cells within the testis, and a similar bias exists in the somatic-cell component of the testis of B6 XY<->XY FVB mice, this could produce the same experimental outcome. Whether the haploid germ-cell defect in *sys* mutant mice is germ-cell autonomous remains unclear. This issue should be resolved by a more direct contemporary experimental method,

involving use of germ-cell transplantation in conjunction with cell lineage markers afforded by ROSA26 mice (Friedrich and Soriano, 1991; Brinster and Avarbock, 1994; McLean *et al.*, 2001).

The FVB/N strain is commonly used for production of random integration transgenic mice (Taketo *et al.*, 1991). A strategy of using C57BL/6 mice as a partner for FVB/N in a chimaeric analysis is attractive due to its common use, and the differences in pigmentation and genetic polymorphism associated with the different origins of the Y chromosome in these strains. The results of this study indicate that this choice of strain combination is unsuitable when the experimental design requires generation of a balanced male, or female, germ line.

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