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High incidence of XXY and XYY males among the offspring of female chimeras from embryonic stem cells

(sex chromosomes/karyotype/aneuploidy/XX/XY females)

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ABSTRACT **Injecting male embryonic stem cells into the blastocoel of female embryos occasionally produces female chimeras capable of transmitting the embryonic stem cell genome. In our experiments several embryonic stem cell-derived male offspring from female chimeras were observed to be infertile. Karyotypic analysis of these infertile animals revealed aneuploidy. We examined the karyotypes of an additional 14 offspring not selected for infertility (3 females and 11 males) that had received the embryonic stem cell genome from 5 transmitting female chimeras. The 3 females and 5 of the males had normal karyotypes. Six of the males exhibited nonmosaic aneuploidy, which included four XXY karyotypes, one XYY karyotype, and an X,i(Y) karyotype. The high incidence of XXY and XYY males supports previous evidence for aberrant pairing and segregation of X and Y chromosomes when they are present in oocytes.**

In the course of producing mice with alterations of specific genes, modified embryonic stem (ES) cells are injected into the blastocoel cavity of wild-type mouse blastocysts. Surgical transfer of injected blastocysts into pseudopregnant recipients and their continued development results in chimeric mice, which are bred to establish germ-line transmission of the genetic alteration (1–4). Usually, the injected ES cells are male, whereas the recipient blastocyst can be either male or female. The injection of male ES cells into a female blastocyst frequently converts the developing embryo to a phenotypic male chimera, thereby reducing the number of female chimeras born (1). We have found that female chimeras will transmit the ES cell genome, although at a lower frequency than is seen in male chimeras. In addition we have observed sterility in about half of the ES cell-derived male offspring but not in the female offspring of female chimeras. To further our understanding of this phenomenon and to determine the frequency with which it occurs, we have accumulated karyotypic and/or genotypic data on 23 ES cell-derived offspring from transmitting female chimeras and report here that the frequency of sex chromosome aneuploidy is high among the male offspring.

MATERIALS AND METHODS

ES Cell-Derived Mice. The homologous recombination experiments leading to the mice described here have been reported elsewhere (5–8) or are unpublished results (S.K.B., D. Cook, and J. Krege). The parent cell line for all targeted cell lines was the E14TG2a ES cell line derived from strain 129/Ola (4). Briefly, targeted ES cells were injected into the blastocoel of C57BL/6J blastocysts, and the blastocysts were surgically transferred to the uteri of pseudopregnant CD1 females (Charles River Breeding Laboratories). Chimeric pups, recognizable by their patchy coat color, were then bred with C57BL/6J mice (The Jackson Laboratory). Successful transmission of the ES cell genome produces pups with the dominantly inherited agouti coat color typical of the F_1 hybrid between 129/Ola and C57BL/6J.

Fibroblast Cultures. A piece of ear ≈5 mm^2 in size was biopsied, minced with a scalpel, and placed in DMEM medium supplemented with 4500 mg of glucose per liter, 10% (vol/vol) heat-inactivated fetal bovine serum, 100 μM 2-mercaptoethanol, 2 mM L-glutamine, and 400 units of type IV collagenase (Sigma) per ml at 37°C and 5% CO_2/95% air. After 24 hr, cells were dispersed, harvested, resuspended in the above medium without collagenase, and plated on standard tissue culture plates. Because of the large inoculum of cells liberated by collagenase treatment, cultures were ready to pass within 4–6 days. To minimize karyotypic artifacts, the cultures were studied after very few passes (usually two or three).

Karyotypic Analysis. Chromosome analyses were performed on trypsin/Giemsa-banded metaphase cells prepared according to standard methods of suspension harvest and hypotonic treatment (9). A minimum of 11 cells were analyzed after detection of an abnormal karyotype. In cultures exhibiting a normal karyotype, a minimum of 20 cells were examined. If an aneuploid cell was found among the first 20, an additional 30 (minimum) were examined.

Southern Blot Analysis. Genomic DNA was digested with *Bam*HI (New England Biolabs), electrophoresed on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a 150-bp *Rsa* I fragment from intron 3 of the murine hypoxanthine phosphoribosyl-transferase (HPRT) gene using standard techniques (10).

RESULTS

In the course of earlier gene targeting experiments, we had observed that germ-line transmission of the ES cell genome through female chimeras occurred occasionally, but subsequent breeding of the ES cell-derived offspring from female chimeras revealed several instances of sterility. Of five sterile male mice seen in these preliminary experiments (Table 1, group I), one had an XYY karyotype (Fig. 1*A*), two had XXY karyotypes (Fig. 1*B*), one had a trisomy 3 karyotype (Fig. 2), and one was a mosaic (six of 42 cells contained 41 chromosomes and the remainder contained the normal 40).

To further investigate the incidence of aneuploidy, we analyzed a second unselected group, chosen without reference to their fertility, of ES cell-derived offspring from transmitting female chimeras. During an 18-month period, 136 chimeric mice were generated. Forty-five of the chimeras (33%) were females, but only 5 of these females (11%) transmitted the ES cell genome to their offspring (agouti coat color). Almost all of the agouti pups born to chimeric females (range, 1–6 pups per female) were in the first two litters. Two of the chimeric

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Abbreviations: ES, embryonic stem; HPRT, hypoxanthine phosphoribosyltransferase.

Table 1. Male offspring of female chimeras

Mouse	Karyotype	X genotype	Fertility
Group I			
338M	41, XYY	ND	Sterile
5050	41, XXY	ND	Sterile
340M	41, XXY	ND	Sterile
341M	41 XY, +3	ND	Sterile
339M	40, XY/41, XY, +?	ND	Sterile
Group II			
5906	41, XYY	E/	ND
1135	41, XXY	ND	ND
2300	41, XXY	E/B	ND
5907	41, XXY	E/B	ND
9774	41, XXY	E/B	ND
9775	ND	E/B	ND
8459	40, X,i(Y)	/B	Sterile
3202	40, XY	/B	Fertile
5901	40, XY	/B	Fertile
5927	40, XY	E/	ND
5928	40, XY	E/	Fertile
9103	40, XY	E/	ND
1001	ND	E/	ND
1021	ND	E/	ND

Group I animals were ascertained due to sterility. Group II animals were unselected progeny. B, C57BL/6; E, E14TG2a; ND, not determined.

females produced only agouti pups and were subsequently infertile, suggesting that only the ES cell component contributed to the germ line. However, three of the female chimeras also had pups originating from the C57BL/6J component of the chimera (black coat color) in the early litters, and they continued to have pups of this coat color in later litters. Of 19 agouti pups born, 5 were females and 14 were males.

Chromosome analyses were performed on 3 female and 11 male agouti offspring. The female mice (data not shown) and 5 of the male mice (Table 1, group II) had normal karyotypes. Among the 6 males with abnormal karyotypes, one had an XYY karyotype and four had XXY karyotypes. The remaining male had a karyotype in which a Y-derived isochromosome, i(Y), replaced the normal Y chromosome (Fig. 1*C*). In addition, analysis of DNA from a male for whom chromosome analysis was not performed (no. 9775) demonstrated that he had hybridizing fragments from two different X chromosomes, thus making it likely that he also had an XXY karyotype (see below).

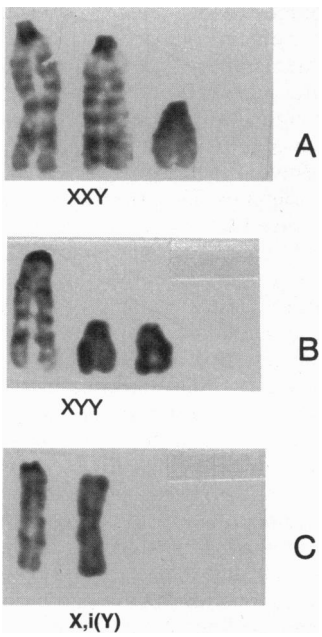

FIG. 1. Metaphase Giemsa-banded sex chromosomes from mice with XXY (*A*), XYY (*B*), and X,i(Y) (*C*) karyotypes.

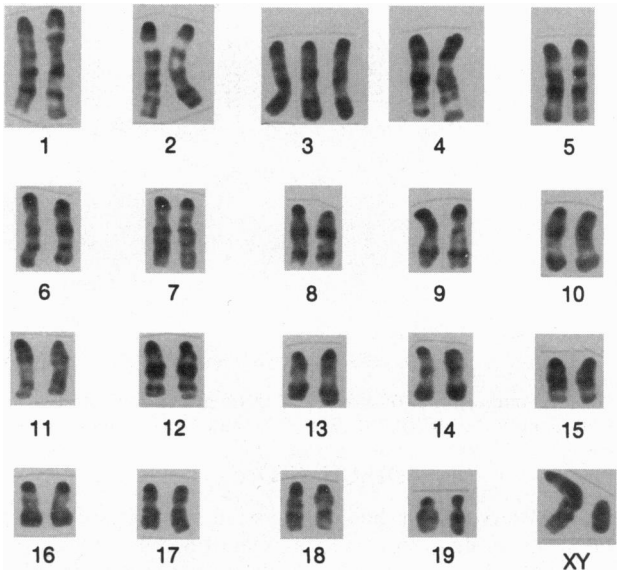

FIG. 2. Metaphase G-banded chromosomes of mouse no. 341M with a trisomy 3 karyotype.

Because of the similarity of Y chromosomes in standard inbred strains of mice (11), it is currently not possible to demonstrate the parental origin of the Y chromosome in the males. However, the parental origins of the X chromosomes could be determined. The E14TG2a ES cell line from which all the injected ES cell lines were derived is a 6-thioguanine-resistant subclone of the E14 ES cell line that has a well-characterized deletion at the HPRT locus, which results in a restriction fragment length polymorphism between E14TG2a and C57BL/6J mouse genomic DNA. An additional allele, T2 (no. 3201), is generated by a homologous recombination event at this locus. We were therefore able to use these restriction fragment length polymorphisms to determine the parental origin of the X chromosomes in the agouti pups from the chimeras. All four of the females and all four of the XXY males (no. 2300 not shown) exhibited a 7-kb plus either a 9.5- or an 11.5-kb hybridizing fragment corresponding to the C57BL/6J HPRT allele plus either the E14TG2a or the T2 HPRT allele, respectively (Fig. 3). This hybridization pattern confirms the expected inheritance of one X chromosome from each parent.

The origin of the Y chromosome in the four XXY mice is presumed to be the ES cell component of the female chimera because there is no evidence for a significant level of XY nondisjunction in the C57BL/6J strain. The one XYY mouse analyzed by Southern blotting (no. 5906) received its X chromosome from the chimeric dam and most likely also received one Y chromosome from her for the reasons just discussed; the other Y chromosome would be from the C57BL/6J sire. The male with the i(Y) chromosome (no. 8459) inherited an X chromosome from his B6 sire, as evidenced by the 7-kb hybridizing fragment, implying that the i(Y) chromosome must have been derived from the chimera. Five karyotypically normal males and two other males whose karyotypes were not studied had a single X chromosome from either the B6 sire ($n = 2$) or the chimeric dam ($n = 5$). The two karyotypically normal males receiving the X chromosome from their C57BL/6J sires must have received their Y chromosomes from the chimeric dam, thereby attesting to the viability of Y chromosome-containing oocytes.

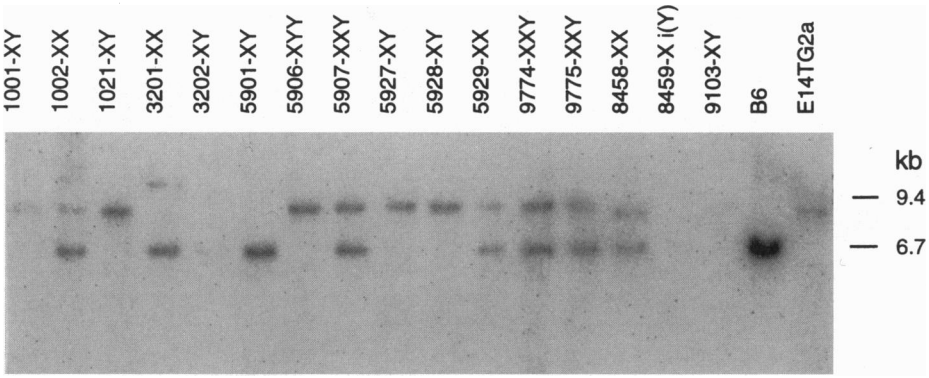

FIG. 3. Southern blot of mouse tail DNA hybridized with a probe from the murine HPRT locus. Hybridizing fragments of 7 kb, 9.5 kb, and 11.5 kb identify the C57BL/6J, E14TG2a, and T2 X chromosomes, respectively.

DISCUSSION

We have observed germ-line transmission from 11% of female chimeras generated after injecting male ES cells into blastocysts. When breeding the ES cell-derived (agouti) offspring from these females, we noticed sterility in about 50% of the male offspring. In contrast, 27% of male chimeras transmitted the ES cell genome, resulting in thousands of agouti pups with no documented sterility.

Our present study shows that the male offspring of the transmitting XX/XY female chimeras have a high rate of aneuploidy. Thus 6 (43%) of 14 unselected male offspring from the female chimeras had abnormal karyotypes, 5 of which were XXY or XYY. Although we did not test the fertility of all animals in our experiments, our experience and that of others (12–16) indicates that XXY and XYY mice are sterile. The high incidence of infertility in the male offspring is therefore explained by the high incidence of sex chromosome aneuploidy. Nevertheless these observations and those of others (14, 17–21) show that Y and XY ova certainly can exist and can form viable XY, XXY, and XYY animals after normal fertilization. However, although we have observed the transmission of the XY genome through five female chimeras, this transmission is infrequent and the resulting agouti pups are born predominately in the early stages of the reproductive life span. Previous investigators have also reported that the fertility of XY females is often reduced as evidenced by their smaller litters and limited reproductive life span (19, 20). XY^{POS} females, where the Y^{POS} is derived from *Mus domesticus poschianinus*, are infertile (22, 23).

There are two possible explanations for the large number of karyotypically abnormal males among the offspring of transmitting female chimeras. The first explanation, which is the most likely for the male offspring that are XXY and XYY, is a failure of the ovary to support normal X–Y segregation. The pairing behavior of X and Y chromosomes during female meiosis in the mouse has been observed in the context of three unique XY females, XY^{POS}, and XY^{*X} (20), and $XY^{Tdy\text{-}m1}$ (24). In the XY^{POS} female, XY pairing in the oocyte is severely reduced (<20% of meioses studied). Similarly, in the XY^{*X} female (14), the X and Y chromosomes were paired in only 63% of meioses studied. Interestingly, pairing of sex chromosomes is 100% in $XXY^{*X}Y^{*X}$ females, with XX and $Y^{*X}Y^{*X}$ pairing configurations observed. This suggests that the pairing failure is not the consequence of some abnormality in the pseudoautosomal region but is rather due to a failure of the X chromosome to pair (with a Y chromosome) when pairing is restricted to the pseudoautosomal region (21). Observation of synaptonemal complex formation during pachytene in $XY^{Tdy\text{-}m1}$, $XXY^{Tdy\text{-}m1}$, and $XY^{Tdy\text{-}m1}Y^{Tdy\text{-}m1}$ oocytes again reveals abnormalities. A higher percentage of abnormal cells are found earlier in pachytene, suggesting that pairing failure can cause oocyte loss (24).

If segregation of the X and Y chromosomes during meiosis in the ovary is erratic, with nondisjunction as likely as normal segregation, at least two-thirds of the viable offspring should be male, since the ES cell-derived gametes of the dam could be X, Y, XY, or 0. [YY and 0Y embryos would not be viable, and the birth of X0 females is often less frequent than expected (20).] In agreement with this expectation, we observed 14 males and only 5 females in our experiments, thereby supporting nondisjunction as an explanation for the sex chromosome-based aneuploidy. A χ^2 analysis indicates that our observed data represent a statistically significant difference from a 1:1 ratio of males to females ($P < 0.05$). Similar ratios of males to females, and of karyotypically normal males to sex-chromosome aneuploid males, are seen in the offspring of $XY^{Tdy\text{-}m1}$ (19, 24) and XY^{*X} females (14, 20, 21).

A second possible explanation for the frequent occurrence of abnormal karyotypes and consequent sterility in the offspring of female chimeras involves a karyotypic instability in the ES cell line. Karyotypic analysis of the E14TG2a ES cell line and of the several subclones derived from it shows that, like other ES cell lines (25), they are prone to instability. However, if ES cell karyotypic instability were a major contributor to the observed aneuploidy, one would expect to find aneuploidy and sterility in the female offspring of the female chimeras and among the offspring of transmitting male chimeras. Although we karyotyped only three female offspring from female chimeras, they were normal. Similarly, although we have not studied the karyotypes of offspring from transmitting male chimeras, we have not noticed sterility in them. Nevertheless three of the mice that we have studied here have karyotypes suggestive of some general karyotypic instability, so that possibility remains. Thus the additional chromosome 3 of mouse 341M, the additional autosome in some of the cells from mouse 339M, and the i(Y) chromosome of mouse 8459 are all likely to have originated in the ES cell line. Mouse 341M is notable in another context: it demonstrates that trisomy 3 is not always lethal in the mouse, despite previous reports that it causes lethality by gestational day 12 (26).

From the standpoint of ES cell technology, female chimeras are in general less valuable than the males, although our data show that they can transmit the ES cell genome to their offspring. Their female offspring will breed normally, but only about 50% of their male offspring will be fertile, because of the high frequency of aneuploidy and consequent sterility in the male offspring. We have suggested two likely causes for this sterility: aberrant pairing and segregation of the X and Y chromosomes during female meiosis and chromosomal instability of ES cell lines. Although female chimeras are clearly less able than male chimeras to lead to an ES cell-derived mouse line, the female chimeras raise some provocative questions

concerning the reproductive biology of chimeric animals and the differences between male and female meiosis.

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1. Bradley, A., Evans, M., Kaufman, M. H. & Robertson, E. (1984) *Nature (London)* **309,** 255–256.
2. Robertson, E., Bradley, A., Kuehn, M. & Evans, M. (1986) *Nature (London)* **323,** 445–448.
3. Gossler, A., Doetschman, T., Korn, R., Serfling, E. & Kemler, R. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9065–9069.
4. Hooper, M., Hardy, K., Handyside, A., Hunter, S. & Monk, M. (1987) *Nature (London)* **326,** 292–295.
5. Koller, B. H., Marrack, P., Kappler, J. W. & Smithies, O. (1990) *Science* **248,** 1227–1230.
6. John, S. W. M., Krege, J. H., Oliver, P. M., Hagaman, J. R., Hodgin, J. B., Pang, S. C., Flynn, G. & Smithies, O. (1995) *Science* **267,** 679–681.
7. Homanics, G. E., de Silva, H. V., Osada, J., Zhang, S. H., Wong, H., Borensztajn, J. & Maeda, N. (1995) *J. Biol. Chem.* **270,** in press.
8. Kim, H. S., Krege, J. H., Kluckman, K. D., Hagaman, J. H., Hodgin, J. B., Best, C. F., Jennette, J. C., Coffman, T. M., Maeda, N. & Smithies, O. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2735–2739.
9. Seabright, M. (1971) *Lancet* **ii,** 971–972.
10. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
11. Bishop, C. E., Boursot, P., Baron, B., Bonhomme, F. & Hatat, D. (1985) *Nature (London)* **315,** 70–72.
12. Cattanach, B. M. & Pollard, C. E. (1969) *Cytogenetics* **8,** 80–86.
13. Evans, E. P., Beechey, C. V. & Burtenshaw, M. D. (1978) *Cytogenet. Cell Genet.* **20,** 249–263.
14. Hunt, P. A. & Eicher, E. M. (1991) *Chromosoma* **100,** 293–299.
15. Endo, A., Watanabe, T. & Fujita, T. (1991) *Genome* **34,** 41–43.
16. de Boer, P., de Jong, J. H. & van der Hoeven, F. A. (1991) *Cytogenet. Cell Genet.* **56,** 36–39.
17. Ford, C. E., Evans, E. P., Burtenshaw, M. D., Clegg, H. M., Tuffrey, M. & Barnes, R. D. (1975) *Proc. R. Soc. London B* **190,** 187–197.
18. Evans, E. P., Ford, C. E. & Lyon, M. F. (1977) *Nature (London)* **267,** 430–431.
19. Lovell-Badge, R. & Robertson, E. (1990) *Development (Cambridge, U.K.)* **109,** 635–646.
20. Hunt, P. (1991) *Development (Cambridge, U.K.)* **111,** 1137–1141.
21. Hunt, P. & LeMaire, R. (1992) *Am. J. Hum. Genet.* **50,** 1162–1170.
22. Eicher, E. M., Washburn, L. L., Whitney, J. B., III, & Morrow, K. E. (1982) *Science* **217,** 535–537.
23. Taketo-Hosotani, T., Nishioka, Y., Nagamine, C. M., Villalpando, I. & Merchant-Larios, H. (1989) *Development (Cambridge, U.K.)* **107,** 95–105.
24. Mahadevaiah, S. K., Lovell-Badge, R. & Burgoyne, P. S. (1993) *J. Reprod. Fertil.* **97,** 151–160.
25. Robertson, E. J. (1987) in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, ed. Robertson, E. J. (IRL, Washington, DC), pp. 71–112.
26. Gropp, A. (1982) *Virchows Arch.* **395,** 117–131.