Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8927–8931, November 1989
Genetics

Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells

(gene targeting/chimeras)

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Contributed by Oliver Smithies, August 25, 1989

ABSTRACT **Embryonic stem cells (derived from 129/Ola mice) containing a mutant hypoxanthine phosphoribosyltransferase gene that had been corrected *in vitro* in a planned manner by homologous recombination were injected into blastocysts obtained from C57BL/6J mice. The injected blastocysts were introduced into pseudopregnant female mice to complete their development. Eleven surviving pups were obtained. Nine were chimeras: six males and three females. Two of the males transmitted the embryonic stem cell genome containing the alteration in the hypoxanthine phosphoribosyltransferase gene to their offspring at high frequencies. These experiments demonstrate that a preplanned alteration in a chosen gene can be made in the germ line of an experimental animal by homologous recombination in an embryonic stem cell.**

Mutations in the germ lines of experimental animals have provided investigators with powerful tools for studying normal and abnormal biology. Such mutations have been obtained by a variety of means, including irradiation; treatment with chemical mutagens, viral agents, or transposable elements; and microinjection of DNA. A severe limitation of all these procedures is their inability to control exactly either the specific mutations that are produced or the target genes that are altered. One step in removing these limitations was provided by Smithies *et al.* (1) who used homologous recombination to create a specific preplanned alteration in a chosen native chromosomal gene (gene targeting) in tissue culture. A second step was provided by the demonstration that embryonic stem cells (ES cells) (2) can be used to transfer mutations isolated in tissue culture to the germ line (3, 4). In this procedure, mutant ES cells isolated in tissue culture are injected into normal blastocysts, which are then returned to pseudopregnant females for continued development. Some of the chimeras resulting from the injected blastocysts are chimeric in their gonads and able to transmit the mutation through the germ line to their progeny. A further step in opening the route to planned genetic alterations in experimental animals is the demonstration by several groups (5–10) of gene targeting in ES cells. The combination of gene targeting with germ-line transfer has been reported by Thompson *et al.* (11). They obtained a male mouse chimera that transmitted to its progeny a hypoxanthine phosphoribosyltransferase (HPRT) gene corrected by targeting, although an unanticipated deletion occurred during or after the targeting event. We report here that the whole process can be accomplished without such complications; we have obtained chimeric males that transmit to their offspring exactly the planned alteration of the chosen target gene (HPRT) that we executed by homologous recombination in ES cells (6).

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MATERIALS AND METHODS

Cell Culture. ES cells were cultured on feeder layers prepared from primary embryonic fibroblasts, in the presence of Dulbecco's modified Eagle's medium containing 15% (vol/vol) fetal calf serum and 0.1 mM 2-mercaptoethanol, but without antibiotics (12). The cultures were regularly checked for mycoplasma contamination. They were passaged at ≈3-day intervals onto feeder layers treated with mitomycin (10 μg/ml) for ≈2 hr at 37°C.

Embryo Manipulations. Blastocysts were collected from 3- to 4-week-old C57BL/6J mice, superovulated as described (13). Freshly trypsinized ES cells, 2–3 days after passage, were resuspended in M2 medium (13) and introduced into blastocysts by microinjection (10–15 cells per blastocyst) at room temperature. Injected blastocysts were returned, without further incubation, to the uteri of pseudopregnant females that had been mated to vasectomized males 2.5 days previously.

Animal Husbandry. The animals used in the successful experiments described here were maintained in sterilized microisolator cages using sterile food and sterile bedding. Animals were handled with gloves, and cage transfers were carried out in a laminar flow hood using the procedures recommended by the manufacturers of microisolator cages.

Southern Blot. Genomic DNA was prepared from ES cells, tissues of chimeras, and from their offspring. After these DNA samples had been digested with *Hin*dIII and electrophoresed in an agarose gel, a Southern blot of the gel was prepared and hybridized to a ^{32}P-labeled probe made from pUC18 DNA. This probe will detect genomic copies of the plasmid used to correct the HPRT gene by homologous recombination.

RESULTS

Planned Alteration. The ES cell lines that we used for the present experiments were derived from a male HPRT$^-$ ES cell line E14TG2a (3) in which a single deletion has removed the promoter and exons 1 and 2 of the HPRT gene. The promoter and exons missing in E14TG2a were restored by homologous recombination using gene targeting with a 12.4-kilobase-pair (kbp) correcting plasmid, as reported by us (6); HPRT$^+$ cells were selected in hypoxanthine/aminopterin/thymidine (HAT)-containing medium. A total of 19 HAT-

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

resistant colonies from these earlier experiments have now been analyzed by genomic Southern blots (including 5 already reported). Eighteen were altered in the planned manner. Five of these 18 had the simplest alteration, in which a single copy of the 12.4-kbp correcting plasmid was inserted by a simple homologous crossover into the HPRT locus without any evidence of gene conversion (see Fig. 2). The other 13 had single copies of the correcting plasmid inserted by homologous crossover accompanied by gene conversion. (for more detail, see ref. 6). One (ES98-12) of the 5 ES cell lines with a simple crossover was arbitrarily chosen for most of our tests (except for some initial trials with ES98-2), and it is the planned gene correction in ES98-12 that has been transmitted through the germ line. We did not find it necessary to test any of the other corrected ES cell lines.

Initial Trials. The ES cell line 98-2 derived from E14TG2a and corrected in the same manner as ES98-12 was injected into blastocysts collected from outbred ICR albino female mice. Chimeras were obtained, but no germ-line transmission of the ES cell genomes was observed, as judged by the coat colors of their progeny. This first set of experiments was terminated when the degree of chimerism dropped precipitously and we found mycoplasma contamination in the specific isolate of ES cells that we were using. The experiments were repeated using a different, mycoplasma-free, ES cell isolate (ES98-12) having the same planned alteration in the HPRT gene. The cells were cultured without antibiotics to allow early detection of any contamination. We also changed the source of recipient blastocysts in this second set of experiments to C57BL/6J female animals, based on a personal communication from Colin Stewart (European Molecular Biology Laboratory, Heidelberg) that he had had better success with this strain and because none of our ICR-derived animals, even those that were clearly chimeric, transmitted the ES cell genome. The second set of experiments was terminated when several chimeras died at weaning of diarrhea. Their nonchimeric littermates survived. C57BL/6J mice, the source of the recipient blastocysts, are more resistant to some infections than are 129 mice (14), the source of the ES cells. A third set of experiments was therefore initiated, repeating the conditions used in the second set, except that the animals were kept in isolation cages. This third set was successful and provided the results reported below.

Detection of Chimeras and Germ-Line Descendants of the ES Cells. Pups derived from blastocysts injected with ES98-12 cells were judged for coat color chimerism by inspection. Strain 129/Ola mice, from which the E14TG2a cell line (3) and thence the ES98-12 cell line were derived, carry mutations at three loci known to affect coat color: *A*, *c*, and *p*. The 129/Ola mice are homozygous for the dominant allele A^w at the agouti locus, the recessive allele c^{ch} at the *c* locus, and the recessive allele *p* at the *p* locus (11); they are creamy-white colored with pink eyes. Strain C57BL/6J mice were the source of the recipient blastocysts; they are solid black in color with black eyes. Chimeras between these two strains are identified by the presence of lighter patches of fur on a black background. The color of the patches varies depending on which layers of epidermis are ES cell-derived and which are recipient blastocyst-derived. Agouti patches show the indirect inhibitory effects of the dominant A^w gene in ES cell-derived mesodermal cells on the production of black pigment by melanocytes. Light yellow patches show the direct effects of the c^{ch} and *p* genes on the production and packaging of the pigments themselves. Agouti and/or yellow patches can be seen in a given chimeric animal. Animals strongly chimeric for coat color can be almost uniformly yellowish brown or cream colored with black eyes.

The male chimeras described below were mated to C57BL/6J females to test for germ-line transmission of the ES cell genome having the corrected HPRT gene. Pups from sperm derived from the recipient blastocysts are black; pups from sperm derived from the ES98-12 cells are uniformly agouti (because A^w is dominant). Both types of pup have black eyes. The recessive c^{ch} and *p* alleles do not appreciably affect the coat color when heterozygous.

Table 1. Blastocyst transfers with the HPRT$^+$ ES cell line 98-12

Test	Surviving pups, no.	Number and sex of chimeras	Transmitting to germ line,* no. and sex of animals
3†	2	2 females	—
4	4‡	1 male, 1 female§	1 male
5	5	5 males§	1 male
Total	11	6 males, 3 females (82)	2 males

The number in parentheses is the percent of surviving pups that are chimeras.
*Pups were scored for coat color chimerism and mated when mature to test for germ-line transmission of coat color markers from ES98-12 to progeny.
†In tests 1 and 2, too few blastocysts were obtained from the superovulated females to warrant completion of the procedure.
‡Two animals in this group were not chimeras.
§One animal in this group died with a tumor.

Production of Chimeras. Table 1 shows the results obtained with ES98-12 cells injected into C57BL/6J blastocysts and returned to pseudopregnant mothers. The proportion of resulting chimeras (82% of surviving pups) compares favorably with those reported by previous investigators using untreated ES cells (15). Note the occurrence of a tumor in two animals. Examination of the tumors showed them to be composed virtually completely of undifferentiated cells, as judged by their cytology, derived from the injected ES cells, as judged by their DNA (see Fig. 2).

Targeted ES Cells Contribute to the Germ Line. Six chimeric males were obtained and tested for their ability to transmit the ES cell genome containing the correctly targeted HPRT gene to their progeny. One of the six was a runt and abnormal in appearance; he failed to reproduce. A second died with a tumor (see Table 2). Two had only black offspring. Two had litters with coat colors indicating that both donor ES98-12 cells and the recipient blastocysts had participated in sperm production. The reproductive histories of the four fertile males are given in Table 2. The proportion of progeny with agouti (ES cell derived) and black (recipient blastocyst derived) coats indicates that >50% of the progeny from the males 4.1 and 5.1 received the ES cell genome. The apparent excess of agouti males over agouti females (21 males/9 females) in this limited sample requires further investigation.

A photograph of the four fertile males is shown in Fig. 1 *Upper*, and a photograph of a family illustrating transmission

Table 2. Breeding history of male chimeras

Chimera	Pups, no.	Agouti coat, number and sex of animals	Black coat, number and sex of animals*
4.1	9	5 males, 1 female	3
	7	0	7
	9	5 males, 3 females	1 male
	8	3 males, 1 female	2 males, 2 females
5.1	10	2 males, 2 females	3 males, 3 females
	9	6 males, 2 females	1 female
5.2	7	0	7
	10	0	10
5.3	7	0	7
	3	0	3
	8	0	4 males, 4 females

*Some black pups were discarded without sexing.

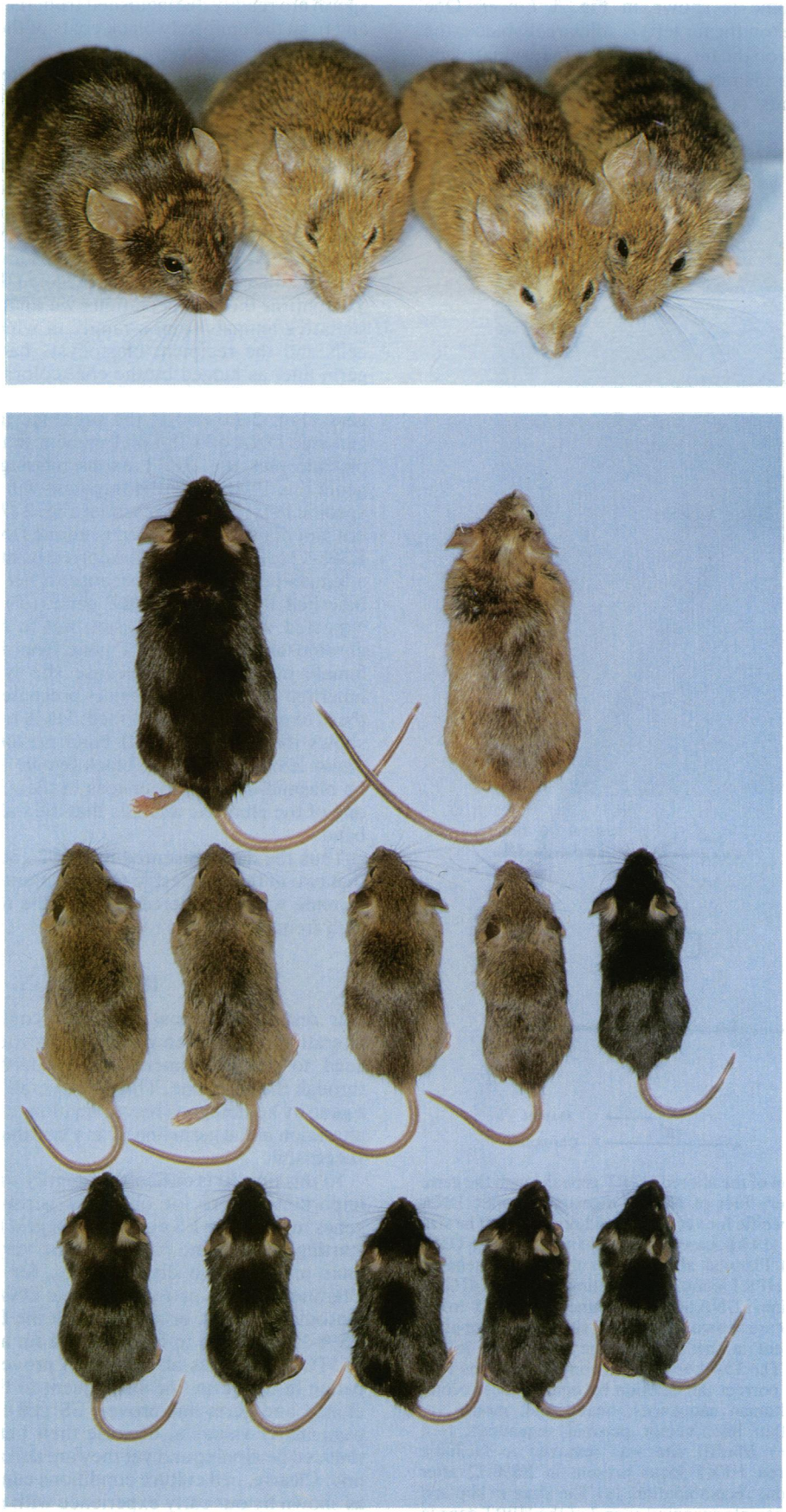

FIG. 1. (*Upper*) Four fertile male chimeras (from left to right: 5.3, 4.1, 5.1, and 5.2 in Table 2) resulting from C57BL/6J blastocysts injected with ES98-12 cells. The two males in the center transmitted the ES98-12 genome to their progeny (see text and Table 2). (*Lower*) One of the transmitting males, 5.1, (*Right top*) mated to a C57BL/6J female (*Left top*). In this family, four nonvariegated agouti pups (brown in the photograph) received ES98-12 genomes from their lightly variegated father; six black pups received C57BL/6J genomes from him.

of the ES cell genome is shown in Fig. 1 *Lower*. One nontransmitting male (on the far left) is still largely black; the other nontransmitter (on the far right) has more residual black color than the two transmitting males (in the center). The uniformly agouti coats of the pups from ES cell-derived sperm are readily distinguished from the coats of their fathers and from the uniformly black coats of the pups from recipient blastocyst-derived sperm.

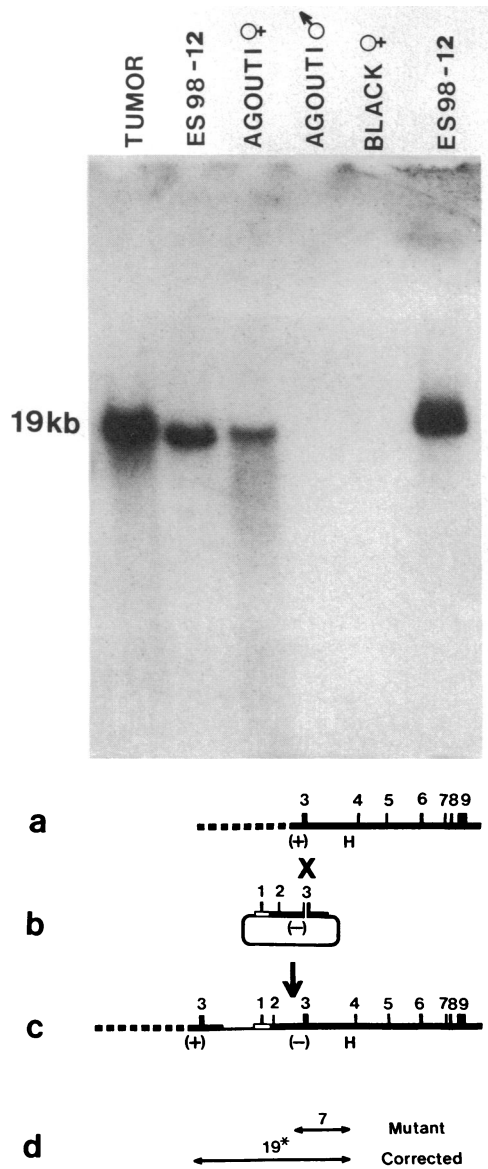

FIG. 2. Transmission of the altered HPRT gene through the germ line. (*Upper*) A Southern blot of *Hin*dIII-digested genomic DNA hybridized to a probe specific for vector plasmid sequences. The size of the hybridizing band [19 kilobases (kb)] and sources of the DNA are indicated. (*Lower*) Planned alteration in the HPRT gene of ES98-12 (see ref. 6). (*a*) HPRT locus in the deletion mutant E14TG2a. The interrupted heavy line is DNA an undetermined distance 5′ to the HPRT locus. Exons 3–9 are shown. H, *Hin*dIII site used in mapping; (+), *Hin*dIII site present in the chromosome but absent in the correcting plasmid. (*b*) The 12.4-kbp plasmid containing exons 1, 2, and 3 that was used to correct the deletion by homologous recombination. Open box, human sequences; heavy line, mouse sequences; continuous thin line, vector plasmid sequences; (−), position where the (+) *Hin*dIII site was removed to facilitate mapping. (*c*) The altered HPRT locus present in ES98-12 after correction by homologous recombination. (*d*) The sizes in kbp and locations of *Hin*dIII fragments spanning exon 3 of the HPRT gene in the deletion mutant E14TG2a and in ES98-12, which contains the corrected HPRT gene. The asterisk indicates that the 19-kbp *Hin*dIII fragment but not the 7-kbp fragment will hybridize to a probe detecting the vector plasmid.

Transmission of the Preplanned Alteration in the HPRT Gene to Offspring of the Chimeras. Since HPRT is an X chromosome-linked gene, only agouti female pups are expected to inherit the altered gene from their chimeric fathers (their agouti coats show that they inherited the ES98-12 genome; their sex shows that they inherited an X chromosome from their fathers). Agouti male pups should not inherit the altered gene (they received the ES98-12 genome from their fathers, but not an X chromosome); nor should black pups of either sex (they did not receive the ES98-12 genome). To confirm these expectations we analyzed DNA of representative animals from a family in which both the ES98-12 cells and the recipient blastocysts had contributed to the germ line, as judged by the coat colors of the pups.

The best single DNA indicator of a correctly altered HPRT gene (Fig. 2 *Lower*) is the presence in *Hin*dIII digests of genomic DNA of a 19-kpb fragment that includes the vector plasmid plus the HPRT exons missing in the uncorrected gene; this 19-kbp *Hin*dIII fragment will hybridize to a probe specific for the vector plasmid. Fig. 2 *Upper* shows a Southern blot of *Hin*dIII-digested genomic DNA from (*i*) the donor ES98-12 cells containing the correctly altered HPRT gene, (*ii*) a female pup expected, because of her agouti coat, to have inherited the altered HPRT gene, (*iii*) an agouti male pup expected, because of his sex, not to have inherited the X chromosome-linked HPRT gene from his father, and (*iv*) a female pup expected, because she is black, not to have inherited the gene. The results presented in Fig. 2 show that these expectations are fulfilled: DNA from the agouti female shows the 19-kbp *Hin*dIII band present in DNA from the donor ES98-12 cells; the black female and agouti male have no plasmid-derived sequences in their DNA. A tumor from one of the chimeric animals that died also shows the 19-kpb band.

Thus the data presented in Fig. 2 and in Table 2 establish that two of the four fertile male chimeras transmit the ES cell genome with the altered HPRT gene to their offspring at a high frequency.

DISCUSSION

Our results show that ES cells, containing a preplanned alteration of a chosen gene made by gene targeting, can be used to obtain chimeras that transmit the altered gene through the germ line. Thus the overall process of modifying a gene by homologous recombination to achieve a preplanned alteration in the germ line of an experimental animal has been successful.

At this time, it is difficult to identify with certainty the most important factors for obtaining germ-line transmission of genes modified in ES cells. We suggest that the choice of the starting ES cell line is likely to be very important. ES cell lines may become divergent by, for example, epigenetic alterations in the patterns of their DNA methylation, chromosomal changes, or mutations at the DNA sequence level. ES98-12 is known to be derived from a cloned ES cell line, E15TG2a, that has already been proven capable of participating in the germ line subsequent to this cloning (3). Such cloned and germ-line-proven ES cell lines are likely to be particularly valuable, because their heterogeneity has been reduced by cloning and yet they are still able to enter the germ line. Clearly, cell culture conditions can introduce problems, as shown by our early experience with mycoplasma contamination. We have also tried to avoid procedures generally recognized as likely to propagate undesirable changes in an ES cell line, such as unnecessary culture of the cloned ES cells prior to their use, long intervals between passaging the

cells to new feeder layers, or bottlenecks in cell populations. Cells that lose their ability to differentiate *in vitro* when cultured without feeder layers may become incapable of differentiating *in vivo*, and so we have occasionally tested our ES cells for retention of this ability (12). The mouse strains and combinations of strains from which the ES cells and recipient blastocysts are derived may be important. Finally, the use of ES cells derived from mice of strain 129 causes the resulting chimeras to be particularly susceptible to some infections, as we observed. Animal husbandry is, therefore, important.

The use of homologous recombination to alter chosen genes in a preplanned way in animal germ lines is likely to be generally applicable. Homologous recombination has been used to modify genes in ES cells that are probably not expressed (16), and genes for which no direct selection is available (8, 9, 10). The procedure is likely to be applicable to species other than the mouse as appropriate ES cell lines become available (17).

We thank Mrs. Natalie Borenstein and Ms. Denise Dolenak for careful tissue culture work, Ms. Tracey Barnes and Ms. Kristen Kesler for animal husbandry, Dr. James Pick and his staff in the Division of Laboratory Animal Medicine, University of North Carolina, for advice and help, and Drs. Achim Gossler and Colin Stewart for helpful suggestions. B.H.K. is a fellow of the Leukemia Society of America. L.J.H. is a trainee on Training Grant GM07215 from the National Institutes of Health. Our work was supported by Grants HL42630 to N.M. and HL37001 and GM20069 to O.S. from the National Institutes of Health.

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