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Methodology article

Generation of single-copy transgenic mouse embryos directly from ES cells by tetraploid embryo complementation

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Abstract

Background: Transgenic mice have been used extensively to analyze gene function. Unfortunately, traditional transgenic procedures have only limited use in analyzing alleles that cause lethality because lines of founder mice cannot be established. This is frustrating given that such alleles often reveal crucial aspects of gene function. For this reason techniques that facilitate the generation of embryos expressing such alleles would be of enormous benefit. Although the transient generation of transgenic embryos has allowed limited analysis of lethal alleles, it is expensive, time consuming and technically challenging. Moreover a fundamental limitation with this approach is that each embryo generated is unique and transgene expression is highly variable due to the integration of different transgene copy numbers at random genomic sites.

Results: Here we describe an alternative method that allows the generation of clonal mouse embryos harboring a single-copy transgene at a defined genomic location. This was facilitated through the production of Hprt negative embryonic stem cells that allow the derivation of embryos by tetraploid embryo complementation. We show that targeting transgenes to the *hprt* locus in these ES cells by homologous recombination can be efficiently selected by growth in HAT medium. Moreover, embryos derived solely from targeted ES cells containing a single copy LacZ transgene under the control of the α-myosin heavy chain promoter exhibited the expected cardiac specific expression pattern.

Conclusion: Our results demonstrate that tetraploid embryo complementation by F3 *hprt* negative ES cells facilitates the generation of transgenic mouse embryos containing a single copy gene at a defined genomic locus. This approach is simple, extremely efficient and bypasses any requirement to generate chimeric mice. Moreover embryos generated by this procedure are clonal in that they are all derived from a single ES cell lines. This facilitates the comparative analysis of lethal alleles and thereby advances our ability to analyze gene function in mammals.

Background

The analysis of gene function during mammalian embryonic development relies heavily on the use of transgenic mice. However, expression of alleles that result in embryonic lethality cannot be studied using regular transgenic procedures because lines of founder mice cannot be established. Analysis of such alleles in mammals has historically relied upon transient transgenic procedures [1]. In this approach, fertilized eggs are injected with a transgene construct and allowed to develop to a pre-determined embryonic stage *in utero* before being collected for phenotypic analysis. Using this method a variable number of transgenes integrate into random genomic loci. Unfortunately, the local chromosomal environment can affect expression of the integrated transgene and this can result in unpredictable transgene expression. Moreover, because no founder line is established it is not possible to repeat an analysis with a specific transgenic mouse embryo.

The problem of random transgene integration can be overcome by targeting transgenes to a specific genomic locus by homologous recombination in ES cells [2–4]. A method that allows the introduction of single copy transgenes into the X-linked *hprt* locus has been described previously by Bronson *et al*[2]. In this approach single-copy transgenes are introduced 5' of the *hprt* locus by homologous recombination in ES cells. This is an extremely efficient procedure because targeted clones have reconstituted the *hprt* gene and are selected on the basis of growth in HAT medium. Following this method, upward of 90% of HAT resistant ES cell clones contain correctly targeted transgenes [2]. The *hprt* locus is a particularly suitable site for the integration of transgenes because it exists as an X-linked gene present as a single copy in male ES cells. Moreover, the *hprt* gene is ubiquitously expressed and so provides a favorable chromatin environment for transgene expression. Indeed, it has been demonstrated that the level of expression of transgenes inserted into the *hprt* locus is directed solely by exogenous transcriptional regulatory elements [2]. Furthermore, mice derived from independent ES cells clones containing a transgene expressed from the same promoter all exhibit comparable levels of expression [2,4–6].

Although the targeting of constructs to the *hprt* locus facilitates consistent expression of transgenes it does not overcome the need to generate founder lines of transgenic mice. However, pioneering work by Nagy *et al* has shown that embryos and mice can be derived directly from R1 ES cells by aggregating them with tetraploid embryos [7,8]. This suggests that controlled expression of transgenes encoding possible gain-of-function and developmental-lethal alleles could be achieved by generating embryos and/or mice from ES cells containing transgenes inserted into the *hprt* locus. Unfortunately, the generation of mice and embryos by tetraploid aggregation from available Hprt negative ES cell lines has proven to be inefficient and unreliable (S.A.D. unpublished). In this report, we describe the generation of a new Hprt negative ES cell line that is compatible with the tetraploid aggregation procedure. Moreover, we demonstrate that it facilitates efficient targeting of transgenes to the *hprt* locus as well as the subsequent generation of clonal transgenic mouse embryos that express the transgene in a promoter-dependent manner.

Results

Nagy *et al* demonstrated that embryos and mice could be derived solely from R1 ES cells by aggregating them with tetraploid embryos [7,8]. Although we were able to repeat these data using R1 ES cells we were unable to generate midgestation embryos from E14TG2a-derived strains of *hprt* negative ES cell lines using the same approach [9–11]. We believed it was unlikely that Hprt itself was required to generate ES cell-derived embryos because *hprt* knockout mice are viable and fertile [12]. E14TG2a-derived ES cell lines contain a large spontaneous deletion encompassing 55 kb of the *hprt* locus [13]. Therefore, it seemed possible that sequences within this region were necessary to maintain ES cell pluripotency. However, we believed a more likely scenario was that the available lines contained unrelated genetic or epigenetic alterations that were incompatible with the aggregation procedure. Consistent with this interpretation, Eggan *et al* have recently shown that viable offspring are more efficiently produced through tetraploid-mediated cloning from outbred ES cell line compared to inbred ES cell lines [14]. We, therefore, believed it likely that aggregation-compatible *hprt* negative ES cells could be generated by targeted mutation of the *hprt* gene in R1 ES cells. To test this we generated a targeting vector to replace exon 1 of the mouse *hprt* gene with a *neomycin phosphotransferase (neo)* gene that conferred resistance to te pharmacological inhibitor G418 in ES cells (fig 1a). Following transfection of this plasmid into R1 cells 192 clones that were resistant to G418 were collected and grown in the presence of 6-thioguanine to select for loss of Hprt activity. Of the original 192 G418-resistant clones that were harvested only one, named F3, was also resistant to 6-thioguanine and sensitive to growth in HAT medium (fig 3b). The integrity of the targeting event was next confirmed by Southern blot analysis of F3 genomic DNA. As shown in fig 1a the correct integration of Neo at the *hprt* locus was predicted to delete a BamH1 restriction endonuclease cut-site from intron 1 and replace it with a BamH1 site that is unique to the mutant *hprt* allele. This restriction fragment polymorphism could be detected using an *hprt-specific* probe (fig 1a). The wild type BamH1 fragment was predicted to be 7.0 kb based

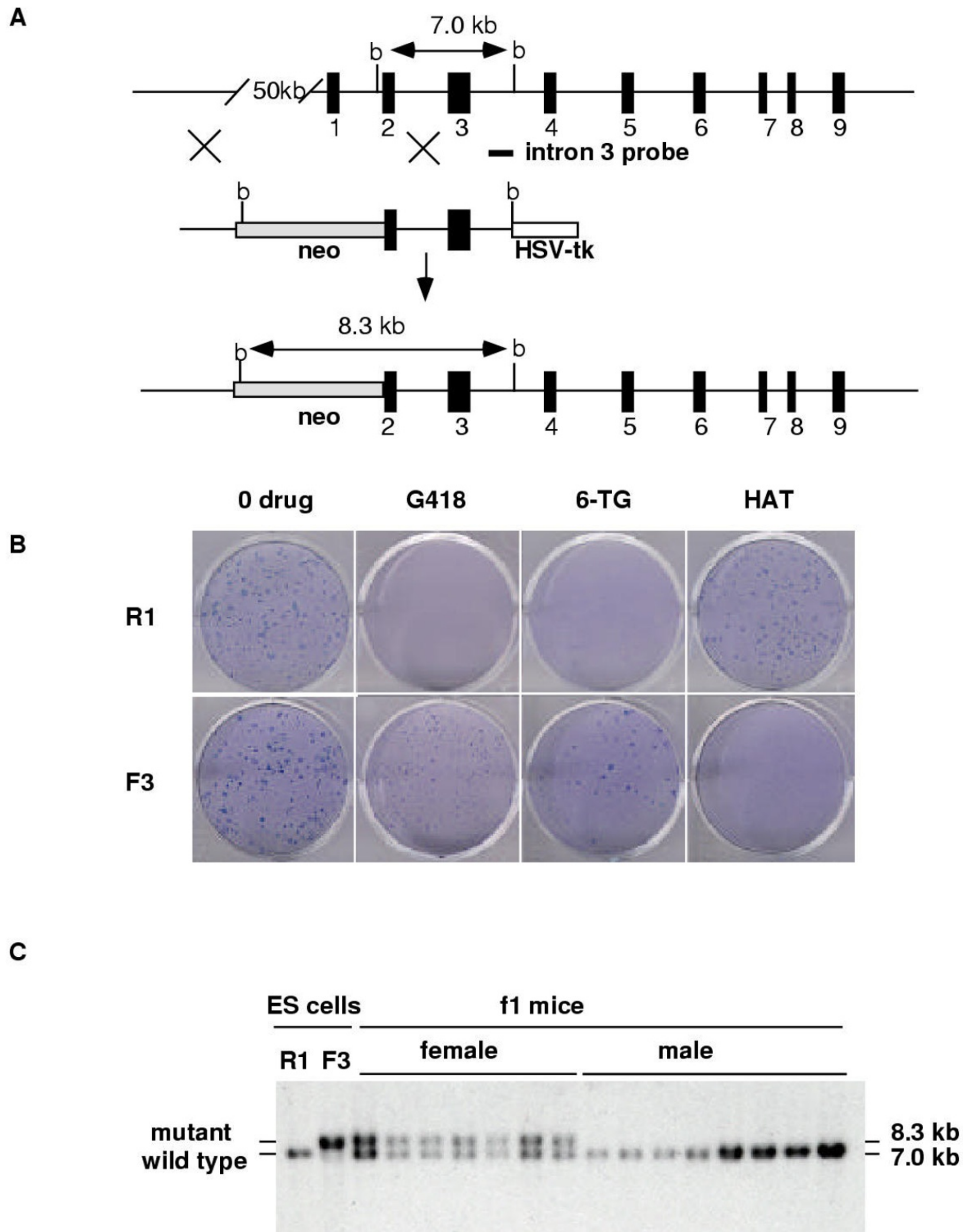

Figure 1
Generation of HPRT negative ES cells. A) Schematic representation of the *hprt* genomic locus, targeting vector and mutant allele. The relative positions of exons (black boxes), *BamH1* restriction endonuclease cut sites (b), and genes encoding *neomycin phosphotransferase* (neo) and HSV thymidine kinase (HSV-tk) selectable markers, as well as the position of the Southern blot probe (intron 3 probe) are shown. B) Growth of R1 *hprt* positive and F3 *hprt* negative ES cells in medium supplemented with G418, 6-TG, HAT or without drug (0 drug). Drug resistant ES cells are visible as blue colonies after staining with methyl green . C) Southern blot analysis of genomic DNA from R1 and F3 ES cells as well from the offspring of chimeric mice generated from F3 cells. The wild type *hprt* allele can be identified using intron 3 probe as a 7.0 kb *BamH1* fragment and the targeted allele (mutant) as a 8.3 kb fragment.

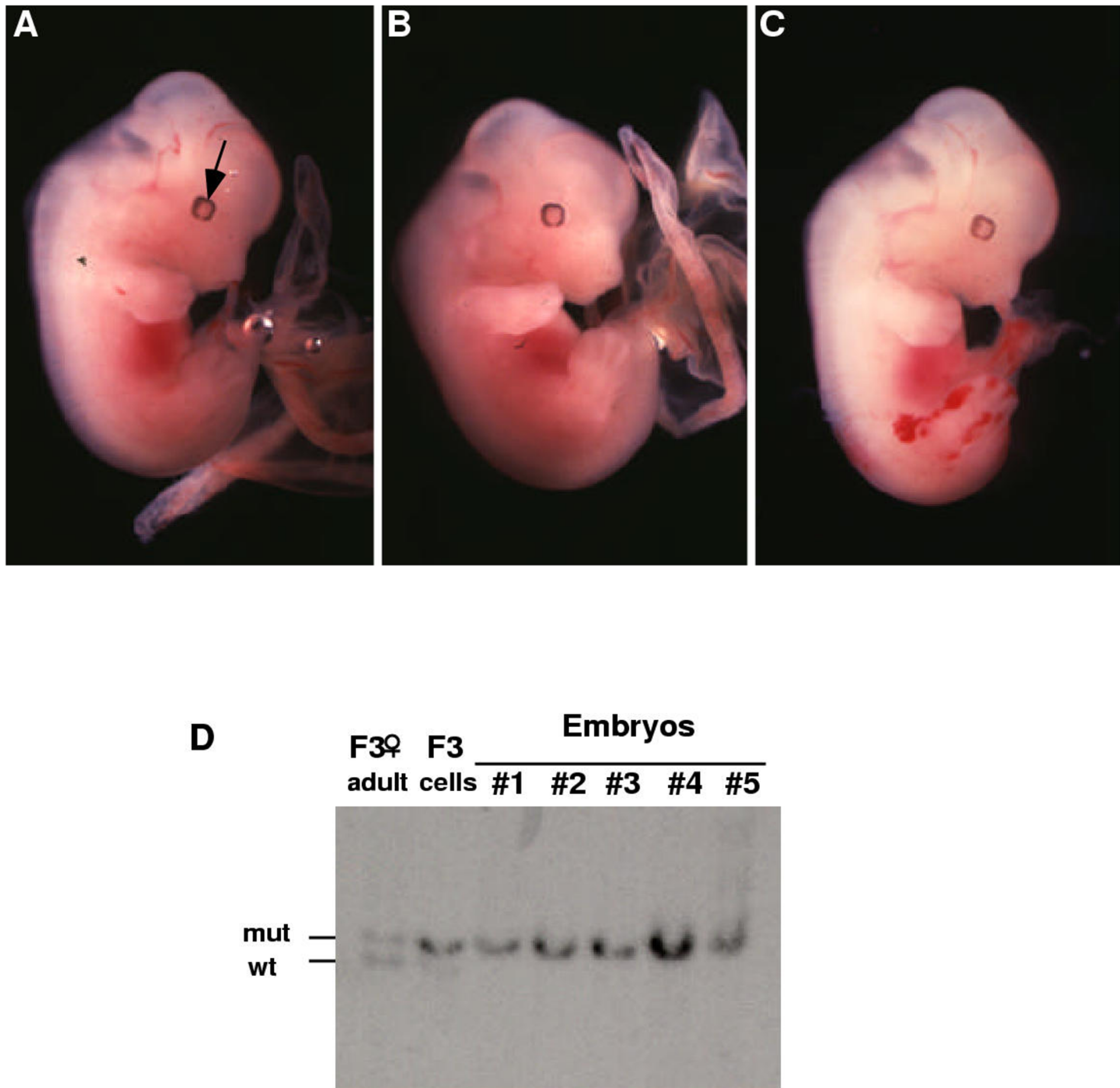

Figure 2
Embryos derived from Hprt-deficient ES cells by tetraploid aggregation. A-C) Photographs of embryos at E13.0 derived from F3 cells. Pigmentation can be seen in the eyes (arrow) reflecting their ES cell origin. D) Southern blot analyses of *BamH1*-digested genomic DNA isolated from five (#1 – #5) F3 ES cell-derived embryos that was hybridized to a radiolabeled intron 3 probe. Only the mutant allele is detected in F3 ES cell derived embryos. The wild type allele can be seen in female F3 offspring as discussed in the text.

on sequence analysis while the mutant fragment should be 8.3 kb. Fig 1c shows that digestion of wild-type R1 ES cell DNA with BamH1 did indeed generate a 7.0 kb fragment that could be detected with intron 3 probe. In contrast, F3 cells were shown to have replaced this fragment with an 8.3 kb BamH1 fragment thereby confirming the integrity of the targeting event.

For F3 cells to be a suitable vehicle for the generation of transgenic mice they should be competent to generate

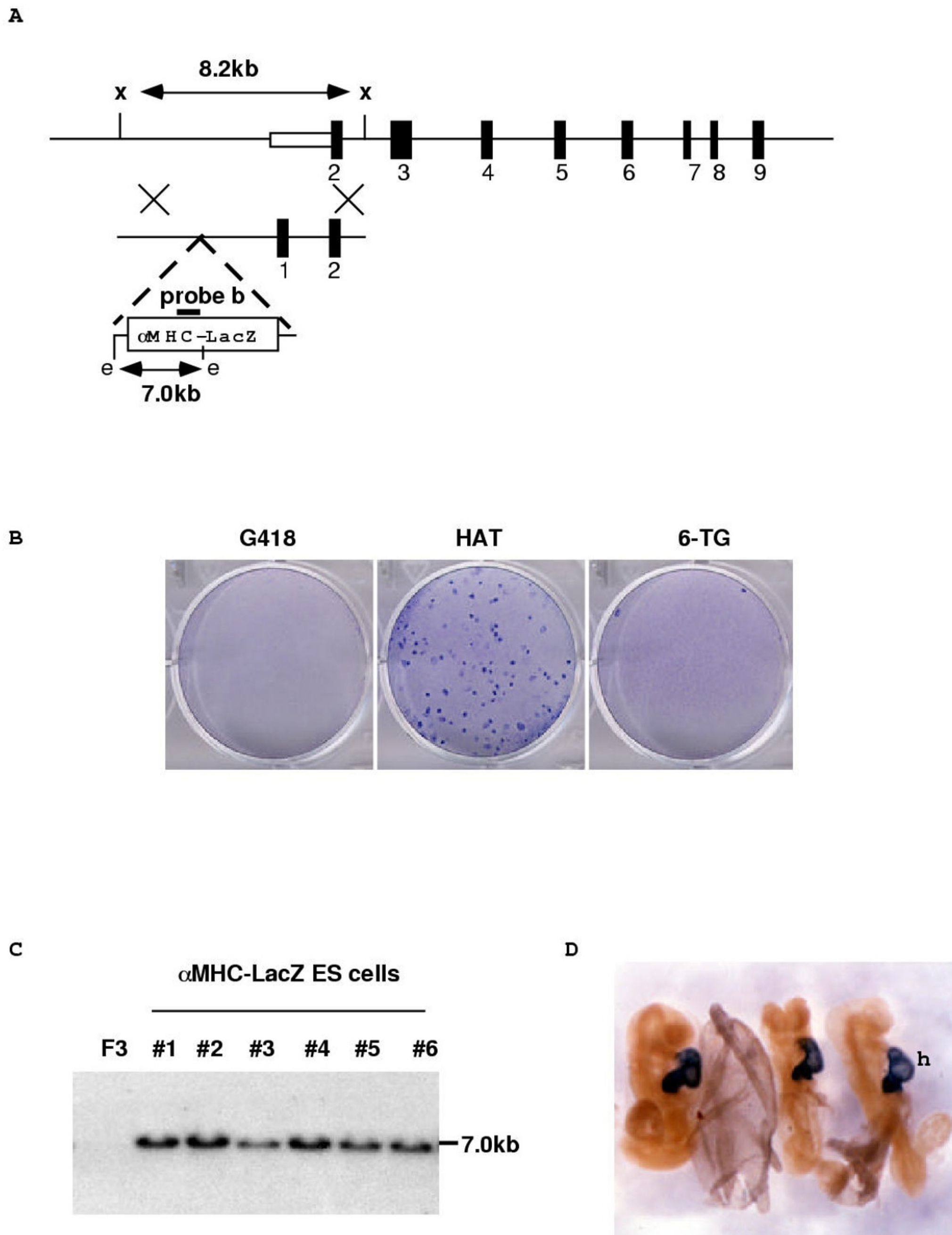

Figure 3
A) Schematic representation of the *hprt* genomic locus and targeting vector with α-MHC-LacZ insert. The position of a Southern blot probe (probe b) containing LacZ specific sequences relative to *XbaI* (x) and *EcoRV (e)* restriction enzyme cut sites is shown. B) Resistance to growth in HAT and sensitivity to G418 and 6-TG was restored in correctly targeted α MHC-LacZ ES cell lines. C) Southern blot analysis of genomic DNA from non-targeted F3 ES cells as well as from six targeted HAT-resistant ES cell lines generated from F3 cells is shown. The targeted allele is identified using probe b as a 7.0 kb *EcoRV* fragment. α MHC-LacZ ES cell line #4 was used to generate embryos shown in D. D) E8.5 embryos derived from α-MHC-LacZ targeted ES cells by tetraploid aggregation. β-galactosidase expression was identified by x-gal staining (blue). Expression was restricted to the developing heart (h).

chimeric mice that can transmit alleles through the germline. To test the "germline potential" of F3 cells we first generated chimeric mice by aggregating F3 ES cells with CD-1 morulae and allowing them to develop to term *in utero*. The resulting chimeric males were bred with CD-1 females and the genotype of offspring determined by Southern blot analysis of genomic DNA from mouse tails. Fig 1c shows that agouti F1 female mice contained both the mutant (paternal) and wild type (maternal) *hprt* alleles. In contrast, the agouti F1 male mice contained only a wild-type *hprt* allele inherited from the maternal X-chromosome as expected. From this we were able to conclude that F3 *hprt* null ES cells are indeed competent to transmit alleles through the germline and that the resulting offspring were fertile and viable as reported previously [12].

We next addressed whether embryos could be generated solely from F3 Hprt negative ES cells by aggregating these cells with tetraploid CD-1 embryos and allowing them to develop *in utero* until day 13 of gestation (El 3). Figs 2a,b,c show that F3 cells could indeed give rise to normal embryos (n = 10). All embryos exhibited characteristic features of midgestation stages of development including normal rostral-caudal patterning, beating hearts, normal limb formation including the formation of fingers. F3 cells derive from the agouti (pigmented) SV129/J strain of mice. In contrast, albino CD-1 mice donated the tetraploid morulae used in the aggregation procedure. The finding, shown in figs 2a,b,c, that all the resulting embryos displayed clear retinal pigmentation strongly implied that the embryos were F3 ES cell-derived. We confirmed this by determining the genotype of the embryos by Southern blot analyses of genomic DNA. Fig 2d shows that all embryos contained a restriction fragment polymorphism that was indicative of deletion of *hprt* exon1. No wild type *hprt* allele was detected confirming that the embryos were generated entirely from F3 cells. These data demonstrate that the *hprt* gene is not essential for the generation of embryos from ES cells by tetraploid aggregation and that F3 cells should could provide a suitable vehicle for the direct generation of transgenic mouse embryos.

Previously it has been shown that a targeting vector, pMP8, can correct the loss of Hprt activity in E14TG2a ES cells that lack exons 1 and 2 of the *hprt* gene [2,15]. The design of this vector implied that it should also be capable of correcting the *hprt* exon 1 deficiency in F3 cells and could, therefore, be used to introduce transgenes into the *hprt* locus by homologous recombination. To test this we generated a modified vector based on pMP8. The practical features of this modification are that it removes the lacZ sequences in the vector backbone and reduces the overall size of the vector, both of which improve subsequent cloning steps. This vector, pMP8NEBΔLacZ, contains a 5' arm of homology consisting of 3.4 kb of mouse 5' *hprt* genomic DNA flanking a unique Not1 restriction endonuclease cut site. The 3' arm of homology contains 1.8 kb of the human *hprt* gene that includes exon1 and 2.9 kb of mouse genomic sequence that includes exon 2 (fig 3a) as described previously [15]. Homologous recombination at the modified *hprt* locus in F3 cells should substitute genomic DNA containing *hprt* exons 1 and 2 for *neo* thereby reconstituting a gene encoding active Hprt (fig 3a). Moreover, only cells having undergone a successful homologous recombination event should become HAT resistant and carry the targeted transgene. To assess the efficiency of this approach we attempted to target a LacZ reporter transgene whose expression is controlled by the promoter from the alpha myosin heavy chain (αMHC) gene to the modified *hprt* locus in F3 cells. We chose the αMHC promoter because others have demonstrated, using traditional transgenic procedures, that it directs high levels of reporter gene expression specifically in cardiac tissue of early mouse embryos [16–18]. Cells were selected for resistance to HAT following electroporation of the *αMHC-LacZ* transgene-containing plasmid. 16 HAT resistant cells lines were collected and their genotype determined by Southern blot analysis. Fig 3c shows that, in contrast to F3 cells, a probe that specifically recognizes the LacZ transgene identified a 6.7 kb EcoRV fragment in HAT-resistant ES cell lines. Of the 16 HAT-resistant colonies retrieved 15 contained LacZ. Furthermore, fig 3b shows that these cells had become G418 and 6-thioguanine sensitive confirming that the *neo* gene had indeed been replaced with *hprt*-coding sequences in the F3 cells. These data confirm that this selection strategy is extremely efficient in identifying ES cell clones containing correctly targeted transgenes [2].

The ultimate use of this procedure is to generate clonal transgenic mouse embryos directly from the modified ES cells. Success in this would provide a system in which to express alleles with potentially lethal phenotypes in the developing embryo. We, therefore, determined whether embryos could be generated from the F3 cells that contained an αMHC-LacZ transgene and whether these embryos appropriately expressed β-galactosidase. E8.5 embryos were generated from three independent αMHC-LacZ cell lines and stained for expression of β-galactosidase. Representative embryos, shown in fig 3d all developed normally and expressed high levels of β-galactosidase in the developing heart, mimicking the expression of endogenous αMHC [16]. Importantly, none of the embryos exhibited ectopic patterns of expression lending further support to the proposal that expression of transgenes introduced to the *hprt* locus is primarily depend-

ent on the accompanying transcriptional regulatory elements [2].

Discussion and conclusions

Here we have described the generation of an ES cell line and a procedure that will allow the phenotypic analyses of transgenic embryos expressing lethal alleles. This procedure is an important addition to the available repertoire of molecular genetic techniques available to developmental biologists because it overcomes several deficiencies associated with alternative transient transgenic approaches normally used to study gain-of-function and dominant-negative alleles. Traditional procedures rely on the generation of transgenic embryos through micro injection of fertilized mouse eggs. Using this approach an unpredictable number of transgene-copies are incorporated at a random location in the recipient genome. This can result in ectopic expression of the transgene because both copy number and the site integration can influence transgene expression.

In the approach described here, a targeting vector that contains exon 1 of the *hprt* gene along with a specific transgene is used to reconstitute Hprt activity in Hprt negative ES cells. Successful targeting of the transgene to the *hprt* locus in Hprt negative ES cells can be selected by growing transfected cells in HAT medium. The efficiency of this selection procedure is such that close to all HAT resistant colonies contain a correctly targeted transgene. Moreover, because the transgene is introduced into the *hprt* locus its expression is dependent upon the inclusion of specific transcriptional regulatory elements and remains relatively unaffected by the local chromatin environment [2]. A major advantage of this targeted transgenic approach compared to traditional transgenics is that transgene expression is predictable so expression at ectopic sites is largely avoided. The availability of F3 Hprt negative ES cells allows transgenic embryos to be derived directly from transgenic ES cells by aggregating the cells with tetraploid embryos and allowing them to develop *in utero*. Each embryo is, in effect, clonal because it is generated from a single clonal ES cell line. This allows reproducible comparison of the phenotype generated by a given transgene at different developmental stages. Moreover, because the transgene is present as a single copy at a known site of integration it circumvents the need to generate many different transgenic lines from a given construct as is the case when using a transient transgenic procedure.

There are many potential uses for this method of generating transgenic mouse embryos. The most obvious is that it provides a mechanism for generating embryos that express alleles that are predicted, or known, to be detrimental to normal embryonic development, for example dominant-negative or gain-of-function alleles. Indeed, the expression of dominant negative alleles that can potentially disrupt multiple members of a gene family has been used extensively in Xenopus laevis and has, for example, helped elucidate the action of fibroblast growth factors during mesoderm formation [19]. Such alleles are particularly useful when a null allele in the same gene causes a disruptive phenotype prior to the stage of development of interest. Choosing a promoter that expresses the dominant negative allele later in development or in a specific cell type could potentially circumvent any early phenotype associated with a null allele.

Another important use of this procedure could be to identify transcriptional regulatory regions that control expression of specific genes during development. Transgenic mice have been extensively used to identify regulatory elements that control gene expression *in vivo*[20,21]. This has, in turn allowed the elucidation of regulatory networks that control cell differentiation during development. In this regard, the use of F3 cells coupled with targeting transgenes to the *hprt* locus offers two major advantages over traditional transgenic approaches. The first is that it is extremely rapid. Once the targeting construct is in hand it takes approximately four to five weeks to generate transgenic mouse embryos. The second advantage is due to the presence of the transgene at a known and constant integration site. This potentially allows the comparison of levels of reporter gene expression driven by different promoters thereby offering a quantitative *in vivo* transgenic assay.

In sum, the availability of tetraploid aggregation-competent ES cells that support targeting of transgenes to the *hprt* locus should facilitate the rapid analysis of transcriptional regulatory elements *in vivo* as well as the phenotypic characterization of alleles that are incompatible with normal development. We, therefore, believe that F3 cells provide a powerful new tool for molecular geneticists.

Materials and Methods

Plasmids

pΔHprt

The targeting plasmid used to delete *hprt* exon1 was called pΔHprt to indicate this deletion. It was derived from the plasmid pOS.DUP/DEL that contained the *neomycin phosphotransferase (neo)* gene that confers resistance to the pharmacological inhibitor G418 and also contains the Herpes Simplex Virus *thymidine kinase (HSV-TK)* that confers sensitivity to gancyclovir. Importantly the *neo* gene is flanked by *loxP* sequences permitting loss of G418 resistance where useful. pΔHprt was constructed by introducing a 2.5 Kb BamHI/HindIII

fragment of 5' *hprt* genomic DNA (E14TG2a origin) from the plasmid pMP8 into the Hpa1 site of pOS.DUP/DEL [15]. The ends of the fragments were made blunt using Klenow. The 3' arm of homology was introduced into the unique Bcl1 site of the previous plasmid as a 7 Kb BamHI fragment from pMP1(SV129 origin) that contained murine *hprt* exons 2 and 3.

pMP8NEBΔLacZ

The plasmid pMP8NEBΔLacZ was generated by ligating an 8 kb Xba1 fragment from pMP8 into the Xba1 site of pNEB193 (New England Biolabs) from which LacZ sequences had been removed as a NarI/EcoRI fragment [15]. This *hprt* replacement vector contains a 5' arm of *hprt* homology consisting of 3.4 kb of mouse 5' *hprt* genomic DNA flanking a unique Not1 cloning site. The 3' arm of homology contains 1.8 kb of the human *hprt* gene that includes exon1 and 2.9 kb of mouse genomic sequence that includes exon 2. A detailed description of the pMP8 targeting vector, from which these sequences were derived, has been described previously [15]. A partial nucleotide sequence of pMP8NEBΔLacZ is available from S.A.D. upon request.

pαMHC-LacZ-Hprt

This targeting vector was constructed by inserting a 9.7 kb αMHCLacZ expression cassette into the NotI site of pMP8NEBΔLacZ. The 9.7 kb αMHCLacZ expression cassette was constructed by inserting a 3.6 kb SalI/HindIII fragment from pLacF containing a *lacZ* gene that incorporates a nuclear localization signal into the SalI/HindIII site of an αMHC expression module.

ES cell culture, gene targeting and selection

All ES cell lines were cultured on mitotically inactivated primary embryonic fibroblasts in ES cell medium supplemented with recombinant leukemia inhibitory factor (LIF) as described elsewhere [22]. F3 cells were generated by introducing 100 μg of Not1 digested pΔHPRT plasmid into 1.5×10^8 R1 ES cells by electroporation at 250 volts/500μf/resistance 8 using a BTX ECM600 electroporation system [7]. Cells containing *neo* were selected by supplementing the ES cell medium with 250 μg/ml Geneticin (G418, Gibco-BRL) and negative selection against *Hsv-tk* gene expression was achieved by including 2 μM gancyclovir (Roche). To identify cells that had lost HPRT activity G418/gancyclovir-resistant colonies were collected and expanded in 96 well plates in the presence of 250 μg/ml 6-thiogaunine. Introduction of transgenes into the *hprt* locus of F3 cells was achieved using the following procedure. F3 cells were grown for two passages in media containing 250 μg/ml 6-thioguanine. 100 μg of linear targeting vector plasmid was introduced into 1.5×10^8 F3 ES cells by electroporation. Cells were grown for 2 days before adding HAT (Sigma) to the growth medium. HAT resistant colonies were harvested after 10 days growth in HAT-containing medium.

Tetraploid and diploid embryo aggregation

ES-cell derived embryos were generated by aggregating clumps of around twenty ES cells with individual eight cell embryos or four cell embryos that had been made tetraploid by electrofusion as described previously [7,9,23]. ES cell:embryo aggregates were cultured overnight and those that formed blastocysts were transferred to the uteri of psuedo-pregnant females surrogate mothers. To generate chimeric mice the embryos were allowed to develop to term. ES cell-derived embryos were harvested at pre-determined developmental stages and DNA prepared for Southern blot analysis or fixed and processed to identify β-galactosidase expression using standard protocols.

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References

1. Sasaki H, Hogan BLM: **HNF-3β as a regulator of floor plate development.** *Cell* 1994, **76**:103-115
2. Bronson SK, Plaehn EG, Kluckman KD, Hagaman JR, Maeda N, Smithies O: **Single-copy transgenic mice with chosen-site integration.** *Proc Natl Acad Sci USA* 1996, **93**:9067-9072
3. Soukharev S, Miller JL, Sauer B: **Segmental genomic replacement in embryonic stem cells by double lox targeting.** *Nucleic Acids Res* 1999, **27**:e21
4. Evans V, Hatzopoulos A, Aird WC, Rayburn HB, Rosenberg RD, Kuivenhoven JA: **Targeting the Hprt locus in mice reveals differential regulation of Tie2 gene expression in the endothelium.** *Physiol Genomics* 2000, **2**:67-75
5. Cvetkovic B, Yang B, Williamson RA, Sigmund CD: **Appropriate tissue- and cell-specific expression of a single copy human angiotensinogen transgene specifically targeted upstream of the HPRT locus by homologous recombination.** *J Biol Chem* 2000, **275**:1073-1078
6. Guillot PV, Liu L, Kuivenhoven JA, Guan J, Rosenberg RD, Aird WC: **Targeting of human eNOS promoter to the Hprt locus of mice leads to tissue-restricted transgene expression.** *Physiol Genomics* 2000, **2**:77-83
7. Nagy A, Rossant J: **Production of completely ES cell-derived fetuses.** *In: Gene targeting: A practical approach Edited by Joyner A. pp. 147–179. Oxford, UK: Oxford Unviersity Press;* 1993147-179
8. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC: **Derivation of completely cell culture-derived mice from early passage embryonic stem cells.** *Proc. Natl. Acad. Sci. USA* 1993, **90**:8424-8428
9. Duncan SA, Nagy A, Chan W: **Murine gastrulation requires HNF-4 regulated gene expression in the visceral endoderm: tetraploid rescue of HNF-4-/- embryos.** *Development* 1997, **124**:279-287
10. Duncan SA, Navas AM, Dufort D, Rossant J, Stoffel M: **Regulation of a transcription factor network required for cell differentiation and metabolism.** *Science* 1998, **281**:692-695
11. Li J, Ning G, Duncan SA: **Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha.** *Genes Dev* 2000, **14**:464-474
12. Hooper M, Hardy K, Handyside A, Hunter S, Monk M: **HPRT-deficient (Lesch-Nyan) mouse embryos derived from germline colonization by cultured cells.** *Nature* 1987, **326**:292-295
13. Tsuda H, Maynard-Currie CE, Reid LH, Yoshida T, Edamura K, Maeda N, Smithies 0, Jakobovits A: **Inactivation of the mouse HPRT lo-**

cus by a 203-bp retroposon insertion and a 55-kb gene-targeted deletion: establishment of new HPRT-deficient mouse embryonic stem cell lines. *Genomics* 1997, **42**:413-421

14. Eggan K, Akutsu H, Loring J, Jackson-Grusby L, Klemm M, Rideout WM 3rd, Yanagimachi R, Jaenisch R: **Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation.** *Proc Natl Acad Sci USA* 2001, **98**:6209-6214
15. Reid LH, Shesely EG, Kim HS, Smithies 0: **Cotransformation and gene targeting in mouse embryonic stem cells.** *Mol Cell Biol* 1991, **11**:2769-2777
16. Lyons GE, Schiaffino S, Sassoon D, Barton P, Buckingham M: **Developmental regulation of myosin gene expression in mouse cardiac muscle.** *J Cell Biol* 1990, **111**:2427-2436
17. Gulick J, Subramaniam A, Neumann J, Robbins J: **Isolation and characterization of the mouse cardiac myosin heavy chain genes.** *J Biol Chem* 1991, **266**:9180-9185
18. Palermo J, Gulick J, Colbert M, Fewell J, Robbins J: **Transgenic remodeling of the contractile apparatus in the mammalian heart.** *Circ Res* 1996, **78**:504-509
19. Amaya E, Musci TJ, Kirschner MW: **Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos.** *Cell* 1991, **66**:257-270
20. Sasaki H, Hogan BL: **Enhancer analysis of the mouse HNF-3 beta gene: regulatory elements for node/notochord and floor plate are independent and consist of multiple sub-elements.** *Genes Cells* 1996, **1**:59-72
21. Epstein DJ, McMahon AP, Joyner AL: **Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms.** *Development* 1999, **126**:281-292
22. Joyner AL: *Gene Targeting: A practical approach. Oxford: Oxford University Press;* 1993
23. Nagy A, Gocza E, Diaz EM, Prideaux VR, Ivanyi E, Markkula M, Rossant J: **Embryonic stem cells alone are able to support fetal development in the mouse.** *Development* 1990, **110**:815-821

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