Perspectives Series: Molecular Medicine in Genetically Engineered Animals

Targeted Mutagenesis: Analysis of Phenotype without Germ Line Transmission

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Summary

The available techniques for directed gene manipulation in the mouse are unprecedented in any multicellular organism and make the mouse an invaluable tool for unraveling all aspects of mammalian biology. To realize fully the potential of these genetic tools requires that phenotypic analysis be efficient, rapid, and complete. Genetic chimeras and mosaics, in which mutant cells are mixed with wild-type cells, can be used to augment standard analysis of intact mutant animals and alleviate the time required and the expense involved in generating and maintaining multiple strains of mutant mice.

Introduction

The mouse has long been the most widely used mammal for studying development and cellular physiology and has provided many useful models for studying human disease. In the last few years, the strength of the mouse as a model system has been dramatically augmented by the development of powerful techniques for manipulating the mouse genome. Most notable among these has been targeted mutagenesis (1), which allows directed alterations to be made and introduced into the mouse germ line. Initially, these mutations were fairly simple gene disruptions, but there are now more sophisticated techniques under development to generate subtle mutations, lineage-specific and inducible mutations, and large-scale genome alterations (2).

With all these diverse capabilities available to generate targeted alterations in the mouse genome, one of the challenges ahead will be determining how to analyze the phenotypic effects in depth in an efficient and cost-effective manner. Transmission through the germ line and examination of the phenotype of the resulting homozygous mutant mice is slow and laborious. Further, the phenotypes may be confusing and the exact site of action of the mutation hard to discern. Generation of homozygous mutant embryonic stem (ES) cells provides a tool to overcome both of these problems. Mutant ES cells allow study of effects on differentiation in vitro, can be used to generate mutant ES-derived embryos to determine phenotype without germ line transmission, and can be "rescued" in chimeras of various sorts, revealing aspects of the phenotype not apparent in the mutants themselves.

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Generation of homozygous mutant ES cells

There are various methods for generating homozygous mutant ES cells once a heterozygous targeted mutant cell line has been generated (Fig. 1). For cell lines in which the targeted allele contains the neomycin resistance gene, the simplest method is to increase the concentration of the antibiotic, G418, thus selecting for cells that have higher neo^r activity (3) (Fig. 1 *A*). Most cells that survive high-dose selection have duplicate copies of the *neo*^{r} allele and lack the wild-type allele. The exact mechanism of reduction to homozygosity in these cells is not clear but seems likely to involve complete or partial chromosome loss and reduplication, rather than a localized gene conversion, in most cases. Some caution should be applied in using such cells as the sole determinant of mutant phenotype, since any chance mutations linked to the targeted allele may also be rendered homozygous and any parental imprinting effects on the chromosome may also become evident.

Direct targeting of the second allele is preferable but entails more work. A second targeting vector carrying a different drug selection marker, such as hygromycin or puromycin resistance, can be made and used to generate homozygous cells (Fig. 1 *B*). The necessity of making a second targeting vector can be avoided by ensuring that the *neo* gene in the first vector is surrounded by loxP sites, the recognition sequence of the Cre site-specific recombinase (4). Heterozygous cells can then be transiently transfected with a Cre-expressing vector to excise *neo* and retargeted with the same *neo*-containing vector (5) (Fig. 1 *C*). With the current excellent ES cells available, cells that have gone through these multiple manipulations remain fully pluripotent.

Finally, new ES cell lines can be made directly from homozygous embryos, after the original mutation has been transmitted through the germ line (Fig. 1 *D*). This does not, of course, provide any assistance in the early analysis of the mutant phenotype, but it does provide cell lines that are unsullied by any defects that may have arisen during passage in culture, and it allows introduction of other marker genes or mutations onto the mutant background by intercrossing mouse lines.

Generation of completely ES-derived fetuses

When ES cells are combined with tetraploid mouse embryos, fetuses and, in the best case scenario, viable offspring can be generated in which all tissues are ES derived (6, 7). This situation is brought about by the impaired capacity of tetraploid cells to contribute to the embryonic lineages, such that survival is only possible if the ES cells take over the embryonic compartment. ES cells have limited ability to make trophoblast and primitive endoderm, the extraembryonic lineages, but the tetraploid cells can survive and populate these lineages. The end result is a fetus with an ES-derived embryonic compartment and tetraploid-derived extraembryonic lineages (Fig. 2). Clearly, this approach has the potential to allow examination of mutant phenotypes derived from genetically altered ES cells very rapidly without the need for germ line transmission and further breeding.

Can this potential be realized in practice? We have shown that the ability of ES cells to generate viable offspring after tetraploid aggregation is very dependent on the quality of the cell line used; survival of ES-derived fetuses beyond term has only been achieved with early passage or selected subclones of cells (7). Thus, this approach is unlikely to be very effective for phenotypes that are viable as homozygotes. However, survival of ES-derived fetuses is much better earlier in gestation, so that ES–tetraploid aggregations can be a valid way of analyzing

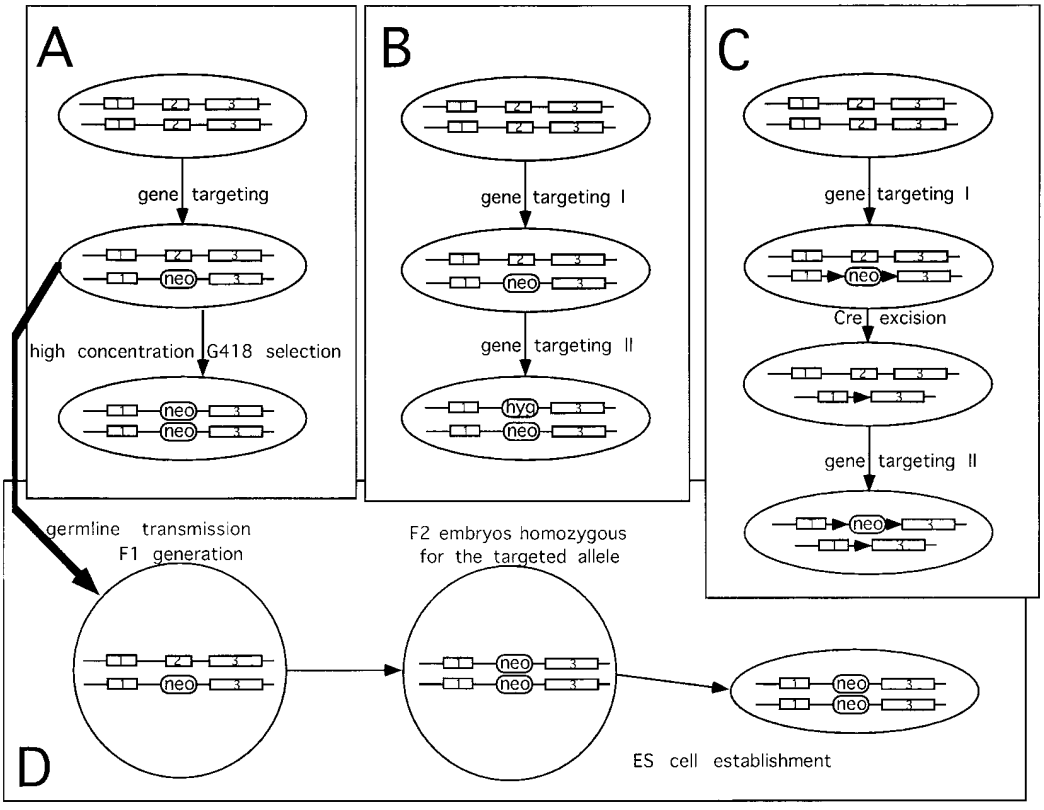

Figure 1. Different strategies to generate homozygous mutant ES cell lines. (*A*) High concentration G418 selection; (*B*) double targeting using different selectable markers; (*C*) removal of the selectable marker by Cre recombinase and targeting again with the same target vector; (*D*) establishment of new ES cell line from F2 embryos homozygous for the mutation.

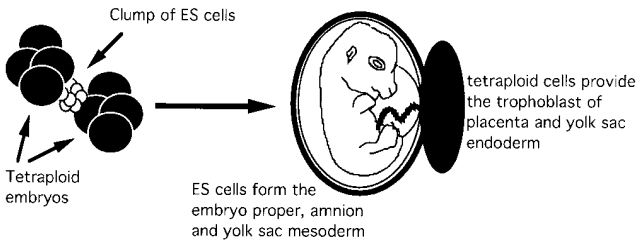

Figure 2. Lineage separation of the tetraploid embryonic and mouse ES cells in chimeras.

embryonic lethal phenotypes. Tetraploid cells are excluded from the embryonic compartment soon after implantation (8), so phenotypes that affect embryonic lineages (but not extraembryonic) between implantation and birth are accessible to analysis. We have recently been able to show that we can replicate the homozygous phenotypes observed in *HNF3β* (9, 10) and *FGFR1* (11, 12) mutants after aggregation of homozygous mutant cells with wild-type tetraploid embryos, thus validating the future use of this approach as a primary screen for mutant phenotype. The embryonic lethal phenotype resulting from complete loss of function of the endothelial growth factor, VEGF, has recently been analyzed by ES–tetraploid aggregation (12a), allowing complete analysis of the mutant phenotype within a much shorter time-frame than by standard analysis. In this particular case, the homozygous phenotype was inaccessible by standard breeding, because heterozygosity for the mutation was also embryonic lethal. The ES–tetraploid system is the only method to assess the effect of homozygosity in such cases.

Again, the availability of excellent ES cells worldwide and the high costs of maintaining mouse breeding stocks should lead to an increased use of this technique in the future, not only for mutant analysis but also for rapid analysis of all kinds of genetic manipulations performed in wild-type or mutant cells.

Lineage-specific mutant analysis

Lineage-specific effects of mutations can be studied by taking advantage of the ability of ES cells to populate lineages that are developmentally compromised in the host embryo. In tetraploid aggregates, it is the whole epiblast lineage that is compromised, making it possible to separate the effects of a particular mutation on the embryonic versus extraembryonic lineage. For example, mutation of the *Mash2* transcription factor gene, which is predominantly expressed in the extraembryonic trophoblast, leads to fetal death, apparently from placental insufficiency in mid-gestation (13). *Mash2* mutant embryos were rescued by aggregation with tetraploid embryos, simultaneously proving that lethality was a direct consequence of trophoblast failure and showing that, in this case, the gene had no obvious role in any other lineage.

There are a number of mutations described in the literature that result in embryonic lethality as a result of likely extraembryonic lineage defects, precluding analysis of later embryonic or adult roles for the genes. For example, mutation in the HNF4 transcription factor causes early embryonic lethality (14), apparently as a result of primitive endoderm defects, precluding analysis of its proposed role in the liver. Similarly, mutation in the EGF receptor, a signaling molecule likely to be used in many different tissues, leads to problems at different stages of gestation, dependent on genetic background (15–17). Again, the main defect causing lethality seems to be in the proliferation of the trophoblast lineage. In both these cases, and others, the ES–tetraploid rescue should allow rescue of the early lethality and elucidation of later defects.

Combination of mutant ES cells with embryos from strains with cell-autonomous defects in specific lineages within the embryo itself can provide more limited lineage-specific rescue, which can be very useful in delineating the roles of a given gene in a particular lineage. The most widely used system of this sort to date is the so-called RAG2 blastocyst complementation assay (18). Mice lacking either RAG1 or RAG2, the recombination-activating genes, contain no mature T or B lymphocytes, because of the failure of VDJ recombination (19, 20). This defect can be rescued by introduction of wild-type ES cells into RAG2-deficient blastocysts: the entire mature lymphocyte population of the resulting chimeras is ES derived. This assay has been used both to examine the effects on lymphopoiesis of mutations that are embryonic lethal in the homozygous state (e.g., *Vav* references 21–23; c-*jun* reference 24) and to analyze rapidly, without germ line transmission, the phenotypic effects of known immune function genes (e.g., *CD40*).

The general approach exemplified by the RAG2 complementation assay could be applied to other lineages, if suitable deficient strains can be identified. Lineage deficiencies that are homozygous viable, like the RAG2 deficiency, would be most suitable, but even deficiencies that are homozygous lethal could be used, provided that easy genotype assays can be developed to identify the one in four chimeras that would have the reconstituted lineage. A number of mutations have been reported to lead to complete loss of certain tissues or organs, e.g., loss of pancreas in insulin promoter factor-1 mutants (25), loss of endothelial cells in *flk1* mutants (26), and loss of cerebellum in *En1* mutants (27). These kinds of mice might be suitable hosts for lineage-specific rescue, if the defects are truly cell autonomous.

Diploid chimera analysis

Although the use of compromised embryos as chimeric partners can be a powerful way of analyzing lineage-specific defects, the generation of chimeras between mutant ES cells and normal diploid wild-type embryos has broader application. When ES cells are aggregated with eight-cell embryos or injected into blastocysts, they become finely intermixed with host cells, generating mosaic embryos and mice in which all cell and tissue types are likely to contain contributions from the two founding cell populations (28). As has been demonstrated over many years in mice and other organisms, such mosaics allow analysis of several aspects of mutant cell behavior not necessarily apparent in the mutants themselves. In the past, two difficulties arose in applying such analysis to lethal mouse mutants. One was the fact that only one quarter of the offspring of a heterozygous cross would be the required homozygous mutants, making identification of the mutant chimeras difficult. Homozygous ES cells solve that problem. The second was the availability of suitable independent cell lineage markers to follow the distribution of normal and mutant cells. While this problem is not completely solved, there are now a number of good cell marker systems, the most common of which is the use of mice carrying the *E. coli β-galactosidase* gene inserted into a widely expressed locus (29).

What kind of information can be gleaned from chimera analysis? First, the cell autonomy of the defect can be ascertained. For example, embryos homozygous for the 413d retroviral insertion die at gastrulation with severe problems in mesoderm generation (30). However, chimeras made with homozygous ES cells survive and show contributions of mutant cells in many different lineages, suggesting that the defect in 413d is not cell autonomous (30). 413d is an insertion in the TGF-β–related *nodal* gene (31), a secreted factor, consistent with the non–cell autonomous nature of the mutation.

Second, more information on the lineage specificity of a defect can be obtained by detailed analysis of mutant cell distribution in chimeras. Exclusion of mutant cells from a given lineage implicates the gene in that lineage. For example, mutation of the *twist* transcription factor leads to failure of neural tube closure (32). Analysis of the distribution of mutant ES cells in chimeras showed that they were excluded from cranial mesenchyme but not neural crest or neural tube, pinpointing a specific defect in the head mesenchyme as the cause of the defects observed.

Third, defects in cell behavior can be seen much more clearly in chimeras than in mutants. Primary defects in cell adhesion or cell migration may become much more apparent when the mutant cells are forced to interact with wild-type cells. For example, the mouse *Brachyury* (T) mutation results in a truncation of the posterior axis and notochord defects (33). Chimeras with marked *T/T* ES cells show an accumulation of mutant cells at the primitive streak and a relative paucity of cells in the nascent mesoderm populations (34). This suggests that one of the main roles of the *Brachyury* gene is to regulate the changing cell adhesion properties necessary for normal gastrulation.

Fourth, rescue of mutant cells in chimeras can allow examination of possible later roles for the gene in question. For example, mutation of GATA-2, a hematopoietic transcription factor, causes lethality in mid-gestation because of severe anemia, suggesting that GATA-2 is required for yolk sac hematopoiesis (35). However, the embryos die too early to reveal any effects on definitive hematopoiesis. Chimeras made with homozygous mutant cells allowed such analysis and showed that definitive hematopoiesis was severely affected, too, implicating GATA-2 as a critical gene in the proliferation of hematopoietic progenitors.

Mosaic analysis: lineage-specific gene knockout and gene repair

Generation of chimeras with mutant ES cells can be a very powerful way of dissecting the multiple roles of genes in development and differentiation. However, the new techniques of genome alteration themselves offer the opportunity to generate mosaic mice with lineage-specific genetic alterations, thus extending the power of this approach. This technology requires two steps. First, a specific alteration is made by homologous recombination in a gene of interest that introduces two loxP sites, either around an essential part of the gene, leaving the gene functional (Fig. 3 *A*), or around an integrating exogenous sequence, interfering with the gene function (Fig. 3 *B*). Second, the Cre recombinase under the control of a lineage-specific promoter is introduced into embryos homozygous for

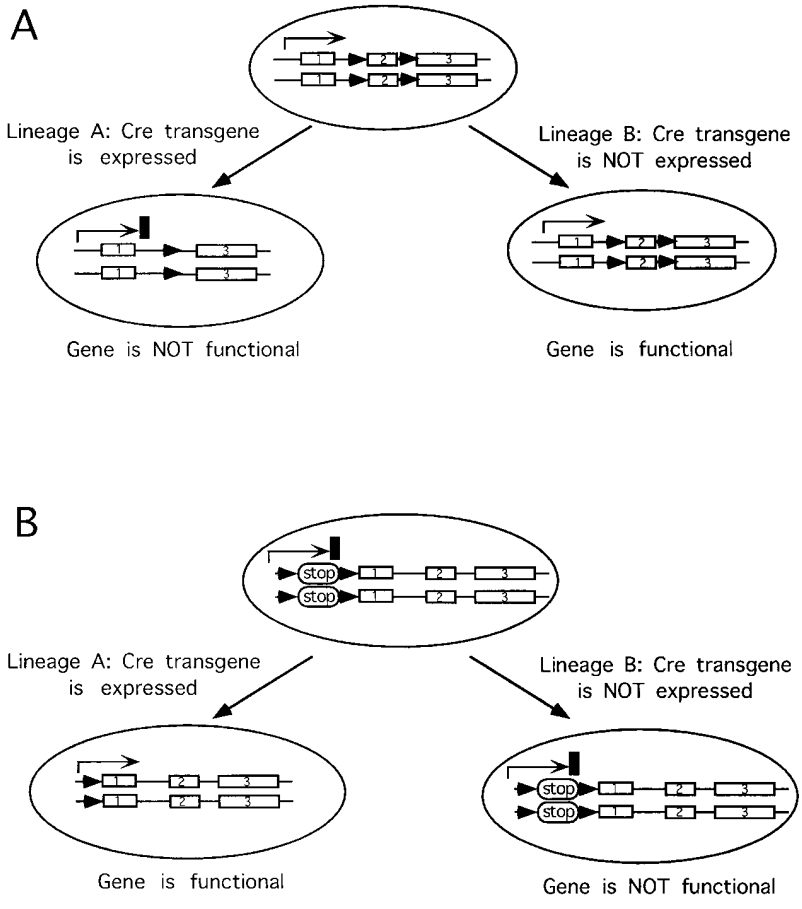

Figure 3. Basic schemes for lineage-specific gene knockout (*A*) and gene repair (*B*) to create genetic mosaicism in the mouse.

the above alteration. The recombinase then excises the essential part (Fig. 3 *A*, lineage-specific gene knockout) or the interfering part (Fig. 3 *B*, lineage-specific gene repair) of the gene, creating a mosaic embryo or animal. Both of these approaches are theoretical extensions of the chimeric complementation assays. They remove the limitation of the complementation chimeras that presently exists because of the small number of available mutant hosts with cell-autonomous ablations. The approach of a lineage-specific knockout or repair is limited only by the need for reliable tissue/cell type–specific expression of the Cre recombinase at a level providing high-fidelity excision in the targeted lineage and gene.

There have been relatively few published cases as yet of successful application of this approach (36, 37), but the creation of an easily accessible panel of *Cre* transgenic lines would be extremely useful for the increasing number of researchers wishing to attempt lineage-specific excision or repair. To this end, one of us (A. Nagy) is organizing a data base listing the *Cre* transgenic lines made, planned, and being produced in many laboratories.

Still more things to do with mutants

Not only do mutant ES cells allow sophisticated phenotypic analysis in vivo without germ line transmission, but they can, of course, be used to study effects on differentiation in vitro under various conditions, as already described in this series (38). From such differentiating ES cells, it may be possible to isolate other lines of more restricted stem cells that could be extremely useful tools in dissecting genetic hierarchies of lineage control. Mutant primary fibroblasts and other cells and tissues can also be obtained directly from mutant embryos, even those showing early lethality, and can be very informative in understanding the cell biology underlying a mutant defect. Such analysis need not mean maintaining large numbers of heterozygous mice, since essentially unlimited supplies of homozygous embryos can be generated by ES–tetraploid aggregation. In the future, we can expect to see increased exploitation of techniques of experimental embryology and cell biology, in combination with the power of targeted mutagenesis, to provide unprecedented insights into all aspects of mammalian biology.

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