Minireview

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Altering Mice by Homologous Recombination Using Embryonic Stem Cells*

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The ability to make genetic changes in the mouse germline in a predetermined way has resulted from the combination of two technologies developed during the past 10 years. The first of these technologies is the mouse embryonic stem (ES)[1] cell system. ES cells (1, 2) are derived from the inner cell mass of a mouse blastocyst and remain undifferentiated under suitable tissue culture conditions. Yet when these *in vitro* cultured cells are introduced into a blastocyst for continuation of their development *in utero* they can yield mouse chimeras able to transmit the ES cell genome to their offspring (3). Genetic changes introduced into ES cells in tissue culture can therefore be transferred into live mice (4–6) by breeding the chimeras and screening offspring for the ES genotype (usually by coat color). The second technology, gene targeting, uses homologous recombination to alter a chosen gene in a predetermined way (7, 8). Gene targeting in ES cells provides the capability of selectively altering genes in the mouse (9, 10). In what follows we consider various applications of this process. Our review does not attempt to reference all reported applications of gene targeting to alter genes in animals but rather to exemplify the major categories of its usage. Other recent reviews include Refs. 11–16.

Gene Disruptions

The most frequent application of gene targeting is the "gene knockout," an event mediated by homologous recombination, resulting in the disruption of a specific gene. There are two main types of gene-targeting vectors, insertion and replacement, with several design options available. Insertion (O-type) vectors mediate a homologous recombination event with a single crossover, during which the regions of homology are duplicated (see Fig. 1); this duplication of sequences is essential for some types of gene targeting, such as "hit and run"/"in-out" and gene duplication (see below). Replacement (Ω-type) vectors are more commonly used for simple gene disruptions (Fig. 1*B*). The two regions of homology (the arms of the Ω), preferably designed to eliminate some of the target gene, are generally separated by a selectable marker gene such as a *neo*^{R} gene in the loop of the Ω (**S1** in Fig. 1*B*).

Vectors are usually transferred to ES cells via electroporation, and cells that contain the targeting construct are identified by their survival in the presence of a drug such as G418 (an aminoglycoside related to neomycin). This is referred to as *positive* selection. The fact that most cells surviving the **S1** selection will have integrated the targeting vector randomly by non-homologous recombination into the genome, rather than at the target locus by homologous recombination, led to the development of a negative selection scheme (17). Inclusion of another selectable marker (**S2** in Fig. 1*B*) in the targeting vector just outside one of the regions of homology allows for concomitant *negative* selection. The herpes simplex thymidine kinase (TK) gene in conjunction with the drug ganciclovir has been very successful in this context, as was originally described (17). Most of the cells taking up the targeting vector randomly into their genomes retain and express the TK gene; they are consequently killed by ganciclovir. In the much smaller proportion of cells that integrate the vector by homologous recombination, the TK gene is lost in the homologous crossover event, allowing the targeted cells to survive in the presence of ganciclovir. Despite this double enrichment for targeted cells, a DNA screening method is usually required to identify correctly targeted cells. When the targeting frequency is high, the screening can be done by Southern blot analysis. In less favorable cases, a polymerase chain reaction-based recombinant fragment assay is more efficient (18).

Several hundred different mutant mouse lines have now been generated by the gene disruption mode of targeting in ES cells. While most of the disruptions result in a null allele, this is not always the case. Depending on the details of the targeting scheme, the resulting mice may produce no functional gene product, a truncated product, or an alternatively spliced product. There is a difference in the severity of the phenotype between mice unable to synthesize the cystic fibrosis transmembrane receptor gene product (19–21) and mice having a disrupted gene that can be alternatively spliced and is consequently "leaky" (22). Similarly, homozygotes for two different targeted mutations in the N-*myc* gene result in either no detectable N-*myc* (23–25) or 25% of normal levels of the protein (26). The null mutation is lethal at embryonic day 10.5; homozygotes for the leaky mutation display perinatal lethality. Compound heterozygotes of the null mutation and the leaky mutation have N-*myc* levels approximately 15% of normal and have a correspondingly intermediate phenotype (27).

Not surprisingly, the phenotypes of mice with null mutations range from having little effect on the health and viability of the mutant animal to being lethal in early embryonic life. An example of the former class is the mice resulting from inactivation of the gene coding for β_2-microglobulin, a protein subunit of the major histocompatibility class I molecules (28). These mice have only very low levels of class I proteins on cell surfaces and a virtual absence of CD8+ T-cells, which are dependent on class I proteins for their development in the thymus; yet the animals are born normal and show no obvious phenotype. Despite the easily detectable alterations in the immune system, only when the animals were studied more extensively did it become apparent that the mice have increased susceptibility to a small subset of infectious agents (29). In contrast, disruption of the gene coding for GATA-1, an erythroid-specific transcription factor, proved to be lethal in early fetal development (30). In such cases of lethality, ES cells that are homozygous or hemizygous for a given targeted mutation can be injected into blastocysts and their fate can be determined. In this example, in which, the gene for GATA-1 is X-linked and the ES cells were male, the hemizygous mutant ES cells contributed to most tissues, but only cells of the wild-type host blastocyst were detected in the erythroid compartment of the resulting chimeric embryos. Thus, lack of the GATA-1 gene product precludes normal erythroid development, although the mutant cells can contribute normally to many other tissues.

In most cases a disrupted gene will be autosomal so that the use of chimeras to analyze severe mutations will be most informative if the ES cells are first made homozygous for the disruption. This has been achieved in two ways. One way is to use a hygromycin resistance gene as a selectable marker for targeted disruption of the second allele (31, 32). Another way of obtaining ES cells containing homozygous disruptions is by selecting cells that survive in the presence of an increased concentration of G418; often among the survivors are colonies that have two copies of the *neo*^{R} gene as a consequence of becoming homozygous for the mutation (33, 34). Homozygous gene disruptions in ES cells obtained by either method are valuable reagents for *in vitro* studies (see below) or for direct injection into blastocysts.

Gene Modifications

While gene disruptions demonstrate the effect of the absence of gene function during normal development and can model human

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[1] The abbreviations used are: ES, embryonic stem; TK, thymidine kinase; HPRT, hypoxanthine phosphoribosyltransferase; HAT, hypoxanthine-aminopterin-thymidine.

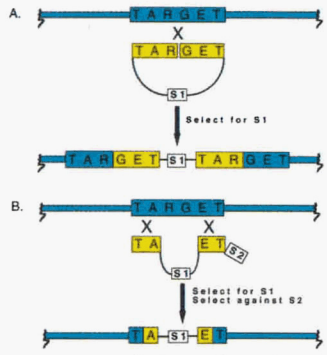

FIG. 1. ***A* represents a generic insertion (O-type) targeting event, and *B* represents a generic replacement (Ω-type) targeting event.** The following conventions are shared by all figures. The endogenous chromosome and the target locus are shown in *blue* and the targeting constructs in *yellow* or *gray*. Continuity with chromosomal DNA is indicated by *vertical jagged lines*. Plasmid sequences and selectable markers are shown in *black* and *white*. The resulting targeted locus is shown below the *arrow*. Selection for **S1**, or against **S2**, does not influence the recombination event but allows the selective survival of cells that have undergone the desired event. Note that the crossovers (*X*) can occur anywhere within the region of homology; the crossover points depicted are arbitrary.

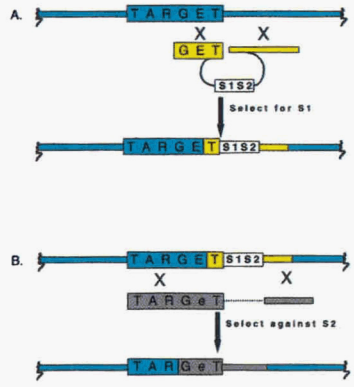

FIG. 2. **The incorporation of a subtle change (“*E*” to “*e*”) and the subsequent removal of selective sequences using a repeatable targeting scheme.** The final locus is the result of two consecutive targeting events, both of the replacement type. The first event (*A*), which is a conventional targeting event that introduces **S1** and **S2** near the target gene, need not be repeated. The second targeting event (*B*) can be efficiently repeated to incorporate many different types of changes, all accompanied by removal of the selectable marker sequences.

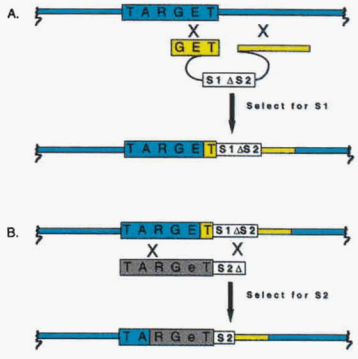

FIG. 3. **The incorporation of a subtle change (“*E*” to “*e*”) using the recombination-dependent positive selection in a repeatable targeting scheme.** The final locus is the result of two consecutive targeting events (*A* and *B*), both of the replacement type. The first conventional targeting event (*A*) introduces **S1** and **ΔS2** near the target gene. The second targeting event (*B*) can be efficiently repeated to incorporate many different types of changes. Note that positive selection is used in this repeatable step and that **S2** function is only generated by homologous recombination allowing detection of difficult targeting events.

disorders caused by recessively inherited single gene defects, they generally are not suitable for analyzing or modeling the effects of an altered gene product. However, homologous recombination can be used to modify gene products in a variety of increasingly sophisticated ways. In a simple form the targeting construct is of the replacement type, having a positively selectable gene inserted either upstream of the promoter of the target gene or downstream of the poly(A) addition site. The method has been used in tissue culture to change the human sickle cell β^S globin mutation to the normal β^A form (35). The method has also been used to create a single amino acid change in the proopiomelanocortin gene in ES cells (36).

A colorful variation on this method involves the insertion of the marker gene *lacZ* into the proto-oncogene *int-2* by homologous recombination, creating an *int-2-lacZ* fusion gene (37). The resulting *lacZ* expression parallels the expression of wild-type *int-2* in *in vitro* differentiated ES cells. This type of experiment is an effective way of observing the expression patterns of a particular gene and can also mark certain cell types for the study of cell lineage *in vivo*.

Repeated Targeting at a Single Locus

In many situations, it is desirable to study a series of changes in the structure of a target gene. The difficulty of obtaining multiple conventional targeting events has resulted in several schemes for repeatedly targeting a locus (38–41). All these schemes employ conventional gene targeting to convert the target locus into a modified form, which then allows a second targeting event to be carried out repeatedly with the help of a more favorable system of selection. Fig. 2 illustrates the scheme employed by Askew *et al.* (38) in their “tag and exchange” system and by Stacey *et al.* (39) and Wu *et al.* (40) in their “double replacement” system. The first targeting step, which need only be carried out once, uses conventional gene targeting with positive selection for **S1** to introduce a negatively selectable marker gene **S2** into or close to the target gene. The second targeting step, which is readily repeated with minor variations, then uses negative selection against the function of **S2** to obtain the altered locus. Askew *et al.* (38) chose the neo^R gene for **S1** in conjunction with G418 selection and the herpes simplex virus TK gene for **S2** in conjunction with ganciclovir selection. A potential drawback when using the TK gene for **S2** is that males having a TK gene expressed in their testes are likely to be infertile (42). Consequently, it may be difficult to test the germline competence of the tagged ES cells and to obtain useful animals before completing step 2. Instead of **S1** and **S2**, Stacey *et al.* (39) used a single gene (a hypoxanthine phosphoribosyltransferase (HPRT) minigene) in $HPRT^-$ ES cells in conjunction with hypoxanthine-aminopterin-thymidine (HAT) medium for the first step positive selection and used 6-thioguanine medium for the second step negative selection. (When an HPRT minigene is used the ES cells must be $HPRT^-$ (6).)

Schemes of the type shown in Fig. 2 have the advantage that the final modified locus does not retain any sequences derived from **S1** or **S2** (see also below). A potential disadvantage is that *loss* of **S2** gene function can occur in a variety of ways in addition to the gene loss accompanying the desired second step recombination. Judging from the published numbers of non-targeted colonies observed after the second step, this disadvantage is less important with the HPRT minigene than with the TK gene. This might be expected, since positive selection to ensure retention of HPRT gene function can be maintained right up to the time of the second electroporation.

Fig. 3 illustrates a “plug and socket” system developed at the University of North Carolina, which employs recombination-dependent positive selection for *gain* of function in both steps (41). The neo^R gene in conjunction with G418 selection is used conventionally as **S1** for the first step. **S2**, in this case an HPRT minigene, is divided into two individual non-functional but overlapping parts. One part, **ΔS2** (the “socket”) is non-functional because it lacks the 5′ end of the HPRT gene; it is on the first targeting vector and so is introduced near the target locus in step one. The other part of the HPRT minigene, **S2Δ** (the “plug”) is non-functional because it lacks the 3′ end of the HPRT gene; it is on the second targeting vector. Only when these two recombine by homologous recombination between their overlapping portions is a complete functional **S2** gene obtained, making the targeted cells HAT-resistant. Many different plugs can be inserted into the same socket. The chief advantage of the scheme is the power of this positive selection for recombinants between **S2Δ** and **ΔS2**, neither of which can become functional without homologous recombination. This precludes background colonies due to non-homologous events, and as a result difficult second targeting events can be achieved. Disadvantages are that the gene **S2** remains near the target locus at completion of the

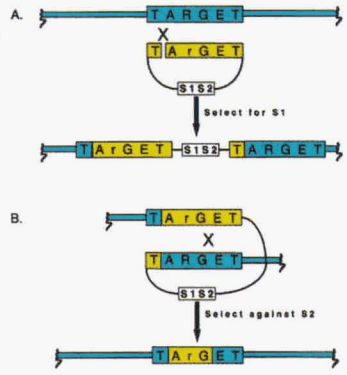

FIG. 4. **A hit-and-run/in-out targeting event that changes "*R*" to "*r*" in two steps.** The first step (*A*) is a conventional insertion (O-type) targeting event that adds the sequence **TArGET** to the endogenous locus; targeted cells survive selection *for* S1. The second step (*B*) is an intrachromosomal recombinational event that can occur spontaneously, although at a low frequency; recombinant cells survive selection *against* S2. The substitution of "*r*" for "*R*" results when crossovers occur as depicted by the *X*.

targeting, and constraints on the placement of the **S2** gene may restrict the types of change that can be effected.

Eliminating Selectable Marker Sequences

The problem of modifying a target gene without leaving any of the selectable marker sequences in the locus has been approached by Hasty *et al.* (43) in their "hit and run" scheme and by Valancius and Smithies (44) in their "in and out" scheme, both of which are illustrated in Fig. 4. The vector introduces the desired modification together with a negatively selectable gene **S2** as a consequence of a conventional insertion (O-type) targeting event; this event is achieved with the help of positive selection for the marker gene **S1**. Note that (as in all insertion type events) the homologous sequences are duplicated during this step. The second event, which eliminates the selectable gene, occurs as a consequence of a spontaneous intrachromosomal (or sister chromatid) exchange between the sequences that were duplicated during the first insertion event. Although spontaneous deletions of this type are infrequent events, selection against **S2** function allows their recovery.

Hasty *et al.* (43) used the *neo*^{R} gene for **S1** and the TK gene for **S2**. Valancius and Smithies (44) used a single HPRT minigene for **S1** and **S2** with HAT medium for the positive selection and 6-thioguanine-containing medium for the negative selection. Either method can be used to generate a wide range of mutations, from single base substitutions to the exchange or removal of large regions of regulatory or coding sequences, without leaving any unwanted sequences in the final modified locus.

Another method of eliminating selectable marker sequences takes advantage of a site-specific recombinase system. Both the FLP recombinase system from yeast (45, 46) and the Cre-*loxP* system from bacteriophage P1 (47) have been demonstrated to function in ES cells with similar applications. Targeting constructs for this type of application incorporate the necessary cis-acting sequences so that they flank the selectable marker. After conventional targeting of the locus by homologous recombination, the ES cells are transfected with a vector expressing the recombinase. Cells that have undergone site-specific recombination and have therefore deleted the marker sequences can be identified by Southern analysis or polymerase chain reaction. In contrast to the "in and out" and "hit and run" strategies, the recombinase systems leave behind a single copy of the recombination sequences, approximately 50 base pairs in length, but recombinase-mediated excision of selectable marker sequences is very efficient.

It is important to remember that the structure and behavior of each individual locus, as well as the specific genetic modification that is desired, will influence the type and design of the targeting vector; all methods are not suitable for all applications. Experiments involving removal of selectable marker sequence are more complicated than those in which the selectable marker remains. Marker removal may not be necessary in many situations.

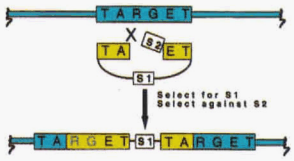

FIG. 5. **The duplication of a locus via a gap-repair targeting scheme.** Sequences generated as a result of repairing the gap are shown in *yellow* with *blue lettering*. The gap can be >15 kilobases.

Gene Duplications

Complex genetic diseases, such as atherosclerosis and essential hypertension, have a mixed genetic and environmental etiology. Many genes contribute to the wide range of phenotypes observed. A general method for investigating in animals the effects of increasing the levels of a gene product without altering the nucleotide sequence, chromosomal location, or regulatory elements of the gene of interest is likely to be of great value in studying these complex diseases. Fig. 5 illustrates a "gap-repair" targeting scheme that generates a tandem duplication of a gene, thereby allowing the production of animals with three or four gene copies. The targeting construct is an insertion (O-type) vector that uses positive selection (**S1**) and negative selection (**S2**) to accomplish the targeted gene duplication. Several features of the targeting construct are important. The construct must include two regions of homology, one from upstream (5′) and one from downstream (3′) of the target locus. (**TA** and **ET** represent these sequences in Fig. 5.) In order to maximize the probability that both copies of the tandemly duplicated gene will be functional, **TA** and **ET** must extend far enough upstream and downstream of the target gene so that all known regulatory sequences lie between their respective 5′ and 3′ ends. Because double strand gap repair can fill in a gap during an O-type targeting event (48, 49), the construct can include a large gap and so can be considerably shorter in length than the gene that is to be duplicated.

A duplicated gene can mimic up-regulation of an endogenous gene, allowing the effects of an increase in gene product to be investigated. Duplicated genes, produced by gap-repair, have a distinct advantage over randomly integrated transgenes in that they are created at the original locus. Consequently they are expected to share the same chromosomal effects (heterochromatization, methylation, DNase hypersensitivity, etc.) as the normal gene. Comparison of mice from a line carrying a duplication of a gene of interest with mice from a line carrying a disruption of the same gene allows one to observe animals having from zero to four copies of the desired gene. Application of this scheme to the gene coding for angiotensinogen has resulted in first generation (F1) animals with steady state plasma angiotensinogen levels ranging from 35% (1 gene copy) to 124% (3 gene copies) of normal levels (50). The second generation (F2) animals provide zero and four copy animals having no plasma angiotensinogen and 148% of normal levels.[2]

Single-copy, Single-position Transgenes

A tremendous amount of valuable information has come from studies of transgenic animals made by injecting DNA into the pronucleus of fertilized eggs (for review see Ref. 51). However, in any situation where exogenous DNA is integrated randomly into the genome, there are three potential problems: insertion of the transgene into an essential gene, variable transcription of the transgene due to position effect, and unpredictable copy number. Targeting of transgenic sequences to a specific locus by homologous recombination in ES cells avoids these three hazards and facilitates the production of many different types of transgenes.

The HPRT locus is a very useful locus for such targeted transgenes and can take advantage of the partially deleted HPRT locus available in the E14TG2a ES cell line (6). The junction sequences of the partial deletion are well characterized and have been used previously to create an Ω-type targeting vector (52). This vector has 5′ and 3′ homology fragments from 5′ and 3′ of the deletion breakpoint, respectively. The central portion of the targeting construct is arranged to contain both the missing portion of the HPRT gene and

[2] H. S. Kim, J. H. Krege, K. D. Kluckman, J. R. Hagaman, J. B. Hodgin, C. F. Best, J. C. Jennett, T. M. Coffman, N. Maeda, and O. Smithies, submitted for publication.

the coding and regulatory sequences of the desired transgene. Introduction of sequences complementary to the deletion restores function to the HPRT gene; homologous recombination events at the locus can therefore be directly selected in HAT media. The overall result is the insertion of a single copy of the transgene into the HPRT locus. Several different regulatory and coding sequences have been inserted into the HPRT locus using this method and lead to moderate levels of promoter-specific transcription in the resulting mice.[3] Study of the effect of changing the promoters is then possible free from the complications of position effects and variable copy numbers.

Advantages of ES Cells

The major advantages of using ES cells for genetic modifications are that they are easy to culture, are readily manipulated by homologous recombination *in vitro*, are amenable to a wide variety of selection and screening schemes to detect relatively rare homologous recombination events, and can be used to make genetic changes in the mouse. They have several other advantages. For example, chimeras generated by the injection of ES cells into blastocysts provide an elegant way of directly comparing the fate of modified cells and wild-type cells in different cell lineages. Hermiston *et al.* (53) have demonstrated the use of such animals in the analysis of intestinal epithelial cell lineages. Intestinal crypts are derived from the differentiation of single cells in the intestinal mucosa, and cells derived from wild-type and mutant cell lineages of a chimeric animal can be observed in adjacent crypts. Similarly, modified ES cells can be injected into blastocysts derived from a genetically modified mouse line allowing direct *in vivo* comparison of two different mutant lineages, as in the RAG-2-deficient blastocyst complementation assay described by Chen *et al.* (54). (The RAG-2-deficient blastocysts provide a B- and T-cell negative background against which lymphoid-specific modifications can be analyzed.)

Another important feature of ES cells is their ability to differentiate into a variety of tissue types *in vitro* via the formation of embryoid bodies (55). All three germ layers have been observed, and several different types of cells can be derived from embryoid bodies, including hematopoietic cells, nerve cells, myocardium, and cartilage (56). It seems likely that further knowledge of the role of various culture components in determining the efficiency and specificity of differentiation of ES cells into specialized tissues will result in a number of very valuable assay systems in which the wide variety of targeted modifications achievable in these cells can be evaluated *in vitro*, alleviating the need to go through the complex steps necessary to generate an animal.

Conclusion

Manipulation of the mouse germline through the genetic modification of embryonic stem cells is clearly a very useful and extremely versatile tool for analyzing mammalian development and physiology and for creating models of human diseases. In addition to the widely utilized method of gene disruption, homologous recombination can mediate many other types of genetic changes, including both qualitative and quantitative modifications, making it practical to analyze the effects of many types of genetic changes on complex traits. The versatility of genetically modified ES cells is facilitating the rapid expansion of a very informative pool of induced mutations in mice and is allowing important questions to be addressed *in vivo* regarding gene expression and protein function and their impact on normal and disease states.

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