replicating the infrequent (1 in 50,000) but simply inherited human genetic disease cystic fibrosis, and some of the hemoglobinopathies (1 in 100–1,000), and progressing to using the method to decipher the genetic complexities of much more common but also much more complex conditions, such as atherosclerosis (1 in 2) and hypertension (1 in 5)[30]. We had an important conceptual change early in our studies of the genetic complexities of hypertension. This was a shift from considering absence of gene function as a principal cause of disease (as is the case with the uncommon disease cystic fibrosis) to considering the possibility that inherited quantitative variations (perhaps even normal variations) in gene expression might be more important in causing the complex common conditions. To investigate this possibility experimentally, we devised a 'gene-titration' method, in which two complementary forms of homologous recombination are used to vary the number of copies of a candidate gene from one through four[31]. The 'one-copy' animals are heterozygous for a wild-type allele and a deleted copy. The 'three-copy' and 'four-copy' animals use a complete tandem gene duplication reminiscent of *Hp2*. In the most dramatic of these experiments, the resulting gene expression varies linearly with copy number from ½× to 2× normal. With the current emphasis on single-nucleotide polymorphisms and functional genomics, it is likely that mice obtained by homologous recombination will prove to be of great value in establishing whether a genotype associated in humans with a complex phenotype could in fact cause the condition.

Obviously I continue to enjoy using the tool for which we are being honored to solve problems of interest to me. And when I open any current issue of the main journals covering biological science, I am very likely to have the vicarious enjoyment of seeing some other investigators' use of homologous recombination to modify their chosen gene in the mouse genome.

Generating mice with targeted mutations

Mario R. Capecchi

Mutational analysis is one of the most informative approaches available for the study of complex biological processes. It has been particularly successful in the analysis of the biology of bacteria, yeast, the nematode worm *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. Extension of this approach to the mouse, though informative, was far less successful relative to what has been achieved with these simpler model organisms. This is because it is not numerically practical in mice to use random mutagenesis to isolate mutations that affect a specified biological process of interest. Nonetheless, biological phenomena such as a sophisticated immune response, cancer, vascular disease or higher-order cognitive function, to mention just a few, must be analyzed in organisms that show such phenomena, and for this reason geneticists and other researchers have turned to the mouse. Gene targeting, the means for creating mice with designed mutations in almost any gene[32], was developed as an alternative to the impractical use of random mutagenesis for pursuing genetic analysis in the mouse. Now gene targeting has advanced the genomic manipulations possible in mice to a level that can be matched only in far simpler organisms such as bacteria and yeast.

The development of gene targeting in mice required the solution to two problems: How to produce a specific mutation in a chosen gene in cultured mammalian cells, and how to transfer this mutation to the mouse germ line. Oliver Smithies' laboratory and mine worked independently on solutions to the first problem. Martin Evans' laboratory provided the basis for a solution to the second problem.

Early experiments

Our entry into what became the field of gene targeting began in 1977. At that time, I was attempting to improve the efficiency with which new genes could be introduced into mammalian cells. It had just been demonstrated by Wigler and Axel that cultured mammalian cells deficient in thymidine kinase (Tk^-) could be transformed to Tk^+ status by the introduction of a functional copy of the herpes thymidine kinase gene (*HSV-tk*)[33]. Although an important advance for the field of somatic cell genetics, their protocol—the use of calcium phosphate co-precipitation to introduce the DNA into cultured cells—was not efficient. With this method, incorporation of functional copies of *tk* occurred in only one per million cells exposed to the DNA–calcium phosphate co-precipitate. Using a similar selection scheme, I sought to determine whether I could introduce a functional *tk* into Tk^- cells using very fine glass needles to inject DNA directly into nuclei[34]. This procedure proved extremely efficient. One cell in three that received the DNA stably passed the functional *tk* to its daughter cells. The high efficiency of DNA transfer by microinjection made it practical for investigators to generate transgenic mice containing random insertions of exogenous DNA. This was accomplished by injection of the desired DNA into nuclei of one-cell zygotes and allowing these embryos to come to term after surgical transfer to foster mothers[35–39].

Efficient functional transfer of *HSV-tk* into cells required that the injected *tk* be linked to other short viral DNA sequences[34]. It seemed plausible that highly evolved viral genomes might contain bits of DNA that enhance their ability to establish themselves within mammalian cell genomes. I searched the genome of the lytic simian virus SV40 for the presence of such sequences and found one near the origin of viral DNA replication. When linked to *HSV-tk,* it increased the transforming capacity of the injected *tk* by 100-fold. I showed that the enhancement did not seem to result from independent replication of the injected *HSV-tk* DNA as an extra-chromosomal plasmid, but rather that the efficiency-enhancing sequence was either

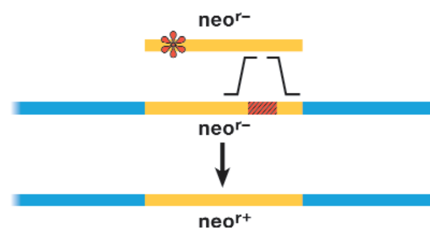

Fig. 1 Regeneration of a functional *neo*^{r} by gene targeting. The recipient cell contains a defective *neo*^{r} with a deletion mutation (*). The targeting vector contains a 5′ point mutation. With a frequency of 1 in 1,000 cells receiving an injection, the deletion mutation in the chromosomal copy of *neo*^{r} is corrected with information supplied by the targeting vector.

increasing the frequency with which the exogenous DNA was integrated into the host genome, or increasing the probability that *tk,* once integrated, was being expressed in the recipient cells. These experiments were completed before the idea of gene expression 'enhancers' had emerged and contributed to the definition of these special DNA sequences[40]. The emerging idea of enhancers profoundly influenced our contributions to the development of gene targeting by alerting us to the importance of using appropriate enhancers to mediate expression of newly introduced selectable genes regardless of the inherent expression characteristics of the host site to which they were targeted.

Homologous recombination

The observation I found most fascinating from these early DNA microinjection experiments was that when many copies of the *tk* plasmid were injected into cells, they were integrated in only one or two loci within any host cell's chromosome, and that multiple copies at those random sites were always present as head-to-tail concatemers. We reasoned that such highly ordered concatemers could only be generated either by replication (for example, a rolling circle-type mechanism) or by homologous recombination between plasmids. We proved that they were generated by homologous recombination[41]. This conclusion was very significant because it demonstrated that mammalian somatic cells contained an efficient enzymatic machinery for mediating homologous recombination. The efficiency of this machinery became evident from the observation that when more than 100 *tk* plasmid molecules were injected per cell, they were all incorporated into a single, ordered, head-to-tail concatemer. It was immediately apparent that if we could harness this efficient machinery to accomplish homologous recombination between a newly introduced DNA molecule of our choice and the same DNA sequence in a recipient cell's genome, we would have the ability to mutate or modify almost any cellular gene in any chosen way.

Our next step in the quest for gene targeting required our becoming familiar with this machinery; specifically, with its substrate preferences and reaction products. By examining recombination between co-injected DNA molecules, we learned, among other things, that linear DNA molecules were the preferred substrate for homologous recombination; that recombination was cell cycle-dependent, showing a peak of activity in early S phase; and that although both reciprocal and nonreciprocal exchanges occurred, there was a distinct bias toward the latter[42–44]. These results contributed substantially to our choice of experimental design for the next stage of this quest: the detection of homologous recombination between newly introduced, exogenous DNA and its chromosome homolog.

In 1980, we submitted a grant proposal to the National Institutes of Health to test the feasibility of gene targeting in mammalian cells; these experiments were rejected on the grounds that there was only a vanishingly small probability that the newly introduced DNA would find its matching sequence within a host cell genome. Despite the rejection, I decided to continue this line of experimentation. Aware that the frequency of gene targeting was likely to be low, and that the far more common competitive reaction would be insertion of the targeting vector at various sites other than the target locus, we proposed to use selection to eliminate cells not containing the desired homologous recombination products. The first test (Fig. 1) used artificially introduced chromosomal target sites. The first step of this scheme required generation of cell lines containing random insertions of a defective neomycin-resistance gene (*neo*^{r}) containing either a deletion or a point mutation. In the second step, target vector DNA carrying defective *neo*^{r} genes with different mutations was introduced into cells of those lines. Homologous recombination between *neo*^{r} sequences in the targeting vector and recipient genome could generate a functional *neo*^{r} from the two defective parts, producing cells resistant to the drug G418, which is lethal to cells without a functional *neo*^{r}.

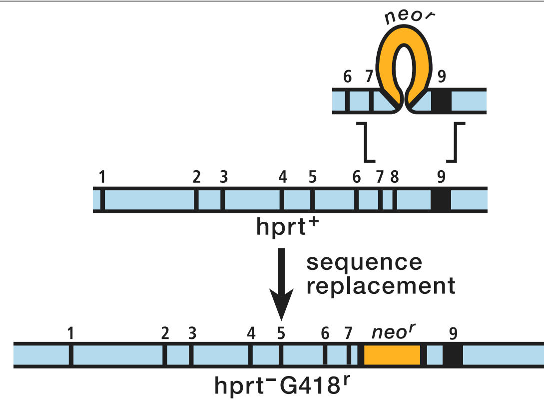

Fig. 2 Disruption of *Hprt* by gene targeting. The vector contains *Hprt* sequences disrupted in the eighth exon by *neo*^{r}. After homologous pairing between the vector and genomic sequences, a homologous recombination event replaces the genomic sequence with vector sequences containing *neo*^{r}. These cells are able to grow in medium containing the drugs G418 and 6-TG.

In the first step, we generated recipient cell lines containing single copies of the defective *neo*^{r}, lines containing multiple copies of the gene in head-to-tail concatemers and, by inhibiting concatemer formation, lines with multiple defective *neo*^{r} targets, each located on separate chromosomes. These different recipient cell lines allowed us to evaluate how the number and location of targets within the recipient cell's genome influenced the targeting frequency. By 1984 we had good evidence that gene targeting in cultured mammalian cells was indeed possible[45]. At this time I resubmitted our grant to the same National Institutes of Health study section that had rejected our earlier grant proposal and their critique began with the phrase "We are glad that you didn't follow our advice."

To our delight, correction of the defective chromosomal *neo*^{r} occurred at an absolute frequency of 1 per 1,000 cells receiving an injection. This frequency was not only higher than we expected, but allowed us to accomplish multiple analyses of the experimental parameters that could influence the gene-targeting reaction[44]. An additional important lesson from these experiments was that all chromosomal target positions analyzed seemed to be equally accessible to the homologous recombination machinery, indicating that a large fraction of the mouse genome could be modified by gene targeting.

At this time, Oliver Smithies and his colleagues reported their classic experiment of targeted modification of the β-globin locus in cultured mammalian cells[18]. This elegant experiment demonstrated that it was feasible to disrupt an endogenous gene in cultured mammalian cells. Having established that gene targeting could be achieved in cultured mammalian cells and having determined some of the parameters that influenced its frequency, we were ready to extend the approach to the whole mouse. The low frequency of targeted

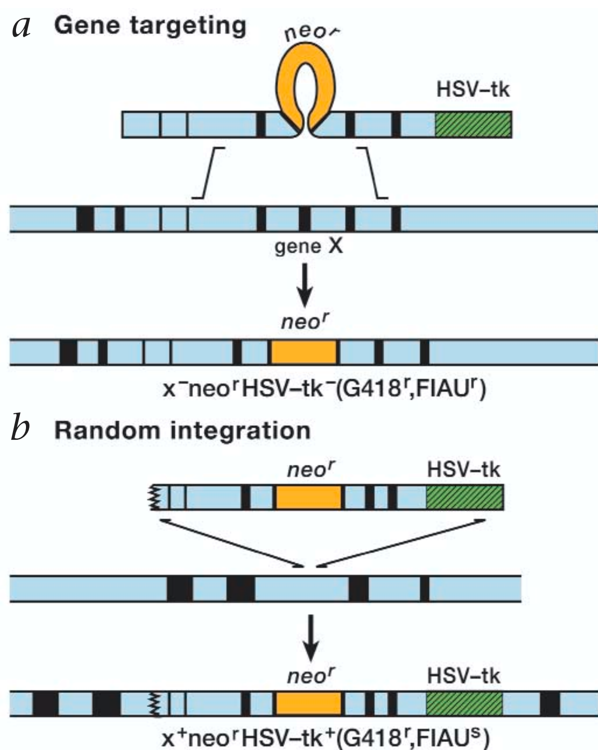

Fig. 3 The positive–negative selection procedure used to enrich for ES cells containing a targeted disruption of gene X. ***a***, The replacement-type vector contains an insertion of *neo*^{r} in an exon of gene X and a linked *HSV-tk* at one end. It is shown pairing with a chromosomal copy of gene X. Homologous recombination between the targeting vector and the cognate chromosomal gene results in the disruption of one genomic copy of gene X and the loss of the vector's *HSV-tk*. Cells in which this event has occurred will be $X^{+/-}$, *neo*$^{r+}$, *HSV-tk*$^-$ and will be resistant to both G418 and FIAU. ***b***, Integration of the targeting vector at a random site of the ES cell genome by non-homologous recombination. Because non-homologous insertion of exogenous DNA into the chromosome occurs through the ends of the linearized DNA, *HSV-tk* will remain linked to *neo*^{r}. Cells derived from this type of recombination event will be $X^{+/+}$, *neo*$^{r+}$ and *HSV-tk*$^+$ and therefore resistant to G418 but killed by FIAU. The nucleoside analog FIAU specifically kills cells with functional *HSV-tk* genes, but is not toxic to cells with only cellular *Tk*.

homologous recombination relative to random integration of the targeting vector into the recipient cell genome made it impractical to attempt gene targeting directly in one-cell mouse zygotes. Instead, it seemed our best option was to do gene targeting in cultured embryo-derived stem (ES) cells, from which the relatively rare targeted recombinants would be selected and purified. These purified cells, when subsequently introduced into a preimplantation embryo and allowed to mature in a foster mother, would contribute to the formation of all tissues of the mouse, including the germ line.

Gene targeting in ES cells

At a Gordon Conference in the summer of 1984, I heard a discussion from a member of Martin Evans' laboratory about ES cells. They seemed much more promising in their potential to contribute to the formation of the germ line than the previously characterized embryonal carcinoma (EC) cells[12,15]. In the winter of 1985, my wife and I spent a week in Martin Evans' laboratory learning how to derive, culture and generate mouse chimeras from these cells.

In the beginning of 1986, our effort switched to doing gene targeting experiments in ES cells. We also decided to use electroporation as the means of introducing our targeting vectors into ES cells. Although microinjection is orders of magnitude more efficient than electroporation as a means for generating cells with targeted mutations, injections must be done one cell at a time. With electroporation, we could introduce the targeting vector into 1×10^7 cells in a single experiment, easily producing large numbers of transformed cells even with the lower efficiency.

To rigorously determine the quantitative efficiency of gene targeting in ES, we chose as our target locus the hypoxanthine phosphoribosyl transferase gene (*Hprt*). There were two main reasons for this choice. As *Hprt* is located on the X chromosome and the ES cell line that we were using was derived from a male mouse, only a single *Hprt* locus had to be disrupted to yield *Hprt*$^-$ cell lines. Moreover, a good protocol for selecting cells with disrupted *Hprt* genes existed, based on the drug 6-thioguanine (6-TG), which kills cells with a functional *Hprt*. The strategy we used was to generate a gene-targeting vector that contained an *Hprt* genomic sequence that was disrupted in an exon by insertion of *neo*^{r} (Fig. 2). Homologous recombination between this targeting vector and the ES cell chromosomal *Hprt* would generate *Hprt*$^-$ cells that would be resistant to growth in medium containing both 6-TG (killing *Hprt*$^+$ cells) and G418 (killing cells lacking *neo*^{r}). All lines generated from cells selected in this way lost *Hprt* function as a result of gene-targeted disruption of the *Hprt* locus[46]. The Hprt locus provided an ideal locus to further test many variables that could potentially influence the targeting efficiency[46–49].

Because we foresaw that *neo*^{r} would probably be used as a positive selectable gene for the disruption of many genes in ES cells, it was essential that its expression be mediated by an enhancer that would function regardless of its location within the ES cell genome. Here our previous experience with enhancers and the transformation of cultured mouse cells proved of value. We knew from those experiments that the activities of promoter–enhancer configurations are very cell-specific. To encourage such strong *neo*^{r} expression in ES cells, we chose to drive it with a duplicated, mutated polyoma virus enhancer selected for strong expression in mouse embryonal carcinoma cells[46]. Subsequently, the strategy described above of using *neo*^{r} driven by an enhancer that allows strong expression in ES cells, independent of chromosomal location, has become the standard for disruption of most genes in ES cells.

The experiments described above showed that ES cells were good recipient hosts, able to mediate homologous recombination between the targeting vector and the cognate chromosomal sequence. In addition, the drug-selection protocols required to identify ES cell lines containing the targeted disruptions did not seem to alter their pluripotent potential. I believe that this paper was pivotal in the development of the field by encouraging other investigators to begin use of gene targeting in mice as a means for determining the function in the intact animal of the genes they were studying.

The ratio of homologous to non-homologous recombination events in ES cells was found to be approximately 1 to 1,000 (ref. 46). Because the disruption of most genes does not produce a phenotype that is selectable at the cellular level, investigators seeking specific gene disruptions would need either to undertake tedious DNA screens through many cell

colonies to identify the rare ones containing the desired targeting events or to use a selection protocol to enrich for cells containing such events.

In 1988 we reported a general strategy to enrich for cells in which homologous targeting events had occurred[50]. This enrichment procedure, known as positive–negative selection, has two components (Fig. 3). One component is a positive selectable gene, *neo*^{r}, used to select for recipient cells that have incorporated the targeting vector anywhere in their genomes (that is, at the target site by homologous recombination or at random sites by non-homologous recombination). The second component is a negative selectable gene, *HSV-tk*, located at the end of the linearized targeting vector and used to select against cells containing random insertions of the target vector. Thus the 'positive' selection enriches for recipient cells that have incorporated the introduced vector and the 'negative' selection eliminates those that have incorporated it at non-homologous sites. The net effect is enrichment for cells in which the desired targeting event has occurred. The strength of this enrichment procedure is that it is independent of the function of the gene that is being disrupted and succeeds whether or not the gene is expressed in cultured ES cells. Positive–negative selection has become the most frequently used procedure to enrich for cells containing gene-targeting events.

Subsequent development and extension

The use of gene targeting to evaluate the functions of genes in the living mouse is now a routine procedure and is used in hundreds of laboratories all over the world. It is gratifying to be able to pick up almost any major biological journal and find the description of yet another 'knockout' mouse. The *in vivo* functions of well over 7,000 genes have been analyzed with gene targeting, a number that is very impressive given that generation of this large collection of mouse lines with targeted mutations has been accomplished by independent investigators without the benefit of any special government program to fund it.

The gene-targeting protocol is now done as follows: The desired DNA sequence modification is introduced into a cloned copy of the chosen gene by standard recombinant DNA technology. Then, the modification is transferred, by means of homologous recombination, to the cognate genomic locus in ES cells and the ES cell lines carrying the desired alteration are selected. Finally, ES cells containing the altered genetic locus are injected into mouse blastocysts, which are in turn brought to term by surgical transfer to foster mothers, generating chimeric mice that are capable of transmitting the modified genetic locus to their offspring. Figure 4 outlines these steps, from the isolation of cultured ES cell lines containing the desired targeted gene modification to the generation of germline chimeras and their offspring.

So far, gene targeting has been used mainly to disrupt chosen genes to determine their function in mice (that is, to generate 'knockout' mice). However, it can be used to manipulate the mouse genome in any desired manner. For example, an allelic series of mutations in a specific gene can be generated to evaluate the effects of changes resulting from gain-of-function or partial loss-of-function mutations, in addition to those produced by simple, complete loss-of-function mutations. Furthermore, to permit the evaluation of multiple potential functions of a gene, particularly if the loss-of-function allele compromises the embryo at early stages of development, the *Cre-loxP* and *Flp/FRT* site-specific recombination systems, in concert with gene targeting, can be used to generate conditional mutations that restrict the effect of a mutation to specific cells, tissues or temporal periods[51]. In conclusion, a very broad range of genetic manipulations in the mouse has been made available through gene targeting. It is hoped that use of this technology will permit the discovery of essential components underlying even very complex biological phenomena such as higher cognitive function and dysfunction. With the recent publication of the complete sequences for human and mouse genomes, practitioners of gene targeting in mice have a bounty of information for conversion to a functional footing. The transformation of human medicine resulting from the translation of this new knowledge base may make tomorrow's medicine unrecognizable relative to today's practices.

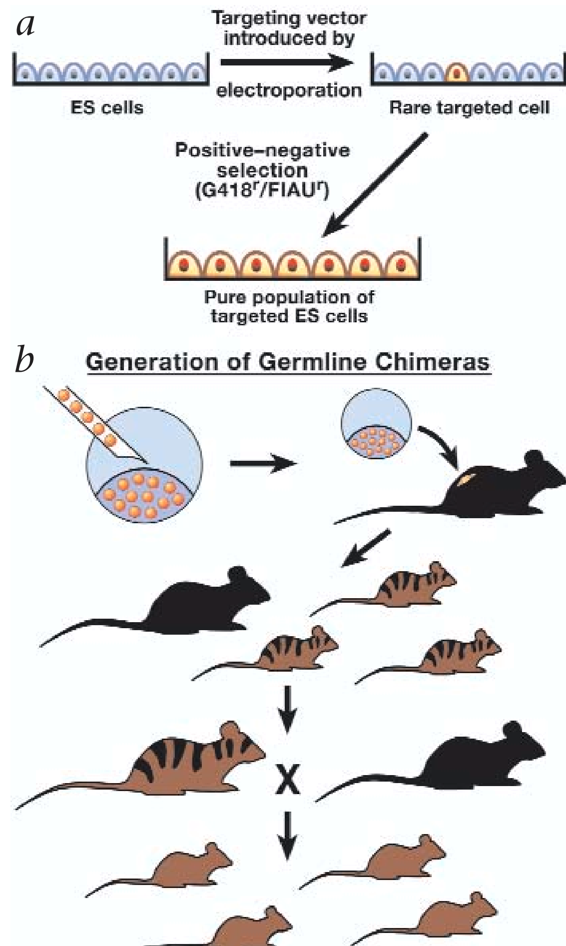

Fig. 4 Generation of mouse germline chimeras from ES cells containing a targeted mutation. ***a***, The first step involves the isolation of a clonal ES cell line containing the desired mutations. Positive–negative selection (Fig. 3) is used to enrich for ES cells containing the desired modified gene. ***b***, The second step is to use those ES cells to generate chimeric mice able to transmit the mutant gene to their progeny. To facilitate isolation of the desired progeny, the ES cells and recipient blastocysts are derived from mice with distinguishable coat color alleles (ES, agouti brown mice; blastocyst, black mice). This permits evaluation of the extent of chimerism by coat color chimerism and evaluation of ES cell contribution to the formation of the germ line by the coat color of the progeny derived from the chimeric animals.

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