RESEARCH PRIZE IN DEVELOPMENTAL BIOLOGY

ESSAY

Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century

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Abstract | Gene targeting in mouse embryonic stem cells has become the 'gold standard' for determining gene function in mammals. Since its inception, this technology has revolutionized the study of mammalian biology and human medicine. Here I provide a personal account of the work that led to the generation of gene targeting which now lies at the centre of functional genomic analysis.

Gene targeting — creating designed genomic modifications — has three enormous advantages relative to other procedures for introducing mutations into mice. First, the investigator chooses which genetic locus to mutate. Second, the technique takes full advantage of all the resources provided by the known sequences of the mouse and human genomes and, third, the investigator has complete control of how to modulate the chosen genetic locus[1]. This last advantage provides the investigator with the ability to design the genetic modification of the chosen locus so as to best address the specific biological question that is being pursued. Such modifications could include the creation of null mutations or HYPOMORPHIC MUTATIONS, the introduction of reporter genes to follow gene expression or determine cell lineage, and/or manipulation to restrict the effects of the mutation to any desired group of cells or organs (spatial restriction) or to any chosen temporal period during the life history of the mouse (temporal restriction). Surprisingly, 20 years after its development, the level of sophistication of genomic manipulations that are currently feasible in the mouse through gene targeting can still only be matched in far simpler organisms, such as bacteria and yeast.

Some investigators have questioned whether such reductionist approaches, which involve inferring gene function from the perturbations of a normal phenotype that are induced by the targeted mutations in one or a small number of genes, have sufficient power to provide significant understanding of how truly complex biological phenomena such as higher cognitive functions are mediated, particularly in an organism as complex as the mouse. Frankly, on more gloomy days, I sometimes raise similar questions myself. However, I am not aware of any other more successful means of dissecting complex biological phenomena into manageable, understandable components. It is to be hoped that through the summation of numerous such components, the desired level of clarity of even very complex biological phenomena will be achieved. Furthermore, when more holistic approaches have been applied to the analysis of the same processes, they have so far failed even more miserably, in my view, to provide significant understanding of these complex topics.

The initial development of gene targeting in mice required the solution to two basic problems. The first and foremost was how to produce specific mutations in a chosen gene in cultured mammalian cells. The second was how to transfer this genetic modification to the mouse germline. Oliver Smithies' laboratory and mine worked independently on solutions to the first problem. Martin Evan's laboratory provided us with an approach for a solution to the second problem. What follows is a description of my laboratory's contributions to the development of gene targeting in the mouse. It is not meant to be comprehensive; it is rather a more personal description of our contributions to this field.

1977–1980: homologous recombination

The discoveries that directed my attention to the development of gene targeting began in 1977. At that time, I was exploring whether I could introduce DNA into nuclei of mammalian cells using extremely small glass needles (with tip diameters of less than one micron). Wigler and Axel had just demonstrated that mammalian cells deficient in thymidine kinase (tk^-) could be transformed into tk^+ cells by exposing these cells to a DNA calcium phosphate co-precipitate containing the herpes virus thymidine kinase (*HSV-tk*; also known as *HHV4gp124*) gene[2]. Although this was an important advance for the field of somatic cell genetics, their protocol was not very efficient. With their procedure, incorporation of functional copies of the *HSV-tk* gene occurred in approximately one per million cells exposed to the DNA calcium phosphate co-precipitate. Using a similar selection procedure, I asked whether I could introduce functional copies of the *HSV-tk* gene into mouse tk^- fibroblasts using very fine glass needles to inject the DNA directly into their nuclei[3]. This procedure proved to be extremely efficient. One cell in three that received the DNA stably passed the functional *HSV-tk* gene to its daughters. One does not often observe an almost 10^6-fold improvement in the efficiency of a process. I first reported these results at a workshop organized by Frank Ruddle in 1978, held in Estarreja, Portugal. The extremely high efficiency of DNA transfer by microinjection made it practical for investigators to use this procedure to generate transgenic mice that contain random insertion of exogenous DNA. This was accomplished by microinjecting the desired DNA into nuclei of 1-cell mouse zygotes and allowing these embryos to come to term after surgical transfer to foster mothers[4–8]. Following this workshop, Frank Ruddle rapidly championed our results throughout the mouse research community.

The efficient transfer of the *HSV-tk* gene into cells by microinjection required the

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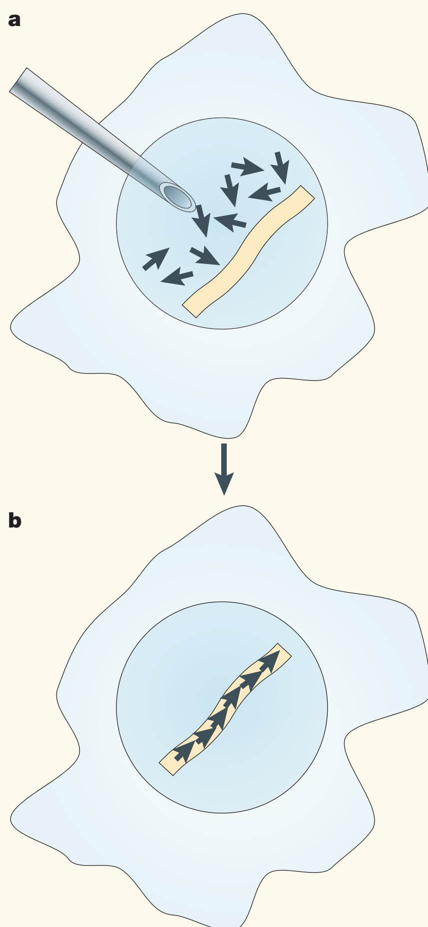

Figure 1 | **Generation of an integrated, head-to-tail concatemer of multiple copies of a plasmid following its injection into nuclei of cultured mammalian cells.** When multiple copies of a DNA sequence (arrows) are introduced into mammalian cells (**a**), they are efficiently integrated into one or a very few random site(s) within the host genome as a concatemer (**b**). Sequence analysis of the concatemer shows that the multiple copies of the DNA sequences are not randomly oriented within the concatemer, but rather are all oriented in the same direction (a head-to-tail concatemer; **b**). We proved that such highly ordered concatemers arose through homologous recombination[11]. This was the first demonstration that generic mammalian somatic cells contain the enzymatic machinery for efficiently mediating homologous recombination.

HSV-tk gene to be linked to other short viral sequences[3]. I reasoned that because many viruses resided within the mammalian genome as part of their life cycle, such viruses might contain within their genome bits of DNA that enhanced their ability to establish themselves within the mammalian genome. I decided to search the genomes of the lytic simian virus, SV40, and the mouse retrovirus, MuLV, for the presence of such sequences and found them in both genomes. When linked to the *HSV-tk* gene, these short sequences increased the transforming capacity of the injected *HSV-tk* gene by 100 fold or more[3]. I showed that the enhancement did not result from independent replication of the injected *HSV-tk* DNA as an extra-chromosomal plasmid, but that the efficiency-enhancing sequences were either increasing the frequency of exogenous DNA integration into the host genome, or increasing the probability that the *HSV-tk* gene, once integrated into the host genome, was expressed in the recipient cells[3,9]. These experiments were carried out before the concept of gene expression 'enhancer sequences' had emerged and contributed to the definition of these special DNA sequences[10]. As I describe below, the emerging concept of enhancers profoundly influenced our contributions to the development of gene targeting; they alerted us to the importance of using appropriate enhancers to mediate the expression of newly introduced selectable genes, regardless of the inherent expression characteristics of the host site to which they were targeted.

However, the observation that I found most fascinating from these early microinjection experiments was that when many copies of the *HSV-tk* plasmid were injected into cells, although integrated randomly into one or two chromosomal sites, they were present within those sites as a highly ordered head-to-tail concatemer (FIG. 1). We reasoned that such highly ordered concatemers could not be generated by a random ligation process, but could be generated either by replication (for example, a rolling-circle-type mechanism) or by homologous recombination between the newly introduced plasmid molecules. We proved that they were generated by homologous recombination[11]. This was the first demonstration that mammalian cells could mediate homologous recombination between newly added exogenous DNA molecules. This conclusion was significant because it showed that mammalian somatic cells, in this case mouse fibroblasts, contained an efficient enzymatic machinery for mediating homologous recombination. The efficiency of this machinery became evident from the observation that when more than 100 *HSV-tk*-carrying plasmid molecules were injected per cell, they were all incorporated into a single, ordered, head-to-tail concatemer. It was immediately clear to me that if we could harness this machinery to carry out homologous recombination between a newly introduced DNA molecule of our choice and the cognate sequence in the recipient cell, we could mutate or modify almost any gene in mammalian cells in any desired manner. Incidentally, we arrived at the above conclusions before knowing that gene targeting would soon be feasible in yeast[12].

In 1980, I submitted a grant proposal to the US National Institutes of Health (NIH) to test the feasibility of gene targeting in mammalian cells, and these experiments were rejected on the grounds that there was only a remote probability that the newly introduced DNA would ever find its matching sequence within a host cell genome. Despite the rejection, I decided to continue this line of experimentation. We knew that the frequency of gene targeting in mammalian cells was likely to be low, and that the insertion of the targeting vector at various random sites in the genome, other than the target locus, would be far more common. We therefore proposed to use selection to eliminate cells that do not contain the desired targeted homologous recombination products. The first step of this scheme required the generation of mammalian cell lines with random insertions of a defective neomycin resistance (*neo*^{r}) gene containing either a deletion or point mutation (FIG. 2). In the second step, target-vector DNA, carrying defective *neo*^{r} genes with mutations that differed from those present at the target site, was introduced into the recipient cell lines. Homologous recombination between *neo*^{r} sequences in the targeting vector and the recipient cell chromosome could generate a functional *neo*^{r} gene from the two defective genes, producing cells that are resistant to the drug G418, which is lethal to cells that lack a functional *neo*^{r} gene[13].

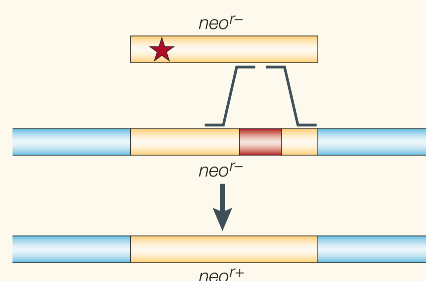

Figure 2 | **Generation of a functional neomycin resistance gene from two defective genes by gene targeting.** The recipient mammalian cultured cell line contains a defective neomycin resistance (*neo*^{r}) gene integrated randomly into one of its chromosomes. This chromosomal copy contains a small deletion (red block) at the 3′ end of the coding sequence. The targeting vector contains a 5′ point mutation (red star). With a frequency of approximately 1 in 1,000 cells that receive an injection of the targeting vector, the chromosomal deletion mutation is corrected by homologous recombination with the exogenously added targeting vector.

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1981–1985: mammalian cells

Before the homologous recombination machinery could be efficiently harnessed to mediate recombination between a chosen exogenous DNA sequence and the cognate sequence in the recipient cell's genome, we had to become familiar with the endogenous homologous recombination machinery. Specifically, did this machinery have substrate preferences; and if so, what were they? What kind of homologous recombination reactions did it prefer to mediate: reciprocal or non-reciprocal, conservative or non-conservative and so on? By examining homologous recombination between co-injected DNA molecules or within preformed heteroduplexes — which the endogenous recombination machinery mediated with great efficiency — we learned, among other things, that linear DNA molecules were the preferred substrates for homologous recombination[13]. We also learned that homologous recombination was cell-cycle dependent, showing a peak of activity during early S-phase[14]. It also turned out that although both reciprocal and non-reciprocal exchange occurred, there was a distinct bias toward the latter[15]. These results contributed significantly 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 chromosomal homologue. By 1984, we had good evidence that gene targeting in cultured mammalian cells was indeed possible[16]. At this time, I resubmitted our grant to the same NIH study section that had previously rejected our earlier grant proposal, and their critique to the second proposal started with the phrase "We are glad that you didn't follow our advice."

To our delight, correction of the defective *neo*^{r} gene occurred at a frequency of 1 per every 1,000 injected cells. This frequency was not only higher than we expected, but allowed us to determine experimental parameters that influenced the gene-targeting reactions[13]. A surprising result was that the targeting efficiency was neither dependent on the input concentration of the targeting vector nor on the number of targets present within the recipient cell genome. Introducing thousands of targeting vector molecules per cell or only one molecule per cell (after correction for cells that receive no molecules per cell) yielded the same targeting frequency. These observations indicated that the rate-limiting step was the presence of the cellular machinery required to mediate the homologous recombination reaction between the exogenous and endogenous DNA sequences. A further important lesson derived from these experiments was that all chromosomal target positions analysed seemed to be equally accessible to the homologous recombination machinery, indicating that a large fraction, if not all, of the mouse genome could be modified by gene targeting.

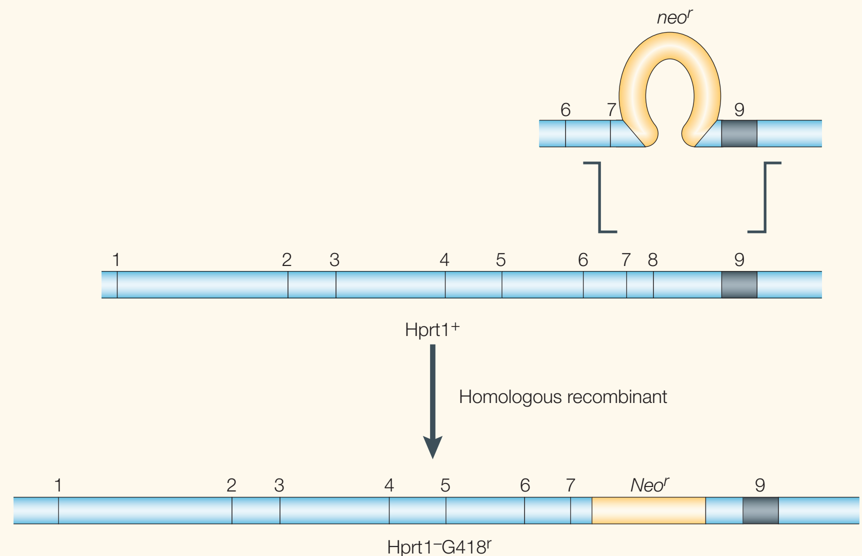

Figure 3 | **Disruption of the endogenous hypoxanthine phosphoribosyl transferase gene by gene targeting in embryonic stem cells.** The targeting vector contains genomic hypoxanthine phosphoribosyl transferase (*Hprt1*) sequences that are disrupted in the eighth exon with the neomycin resistance (*neo*^{r}) gene. Following homologous pairing between the targeting vector and the chromosomal *Hprt1* sequence, a homologous recombination event replaces the genomic sequences with vector sequences that contain the *neo*^{r} gene. Disruption of the endogenous *Hprt1* gene renders the cells *Hprt1*$^-$ because this gene resides on the X chromosome and the embryonic stem cells were derived from a male mouse embryo. The cells are able to grow in a medium that contains the drugs G418 and 6TG (see main text). A hundred percent of the cell lines derived from this procedure have lost Hprt1 function from targeted disruption of this endogenous locus[20]. Therefore, targeted disruption of the *Hprt1* locus using this protocol provides an ideal assay for determining the parameters that influence the gene targeting frequency[20–23].

At this time, Oliver Smithies and his colleagues reported their classic experiment of targeted modification of the haemoglobin-β locus in cultured mammalian cells[17]. 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 homologous recombination (relative to random integration of the targeting vector into the recipient cell's genome) made it impractical to attempt gene targeting directly in 1-cell mouse zygotes. Instead, it seemed that our best option would be to carry out gene targeting in cultured embryonic stem (ES) cells, from which the relatively rare targeted recombinant would be selected and purified. When subsequently introduced into a preimplantation embryo and allowed to mature in a foster mother, these purified cells would contribute to the formation of all tissues of the mouse, including its germline.

1986–1991: embryonic stem cells

At a Gordon Conference in the summer of 1984, I heard a discussion from a member of Martin Evans' laboratory in Cambridge, UK, about mouse ES cells (at that time called EK cells). They seemed to be much more promising in their potential to contribute to the formation of the mouse germline than the previously characterized embryonal carcinoma (EC) cells[18,19]. The former differed from the latter in their tissue of origin. Whereas ES cells were derived from early mouse embryos, EC cells were obtained from mouse tumours. In the winter of 1985, my wife and I spent a week in Martin Evans' laboratory learning how to derive, culture and generate mouse chimaeras from these cells.

In the beginning of 1986, our total laboratory effort switched to doing gene targeting in mouse ES cells. We also decided to use electroporation as the means of introducing our targeting vectors into ES cells rather than microinjection. Although microinjection was

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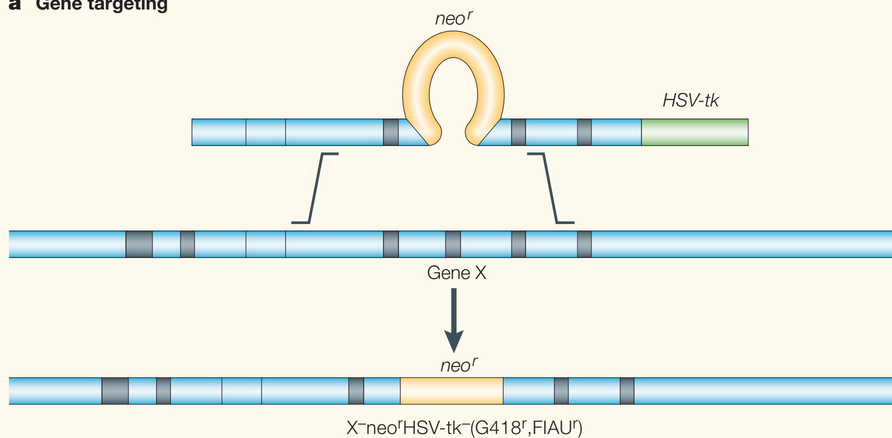

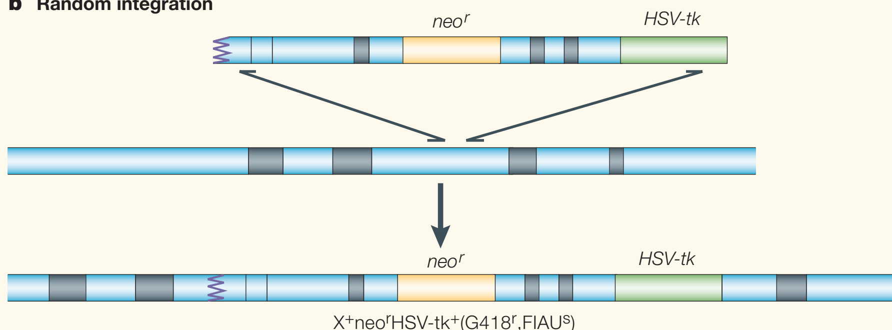

Figure 4 | **Positive–negative selection.** A selection protocol used to enrich for embryonic stem (ES) cell lines that contain a targeted disruption of any chosen gene (here, gene X), regardless of its function or expression in ES cells[30]. **a** | The targeting vector contains an insertion of a neomycin resistance (*neo*^{r}) gene in an exon of gene X and a linked herpes virus thymidine kinase (*HSV-tk*) gene at one end. The vector 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 *HSV-tk* gene. Cells in which this event has occurred will be X$^{+/-}$, neo^{r+} and HSV-tk$^-$, and will be resistant to both G418 and FIAU. **b** | Most frequently, the targeting vector will be integrated into the host cell genome at a random site, through non-homologous recombination. Because non-homologous insertion of exogenous DNA into the host cell chromosome occurs through the ends of the linearized targeting vector, the *HSV-tk* gene will remain linked to the *neo*^{r} gene. 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 (a drug that kills cells containing a functional *HSV-tk* gene, but which is not toxic to cells with only the cellular *tk* gene). ES cells that contain the desired targeting event survive the selection procedure, but cells that contain the much more common random integration of the targeting vector do not.

orders of magnitude more efficient than electroporation as a means of generating cell lines with targeted mutations, injections had to be done one cell at a time. With electroporation, we could introduce the targeting vector into 10^7 cells in a single experiment, easily producing large numbers of targeted cells, even with the lower efficiency. Electroporation was also more likely to be used by other investigators, thereby facilitating the transfer of our findings to other investigators.

To allow further quantitative analysis of the parameters that affect the efficiency of gene targeting in ES cells, we chose the endogenous hypoxanthine phosphoribosyl transferase (*Hprt1*) gene as our target locus. There were two primary reasons for this choice. First, because *Hprt1* is located on the X chromosome and the ES cell line that we were using was derived from a male mouse, only a single *Hprt1* locus had to be disrupted to yield *Hprt1*$^{-/-}$ cell lines. Second, a good protocol for selecting cells with disrupted *Hprt1* genes already existed, which is based on the drug 6-thioguanine (6TG) and kills cells that have a functional *Hprt1* gene. We made a targeting vector that contained an *Hprt1* genomic sequence, which was disrupted in an exon by insertion of a *neo*^{r} gene (FIG. 3). Homologous recombination between this targeting vector and the ES cell chromosomal *Hprt1* gene would generate *Hprt1*$^{-/--}$ cells that would be resistant to growth in media containing both 6TG (killing *Hprt1*$^+$ cells) and G418 (killing cells that lack a *neo*^{r} gene). All lines generated from cells selected in this way lost *Hprt1* function as a result of targeted gene disruption of the *Hprt1* locus[20]. Manipulation of the *Hprt1* locus provided an ideal locus to study the many variables that influence the targeting efficiency[20–23].

Because we foresaw that the *neo*^{r} gene would probably be used as a positive selectable gene for the disruption of many genes in ES cells, it was crucial that its expression be mediated by an enhancer that would function regardless of its location within the ES cell genome. It was here that our previous experience with enhancers proved to be of value. We knew from those experiments that the activity of promoter–enhancer combinations was cell-type specific. To encourage such strong *neo*^{r} expression in ES cells, we chose to drive it with a mutant polyoma-virus enhancer that was selected for strong expression in mouse embryonal carcinoma cells[20]. The already mentioned strategy of using a *neo*^{r} gene, driven by an enhancer that allows strong expression in ES cells, independent of chromosomal target location, has become the standard for disruption of almost all genes in ES cells.

These experiments showed that ES cells were a good recipient host, able to mediate homologous recombination between the exogenous targeting vector and the cognate chromosomal sequence. In addition, the drug-selection protocols required to isolate the ES cell lines that contain the targeted disruption did not seem to alter their PLURIPOTENT potential. I believe that the resulting paper had a pivotal role in the development of the gene-targeting field — it encouraged other investigators to use gene targeting in mice as a means for determining the function of the gene they were studying in intact mice. By the end of the 1980s and the beginning of the 1990s, a host of papers were published that described the function of numerous genes in living mice (see for example REFS 24–29).

The ratio of homologous to non-homologous recombination events in ES cells turned out to be approximately 1 to 1,000, although there is considerable variation from gene locus to gene locus. Because the disruption of most genes does not produce a phenotype that is selectable at the ES cell level, investigators seeking specific gene disruptions would either need to conduct tedious DNA screens through many cell colonies to identify the rare ones that contain the desired targeting events, or use a selection protocol to enrich for cells that contain such events.

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In 1988, we reported a general strategy to enrich for cells in which homologous targeting events had occurred[30]. This enrichment procedure, known as positive–negative selection, uses two components (FIG. 4). One component is a positive selectable gene, such as *neo*^{r}, used to select for recipient cells that have incorporated the targeting vector in their genome (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, such as *HSV-tk*, which is located at the end of the linearized targeting vector and is used to select against cells that contain random insertions of the target vector. Therefore, the 'positive' selection enriches for recipient cells that have incorporated the introduced vector at all sites and the 'negative' selection eliminates those that have incorporated it at non-homologous sites. The net effect is to enrich for ES cell lines 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. This procedure is currently in such common use that it has long-ago ceased to be referenced.

1991 to present

The use of gene targeting to evaluate the function of genes in the living mouse is now a routine procedure and is used in hundreds if not thousands of laboratories all over the world. It is gratifying to be able to pick up almost any of the leading biological journals and find the description of yet another 'knockout' mouse. The *in vivo* functions of well over 7,000 genes have been analysed by gene targeting, which is quite impressive considering that this large collection of mouse lines with targeted mutations has been generated without a concerted government programme to finance it. Now, however, such a programme is being organized by the NIH to generate a collection of mouse lines that contain a targeted disruption in every known mouse gene[31]. FIGURE 5 outlines the most common steps used to generate germline chimaeras that contain a specific targeted mutation.

So far, gene targeting is most commonly used to disrupt a chosen gene in the mouse (a gene knockout). But gene targeting can be used to manipulate any chosen mouse locus in any desired manner. For example, an ALLELIC SERIES of mutations in the same gene can be generated with point mutations in different domains to allow independent

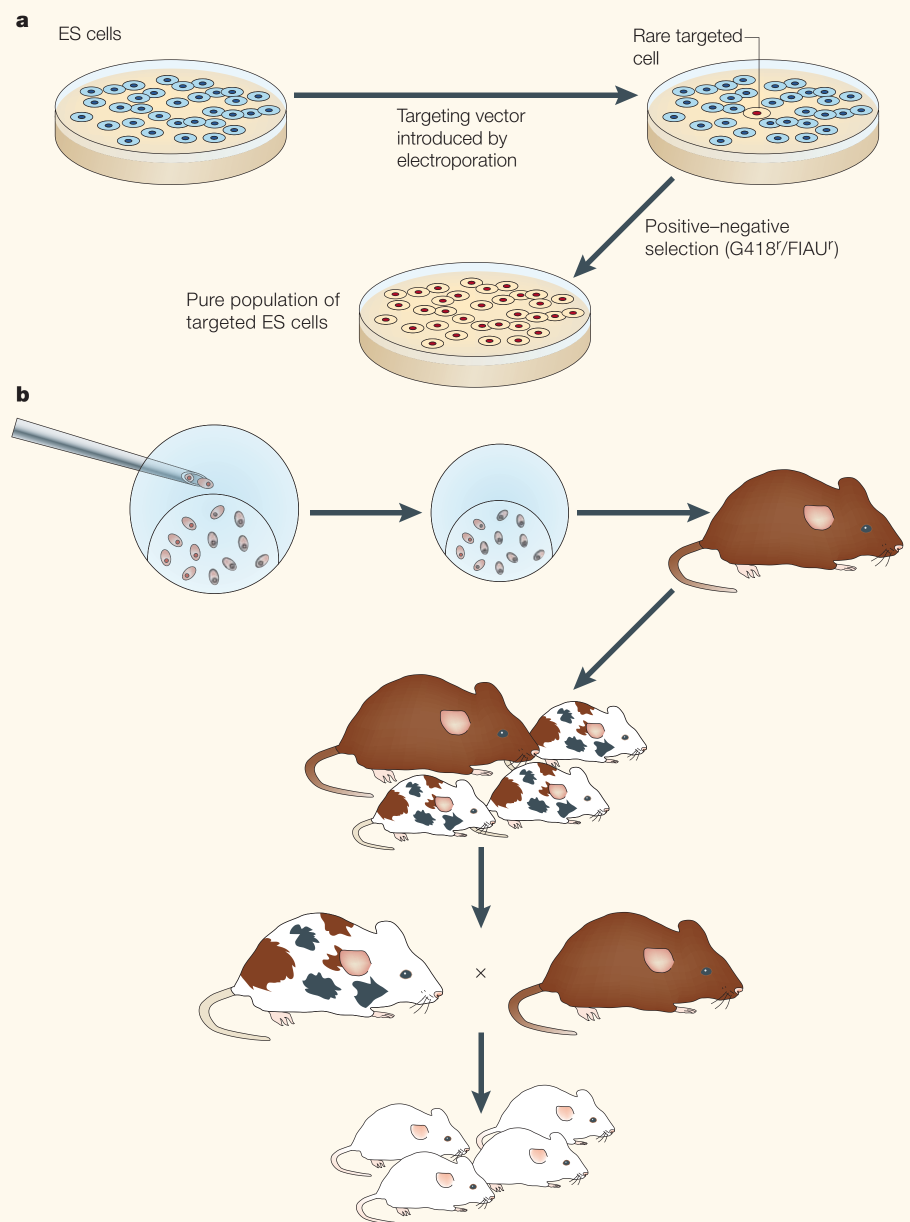

Figure 5 | **Generation of mouse germline chimaeras from embryonic stem cells that contain the desired targeted mutation. a** | The first step involves the isolation of a clonal embryonic stem (ES) cell line that contains the desired mutation. Positive–negative selection (see also FIG. 4) can be used to enrich for ES cell lines that contain the desired modified gene. **b** | The second step is to use these ES cells to generate chimeric mice that are able to transmit the mutant gene to their progeny. This is accomplished by injecting ES cells that contain the desired targeted mutation into a recipient pre-implantation mouse embryo, a blastocyst. These embryos are then surgically transferred to a recipient pseudopregnant foster mother to allow the embryos to come to term. To facilitate isolation of the desired progeny, the ES cells and recipient blastocysts are derived from mice with distinguishable coat-colour alleles (that is, ES cells from agouti brown mice and blastocysts from black mice). The extent of the contribution of ES cells to the formation of the chimeric mouse can be evaluated by visual assessment of coat-colour chimerism. ES cell contribution to the germline can be evaluated by observing the coat colour of the progeny that is derived by breeding the chimeric mouse with black mice.

determination of separable functions that are associated with that gene product. Mutations can also be introduced into the *cis*-regulatory elements that regulate gene activity to restrict or alter its gene-expression profile. Gene targeting, coupled with a designated site-specific recombination system, such as Cre–*loxP* or Flp–*FRT*, can be used to generate conditional mutations to allow separate evaluation of a gene's function in specific tissues and/or at a restricted time[32]. This application of gene targeting will find increasing use in

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Glossary

ALLELIC SERIES
A series of alleles that can be present at the same locus, which produces graded phenotypes.

HYPOMORPHIC MUTATION
A mutation of which the phenotypic effects are less severe relative to a null mutation in the same gene.

PLURIPOTENT
When referring to stem cells, having the capacity to contribute to the formation of all cell types, such as embryonic stem cells.

dissecting complex biological phenomena such as development and higher order neuronal functions or dysfunctions.

We continue to contribute to the repertoire of targeted genomic manipulations that broaden the spectrum of biological phenomena that can be analysed[33–35]. However, most of our current effort is directed at using gene targeting to address medical and biological questions of special interest to our laboratory. This has included the analysis of mice that have loss-of-function and conditional alleles in molecules involved in cell–cell signalling, such as members of the Fgf and Wnt families[36–38], analysis of the role of Hox genes during embryogenesis and in the adult[39–43], and the generation of mouse models for cancers and neuropsychiatric diseases[44–46]. The repertoire of biological phenomena that can be studied through the use of gene targeting is only limited by the imagination of the investigator.

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1. Capecchi, M. R. Generating mice with targeted mutations. *Nature Med.* **7**, 1086–1090 (2001).
2. Wigler, M. *et al.* Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* **11**, 223–232 (1977).
3. Capecchi, M. R. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* **22**, 479–488 (1980).
4. Gordon, J. W., Scangos, G. A., Plotkin, D. J., Barbosa, J. A. & Ruddle, F. H. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl Acad. Sci. USA* **77**, 7380–7384 (1980).
5. Brinster, R. L. *et al.* Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell* **27**, 223–231 (1981).
6. Costantini, F. & Lacy, E. Introduction of a rabbit β-globin gene into the mouse germ line. *Nature* **294**, 92–94 (1981).
7. Wagner, E. F., Stewart, T. A. & Mintz, B. The human β-globin gene and a functional thymidine kinase gene in developing mice. *Proc. Natl Acad. Sci. USA* **78**, 5016–5020 (1981).
8. Wagner, T. E. *et al.* Microinjection of a rabbit β-globin gene in zygotes and its subsequent expression in adult mice and their offspring. *Proc. Natl Acad. Sci. USA* **78**, 6376–6380 (1981).
9. Luciw, P. A., Bishop, J. M., Varmus, H. E. & Capecchi, M. R. Location and function of retroviral and SV40 sequences that enhance biochemical transformation after microinjection of DNA. *Cell* **33**, 705–716 (1983).
10. Levinson, B., Khoury, G., VandeWoude, G. & Gruss, P. Activation of SV40 genome by 72-base pair tandem repeats of Moloney sarcoma virus. *Nature* **295**, 568–572 (1982).
11. Folger, K. R., Wong, E. A., Wahl, G. & Capecchi, M. R. Patterns of integration of DNA microinjected into cultured mammalian cells: evidence for homologous recombination between injected plasmid DNA molecules. *Mol. Cell. Biol.* **2**, 1372–1387 (1982).
12. Hinnen, A., Hicks, J. B. & Fink, G. R. Transformation of yeast. *Proc. Natl Acad. Sci. USA* **75**, 1929–1933 (1978).
13. Thomas, K. R., Folger, K. R. & Capecchi, M. R. High frequency targeting of genes to specific sites in the mammalian genome. *Cell* **44**, 419–428 (1986).
14. Wong, E. A. & Capecchi, M. R. Analysis of homologous recombination in cultured mammalian cells in transient expression and stable transformation assays. *Somat. Cell Mol. Genet.* **12**, 63–72 (1986).
15. Folger, K. R., Thomas, K. R. & Capecchi, M. R. Nonreciprocal exchanges of information between DNA duplexes coinjected into mammalian cell nuclei. *Mol. Cell. Biol.* **2**, 1372–1387 (1985).
16. Folger, K., Thomas, K. & Capecchi, M. R. Analysis of homologous recombination in cultured mammalian cells. *Cold Spring Harbor Symp. Quant. Biol.* **49**, 123–138 (1984).
17. Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A. & Kucherlapati, R. S. Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination. *Nature* **317**, 230–234 (1985).
18. Evans, M. J. & Kaufman, M. H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156 (1981).
19. Bradley, A., Evans, M., Kaufman, M. H. & Robertson, E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**, 255–256 (1984).
20. Thomas, K. R. & Capecchi, M. R. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503–512 (1987).
21. Thomas, K. R., Deng, C. & Capecchi, M. R. High-fidelity gene targeting in embryonic stem cells by using sequence replacement vectors. *Mol. Cell. Biol.* **12**, 2919–2923 (1992).
22. Deng, C. & Capecchi, M. R. Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. *Mol. Cell. Biol.* **12**, 3365–3371 (1992).
23. Deng, C., Thomas, K. R. & Capecchi, M. R. Location of crossovers during gene targeting with insertion and replacement vectors. *Mol. Cell. Biol.* **13**, 2134–2140 (1993).
24. Joyner, A. L., Skarnes, W. C. & Rossant, J. Production of a mutation in mouse *En-2* gene by homologous recombination in embryonic stem cells. *Nature* **338**, 153–156 (1989).
25. Zijlstra, M., Li, E., Sajjadi, F., Subramani, S. & Jaenisch, R. Germ-line transmission of a disrupted β_2-microglobulin gene produced by homologous recombination in embryonic stem cells. *Nature* **342**, 435–438 (1989).
26. Schwartzberg, P. L., Goff, S. P. & Robertson, E. J. Germ-line transmission of a *c-abl* mutation produced by targeted gene disruption in ES cells. *Science* **246**, 799–803 (1989).
27. DeChiara, T. M., Efstratiadis, A. & Robertson, E. J. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* **345**, 78–80 (1990).
28. Koller, B. H., Marrack, P., Kappler, J. W. & Smithies, O. Normal development of mice deficient in β_2M, MHC class I proteins, and $CD8^+$ T cells. *Science* **248**, 1227–1230 (1990).
29. Thomas, K. R. & Capecchi, M. R. Targeted disruption of the murine *int-1* proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* **346**, 847–850 (1990).
30. Mansour, S. L., Thomas, K. R. & Capecchi, M. R. Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* **336**, 348–352 (1988).
31. Austin *et al.* The Knockout Mouse Project. *Nature Genet.* **36**, 921–924 (2004).
32. Gu, H., Marth, J. D., Orban, P. C., Mossmann, H. & Rajewsky, K. Deletion of a DNA polymerase β-gene segment in T cells using cell type-specific gene targeting. *Science* **265**, 103–106 (1994).
33. Bunting, M., Bernstein, K. E., Greer, J. M., Capecchi, M. R. & Thomas, K. R. Targeting genes for self-excision in the germline. *Genes Dev.* **13**, 1524–1528 (1999).
34. Moon, A. M., Boulet, A. M. & Capecchi, M. R. Normal limb development in conditional mutants of *Fgf4*. *Development* **127**, 989–996 (2000).
35. Schmidt, E. E., Taylor, D. S., Prigge, J. R., Barnett, S. & Capecchi, M. R. Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. *Proc. Natl Acad. Sci. USA* **97**, 13702–13707 (2000).
36. Moon, A. M. & Capecchi, M. R. Fgf8 is required for outgrowth and patterning of the limbs. *Nature Genet.* **26**, 455–459 (2000).
37. Barrow, J. R. *et al.* Ectodermal *Wnt3*/βcatenin signalling is required for the establishment and the maintenance of the apical ectodermal ridge. *Genes Dev.* **17**, 394–409 (2003).
38. Boulet, A. M. & Capecchi, M. R. Multiple roles of *Hoxa11* and *Hoxd11* in the formation of the mammalian forelimb zeugopod. *Development* **131**, 299–309 (2004).
39. Davis, A. P., Witte, D. P., Hsieh-Li, H. M., Potter, S. S. & Capecchi, M. R. Absence of radius and ulna in mice lacking *hoxa-11* and *hoxd-11*. *Nature* **375**, 791–795 (1995).
40. Goddard, J. M., Rossel, M., Manley, N. R. & Capecchi, M. R. Mice with targeted disruption of *Hoxb-1* fail to form the motor nucleus of the VIIth nerve. *Development* **122**, 3217–3228 (1996).
41. Greer, J. M., Puetz, J., Thomas, K. R. & Capecchi, M. R. Maintenance of functional equivalence during paralogous Hox gene evolution. *Nature* **403**, 661–665 (2000).
42. Wellik, D. M. & Capecchi, M. R. *Hox10* and *Hox11* genes are required to globally pattern the mammalian skeleton. *Science* **301**, 363–367 (2003).
43. Arenkiel, B. R., Tvrdik, P., Gaufo, G. O. & Capecchi, M. R. *Hoxb1* functions in both motoneurons and target tissues to establish and maintain proper neuronal circuitry. *Development* (2004).
44. Greer, J. M. & Capecchi, M. R. *Hoxb8* is required for normal grooming behavior in the mouse. *Neuron* **33**, 23–34 (2002).
45. Keller, C., Hansen, M. S., Coffin, C. M. & Capecchi, M. R. *Pax3:Fkhr* interferes with embryonic *Pax3* and *Pax7* function: implications for alveolar rhabdomyosarcoma cell of origin. *Genes Dev.* **18**, 2608–2613 (2004).
46. Keller, C. *et al.* Alveolar rhabdomyosarcomas in conditional *Pax3:Fkhr* mice: cooperativity of Ink4a/ARF and Trp53 loss of function. *Genes Dev.* **18**, 2614–2626 (2004).

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Competing interests statement
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