Lasker Basic Medical Research Award

Mouse gene targeting

MARTIN J. EVANS, OLIVER SMITHIES & MARIO R. CAPECCHI

The cultural mouse

MARTIN J. EVANS

I was inspired by biology, particularly by my experience at Cambridge in Christ's College, tutored by David Coombes and in Part II Biochemistry, where I remember in particular such luminaries as Malcolm Dixon and Don Northcote. In that year (1962–1963), a series of lectures at Cambridge by Jacques Monod burst open a new understanding for me and, together with a seminar series organized by Sidney Brenner in his rooms at King's College, inspired me with the new concepts of control of genetic readout through mRNA. I resolved to work in either plant biochemistry or developmental biology. A bout of glandular fever prevented me from taking my final examinations for which I was so eagerly preparing, and resulted in my taking a research assistantship with Elizabeth Deuchar at University College London, on *Xenopus* development. My ambition was to isolate developmentally controlled mRNA, but at that time none of the cloning tools or probes on which we now rely were available. All I could study were double-reciprocally labeled (^{14}C and ^{3}H) profiles of polyribosomes and mRNA from dissected blastula and gastrula ectoderm by sucrose density gradient centrifugation and RNA by agarose electrophoresis. In modern terms, I was looking at animal cap development in culture before induction and after commitment to either a neural or an epidermal ectoderm. At that time I saw two impediments to further progress: the difficulty of getting enough material for biochemical analysis, and the lack of any foreseeable genetics.

I sought a more 'tractable' developmental system and, at the suggestion of Robin Weiss, looked to the possibility of establishing an *in vitro* system of mammalian cell differentiation from mouse teratocarcinomas. In 1967, Leroy Stevens[1] and Barry Pierce[2] both published reviews of their formative studies. Leroy Stevens had developed a strain of mice with a high incidence of spontaneous testicular teratomas (129Sv).These teratomas contain within them a complex mixture of tissue types; some (teratocarcinomas) grow progressively and are serially transplantable in the inbred mouse strain. Barry Pierce, who was interested in the relationship between the tumor-forming stem cells and their non-malignant differentiated products, led a series of experiments converting the tumors to an ascites state, in which they grew as embryoid bodies, and culturing mass populations of cells from these *in vitro*. A pivotal experiment by Kleinsmith and Pierce[3] showed that these tumors could be clonally derived from a single transplanted cell, thus proving that the diverse cell and tissue types arise by differentiation from a single pluripotential stem cell line.

Leroy Stevens sent me breeding stock from his 129 inbred line and also several transplantable tumors that he had established. I established clonally derived tissue culture lines from these and demonstrated that the rounded cells (C "clump cells") depended initially on co-culture with a more flattened epithelioid (E) cell[4]. These C cells were the embryonal carcinoma (EC) stem cells from the tumor. Gail Martin joined my lab, and we were able to show that these spontaneously arising 'E-cells' could be replaced by mitotically inactivated chick or mouse fibroblasts, and that when these diminished or were withdrawn, extensive *in vitro* differentiation occurred. In every case, the differentiation proceeded through the production of a primary embryonic endoderm, and clumps of suspended cells formed recognizable embryoid bodies. Re-attachment of these to a solid surface gave rise to the most splendid and diverse differentiation, with beating cardiac muscle, nerve skin, cartilage and so on[5]. It was apparent, however, that they were undergoing the same first-step differentiation to an embryonic endoderm as did the inner cell mass (ICM) of a mouse embryo[6].

This likeness to the ICM was tested by experiments with Richard Gardner. I well remember transporting cells from University College London to Oxford, where he carefully introduced them into blastocysts. The chimeras we obtained demonstrated a dramatic result, with nearly every tissue of the derived mouse having contributions from the tissue culture cells[7].These cells, however, were not normal. They were derived from serially passaged tumors and had been cloned and cultured for some time. Karyotypically they were remarkably close to normal for mouse tissue cells, but although they had an apparently normal chromosome number they only had one X chromosome and no Y chromosome. Many of the initially normal mice later succumbed to somatic tumors (rhabdomyosarcomas, fibrosarcomas and so on), presumably as a result of the passage-derived mutational load in these cells. We and our colleagues in Oxford, as well as Francois Jacob's laboratory in Paris, tried in vain to recover a euploid XY EC cell line to obtain a perfect germline chimera, but this had to await the direct derivation of the cells from embryos rather than from tumors.

In 1978 I started work in the Department of Genetics at Cambridge University, and many investigations continued to show the close relationships between EC cells and early embryo epiblast. Together with Ten Fiezi, I was able to begin to determine that the main cell surface antigens on the EC cells were carbohydrate epitopes of the glycohalix[8], and Peter Stern, who had recently also moved from University College, London, to Cambridge to Sydney Brenner's laboratory, produced a very useful monoclonal against a cell surface glycolipid: the Forsman antigen. The reaction of this monoclonal antibody with cells of the normal early mouse embryo allowed us to refine the apparent homology between EC cells and cells of the embryonic ectoderm before 6 days of development[9]. Robin Lovell-Badge, using what would now be called a proteomic approach—that is, two-dimensional gels of whole-protein

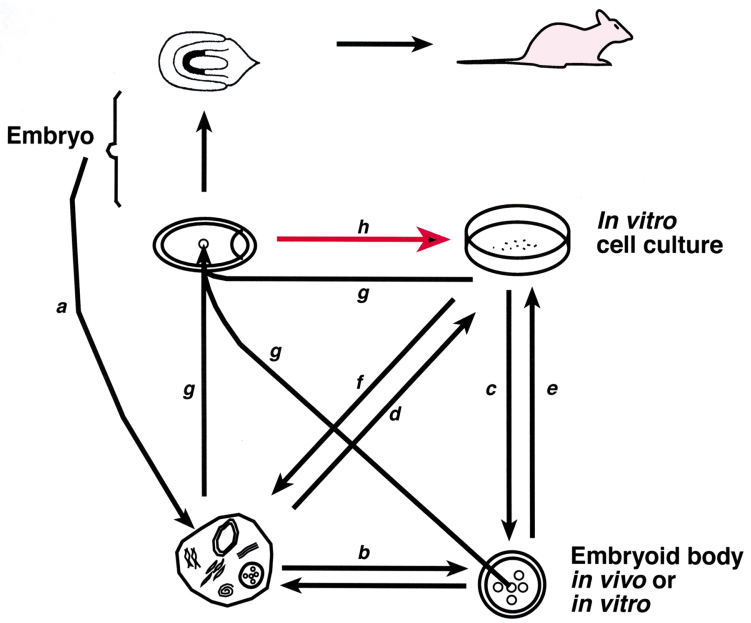

Fig. 1 Establishment in culture of pluripotential cells from mouse embryos (reproduced from ref. 12). All of the known interrelationships of *in vivo* and *in vitro* differentiation and of derivation of EC cells via a tumor are diagrammed in black. The missing link in the network of relationships, which was provided by the experiments reported in this paper is (h) a direct derivation of the cultured stem cells from an embryo.

extracts—showed a remarkably similar protein synthetic profile in EC cells and early embryo epiblast[10]. The stage was set[11].

It was only when I met up with Matt Kaufman in 1980, however, that the breakthrough could be made. I had remained convinced of the power of a genetic approach, but the somatic cell genetic techniques we were able to use with EC cells at that time—cell hybridization and selection together with exploration of variants in differentiative capacity—were 'blunt instruments'. Matt Kaufman was making haploid mouse embryos, and I knew that I could grow cell lines from blastocysts (albeit not pluripotential lines), so we hoped that we would be able to isolate an haploid cell culture from the embryos. (In retrospect, that never proved possible; the cells always doubled up to a diploid condition during isolation in culture.)

Haploid embryos are retarded in growth and have small ICMs, but Matt had a trick to allow them to catch up. By putting them into implantation delay *in vivo,* the size of the ICM could be allowed to increase before implantation. We planned to use such implantationally delayed, haploid-derived embryos to attempt to establish a cell line, and Matt prepared some normal diploid, but delayed, embryos as controls and for me to use for practice. When I cultured these blastocysts as explants in tissue culture, using a medium that had been honed for optimum cloning efficiency of both mouse and human EC cells, I immediately noted an outgrowth of EC-like cells. These were clearly recognizable as the sought-after pluripotential cells, and they passed every test: They formed teratomas *in vivo,* and they differentiated *in vitro*. They bore the cell surface antigens that we expected. They stained strongly positive for alkaline phosphatase, were karyotypically normal and, most importantly, made splendid chimeras. At first we called them 'ED', for 'embryo-derived', and then 'EK', as a slight change from EC and as our initials (Evans–Kaufman). Gail Martin, who derived similar but slightly abnormal cells a year later, coined the term 'embryonic stem cells' or 'ES', the name that has stuck.

Matt and I submitted our original derivation and characterization of the ES cells to *Nature* early in 1981 and it was published in July[12]. Over the next 3 years we studied details of their establishment and maintenance and ability to form chimeras. Liz Robertson took up the challenge of determining what happened in the derivation of the ES cells from the haploid embryos, and demonstrated that the expected XX chromosome composition of the diploidized cell lines was very unstable, with either loss of one X chromosome producing XO cells or, more unexpectedly, partial deletion of one of the two X chromosomes. These deleted X chromosomes helped Sohaila Rasten to identify the site of X inactivation[13]. Allan Bradley joined me first as a final-year-project student and subsequently as a PhD student. He and Liz were most instrumental in bringing the embryo injection technology to our lab and the resulting proof of the germline capability of these cells, which we were able to report in 1983–1984 (refs. 14,15).

Having proven the germline potential of these cells, I sought to develop techniques for their mutagenesis. Richard Man, Richard Mulligan and David Baltimore published their seminal paper on packaging retroviral vectors in 1983 (ref. 16), and in October 1985 I visited the Whitehead Institute for a month of exclusive uninterrupted bench work in Mulligan's lab. We later used the techniques I had learned there to mutagenize hypoxanthine phosphoribosyltransferase; this was our first specific 'designer mutation' in the mouse[17]. When, during the stay, I received a call from Oliver Smithies, I responded that only for him would I break my work in the lab. His paper demonstrating gene targeting by homologous recombination into an endogenous locus in tissue culture cells had just appeared[18]. I took samples of the ES cell cultures to him and spent a delightful weekend in Wisconsin.

Soon after I returned to my lab in Cambridge, Mario Capecchi came for a week's visit to collect cells and

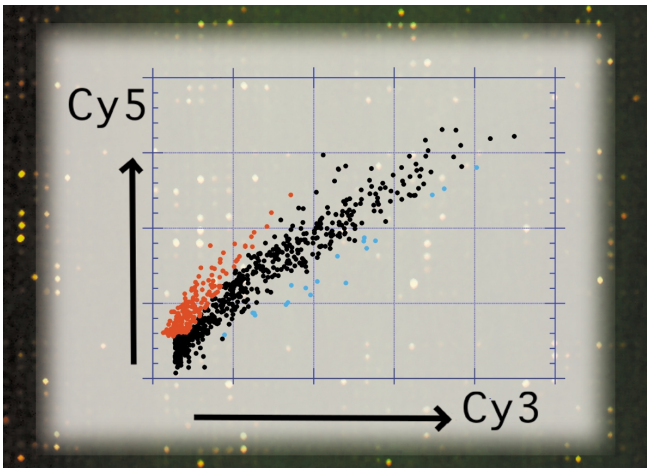

Fig. 2 Gene expression changes associated with ES cell differentiation. Microarray analysis using the NIA 15k c-DNA set as a probe. Cy3 labelling showing RNA from undifferentiated ES cells. Cy5 labelling showing RNA from cells 18 h into differentiation into embryoid bodies. Loci showing significantly (> 2 sd) increased (red) and decreased (blue) expression. (M.J. Evans, S.M. Hunter, P. Kille and S. Turner, unpublished data.)

learn the techniques. The rest of this story is better known. Many hundreds of specifically targeted mouse mutations have been made and the technique, although still not trivial, may now merit no more than a few lines' mention in experimental genetics papers. Almost any specific genetic change may now be generated, selected and verified in culture before being converted to the germ lines of mice, and this is the experimental genetics that is illuminating our understanding of the mammalian genome physiology and human function in health and disease.

I set out to derive a 'tractable' system for following mRNA changes coincident with embryonic cell differentiation. ES cells now provide the culture system and, at long last, methods for genome-wide monitoring of mRNA have come of age in cDNA microarrays. I am now putting the two techniques together, and results are beginning to emerge from this work (Fig. 2).

Forty years with homologous recombination

OLIVER SMITHIES

Toolmakers—and I suspect that the three of us being honored by the Lasker Foundation fit into this category—are fortunate people. They see problems, invent tools to solve them and enjoy the solutions, which often demonstrate new principles that were not part of the original thought. As a bonus, they also enjoy the vicarious pleasure of seeing other people use the same tools to solve very different problems. Yet the invention of an effective scientific tool is rarely an isolated event; there are often many prior experiences that trigger the inventive thought, and there may be various unexpected additional problems to solve before the toolmaker can bring a nascent idea into practice.

The chain of events leading to my contributions to the use of homologous recombination to modify genes in the mouse genome began over 40 years ago as an unplanned consequence of my somewhat serendipitous invention in the 1950s of an earlier tool—high-resolution gel electrophoresis—to solve a completely non-genetic problem. On 26 October 1954, during final pre-publication tests of my starch-gel electrophoresis system (the immediate forerunner of one of molecular biologists' primary tools, polyacrylamide gel electrophoresis), I ran a sample of serum from a female. My notebook (Fig. 1) has the entry that the pattern was "Most odd—many extra components." For about a week I enjoyed the misconception that I had discovered a new way of telling males from females. But this 'sexy' hypothesis soon gave way to the idea that "hereditary factors may determine the serum groups"[19] and, with the help of Norma Ford Walker, who began my education as a geneticist, this was soon proven correct[20]. The field of normal human protein polymorphic variants was seeded!

The hereditary variations we had discovered proved to be in the hemoglobin-binding serum protein haptoglobin, and their details were worked out during a happy collaboration between George E. Connell, Gordon H. Dixon and me in the early 1960s. The haptoglobin alleles *Hp1F* (fast) and *Hp1S* (slow) encoded polypeptides differing by two amino acids, but the third allele, *Hp2,* seemed to be a tandem joining together of sequences from *Hp1F* with sequences from *Hp1S*. The then-chairman of my department at the University of Wisconsin, James F. Crow, on being asked how the *Hp2* allele might have arisen, directed me to the *Bar* locus in *Drosophila* with its fascinating history of repeated 'mutations' resulting from unequal crossing over[21]. This led us to hypothesize that the *Hp2* allele was formed by a unique non-homologous recombinational event that joined the end of *Hp1F* to the beginning of *Hp1S* (ref. 22). *Hp2* therefore contained a small intragenic tandem duplication. The Bar gene in *Drosophila* is also a unique tandem duplication, but it is large enough to be visible when the fly salivary chromosomes are under the microscope. Yet the consequences of the tandemly repeated sequences in *Bar* and in *Hp* are completely comparable. In both cases, subsequent predictable unequal homologous crossing over events occur, which generate a new triplicate product and regenerate the singleton: B–B × B–B leads to B–B–B + B.

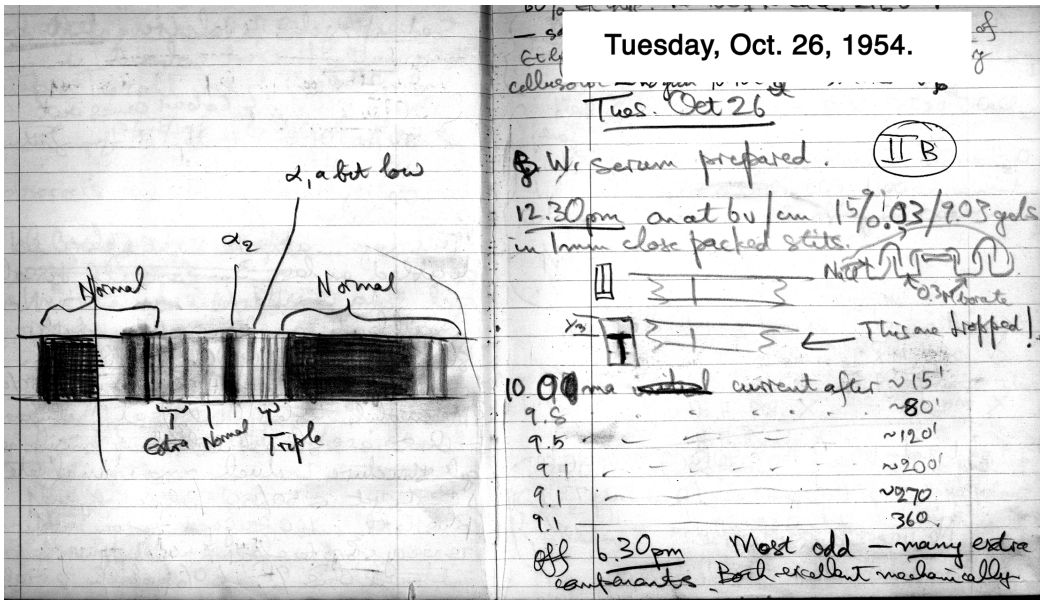

Fig. 1 Pages 97 & 98 from Smithies' lab notebook "Physical IV", 1954.

I found the predictability of homologous recombination seductive, and enjoyed enormously hypothesizing that antibody variability might be achieved by homologous recombination between tandemly arranged sequences[23]. The hypothesis turned out to be incorrect in mammals, but was remarkably close to being correct in chickens. Homologous recombination reappeared in my experimental science in the early days of cloning human genes when we were determining the nucleotide sequences of the two

human fetal globin genes, $^{G}\gamma$ and $^{A}\gamma$. The nucleotide sequence data showed distinct evidence that an exchange of DNA sequences had occurred between these two genes, as a result of homologous recombination in the form of a gene-conversion event[24].

With 20 years of experimental and theoretical exposure to the ubiquity and predictability of homologous recombination in the human genome when suitable sequences were present, and an inexhaustible supply of normal β-globin DNA now available by cloning, a not-unexpected thought kept coming into my mind, namely that correction of the sickle-cell β-globin gene mutation should be possible by homologous recombination between 'corrective' DNA introduced into a cell and a mutated gene present in the cellular genome. However, I had no way of estimating the likely frequency of such an event, which I suspected might be low, nor could I think of any easy way of detecting the event if it occurred. Then in the spring of 1982, I reviewed for my genetics class a paper published in *Nature* 1 April 1982 (ref. 25). The authors of this paper were searching for a mutated gene in a carcinoma cell genome that changes normal cells into focus-forming transformants. Their strategy for isolating this gene was elegant, although complex. But I realized that it could be adapted to determining whether homologous recombination can place corrective DNA into a chosen place in the genome.

I have lost the scraps of paper on which this realization was assembled into a plan, but on 22 April 1982, 3 weeks after the Goldfarb paper was published, I wrote in my lab notebook (Fig. 2) an outline of how I thought a test of corrective gene targeting might be made. In my more than 100 lab notebooks, this page is the one I like best! The principle of the proposed "assay for gene placement" is simple, although its execution was not. It depends on detecting in the genomes of correctly targeted cells the bringing together of DNA sequences present in the targeting construct but not in the genome and DNA sequences that are in the target locus but not in the incoming DNA. Three levels of selection were in the assay. I fondly imagined (probably incorrectly) that the power of my assay was therefore about 10^5 (for the thymidine kinase selection in eukaryotic cells) $\times$ 10^5 (for the *sup F* selection in prokaryotic cells) $\times$ 10^6 (for the β-specific probe hybridization). Even if targeting were random, I should be able to detect it!

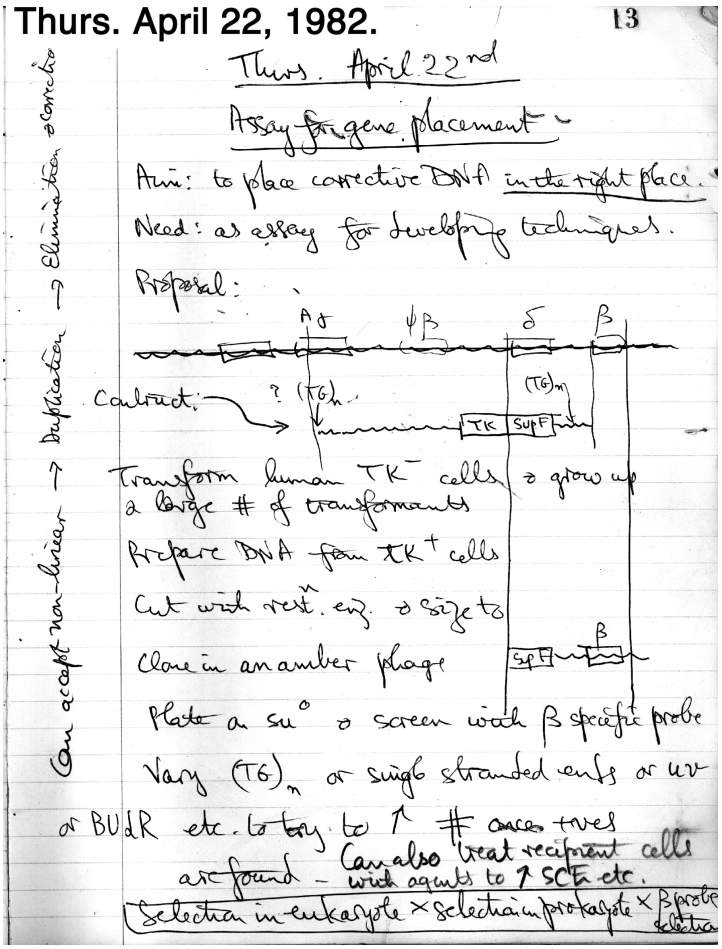

Fig. 2 Page 13 from Smithies' lab notebook γ, 1982.

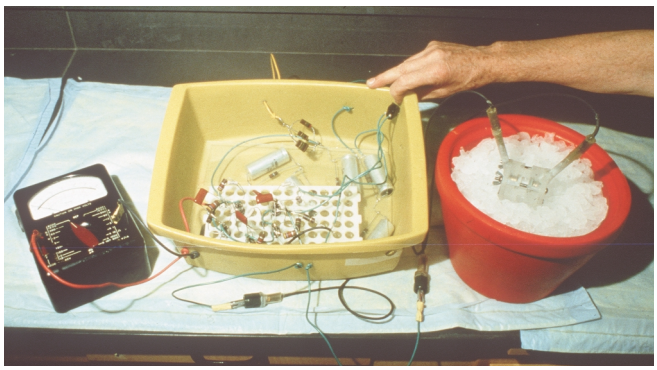

Fig. 3 Electroporater for introducing exogenous DNA into target cells, Smithies' lab notebook ι, 1984.

It took 3 years and the invaluable help of my postdoctoral fellow Ron Gregg, my visiting professor Sallie Boggs, my technician Mike Koralewski and my longtime collaborator Raju Kucherlapati to go from this notebook page to successful proof that homologous recombination can be used to target a chosen gene in a mammalian cell. We first tested the scheme using human bladder carcinoma cells and calcium phosphate–DNA precipitates to introduce the DNA into the cells. We obtained no positive signals with these cells. My graduate student Karen Lyons pointed out that bladder cells might not express a selectable marker when it is introduced into the β-globin locus, as they do not express β-globin. So I began again with modified mouse erythroleukemia cells that do express the β-globin gene. But these cells only grow in suspension and cannot be transformed with calcium phosphate, and electroporators were not yet available commercially. So I designed and built my own, with a plastic bath tub, part of a test tube rack, a glass plate and some silicon-controlled rectifiers (Fig. 3). We used this apparatus for all the key experiments.

The first real, albeit indirect, evidence that the experiment was working was obtained on 30 January 1985, the happy day when we first detected bacteriophages that grew because they had picked up the *supF* gene and that also hybridized to the β-globin probe. We were now fairly certain that planned modification by homologous recombination had been achieved. To obtain direct evidence, over the next 3 months we did a 'sib-selection' procedure (bacteriophage assays on total DNA from decreasingly small pools of cells) until we were down to three 20-colony pools, one of which was still positive by the

bacteriophage assay. Individual colonies from this pool were tested on 18 May 1985 by Southern blot analysis (a direct assay). DNA from one of the colonies produced a hybridizing fragment of the correct size (Fig. 4), and we were 'home'!

I presented the results of our work at a Gordon Conference in 1985, and told the attendees the true story that, as I developed the critical gel autoradiograph, which we knew would provide the first direct test of whether or not the target gene had been modified, I was thinking that we had been a long time (3 years) knowing that our experiment was working only by indirect evidence—much like being an airplane pilot on instruments in the clouds. The autoradiograph was the moment of truth, comparable to the moment when you descend below the clouds and no longer depend on the indirect indications of your instruments: The runway is either there or it is not! The thrill of seeing it never pales. For the remainder of that meeting, other investigators would say, as they pointed to a desired result, "And there is my runway!" We published our results in the 19 September 1985 issue of *Nature*[18].

Nonetheless, our 'runway' was exceedingly difficult to find. In only about one in a million treated cells was homologous recombination achieved. Such a low frequency of gene targeting was not much use for gene therapy. And the assay, like my doctoral-thesis method of measuring osmotic pressures[26], was remarkably good at doing what it was designed to do, but both methods were impossibly laborious. No one, not even me, ever used either again. So, what to do? The first order of business was to try to improve the method. For this we needed an easier target, preferably one whose targeting could be assessed directly. The hypoxanthine phosphoribosyltransferase gene *(HPRT)* was an obvious choice, and so Ron Gregg began a series of attempts to correct a mutated *HPRT* or to inactivate a wild-type copy of the gene using homologous recombination.

We also needed to replace the bacteriophage recombinant fragment assay with something easier. Kary Mullis' new PCR tool could in principle detect recombinants. We could choose one primer specific to the incoming DNA and another primer specific to the target gene. PCR amplification would then only occur when the two primer sequences were juxtaposed by the desired homologous recombination. But, again, there were no commercial PCR machines available. So we made our own out of three old water-baths, home-made controllers and hot water valves used in domestic heating systems[27]. We still use it! Its six hoses look like octopus arms; for obvious reasons we call it 'hexapus'. The ease of this PCR-based recombinant fragment assay made screening for homologous recombinants much less difficult.

Meanwhile, at a 1985 Gordon Conference, I heard Erwin Wagner talk about Martin Evans' embryonic stem cells[12], which after injection into blastocysts can produce living progeny mice. Here was a more promising use of our one-in-a-million targeting skill. We could generate planned mutations or correct existing mutations in tissue culture, even if it took millions of cells, and expect to transfer the alterations into living mice. A visit to Erwin Wagner led to my contacting Martin Evans who, with typical generosity of spirit, personally brought some of his EK CC-1 cells to us in November 1985. My plan was "to use these to get *HPRT*$^-$ by recombination and get chimeras or germline by blastocyst route." Martin also put me in touch with Tom Doetschman, an American postdoctoral fellow wanting to return to the United States, who had personally isolated embryonic stem cells (now called ES cells) while in Rolf Kemmler's laboratory. He joined our group in late 1986.

At this point, Nobuyo Maeda and I attended a conference in Scotland at which Martin Evans and Martin Hooper both reported that they had obtained *HPRT*$^-$ mutant ES cells in tissue culture experiments. Nobuyo recognized that, in the course of helping Ron Gregg, she had already made a construct that could correct either of their *HPRT*$^-$ mutant cells. We told Evans and Hooper about this, and both immediately agreed to collaborate with us: Martin Hooper sent his mutant cells (TG-2a) to us, and we sent our construct to Martin Evans. Tom Doetschman tried Nobuyo's construct on the TG-2a cells. The very first experiment worked, and we published our results in *Nature* 10 December 1987 (ref. 28). But it still took 2 more years of valiant effort, spearheaded by my postdoctoral fellow Bev Koller, to accomplish the mouse blastocyst injections leading to chimeras, to obtain progeny with the altered gene and to report the "Germ-line transmission of a planned alteration made by homologous recombination in embryonic stem cells."[29]

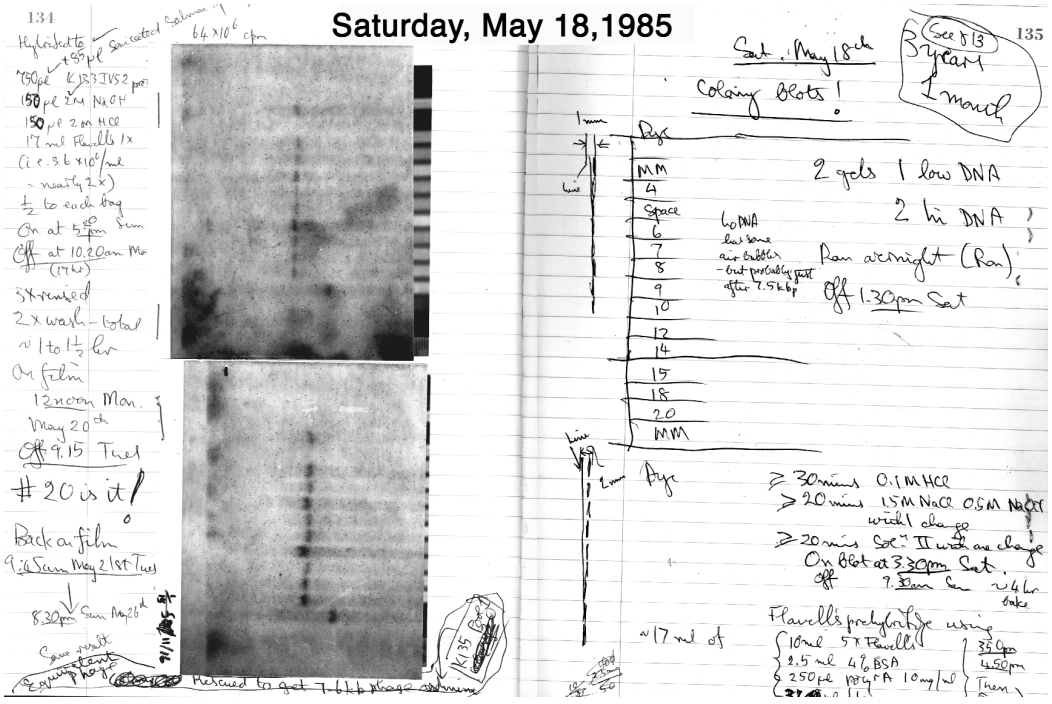

Fig. 4 Pages 134 & 135 from Smithies' lab notebook κ, 1985.

We next turned our attention to problems related to human diseases, beginning by

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

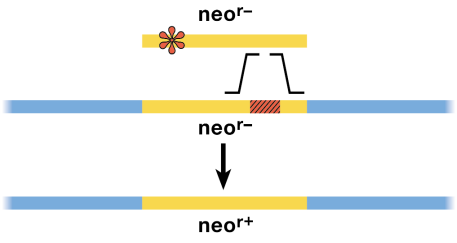

Fig. 1 Regeneration of a functional *neo*^{r} by gene targeting. The recipient cell contains a defective *neo*^{r} with a deletion mutation (*). The targeting vector contains a 5′ point mutation (Q). With a frequency of 1 in 1,000 cells receiving an injection, the deletion mutation in the chromosomal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

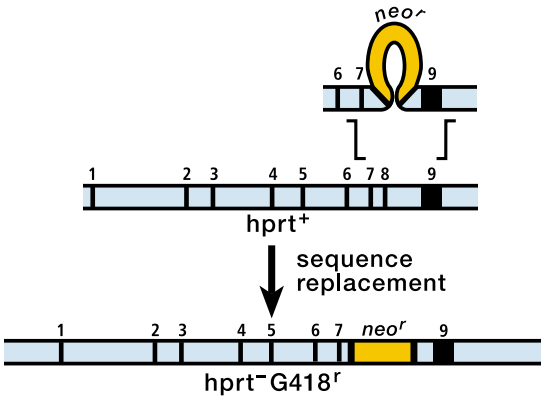

Fig. 2 Disruption of *Hprt* by gene targeting. The vector contains *Hprt* sequences disrupted in the eighth exon by *neo*ʳ. After homologous pairing between the vector and genomic sequences, a homologous recombination event replaces the genomic sequence with vector sequences containing *neo*ʳ. These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

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.

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

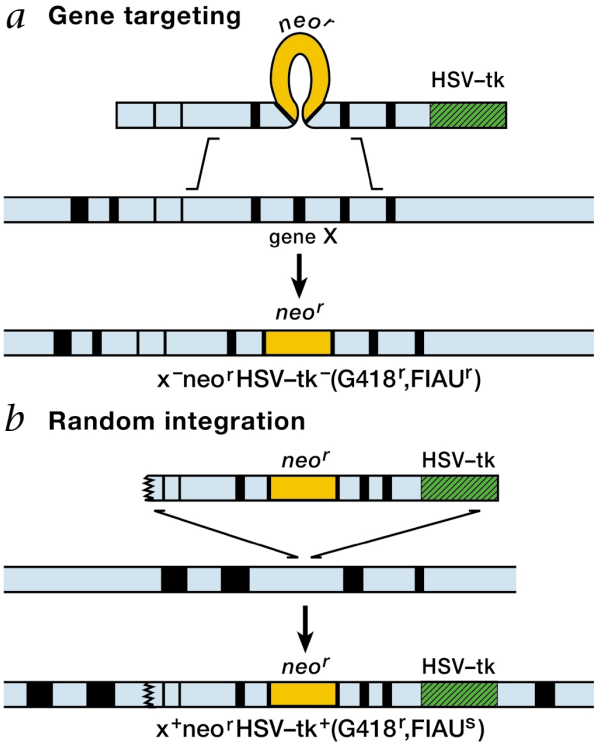

Fig. 3 The positive–negative selection procedure used to enrich for ES cells containing a targeted disruption of gene X. ***a***, The replacement-type vector contains an insertion of *neo*ʳ in an exon of gene X and a linked *HSV-tk* at one end. It is shown pairing with a chromosomal copy of gene X. Homologous 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 essnetial 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.

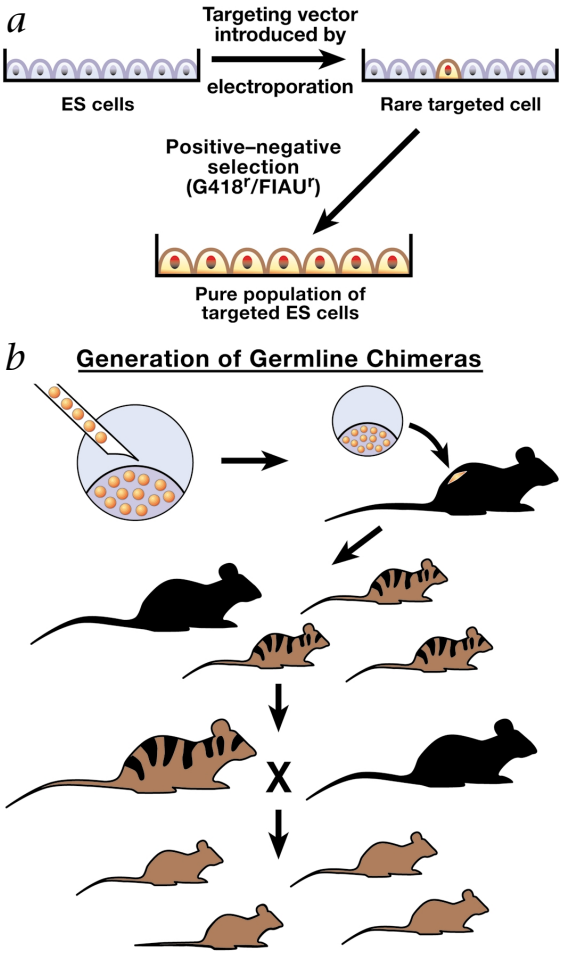

Fig. 4 Generation of mouse germline chimeras from ES cells containing a targeted mutation. ***a***, The first step involves the isolation of a clonal ES cell line containing the desired mutations. Positive–negative selection (Fig. 3) is used to enrich for ES cells containing the desired modified gene. ***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.

1. Stevens, L.C. The biology of teratomas. *Adv. Morphog.* **6**, 1–31 (1967).
2. Pierce, G.B. Teratocarcinoma: Model for a developmental concept of cancer. *Curr. Topics Dev. Biol.* **2**, 223–246 (1967).
3. Kleinsmith, L.J. & Pierce, G.B. Multipotentiality of single embryonal carcinoma cells. *Cancer Res.* **24**, 1544–1551 (1964).
4. Evans, M.J. The isolation and properties of a clonal tissue culture strain of pluripotent mouse teratocarcinoma cells. *J. Embryol. Exp. Morphol.* **28**, 163–196 (1972).
5. Evans, M.J. & Martin, G.R. The differentiation of clonal teratocarcinoma cell culture *in vitro*. In *Roche Symposium on Teratomas and Differentiation* (eds. Solter, D. & Sherman, M.) (Academic Press, New York, 1975).
6. Martin, G.R & Evans, M.J. Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies *in vitro. Proc. Natl. Acad. Sci. USA* **72**, 1441–1445, (1975).
7. Papaioannou, V.E., McBurney, M., Gardner, R.L. & Evans, M.J. The fate of teratocarcinoma cells injected into early mouse embryos. *Nature* **258**, 70–73 (1975).
8. Gooi, H.C. *et al.* Stage-specific embryonic antigen involves 1-3 fucosylated type 2 blood group chains. *Nature* **292**, 156–158 (1981).
9. Stinnakre, M.G., Evans, M.J., Willison, K.R. & Stern, P.L. Expression of Forssman antigen in the post-implantation mouse embryo. *J. Embryol. Exp. Morphol.* **61**, 117–131 (1981).
10. Lovell-Badge, R.H. & Evans, M.J. Changes in protein synthesis during differentiation of embryonal carcinoma cells and a comparison with embryo cells. *J. Embryol. Exp. Morphol.* **59**, 187–206 (1980).
11. Evans, M.J. Origin of mouse embryonal carcinoma cells and the possibility of their direct isolation into tissue culture. *J. Reprod. Fertil.* **62**, 625–631 (1981).
12. Evans, M.J. & Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156 (1981).
13. Rastan, S. & Robertson, E.J. X-chromosome deletions in embryo-derived (EK) cell-lines associated with lack of X-chromosome inactivation. *J. Embryol. Exp. Morph.* **90**, 379–388 (1985).
14. Evans, M.J., Bradley, A. & Robertson, E.J. EK cell contribution to chimeric mice: from tissue culture to sperm. In *Genetic Manipulation of the Early Mammalian Embryo, Banbury Report.* (Cold Spring Harbor Laboratory Press, Plainview, NY, 1983).
15. Bradley, A., Evans, M.J., Kaufman, M.H. & Robertson, E.J. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**, 255–256 (1984).
16. Mann, R., Mulligan, R.C. & Baltimore, D. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**, 153–159 (1983).
17. Kuehn, M.R., Bradley, A., Robertson, E.J. & Evans, M.J. A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. *Nature* **326**, 295–298 (1987).
18. 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).
19. Smithies, O. Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. *Biochem. J.* **61**, 629–641 (1955).
20. Smithies, O. & Walker, N.F. Genetic control of some serum proteins in normal humans. *Nature* **176**, 1265–1266 (1955).
21. Sturtevant, A.H. The effects of unequal crossing over at the Bar locus in *Drosophila. Genetics* **10**, 117–147 (1925).
22. Smithies, O., Connell, G.E. & Dixon, G.H. Chromosomal rearrangements and the evolution of haptoglobin genes. *Nature* **196**, 232–236 (1962).
23. Smithies, O. Antibody variability. *Science* **157**, 267–273 (1967).
24. Slightom, J.L., Blechl, A.E. & Smithies, O. Human fetal $^{G}\gamma$ and $^{A}\gamma$ globin genes: Complete nucleotide sequences suggest that DNA can be exchanged between these duplicated genes. *Cell* **21**, 627–638 (1980).
25. Goldfarb, M., Shimizu, K., Perucho, M. & Wigler, M. Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells. *Nature* **296**, 404–409 (1982).
26. Smithies, O. A Dynamic osmometer for accurate measurements on small quantities of material: Osmotic pressures of isoelectric β-lactoglobulin solutions. *Biochem. J.* **55**, 57–67 (1953).
27. Kim, H.S. & Smithies, O. Recombinant fragment assay for gene targetting based on the polymerase chain reaction. *Nucleic Acids Res.* **16**, 8887–8903 (1988).
28. Doetschman, T. *et al.* Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. *Nature* **330**, 576–578 (1987).
29. Koller, B.H. *et al.* Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cell. *Proc. Natl. Acad. Sci. USA* **86**, 8927–8931 (1989).
30. Smithies, O. & Maeda, N. Gene targeting approaches to complex genetic diseases: Atherosclerosis and essential hypertension. *Proc. Natl. Acad. Sci. USA* **92**, 5266–5272 (1995).
31. Kim, H.-S. *et al.* Genetic control of blood pressure and the angiotensinogen locus. *Proc. Natl. Acad. Sci. USA* **92**, 2735–2739 (1995).
32. Capecchi, M.R. Targeted gene replacement. *Sci. Am.* **270**, 54–61 (1994).
33. Wigler, M. *et al.* Transfer of purified Herpes Virus thymidine kinase gene to cultured mouse cells. *Cell* **11**, 223–232 (1977).
34. Capecchi, M.R. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* **22**, 479–488 (1980).
35. 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).
36. Costantini, F. & Lacy, E. Introduction of a rabbit β-globin gene into the mouse germ line. *Nature* **294**, 92–94 (1981).
37. 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).
38. 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).
39. 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).
40. Levinson, B., Khoury, B.G., VandeWoude, G. & Gruss, P. Activation of SV40 genome by 72-base pair tandem repeats of Moloney sarcoma virus. *Nature* **295**, 568–572 (1982).
41. 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).
42. Folger, K.R., Thomas, K.R. & Capecchi, M.R. Nonreciprocal exchanges of information between DNA duplexes coinjected into mammalian cell nuclei. *Mol. Cell. Biol.* **5**, 59–69 (1985).
43. Wong, E.A. & Capecchi, M.R. Homologous recombination between coinjected DNA sequences peaks in early to mid-S phase. *Mol. Cell. Biol.* **7**, 2294–2295 (1987).
44. 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).
45. Folger, K.R., Thomas, K.R. & M. R. Capecchi. Analysis of homologous recombination in cultured mammalian cells. *Cold Spring Harbor Symp. Quant. Biol.* **49**, 123–138 (1984).
46. Thomas, K.R. & Capecchi, M.R. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503–512 (1987).
47. 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).
48. 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).
49. 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).
50. 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).
51. Gu, H., Marth, J.D., Orban, P.C., Mossmann, H. & Rajewsky, K. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* **265**, 103–106 (1994).

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