

# GENE TARGETING 1977–PRESENT

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by

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## EARLY EXPERIMENTS

My entry into what was going to become the field of gene targeting started in 1977. The size of my laboratory in Utah, devoted to this project, was modest: myself and two competent technicians—my wife Laurie Fraser, and Susan Tamowski. I was experimenting with the use of extremely small glass needles to inject DNA directly into nuclei of living cells. In the laboratory adjacent to ours, Dr. Larry Okun, a neuroscientist, was recording intracellular electrical potentials in cultured neurons from chick dorsal root ganglia. His apparatus for penetrating the cells non-destructively to measure these electrical potentials appeared to be ideal for conversion into a “microsyringe” to allow pumping of defined quantities of macromolecules, including DNA, into mammalian cells in culture. Larry (Figure 1) graciously helped me enormously with the process of conversion. I should further add that Larry Okun, has been over many years, too many to count on one’s fingers and toes, my favorite person to discuss science, politics, and trivia. But his rigorous insight into science, in particular, has been of immeasurable help to me throughout my tenure at the University of Utah. Having enticed me and my wife<sup>(1)</sup> to come to Utah from Boston in the first place, by organizing an unbelievably beautiful 10 day backpacking trip in the nearby Wind River Mountains of Wyoming, along a series of mountain lakes bursting with trout every evening, he owed us quite a bit, and he delivered. Once assembled, the injection apparatus (Figure 2) was quite effective, allowing me to do 1000 nuclear injections per hour of well-defined volumes of solution (in the range of femtoliters) containing chosen macromolecules.

In 1977, Wigler and Axel showed that cultured mammalian cells deficient in the enzyme, thymidine kinase,  $Tk^-$ , could be restored to  $Tk^+$  status by the introduction of functional copies of the herpes virus *thymidine kinase gene* (*HSV-tk*)<sup>(2)</sup>. 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 the cultured cells by phagocytosis—was not very efficient. With their method, stable incorporation of functional copies of HSV-tk occurred in approximately one cell per million cells exposed to the DNA calcium phosphate co-precipitate<sup>(2)</sup>. It seemed that the low efficiency might be



Figure 1. A photograph of Lawrence M. Okun.

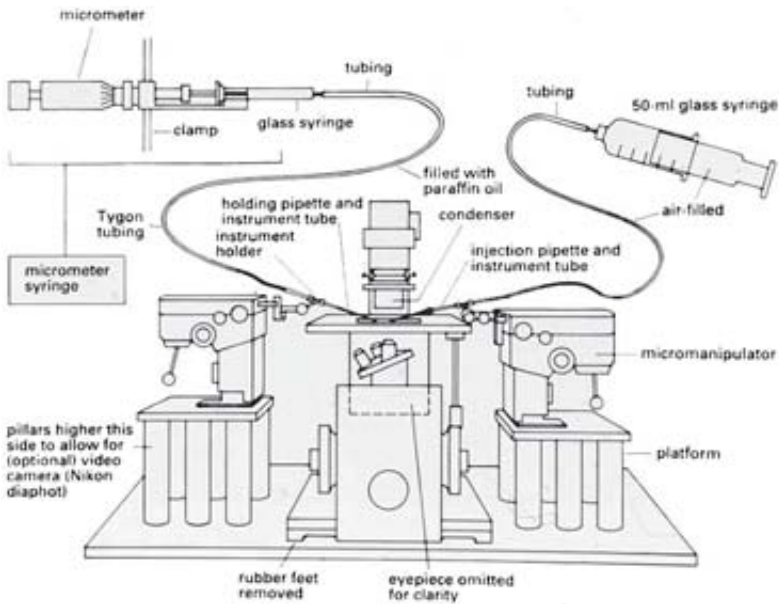


Figure 2. A schematic of the apparatus I used to inject DNA into nuclei of mammalian cells in culture. Micromanipulators are used to guide the needle, tip diameter  $0.1 \mu\text{m}$ , containing the DNA solution into nuclei of living cells while being viewed through a light microscope.

a problem of delivery. Most of the DNA taken up by the cells did not appear to be delivered to the nucleus, where it could function, but instead was destined for lysosomes, where it was degraded. I sought to determine whether I could introduce functional copies of the *HSV-tk* gene directly into nuclei of cultured TK<sup>-</sup> cells using the microinjection apparatus described above. This procedure proved to be extremely efficient; one cell in three that received the DNA stably passed functional copies of the *HSV-tk* gene onto its daughter cells<sup>(3)</sup>. An immediate outcome of these experiments was that the high efficiency of DNA transfer that we observed by microinjection made it practical for investigators to use the same methodology to generate transgenic mice containing random insertions of exogenous DNA. This was accomplished by injection of the desired DNA into nuclei of one-celled mouse zygotes with the resulting embryos allowed to come to term after transfer to the uterus of foster mothers<sup>(4-8)</sup>. The generation of transgenic mice, in which chosen exogenous pieces of DNA have been randomly inserted within the mouse genome, has become a cottage industry.

However, I found that the efficient transfer of functional *HSV-tk* genes into the host cell genome required that the injected *HSV-tk* genes be linked to an additional short viral DNA sequence<sup>(3)</sup>. It seemed plausible to me that highly evolved viral genomes which, as part of their life cycle, resided in the host cell genome, might contain bits of DNA sequence that enhanced their ability to establish themselves within the host cell genome. I searched the genomes of the lytic simian virus, SV40, and the ASV retroviral provirus for the presence of such sequences and found them<sup>(3)</sup>. When linked to the injected *HSV-tk* gene, these sequences increased the frequency with which TK<sup>+</sup> cells were generated by a factor of 100 over that produced by *HSV-tk* DNA injected alone. I showed that this enhancement did not result from independent replication of the injected *HSV-tk* DNA as an extra-chromosomal plasmid, but rather that the efficiency-enhancing sequences were either increasing the frequency with which the exogenous DNA was inserting itself into the host genome or increasing the probability that the *HSV-tk* gene, once integrated into the host genome, was being expressed in the recipient cells. The latter turned out to be correct. These experiments were completed before the idea of gene-expression enhancers had emerged and contributed to the definition of these special DNA sequences<sup>(9)</sup>. Further, the emerging idea of enhancers profoundly influenced our contributions to the development of gene targeting vectors. Specifically, it alerted us to the importance of using appropriate enhancers to mediate expression of newly introduced selectable genes (used to select for successfully altered recipient cells), regardless of the inherent expression characteristics of the host chromosomal sites into which we were targeting those genes<sup>(10)</sup>.

## HOMOLOGOUS RECOMBINATION

Although the ability to introduce exogenous DNA randomly into the host cell genome with very high efficiency by microinjection was itself extremely

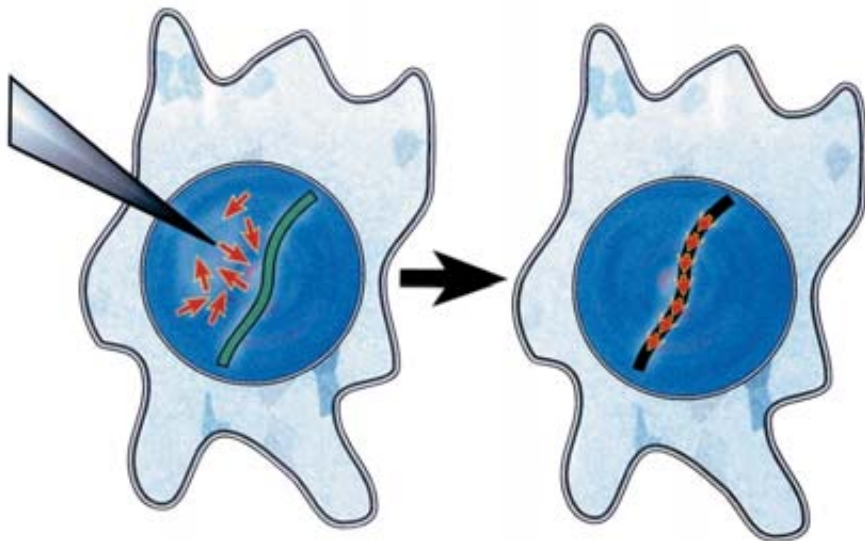


Figure 3. Formation of highly ordered head-to-tail DNA concatemers following introduction of multiple copies of the same DNA sequence into mammalian cell nuclei.

useful, the observation that I found most fascinating from these early DNA-injection experiments was that, when multiple copies of the HSV-tk plasmid were injected into a given cell, though many of them became randomly inserted into the host cell's genome, they would all be found at a single locus, as a highly ordered head-to-tail concatemer (see Figure 3)<sup>(11)</sup>. This was the key observation and stimulus for the targeting project that followed.

It was clear, however, that this project would now progress more rapidly with the efforts of additional investigators. Fortunately, two very gifted post-doctoral fellows, Drs. Kim Folger Bruce and Eric Wong chose to join my group at this time (see Figure 4). It seemed that the highly-ordered concatemers of exogenous genes found at the insertion sites could not arise by a random mechanism, but were likely generated either by replication of the injected DNA before insertion (for example by a rolling circle-type mechanism of DNA replication) or by homologous recombination between the co-injected HSV-tk plasmids. We proved that they were generated by homologous recombination<sup>(11)</sup>. This conclusion was very significant because it demonstrated that mammalian somatic cells contain an efficient enzymatic machinery for mediating homologous recombination. The high efficiency of this machinery became evident from the observation that when more than 100 HSV-tk plasmid molecules were injected per cell, they were all incorporated into a single, ordered, head-to-tail concatemer<sup>(11)</sup>. These experiments were also the first demonstration of homologous recombination between co-introduced DNA molecules in cultured mammalian cells. From these results it was immediately apparent to me that if we could harness this efficient machinery to mediate homologous recombination between a newly introduced DNA molecule of our choice and the same DNA sequence in the recipient



Figure 4. Photographs of Kim Folger Bruce and Eric Wong who worked in my laboratory from 1981–1985 and 1983–1986 respectively.

cell's genome, we would have the ability to mutate any endogenous cellular gene in cultured cells, in any chosen way. It was thus these experiments that provided us the incentive for vigorous pursuit of gene targeting in mammalian cells. Interestingly, these experiments were done prior to our hearing that gene targeting could be readily achieved in yeast<sup>(12)</sup>. The results derived from the analysis of mechanisms of gene targeting in yeast did, however, influence our thinking during subsequent development of gene targeting in mammalian cells<sup>(13–15)</sup>.

The next step in our quest for achieving mammalian gene targeting required our becoming more familiar with the homologous recombination machinery in mammalian cells, for example its substrate preferences and what were the most common reaction products resulting from homologous recombination. At this time Dr. Kirk Thomas also joined my research group as a postdoctoral fellow and became a critical contributor to our research (Figure 5). By examining homologous recombination between co-injected DNA molecules, we learned, among other things, that linear DNA molecules, rather than circular or super-coiled molecules were a preferred substrate for homologous recombination, that the efficiency of homologous recombination was cell-cycle dependent, showing a peak of activity in early S phase; and that, although both reciprocal and non-reciprocal exchanges occurred, there was a distinct bias towards the latter<sup>(16–18)</sup>. These results contributed substantially to our choice of experimental design for the next stage of our quest—the detection of homologous recombination between newly introduced, exogenous DNA molecules and their endogenous chromosomal homologs in recipient cells.



Figure 5. Photograph of Kirk R. Thomas who was in my laboratory from 1983 to 2002 first as a postdoctoral fellow and then as a Senior Scientist of the Howard Hughes Medical Institute.

In 1980, I submitted a grant application to the National Institute of Health proposing to test the feasibility of such gene targeting in mammalian cells. These experiments were emphatically discouraged by the reviewers on the grounds that there might be only a vanishing small probability that the newly introduced DNA would ever find its matching sequence within the host cell genome, a prerequisite for homologous recombination. Despite the rejection, I decided to put all of our effort into continuing this line of research. This was a big gamble on our part. Aware that the frequency of gene targeting to homologous sites was likely to be low and that the far more common competitive reaction would be random insertion of the targeting vector into non-homologous sites of the host cell genome, we proposed to use selection to eliminate cells not containing the desired homologous recombination events. One first test of gene targeting (Figure 6) used, as the chromosomal target, DNA sequences that we had previously randomly inserted into the host cell genome. Thus, the first step in this scheme required generating cell lines containing random insertions of a defective *neomycin-resistance gene* (*neo<sup>r</sup>*) that contained either a small deletion or a point mutation in that gene. In the second step, the targeting-vector DNA also contained a defective *neo<sup>r</sup>* gene, with a mutation that differed from the one present at the host cell target site. Homologous recombination, between the two defective *neo<sup>r</sup>* genes, one in the targeting vector and the second residing in the host cell chromosome, could generate a functional *neo<sup>r</sup>* from the two defective parts and render the cells resistant to the drug G418, which is lethal to cells without a

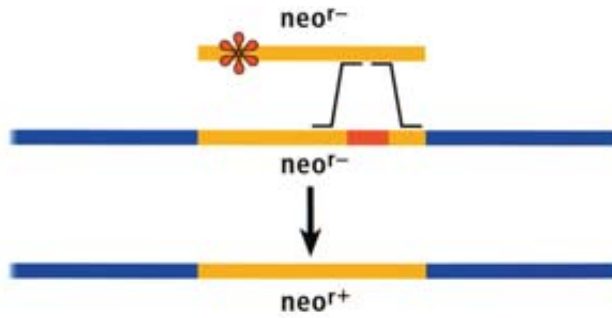


Figure 6. Regeneration of a functional  $neo^r$  gene by gene targeting. The recipient cell contains a defective  $neo^r$  with a deletion mutation (del). The targeting vector contains a 5'-point mutation (\*). With a frequency of approximately 1 in 1000 cells receiving an injection of the targeting vector containing the point mutation, the chromosomal copy of  $neo^r$  is corrected with the information supplied by the targeting vector.

functional  $neo^r$  gene. Thus, successful gene targeting events would yield cells capable of growth in medium containing G418.

For the first step we generated recipient cell lines containing a single copy of the defective  $neo^r$  gene, cell lines containing multiple copies of the defective gene as a head-to-tail concatemer and, by inhibiting concatemer formation, even cell lines containing multiple defective  $neo^r$  genes as single copies inserted in separate chromosomes. These different recipient cell lines allowed us to evaluate how the number and location of the target sites within a recipient cell's genome influenced the targeting frequency. By 1984, we had good evidence that gene targeting in cultured mammalian cells indeed occurs<sup>(19)</sup>. At this time, I submitted another grant application to the same National Institute of Health study section that had rejected our earlier proposed gene targeting experiments. Their response was "We are glad that you didn't follow our advice."

To our delight, correction of the defective chromosomally-located  $neo^r$  genes by homologous recombination with our microinjected gene-targeting vector occurred at an absolute frequency of 1 per 1,000 injected cells<sup>(20)</sup>. This frequency was many orders of magnitude greater than the reversion frequency of the individual  $neo^r$  mutations by themselves. Furthermore, the frequency was not only higher than we expected, particularly considering that the extent of DNA sequence homology between the targeting vector and the target locus was less than 1,000 base pairs, but the relatively high targeting frequency made it practical for us to examine a number of parameters influencing that frequency<sup>(20)</sup>. An important lesson learned from testing the different recipient cell lines was that all of the chromosomal target positions analyzed seemed equally accessible to the homologous recombination machinery, indicating that a large fraction of the mouse genome was likely to be manipulable through gene targeting<sup>(20)</sup>.

At this time, Oliver Smithies and his colleagues reported their classic experiment of targeting modification of the  $\beta$ -globin locus in cultured mam-

malian cells<sup>(21)</sup>. This elegant experiment demonstrated that it was feasible to disrupt an endogenous gene in cultured mammalian cells. Oliver and I pursued gene targeting independently. We had separate visions in mind and different approaches to its implementation. Through the years we have been extremely fortunate in our ability to share expertise and reagents, as well as enjoying each other's fellowship. That is not to say we were not competitive. Science is very competitive and a high premium is placed on being first. Equally important, however, science is also a very communal enterprise in which all are dependent on past and concurrent contributions by many, many other investigators for advances and inspiration. Where would either Oliver's lab or mine have gotten without the ability to generate viable mouse chimeras, initially starting with mouse morulas, and then extending the technology to injected cells from the innercell mass, EC cells, and ES cells into the pre-implantation embryos? The contributions and progression of this technology by Mintz, Gardner, Stevens, Martin, and Evans are apparent<sup>(22-28)</sup>, providing just some examples of the many investigators whose efforts have been essential to our eventual ability to do gene targeting in living mice.

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 gene targeting to the living mouse. The low frequency of gene targeting, relative to random integration of the targeting vector into the recipient cell genome, made it impractical to attempt gene targeting directly in one-celled mouse zygotes. Instead, it seemed our best option was to carry out the gene targeting in populations of cultured embryo-derived stem (ES) cells, from which the relatively rare targeted recombinants could then be selected and purified. These purified cells, when subsequently introduced into preimplantation embryos and allowed to mature in a foster mother, would be expected to contribute to the formation of all tissues of the mouse, including the germ line. Fortunately, at a Gordon Conference in the summer of 1984, when we were ready to initiate these experiments, I heard that Martin Evans had isolated from mouse embryos ES cells capable of contributing in just this way to the formation of the germ line and to do so at a reasonable frequency<sup>(27,28)</sup>. Martin's ES cells appeared to be much more promising in their potential to contribute to the embryonic germline than were the previously described embryonal carcinoma (EC) cells<sup>(24,29)</sup>.

## GENE TARGETING IN ES CELLS

In the winter of 1985, my wife, Laurie Fraser, and I spent a week in Martin Evans' laboratory learning how to derive and culture mouse ES cells, as well as how to generate mouse chimeras from these cells by their addition to recipient preimplantation embryos. Also instrumental in our learning these techniques were Dr. Elizabeth Robertson and Alan Bradley, a postdoctoral fellow and graduate student in Evans' laboratory, respectively. This is an excellent example of how science progresses from the collective sharing of expertise and resources. We have always been grateful for Martin's generosity.



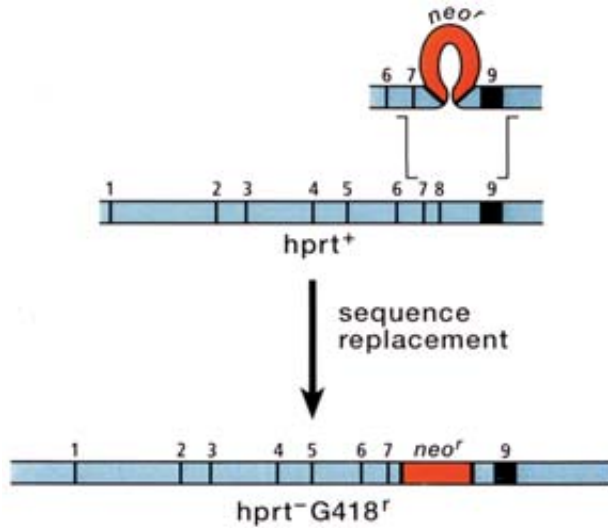


Figure 7. Disruption of *hprt* gene by gene targeting in mouse ES cells. The targeting vector contains genomic sequences from the mouse *hprt* gene disrupted in the eighth exon by *neo<sup>r</sup>*. After homologous pairing between the vector and the cognate sequences in the endogenous *hprt* gene of the ES cell genome, a homologous recombination event replaces the ES cell genomic sequences with vector sequences containing the *neo<sup>r</sup>* gene. The resulting cells are able to grow in medium containing the drugs G418, which kills cells without an inserted functional *neo<sup>r</sup>* gene, and 6-TG, which kills cells with an undisrupted functional *hprt* gene.

In the beginning of 1986, our effort thus shifted to doing gene targeting experiments in mouse ES cells. We decided also to switch to electroporation as a means of introducing our targeting vectors into ES cells. Although microinjection was orders of magnitude more efficient than electroporation as a means for generating cell lines with targeted mutations, injections are done one cell at a time. With electroporation, we could introduce the gene targeting vector into  $10^7$  cells in a single experiment, easily producing large numbers of cells containing targeted mutations, even at the lower efficiency. In addition, it was apparent to us that, as a technology, electroporation would be more readily adopted by other laboratories, relative to microinjection, thereby making gene targeting more user friendly to more scientists.

To rigorously determine the quantitative efficiency of gene targeting in ES cells as well as to evaluate the parameters that affect the gene targeting frequency, we chose as our target locus the *hypoxanthine phosphoribosyl transferase* (*hprt*) gene. There were two reasons for this choice. First, since *hprt* is located on the X chromosome, and the ES cell lines that we were using were derived from a male mouse, only a single *hprt* locus had to be disrupted in the recipient cells to yield *hprt<sup>-</sup>* cell lines. Second, a good protocol for selecting cells with a disrupted *hprt* gene existed, based on the drug 6-thioguanine (6TG), which kills cells with a functional *hprt* gene<sup>(30)</sup>. The strategy we used was to generate a gene-targeting vector that contained an *hprt* genomic sequence that was disrupted by insertion of *neo<sup>r</sup>* in one of the gene's exons

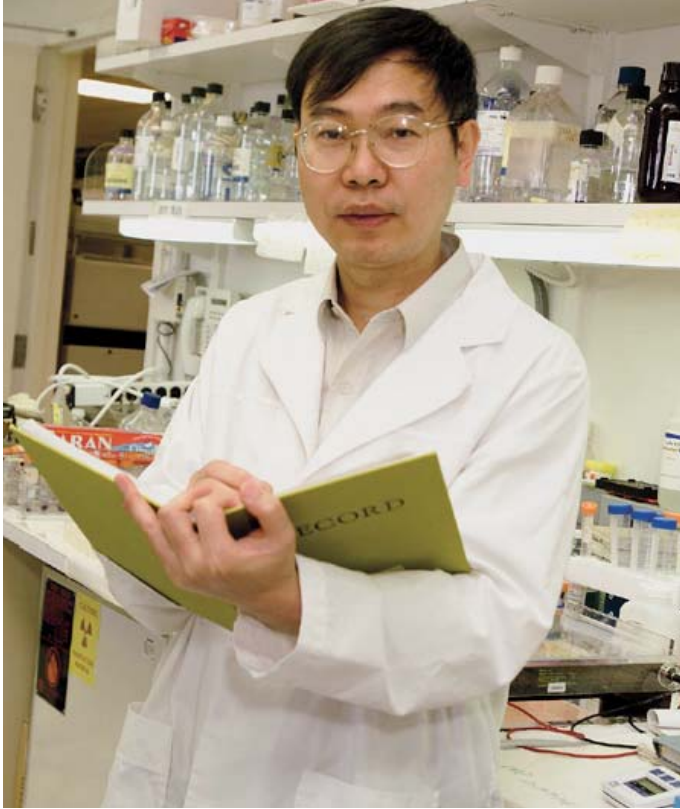


Figure 8. A photograph of Chuxia Deng who worked in my laboratory as a graduate student from 1987 to 1992.

(Figure 7). The exon we chose, exon 8, encodes the active catalytic site for this enzyme. Homologous recombination between this targeting vector and the endogenous *hprt* locus would generate *hprt*<sup>-</sup> ES cells resistant to growth in medium containing both 6TG (killing cells with untargeted *hprt*<sup>+</sup> loci) and G418 (killing cells lacking the inserted *neo*<sup>r</sup> gene, as described above). All cell lines generated from cells selected in this way, had lost *hprt* function as a result of the targeted disruptions of the *hprt* locus<sup>(10)</sup>. Thus, the *hprt* locus provided Chuxia Deng (Figure 8), then a graduate student in our laboratory, an ideal locus for further study of the parameters that influenced the targeting efficiency<sup>(31-33)</sup>.

Because we foresaw that the *neo*<sup>r</sup> gene would probably be used as a positive selectable gene for the disruption of many genes in ES cells, it was essential that its expression be mediated by an enhancer that would function in ES cells, regardless of the expression status of the target locus. Here our previous experience with enhancers proved of value. We knew from those experiments that the activities of promoter-enhancer configurations are very cell-type specific. To encourage strong *neo*<sup>r</sup> expression in ES cells, we chose to drive its expression with a mutated polyoma virus enhancer that had been

selected for strong expression in mouse embryonal carcinoma cells, which we presumed to be similar to mouse ES cells<sup>(10,34)</sup>. Subsequently, the strategy described above namely using a *neo<sup>r</sup>* driven by an enhancer that allows strong expression in ES cells independent of chromosomal locations, has become the standard for the disruption of most genes in ES cells.

The experiments described above showed that mouse ES cells were a good recipient host for gene targeting. In addition the drug-selection protocols required to identify ES cell lines containing the desired gene-targeting event did not appear to alter their pluripotent potential<sup>(10)</sup>. I believe that this paper was pivotal in the development of the field, encouraging other investigators to begin to use gene targeting in mice as a means for determining the function of chosen mammalian genes in the living animal.

The ratio of homologous, i.e. targeted insertions to random insertions at non-homologous sites in ES cells is approximately 1 to 1,000<sup>(10)</sup>. Because the disruption of most genes does not produce a cellular phenotype, that is selectable in cell culture, investigators seeking to disrupt a gene of choice would need to undertake tedious DNA screens through many cell colonies to identify the rare ones containing the desired targeting event.

To address this problem we reported in 1988, a general strategy to enrich cells in which a homologous targeting event has occurred<sup>(35)</sup>. This enrichment procedure, known as positive-negative selection, was derived from an observation in experiments done in our laboratory, namely that linear DNA molecules, when inserted at random sites in the recipient cell's genome, most frequently retain their ends, while sequences inserted at the target site, by homologous recombination, lose non-homologous ends from the original vector (see Figure 9). Further, contrary to expectations from studies of homologous recombinations in yeast, we showed that even blocking both ends of the homology arms of a targeting vector with non-homologous DNA sequences does not reduce the targeting frequency in mammalian cells<sup>(35)</sup>. This approach, correspondingly has two components. One component is a positive selectable gene, *neo<sup>r</sup>* used, as described above, 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 a random site 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 (medium containing the drugs, *gancyclovir* or FIAU, kills cells expressing the *HSV-tk* gene but not cells expressing the endogenous mammalian thymidine kinase gene). Thus the positive selection enriches for recipient cells that have incorporated the targeting vector somewhere in their genome, whereas the negative selection eliminates those that have incorporated it at non-homologous sites. The net effect is enrichment for cells in which the desired homologous 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 ES cells. The validity of the procedure was shown by using it to enrich for ES

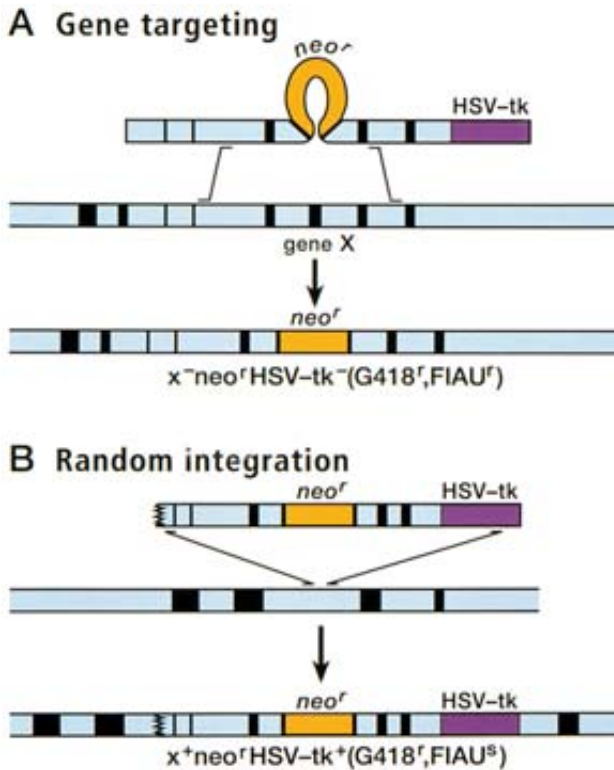


Figure 9. The positive-negative selection procedure used to enrich for ES cells containing a targeted disruption of gene X. a. The linear replacement-type targeting vector contains an insertion of  $neo^r$  in an exon of gene X and a linked HSV-tk gene at one end. It is shown pairing with a chromosomal 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 gene. Cells in which this event has occurred will be  $X^{+/-}$ ,  $neo^r$ , HSV-tk $^-$  and will grow in medium containing G418 and FIAU. The former requires the presence of a functional  $neo^r$  gene and the latter kills cells containing a functional HSV-tk gene. 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  $X^{+/+}$   $neo^{+}$  and HSVtk $^{+}$  and therefore resistant to growth in G418 but killed by presence of FIAU. Cells that have not received a targeting vector, will be  $X^{+/+}$   $neo^{-}$  and HSVtk $^{-}$  and will be killed by the presence of G418. As a consequence this procedure specifically enriches for cells in which a gene targeting event has occurred.

cells containing targeted mutations in the *int2* gene, now known as *Fgf3*<sup>(35)</sup>. These experiments were carried out by Suzi Mansour, a talented postdoctoral fellow in our laboratory (Figure 10) and Kirk R. Thomas (Figure 4). Positive-negative selection has become the most frequently used procedure to enrich for cells containing gene targeting events. Using positive-negative selection we have found that the targeting frequency varies from gene to gene. With genes that exhibit a high targeting frequency, a high percentage of clones obtained after positive-negative selection contain the targeting event. The worst



Figure 10. A photograph of Suzanne Mansour. She worked in my laboratory as a post-doctoral fellow from 1987 to 1992.

cases have been ones in which one in a hundred selected clones contains the desired targeting event. If the targeted gene is one expressed in ES cells, then the targeting frequency at that locus is likely to be high.

#### EXTENSIONS AND MORE RECENT DEVELOPMENTS

The use of gene targeting to evaluate the functions of genes in the mouse is now routine. It is being used in hundreds of laboratories all over the world. Well over 11,000 genes have been disrupted in the mouse using the described procedures. This is quite surprising considering that these disruptions have been done in individual laboratories in the absence of coordinated programs. Now however, there are a number of funded national and international efforts to disrupt every gene in the mouse by gene targeting<sup>(36)</sup>. In addition, hundreds of human diseases have been modeled in the mouse by the use of gene targeting. These models allow study of the pathology of the diseases in much more detail than is possible in humans. In addition, the models provide a vehicle for subsequent development and evaluation of new therapeutic modalities including drugs.

To date, gene targeting has been used primarily to disrupt genes, producing so called “knockout mice.” However gene targeting can be used to alter the sequences of a chosen genetic locus in the mouse in any conceivable manner, thus providing a very general means for “editing” the mouse genome. It can be used to generate gain-of-function mutations or partial loss-of-function mutations. Gene targeting can also be used to restrict the loss of

function of a chosen gene to particular tissues, yielding so-called conditional mutations. This is most commonly achieved by combining exogenous (non-mammalian) site-specific recombination systems, such as those derived from bacteriophages or yeast (i.e. Cre/loxP or Flp/FRT respectively), with gene targeting, to mediate excision of a gene only where the appropriate recombinase is produced<sup>(37-40)</sup>. By control of where Cre- or Flp-recombinase is expressed, for example in the liver, a gene, flanked by loxP or FRT recognition sequences, respectively, can be excised in the desired tissue (e.g. liver). Temporal control of gene function has also been achieved by making the production of the functional recombinase dependent upon the administration of small molecules or even on physical stimuli, such as light<sup>(41-44)</sup>. Such conditional mutagenesis has been very effective for more accurate modeling of human cancers, which are often restricted to particular tissues and even to specific cells within those tissues, as well as being initiated post birth<sup>(45-48)</sup>. In human cancers, the interactions between the host tissues and the malignant cells are often critical to its initiation and progression<sup>(49,50)</sup>. Thus, inclusion of these interactions in the mouse model also becomes critical if the mouse model is to accurately recapitulate the human malignancy.

Gene targeting is an evolving technology and we can anticipate further extensions to its repertoire. To date it has been used primarily to perturb the function of one gene at a time. We can anticipate development of efficient multiplexing systems that will allow simultaneous conditional or non-conditional modulation of multiple genes. We can also anticipate improvements in exogenous reporter genes with parallel improvements in their detection, particularly with respect to capture times, resolution and sensitivity. Such improvements will undoubtedly be necessary if this technology is to make significant inroads in addressing truly complex biological questions, such as the molecular mechanisms underlying higher cognitive functions in mammals.

I have tried to take the reader through a brief, personal journey of my life, my development as a scientist, and our laboratory's development of gene targeting. In the process, I have tried to give credit to those who have helped me along the way to reach our goals. What I have failed to communicate is the enormity of the scientific community and how many scientists actually have helped in untold, countless ways. That list would be in the hundreds and thousands. As a scientist I have been fortunate to have visited many, many laboratories all over the world and to have talked with other scientists about their work and aspirations. It is through these conversations that one's vision broadens and an appreciation of the complexity and beauty of the biological world is reinforced. However the people that have been most influential are the members of my immediate family, Laurie Fraser and Misha Capecchi, my wife and daughter, respectively. Their support has kept me going, their sage advice has kept me from falling down too frequently, their love has made it all worthwhile.

The Nobel Prize has greatly rewarded a major segment of my life and, as a kind of demarcation invites some reflection. I hope that our contributions, among other developments, will be used by many to reduce suffering,

improve our health and extend the productivity and fulfillment of our lives. Equally important, I hope that the new biological insights will yield a better understanding of ourselves as human beings and of our relationship to our environment, so that we may become better stewards of a fragile Earth. We live in a closed system and we have to gain the knowledge that will enable us to live in harmony with it. Neither we nor our planet can any longer afford the ravages of wars. Nor can the planet survive needless consumption. We must learn to distribute our resources more equitably among all peoples. As a scientist, I naturally find myself thinking about the future. As a people, we must learn to become more responsible for the consequences of our activities over much longer periods of time so that future generations may also enjoy this splendid world. It is my hope that science can combine with ethics to permit this.

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