GENE TARGETING: AN HISTORICAL PERSPECTIVE

by

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Our entry into what was going to become the field of gene targeting began in 1977. I was experimenting with the use of extremely small glass needles to inject DNA directly into the nuclei of living cells. The needles were controlled by hydraulically driven micro-manipulators and were directed into nuclei with the aid of a microscope. Using this experimental paradigm, I asked myself whether I could introduce a functional gene into cells by injecting the DNA directly into their nuclei. This procedure turned out to be extremely efficient. One in three cells received the DNA in functional form and went on to divide and stably pass the gene onto its daughter cells (Capecchi, 1980). The high efficiency of micro-injection meant that it was now practical to use this technology to generate transgenic mice by the injection of DNA into one-cell zygotes. The embryos were then allowed to come to term by surgical transfer of the zygote to foster mothers. Indeed, this has become a cottage industry in many laboratories throughout the world (Gordon *et al.*, 1980; Costantini and Lacy, 1981; Brinster *et al.*, 1981; Wagner, E.F. *et al.*, 1981; Wagner, T.C. *et al.*, 1981) . However, generation of transgenic animals in this way involves the introduction of exogenous DNA segments at unpredictable locations in the recipient genome, and not targeted genetic alterations at defined sites.

I was personally fascinated by the following observation from these early micro-injection experiments. Although multiple copies of a DNA segment were integrated into a random location within the host chromosome when they were injected into a cell, they were always present in head-to-tail concatemers. Such highly ordered concatemers could be generated in two ways: (1) by replication, for example by a rolling circle type mechanism; or (2) by homologous recombination. We were able to prove unambiguously that the concatemers were generated by homologous recombination (Folger *et al.*, 1982). The significance of this observation was its demonstration that mammalian cells contain an efficient machinery for mediating homologous recombination. At the time, this was a startling discovery, because it was always assumed that the function of homologous recombination in all organisms, simple or complex, was to ensure broad dissemination of the parental genetic traits to their offspring by shuffling these traits in the germ cells. Finding evidence for this activity in mouse fibroblast cells implied that all cells, somatic as well as germ cells, were capable of mediating homologous recombination. The machinery in somatic cells appeared to be very efficient, since I could inject over 100 copies of a DNA sequence into a cell nucleus and they were all neatly incorporated into a single, ordered, head-to-tail concatemer. I realised immediately that, if I could harness this machinery to carry out homologous recombination between a newly introduced DNA

molecule of our choice and the same DNA sequence in the cell's chromosome, I would have the ability to mutate at will any specific gene in the living cell.

Homologous recombination between two similar DNA molecules involves the breaking and rejoining of these molecules. The exchange is done with such precision that the DNA sequence at the point of exchange is not altered. If one of the DNA molecules has a mutation or alteration relative to the other, then the modification is transferred to the other DNA molecule during the exchange. Gene targeting involves the transfer of a designed alteration in an exogenous DNA sequence to the cognate DNA sequence in the living cell genome via homologous recombination.

We spent the next few years in my laboratory becoming familiar with the machinery that mediates homologous recombination in mammalian cells in order to determine its likes and dislikes so that we could exploit this machinery for our purpose of gene targeting. In 1980, I submitted a National Institutes of Health (NIH) grant application outlining experiments intended to test the feasibility of gene targeting in mammalian cells. This part of the grant was soundly rejected. In the reviewers' opinion, the probability that the newly introduced DNA would ever find its matching sequence within the host genome was very small and, therefore, the experiments were not worthy of pursuit. Despite this rejection, I decided to forge ahead with these experiments using a paradigm that was capable of detecting gene-targeting events at a very low frequency. Once we observed a gene-targeting event, we could optimise the conditions to improve its efficiency. By 1984, we were confident that it was feasible to do gene targeting in cultured mammalian cells, and I presented our work at a memorable symposium on homologous recombination held at the Cold Spring Harbor Laboratory (Folger *et al.*, 1984). We resubmitted our grant application to the NIH. This time, the grant proposal was received with enthusiasm, and the new critique started with the words, "We are glad that you didn't follow our advice".

The next question we pursued was whether gene targeting could be extended to a whole animal, i.e. the mouse. Because of the low frequency of targeting events in mammalian cells, it was clear that doing the experiments directly in mouse zygotes would not be practical. Rather, targeting events had to be identified first in cultured cells to allow purification of a clonal cell line containing the desired gene disruption; these cells in turn could be used to generate mice capable of transmitting the mutation in their germline. I was familiar with the frustrations associated with previous attempts to obtain germline chimeras using embryonal carcinoma (EC) cells. However, in the summer of 1984 at a Gordon Conference, I heard a discussion of EK cells that appeared to be much more promising in their potential for contributing to the germline. EK cells, which were developed in Martin Evans' laboratory in Cambridge, United Kingdom, differed from EC cells in that they were obtained from the early mouse embryo, rather than from mouse tumours (Evans and Kaufman, 1981). In the winter of 1985, I arranged to spend a week in Martin Evans' laboratory to learn how to culture and manipulate mouse EK cells. It was just before Christmas, a marvellous time to be in Cambridge.

By early 1986, our total efforts were focused on EK cells, now known as embryonal stem (ES) cells. We also decided to use electroporation, rather than micro-injection, as a means of introducing the targeting vector into cells. Although micro-injection was orders of magnitude more efficient than electroporation for this purpose, injections had to be done one cell at a time and I was getting tired of doing micro-injections. With electroporation, 10^8 cells could be manipulated in a single experiment. I also thought that the use of the easier electroporation approach would help facilitate the transfer of the gene-targeting technology to other investigators. The gene that we chose to disrupt in ES cells was *hprt*, because it provided us with the luxury of being able to select directly for cells containing the desired disrupted gene. Since the *hprt* gene is located on the X-chromosome and ES cells were

derived from a male mouse, only a single locus had to be disrupted in order to yield *hprt* defective cells. The strategy that we employed was to use a neomycin-resistance gene (*neo*^{r}) to disrupt the *hprt* genomic sequences, then to select for cells resistant to both G418, a neomycin analogue, and 6-thioguanine (6-TG), a drug toxic to cells with a functional *hprt* gene. All such selected cell lines had lost *hprt* enzymatic activity as a result of targeted disruption of the *hprt* locus. These experiments showed that ES cells were indeed able to mediate homologous recombination, and that the selection protocols required to identify cell lines containing the targeted disruption did not alter their pluripotent state in culture (Thomas and Capecchi, 1987). This system also provided a good experimental paradigm for exploring the parameters that affect the efficiency of gene targeting. I believe that this study played a pivotal role in the development of the field by encouraging other investigators to now begin using gene targeting as a means of determining the function of genes in mammals.

Although mammalian cells have the machinery to direct the newly introduced DNA to its endogenous target, we found that the targeting vector was also inserted into random sites within the host genome by non-homologous recombination. The ratio of homologous to non-homologous recombination events is approximately 1 to 1 000. Since disruption of most genes is not expected to produce a phenotype selectable at the cellular level, an investigator seeking a specific disruption must either screen through many colonies of cells in order to identify the rare colony that contains the desired targeting event, or use selections that enrich for cells containing the targeting event. Late in 1986, I conceived of a general strategy to enrich for cells in which a targeting event had occurred. It was based on key observations made during our studies of recombination involving exogenous DNA in mammalian cells. Incorporation of DNA segments at random non-homologous sites involves insertions of a linearised vector through its ends, whereas recombination at a homologous target site involves crossover events occurring only through homologous sequences in the vector. Our strategy based on these observations, and known as positive-negative selection (PNS), uses two components. One component is a "positive selectable" gene, *neo*^{r}, used as a marker to select *for* cells that have incorporated the targeting vector anywhere in the recipient cell genome (i.e. at the target site via homologous recombination or at random sites via non-homologous recombination). The second component is a "negative selectable" gene, located at one end of the linearised targeting vector, used to select *against* cells that contain random insertions of the targeting vector. The net effect is to enrich 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 and succeeds whether or not the gene is expressed in the recipient ES cells (Mansour *et al.*, 1988). PNS is now the most frequently used procedure for enrichment of cells containing gene-targeting events. The origin of the idea for positive-negative selection was not very romantic and involved purely deductive reasoning. Enrichment for cells containing the targeting event could be achieved either by direct selection for cells containing the targeting event or by elimination of cells that contained random insertions of the targeting vector. This fact, coupled with an appreciation of how information is transferred between an exogenous and an endogenous DNA sequence via homologous and non-homologous recombination, naturally led to the concept of positive-negative selection.

Once ES cells with the desired mutation are obtained, how are they used to generate mice with the targeted mutation in all of their cells? Briefly, the ES cells carrying the targeted disruption are injected into an early, pre-implantation mouse embryo, the blastocyst. The blastocyst is then surgically transferred into the uterus of a foster mother to allow the embryo to come to term. ES cells are pluripotent, i.e. capable of giving rise to all cell types in the embryo. In the embryonic environment, these cells participate in forming all mouse tissues, most importantly the germ cells. If the source of the recipient blastocyst and the ES cells are mice of distinguishable coat colours, then

the resulting mouse is recognisable, because its coat will have stripes of both colours. Such mice, with cells of more than one genotype, are known as chimeras. If the chimera is a male, some or all of the sperm are likely to be derived from the ES cells that carry the targeted mutation. On breeding, the mutation will be transmitted, on average, to half of the offspring. These heterozygotes will be healthy in most instances, because their second, undamaged copy of the gene will still function properly. But mating of heterozygotes to brothers or sisters bearing the same mutation yields homozygotes: animals carrying the targeted mutation in both copies of the gene. Such animals will display abnormalities that help to reveal the normal function of the disrupted gene.

The use of gene targeting to evaluate the functions of genes in the living mouse is now a routine procedure. It is very gratifying to be able to pick up almost any major journal in the biological sciences and find the description of yet another gene "knock-out" mouse. In the past five years, the *in vivo* functions of over 300 genes have been determined with this approach. It is relatively easy to project where gene-targeting technology will go in the near future. It will continue to serve as the way to determine the roles of individual genes in mammalian biology. This will be accomplished by the generation of null mutations knocking out the genes of interest. Those investigators who desire deeper insights will generate an allelic series of mutations in a chosen gene to evaluate the effects of partial loss-of-function as well as gain-of-function mutations. To permit evaluation of potential multiple roles of a gene in different tissues, gene targeting will be used to engineer tissue-specific gene disruptions using the cre/loxP system (Gu *et al.*, 1994). Further, the technology soon should become available that will allow the investigator to turn chosen genes on or off in the adult or during any phase of mouse development. Finally, since most biological processes are mediated by interactions among a number of genes, such phenomena will be studied by combining multiple targeted mutations in a single mouse. There is no question that the mouse is a very complex organism. However, the broad range of genetic manipulations that are now available through gene targeting should provide a means for us to begin deciphering even the most complex of biological processes including development and learning.

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