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Thirteen years of manipulating the mouse genome: a personal history

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ABSTRACT In 1974, Dr. Ralph Brinster published a paper describing the consequences of injecting embryonal carcinoma cells, the predecessors of embryonic stem cells, into mouse blastocysts. Despite their early promise, embryonal carcinoma cells would not efficiently populate the germ line of mice. A decade later Elizabeth Robertson and I described the efficient generation of germline chimaeras from cultured embryonic stem cells and shortly afterwards the genetic manipulation of the mouse germline using ES cells. Our demonstration of the potency of Embryonic Stem cells gave birth to a new era in manipulative mouse genetics, one in which endogenous genes can now be mutated at will using gene targeting of retroviral mutagenesis. This review focuses on the development and testing of concepts and techniques during the thirteen years after we knew germline modification of endogenous genes in the mouse would be possible. This period is one in which more and more sophisticated tools for manipulating the mouse germline were developed and implemented. In this review I have taken the rare opportunity to reveal some of my thought processes, frustrations, successes and failures as we moved through this exciting period of rapid technological change. As I look forward to the next thirteen years, I feel that this will be an equally exciting period for manipulative genetics as we struggle to formulate concepts and design experiments that enable us to understand gene function in an era when the sequence of all genes will be known.

KEY WORDS: *embryonic stem cells, gene targeting, hit and run, retroviral mutagenesis, chromosome engineering*

Germline transmission of ES cells

Fifteen years ago, the appearance of a pup with dark eyes in a litter caused great excitement in the Evans' laboratory. This pup was fathered by a male chimaera generated from cultured embryonic stem (ES) cells (Evans, and Kaufman, 1981, Fig. 1). Unbeknownst to us at the time, this germline transmission event signaled the emergence of a new age in mouse genetics. While we were confident that this experimental success was significant, the power of ES cells did not become apparent to the wider scientific community until a few years later when mutations were generated in ES cells in culture and transmitted into the mouse germline. Following the publication of our success in *Nature* (Bradley *et. al.*, 1984). I received a letter from Dr. Ralph Brinster. Unlike the thousands of letters I have received subsequently, this was not a request for materials but had a very simple message, "congratulations". I recall very clearly opening and reading this correspondence in the Tea Room of the University of Cambridge Genetics Department. I was honored that an individual of Dr. Brinster's stature had so selflessly taken the time to write a letter to a graduate student. Clearly, Dr. Brinster recognized the breakthrough.

The experiments which Elizabeth Robertson and I performed with ES cells were conceptually related to, though different in outcome from, experiments which Dr. Brinster had described ten

Abbreviations used in this paper: ES cells, Embryonic stem cells; EC cells, Embryonal carcinaoma cells; PCR, Polymerase chain reaction; Hprt, Hypoxanthine Phosphoribosyl transferase; LTR, Long terminal repeat; HSVtk, Herpes simplex virus thymidine kinase; FIAU, 1- (2'-Deoxy-2'-Fluoro-β-D-Arabinofuranosyl)-5- Iodouracil; LoxP, Locus of crossover P; Cre, Cyclization recombinase; HAT, Hypoxanthine, amniopterin, thymidine.

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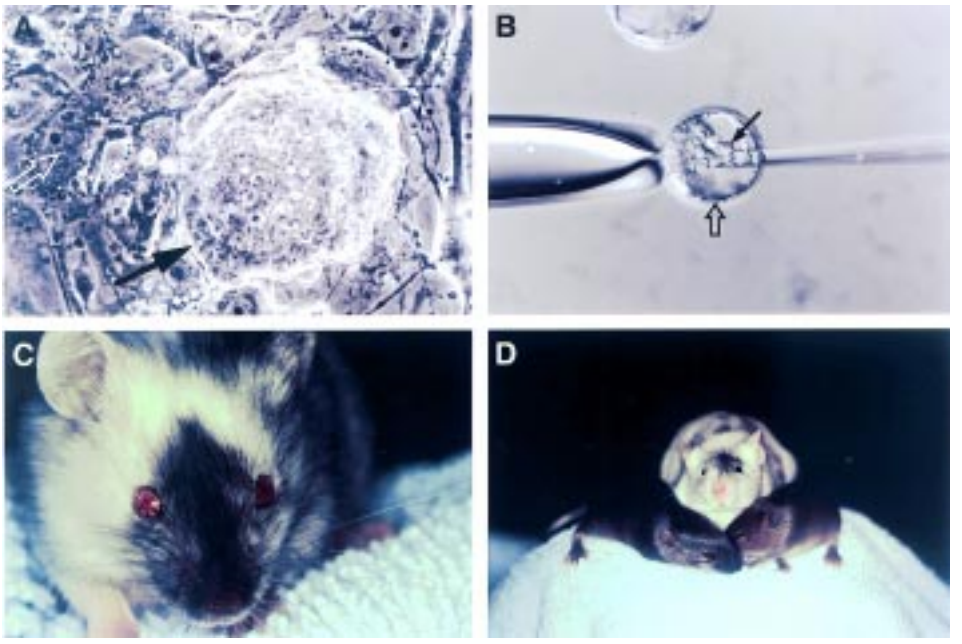

Fig. 1. Embryonic stem cell technology-reconstructing a mouse from cultured cells. **(A)** *ES cells growing on a STO feeder layer. White arrow shows a STO feeder cell nucleus, black arrow points to a colony of ES cells probably containing 1000 cells.* **(B)** *Injection of ES cells (thin arrow) into a 3.5 day blastocyst (thick arrow).* **(C)** *Chimaeric mouse showing contributions from descendants of the injected cells (pigmented) in the eye and melanocytes of the skin.* **(D)** *Germ line transmission from a chimaera demonstrated by the pigmented pups.*

years earlier. In this *Journal of Experimental Medicine* report (Brinster, 1974), Dr. Brinster showed that tumor derived embryonal carcinoma (EC) cells (the predecessors of ES cells) could be regulated by the embryonic environment of the blastocyst and contribute to the somatic tissues of chimaeric mice. This observation was subsequently confirmed by other laboratories (Mintz and Illmensee, 1975; Papaioannou *et al.,* 1975), however despite their early promise, EC cells were destined to diminish in popularity as an experimental system since the descendants of these cells rarely contributed to the germ line. Those successes that were reported could never be confirmed by other laboratories. The hope of being able to reconstruct the mouse germline from cultured EC cells therefore languished for almost ten years until it was eventually revived by our observation of germline transmission from chimeras constructed from cultured ES cells.

The demonstration of the germline transmission of ES cells took place during an exciting time for manipulative mouse genetics. The early-eighties was a time during which a series of landmark papers were published by the Brinster and Palmiter laboratories using transgenic mice generated by pronuclear injection (Brinster *et al.,* 1981,1983; Palmiter *et al.,* 1982). The power of gain of function modification of the mouse germ line as an experimental tool to address and answer important questions was made abundantly clear by these reports and this served as an inspiration to me. In 1984, ES cells offered a potential alternative route for generating transgenic mice, with the distinct advantage of being able to select or screen for a clone with a rare genetic change from millions of cells in culture *before* constructing a mouse. It was clear however that ES cells would not be able to compete with the efficiency with which transgenic mice could be generated by pro-nuclear microinjection, but we believed that we might be able to generate mutations in endogenous genes and thereby determine the function of these genes.

Today this dream has become reality. It is now virtually impossible to open a major journal without coming across one or more papers in which ES cells have been manipulated in culture to construct a loss of function mutation in a gene which is subsequently established in the germ line of mice. Like transgenics generated by pronuclear micro-injection, ES cell technology is now routinely practised in many laboratories to address a diverse array of biological questions. This article will focus on the evolution of ideas and approaches used to modify the genome of ES cells from a personal perspective, we apologize to those individuals whose work is not mentioned in this personal account.

The quest for recessive mutations

Shortly after we obtained germ line transmission of ES cells, we turned our attention to manipulating the genome of ES cells and establishing those mutations in the germ line. Two approaches were adopted, the first was retroviral mutagenesis using the helper free retroviral vectors, the second was homologous recombination.

Retroviral mutagenesis

Retroviral mutagenesis proved to be quite an efficient means of gene transfer in ES cells. However, the recombinant retroviral vectors available at the time had viral promoters which functioned very poorly in ES cells, so that the apparent viral titers on ES cells were greatly reduced compared with those which could be measured using fibroblasts. In order to achieve productive infection it was necessary to infect ES cell cultures with virus repeatedly. By following this strategy, adequate numbers of viral particles were eventually delivered to the culture so that every ES cell was infected multiple times, obviating the need for selection (Fig. 2). In the first instance, pools of ES cells from these experiments were injected into blastocysts to generate chimeras which transmitted and segregated these proviral insertions in the mouse germ line. These experiments provided the first evidence that genetically manipulated ES cells were still totipotent (Robertson *et al.,* 1986).

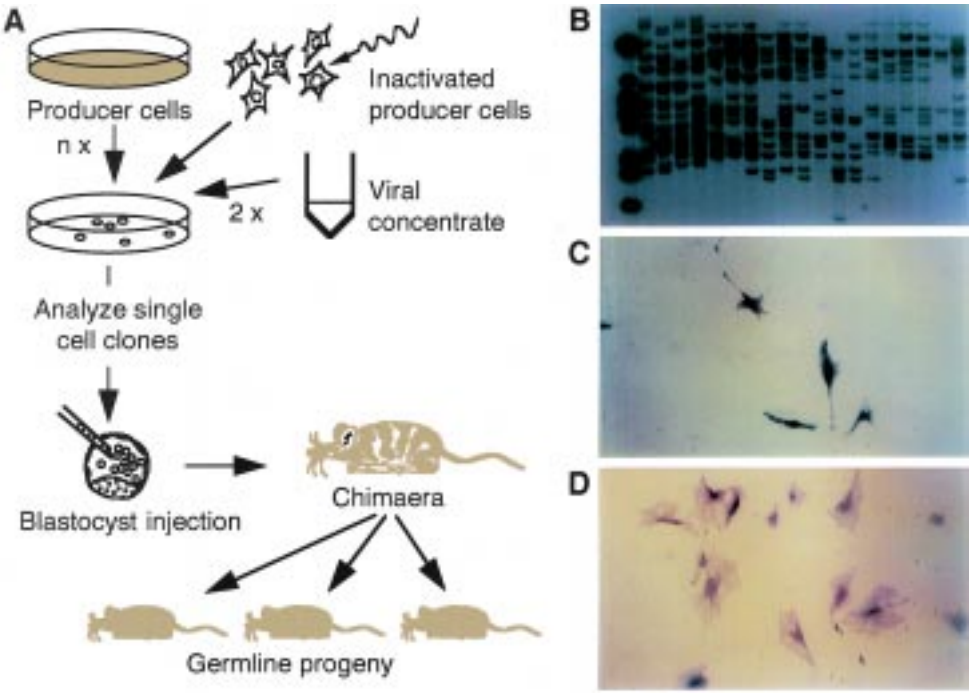

Fig. 2. Using retroviral mutagenesis to modify the mouse genome via ES cell Technology. (A) *The scheme used to demonstrate the germ line transmission of ES cells genomes modified in culture. Since retroviral promoters worked poorly in ES cells and active promoters which worked well in ES cells had not been identified, ES cells were infected multiple times with the virus or treated with a concentrated preparation of virions. The course of the infection could be monitored by Southern blots. Cloning of these cells could be accomplished by injecting the cells into blastocysts since only a few of the injected cells contributed to the germ line in any one chimaeric mouse.* **(B)** *F_1 Germ line progeny carried multiple copies of the provirus integration events distinguished by the use of enzymes which cut genomic DNA to generate unique junction fragments.* **(C** *and* **D)** *Primary fibroblasts from Hprt positive (C) and Hprt negative mice labeled with a ^{3}H-hypoxanthine incorporation assay confirming that the cells from the latter lack functional Hprt (D).*

Subsequently, some of these retroviral insertions were bred to homozygosity to identify integration events that were mutagenic and had inactivated interesting genes (Conlon *et al.*, 1991; Zhou *et al.*, 1993). Due to the random nature of retroviral insertions, only a subset of the integrations are actually mutagenic unless the insertions have been pre-selected in some way. Therefore in our experiments only about 5% of the integrations caused detectable phenotypes when bred to homozygosity.

These large pools of ES cells, carrying multiple independent retroviral insertions per cell, represent a vast collection of mutant cells which can be screened to identify integration events in specific genes (Fig. 2). In the first instance, we decided to recover mutations in a gene in which loss of function mutations could be directly selected in culture. *Hprt* is an X-linked gene, therefore it is present at only a single copy in XY ES cells and loss of function mutations can be directly selected in 6-thioguanine. *Hprt*-negative ES cell clones were recovered with this selection and these clones were used to generate chimeras which were bred to establish the mutant *Hprt* allele in the mouse germ line. Hprt deficient mice were subsequently generated by intercrossing F_1 animals (Kuehn *et al.*, 1987). These mice were the first to be generated with a specific modification of an endogenous gene through the modification of a cell line *in vitro*.

While retro-viral mutagenesis worked well for genes such as *Hprt,* where loss of function phenotypes could be selected in culture, it was hard to adopt this methodology for autosomal genes, where recessive mutations could not be directly selected. We proposed that we could identify clones with a viral insertion in a gene of interest by using a very deep library of ES cell clones each with multiple retroviral insertion events. By infecting at high multiplicity (100 insertions/clone) and generating a library of 10,000 clones theoretically the genome would be saturated with pro-viral insertions at an average density of 1 insertion every 3 kbp. In principle, one could screen such a library of ES cell clones using PCR primers specific for the gene of interest in combination with primers specific for the retroviral LTRs (Fig. 3). An appropriate junction fragment in the pool generated by an insertion in the locus of interest should be amplified with these primers. In principle, mutations in most genes would be accessible in this pool, provided the target locus was of a reasonable size. However, the work required to identify and recover specific clones from the pool is substantial, which is why this technique was not aggressively pursued once it was clear that mutations in endogenous genes could be efficiently generated by gene targeting techniques.

Many of the problems with the first generation of retroviral vectors have now been resolved. Specifically, integrations into genes can be directly selected by the use of read through transcription (von Melchner and Ruley, 1989) or by the use of splice acceptor and splice donor sequences that activate the expression of selectable markers (Friedrich and Soriano, 1991). Moreover, the integration loci are now readily identified which has meant that it is possible to generate vast libraries of ES cell clones each tagged with a unique insertion event (Hicks *et al.,* 1997; Zambrowicz *et al.,* 1998). My original view that retroviral mutagenesis would become the dominant technology for obtaining mutations seems likely to be correct, despite the current popularity of gene targeting. In part, this view has been realized by founding a commercial company to pursue this objective, Lexicon Genetics Inc., where the implementation of gene trapping on a genome wide scale was set as a primary goal.

Gene targeting-the early days

Simultaneously with our retroviral mutagenesis experiments, we began to work on trying to mutate genes by homologous recombination in ES cells. There was evidence in the literature that

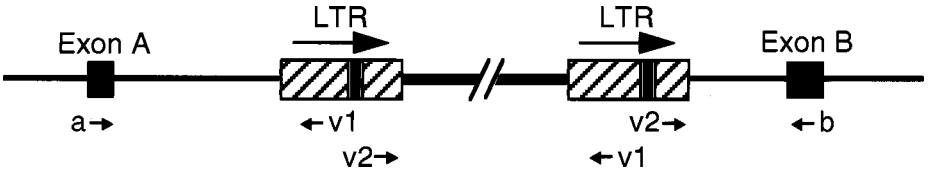

Fig. 3. A PCR screen used to identify retroviral insertion events in known genes in ES cells. *Pools of ES cells were screened for retroviral-exon junction fragments using primers specific to the LTR of the virus (v1 and v2) and primers specific to the exons of the target in the genome (a and b). Using infection ratios of 100 viral genomes per cell, we anticipated that this would give an effective coverage of 1 hit per 3 kb in a collection of 10^4 clones.*

mammalian cells could mediate extra-chromosomal homologous recombination between co-transfected DNA molecules (Kucherlapati *et al.,* 1984). However, there was no evidence that vector-chromosome recombination would be possible. The prevailing views at the time were that the mammalian genome was much too complex for incoming vector DNA to search, find and recombine with a homologous target before the efficient non-homologous recombination pathway effectively inserted the vector into a random location in the genome, how wrong this view was! A decade of gene targeting has revealed to us that homologous integration can often occur just as frequently as random insertion, moreover it is rare for the ratio of targeted to random insertions to be less than 5%.

In 1984, the absence of any published data that gene targeting was possible led me to take the view that strong positive selection for the desired recombination event was essential if our experiments were ever going to succeed. Our first experiments therefore attempted to target a gene which was highly expressed in ES cells *(c-myc)* and to use a selectable marker which lacked it's own promoter and would therefore only be expressed if it was inserted into the transcribed portion of the *c-myc* locus (Fig. 4). This vector failed to yield any targeted clones, to this day I don't understand why, especially since we later targeted *c-myc* using the same genomic clone at a 10% frequency (Davis *et al.,* 1993)!

Around the time my experiments to positively select for targeting at the *c-myc* locus was proving to be unsuccessful, there was emerging evidence that vector-chromosome recombination could occur in mammalian cells in two seminal papers published in 1985 from the Sternberg (Lin *et al.,* 1985) and Smithies (Smithies *et al.,* 1985) laboratories. In the former case, incoming vector DNA restored a defective *HSVtk* gene which had been randomly integrated into the genome while in the latter case, the endogenous β-globin locus was targeted with an insertion vector in human erythroleukemia cells. In the case of the β-globin targeting, the targeted clones could not be selected but had to be identified by an elegant yet very labor intensive sib-selection screen. These experiments suggested that targeted integration would occur at a frequency of 10^{-3} per random integration event. In the following year the Capecchi laboratory began to unravel some of the variables that affected gene targeting using a microinjection and positive selection approach in fibroblasts (Thomas *et al.,* 1986).

Gene targeting-disrupting nonselectable loci

In 1987, both the Capecchi and Smithies laboratories reported successful targeting of the *Hprt* locus in ES cells (Doetschman *et al.,* 1987; Thomas and Capecchi, 1987). These reports of gene targeting in ES cells pursued the same target we used to demonstrate the effectiveness of our retroviral mutagenesis experiments, the X-linked *Hprt* locus, relying on the direct selection for gene targeting events in 6-thioguanine (Thomas and Capecchi, 1987) or HAT (Doetschman *et al.,* 1987). Targeted clones were reported to be recovered at frequencies ranging from 10^{-3} to 10^{-5} per stably transfected cell. Moreover, Thomas *et al.* (1986), reported that efficient recombination seemed to require very large vectors, ideally 10 kb of homology (Thomas and Capecchi, 1987). We did not know this at the time, but these frequencies were several orders of magnitude lower than those we and others were subsequently able to achieve at many different loci.

At the time though, we viewed this data very positively since vector chromosome recombination had been achieved. However, the very low frequencies reported forced us to adopt a variety of elaborate selection and screening strategies for identifying recombinant clones. This included adapting vectors so that we could detect recombinant clones in pools by PCR amplification of junction fragments (McMahon and Bradley, 1990; Soriano *et al.,* 1991), the use of very large homology regions and the use of alternative positive selection schemes such as 3' trapping (Donehower *et al.,* 1992).

Approximately a year later the Cappecchi laboratory published a paper in *Nature* in which selection for targeting into non-selectable loci was achieved (Mansour *et al.,* 1988). This technique known as positive negative selection, heralded as a breakthrough, has become the most widely used technique in the

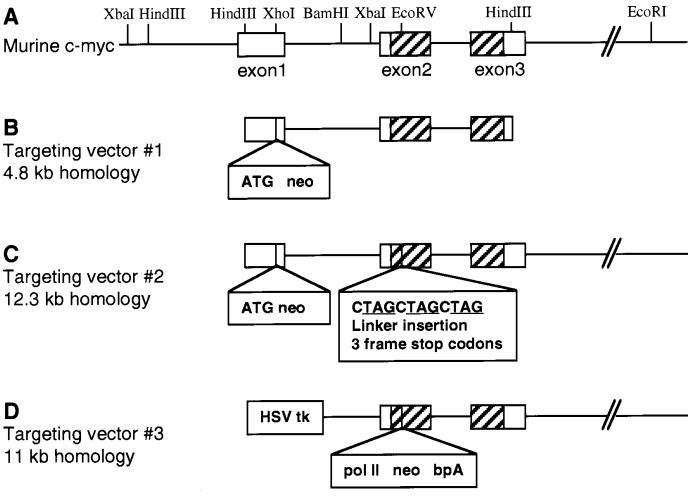

Fig. 4. Targeting the c-myc locus. (A) *depicts the genomic locus.* **(B** *and* **C)** *show two promoter trap targeting vectors that we used in repeated attempts to obtain targeting at the c-myc locus. These vectors failed to give any targeted clones while the vector in panel* **(D)** *targeted at a frequency of 9% of clones analyzed.*

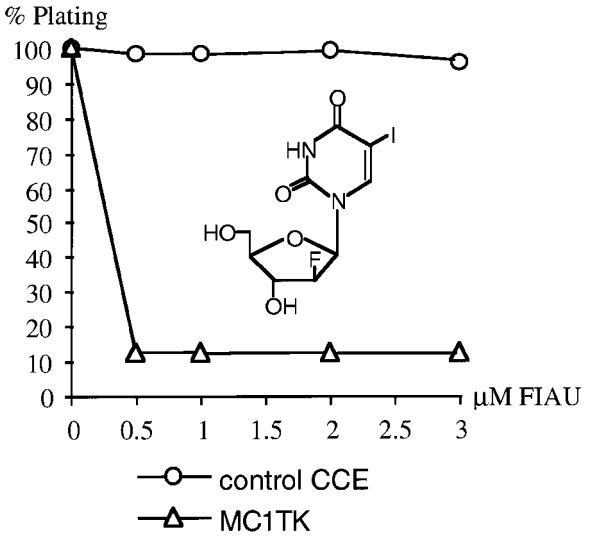

Fig. 5. Testing positive negative selection. *CCE ES cells were transfected with a construct which carried MC1neopA and HSVtk selection cassettes cloned into a plasmid backbone. Colony numbers were assessed after plating in G418 (positive) or G418+FIAU (positive/negative). The negative selection efficiency is calculated as the ratio of the colony number in the different selections (about an 8 fold enrichment) which was the best we ever achieved.*

practice of gene targeting. Ironically the power of the selection achieved with this technique is actually quite modest. In 1988, my two person laboratory tried very hard to repeat the experiments which had been published in *Nature* yet to this day neither we nor any other group I know, have ever achieved the 2,000 fold enrichment reported! The experiment illustrated in Figure 5 was repeated innumerable times under a vast array of varying experimental conditions [including with RV9.1TK, one of the vectors described in Mansour *et al.,* (1988)]. Initially, we believed that the problem lay in the efficiency of the selection. In 1988, the only readily available drug designed for HSV*tk* negative selection was acylovir which was very non-specific and killed wild type-cells almost as efficiently as HSV*tk* expressing cells. We took two approaches to attempt to resolve this problem. We explored the use of alterative Herpes Virues including marmoset and bovine herpes virus *thymidine kinase* genes which exhibited high activity and specificity for several nucleotide analogs which were readily available. Simultaneously, we obtained other analogs which were under development. The best and most specific enrichment we ever achieved was approximately 8 fold using an analog called FIAU! The failure to obtain better enrichments was not a failure of selection, (every FIAU-resistant clone that we have analyzed has lost or mutated the HSV*tk* gene), rather it appears that the enrichments are always quite modest.

Fortunately, the frequency of targeting at most loci (including *Hprt)* is many orders of magnitude higher than the frequencies first reported by Thomas and Capecchi (Thomas and Capecchi, 1987). Even without negative selection, it is not unusual to obtain a ratio of targeted to random insertion events of 5 to 20% in ES cells.

While we were realizing our goals using homologous recombination in ES cells, the Brinster and Palmiter laboratories had embarked on a heroic series of experiments to attempt to obtain productive recombination in zygotes. In these experiments 1841 mice were born including 506 transgenics, of which only one was targeted (Brinster *et al.,* 1989). In much the same way that our early attempts at targeting in ES cells were based on inadequate knowledge of the requirements for efficient recombination, the injected DNA used in these experiments was not a particularly good substrate for productive homologous recombination with the target locus. Subsequently we provided Dr. Brinster's laboratory with a construct that yielded a 10% targeting efficiency in ES cells, however this also proved to be ineffective in mediating recombination following zygote injection. I am frequently asked about the possibility of achieving targeting via pro-nuclear injection, I'm rarely enthusiastic! After all ES cells have proven to be the workhorse vehicle for modification of the mouse genome.

Developing new cell lines

In the process of obtaining ES cell clones with targeted mutations in many different loci, we began to observe dramatic differences in the behavior of these clones when they were used to generate chimaeric mice. Importantly, many of these subclones formed chimeras very inefficiently, and the chimeras that were generated were usually low grade and these never exhibited germ line transmission of their ES cell derived genome. Occasionally, we identified a clone from the same population which exhibited the high chimera forming efficiency characteristics exhibited by its parental population. Other laboratories with whom we were competing on a variety of different projects also ran into the same technical difficulties.

I realized that we were observing the problem of genetic drift in our parental ES cell lines. Abnormal cells were cloned out in the

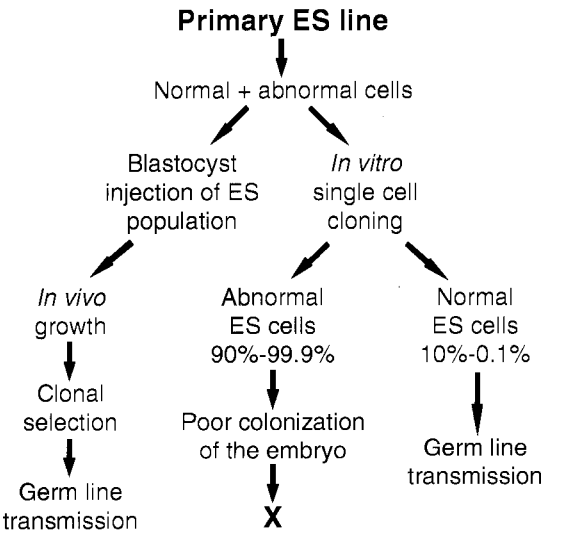

Fig. 6. Clonal variation in ES cell lines. *During the growth of ES cells variants will arise which will overgrow the normal population. These variants will not normally compromise the ability of the parental pool to contribute to the germ line, since usually adequate numbers of "normal" cells are still present in the population. When the parental cells are subcloned, the "abnormal" subclones will be identified, these will not contribute to chimeras or the germ line efficiently. These "abnormal" cells may represent the majority of subclones analyzed. Subclones of normal ES cells will have a much greater efficiency in forming chimeras and germ line transmission than the parental population.*

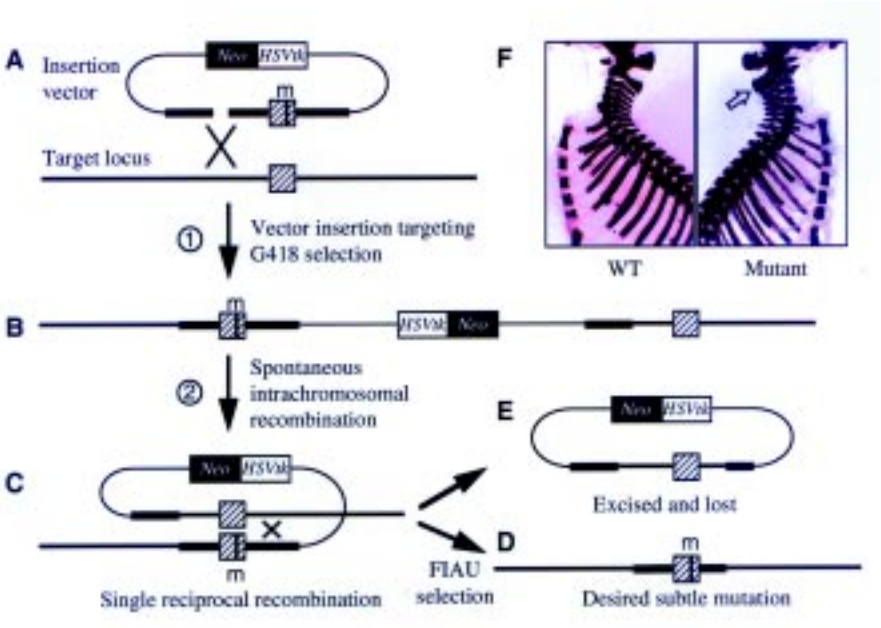

Fig. 7. The Hit and Run targeting scheme which we developed to generate point mutations in the genome (Hasty *et al.,* 1991). (A) *An insertion targeting vector was configured for both positive (neo) and negative (HSVtk) selection. This vector was modified to carry the desired point mutation and was linearized prior to transfection.* **(B)** *Recombinant clones were identified by Southern analysis.* **(C)** *The duplicated sequences in the recombinant locus spontaneously recombine to pop-out the vector either by an intra-chromosomal event (shown) or a sister chromatid pathway (not shown).* **(D)** *The revertant cells are selected for the loss of HSVtk in FIAU and screened for the desired allele.* **(E)** *The excised circle is lost during normal cell replication.* **(F)** *Skeletal preparations of the first mutant mice generated with a point mutation via ES cell manipulation. The embryo on the left is wild type, while the one on the right has a homeotic transformation in the Axis which has been converted to the Atlas (arrow) due to a premature termination in the third helix of the homeobox of HoxB4 (Ramirez-Solis et al., 1993).*

process of selecting and screening for targeted clones, and these were never able to colonize the germ line. The injection of the original populations of these ES cells into blastocysts indicated that normal cells were still present in these cultures, and that these normal cells could still contribute to chimeras even in the presence of large numbers of abnormal ES cells (Fig. 6). Our early recognition of this problem led me to isolate and evaluate new clones of STO feeder cells, one of which (SNL76/7) had dramatically improved qualities for ES cell maintenance compared with the parental cell line. Using these cells as feeder layers three new ES cell lines were isolated (AB1, AB2.1 and AB2.2) which we have used almost exclusively for the last 10 years (McMahon and Bradley, 1990; Soriano *et al.,* 1991; Donehower *et al.,* 1992; Matzuk *et al.,* 1992; Ramirez-Solis *et al.,* 1995). We still obtain very high germline transmission rates with the targeted clones derived from these cell lines. Our chromosome engineering projects require 3 successive subcloning steps on these cell lines, however, this has never proved to be a problem for obtaining germline transmission of these modified chromosomes (Ramirez-Solis *et al.,* 1995).

These new cell lines gave my then very small laboratory a competitive edge in efficiently transmitting various mutations into the mouse germline. When some of the early successes with these cells were published this created an unusual challenge for my small group to distribute these cells to the 1,000-plus laboratories that have now requested them. Distributing materials is usually a pretty thankless task, occasionally one receives a letter of acknowledgment, rarely anything more. My group remembers the thanks we received for dispatching cells to Ralph Brinster, gourmet cheese and French red wine, for once I felt we got the best part of the exchange!

Generating point mutations in the mouse genome

As we began to realize that gene targeting frequencies were actually exceedingly high, we shifted our attention to generating subtle mutations in endogenous genes. One of my concerns at the time related to our ability to interpret mutations we were generating in the *HoxB4* gene, one of the dense cluster of genes in the *HoxB* cluster on mouse chromosome 11. My concern related to the possible effect that the insertion of a selection cassette with strong promoters and enhancers in the *HoxB4* gene might have on the regulation of other genes in the region. To obviate this concern we explored three methods for making point mutations in the genome. The first of these involved co-electroporating two cassettes in to ES cells. One cassette served to facilitate selection for the transfected cells while the second was designed to recombine with the target locus. Although we were able to achieve the desired outcome with this strategy, the propensity of co-transfected DNA to co-integrate at a single locus made this technique very inefficient (Davis *et al.,* 1992). The second technique we explored was to use two rounds of targeting with replacement vectors. This required an initial recombination event to insert a negatively selectable maker into the locus and a second round of recombination to replace the cassette in the target locus with one that contains the desired modification. This technique has since been published by other laboratories (Askew *et al.,* 1993; Stacey *et al.,* 1994) and called tag-and exchange or double replacement gene targeting, respectively. While we could also occasionally obtain the desired recombinations with this technique, I never felt that this method was robust enough to warrant publication.

By contrast, a technique we termed “Hit and Run” proved to be highly effective (Hasty *et al.,* 1991). Simultaneously, the Smithies laboratory reported the same concept which they termed “In and Out” (Valancius and Smithies, 1991). This technique relies on a two step recombination procedure (Fig. 7). In the first step an insertion vector was constructed with the desired point mutation in the homologous sequences. In the case of *HoxB4* this was in the third helix of the homeodomain and was predicted to give a *null* allele. This insertion vector contained both positively and negatively selectable elements in the vector backbone. This insertion

vector was used to recover recombinants in the first step, utilizing the positive selectable marker. In the second step these recombinants were selected for "pop-out" events due to the intrachromosomal or sister chromatid recombination event between the duplication generated by the insertional targeting. These events were selected using the negative selection cassette in the vector to eliminate non-popped out clones. This method proved to be highly efficient and we generated the desired mutation in the *HoxB4* locus. The first mice with an engineered part mutation were described by my group in 1993. As we predicted, the mice exhibited a different phenotype compared with an allele in the same locus in which we had inserted a selectable marker(Ramirez-Solis *et al.,* 1993). We have generated other mutations in the *HoxB* cluster using this technique (Studer *et al.,* 1996). Other groups have since recognized the problem of the long range consequences of the insertion of selectable markers on neighboring transcription units (Olson *et al.,* 1996). These effects can extend over distances greater than 10 kb.

Long range recombination

In 1992, I decided to move the laboratory in a new direction. As far as I was concerned, most of the outstanding issues required for effective gene targeting had been solved and we could make most mutations we desired. At the same time, I saw an opportunity to engineer the mouse so that we could effectively use it in genetic screens. In particular, I had the desire to perform screens in a haploid context, recognizing the power of deficiency screens in *Drosophila* (Bridges, 1917) and the power of haploid genetics in general. However, a major obstacle lay in the way, namely the ability to generate deletions in a directed way in ES cells. We attempted to make large deletions in a single step with the use of standard replacement vectors. Although we succeeded in making 19 kb deletions at the *Hprt* locus (Zhang *et al.,* 1994), it was never possible for us to obtain larger deletions at an autosomal locus with this technique, even with the use of very powerful selection schemes analogous to those used at the *Hprt* locus. Therefore a different technique to generate and select clones with the desired deletions needed to be developed.

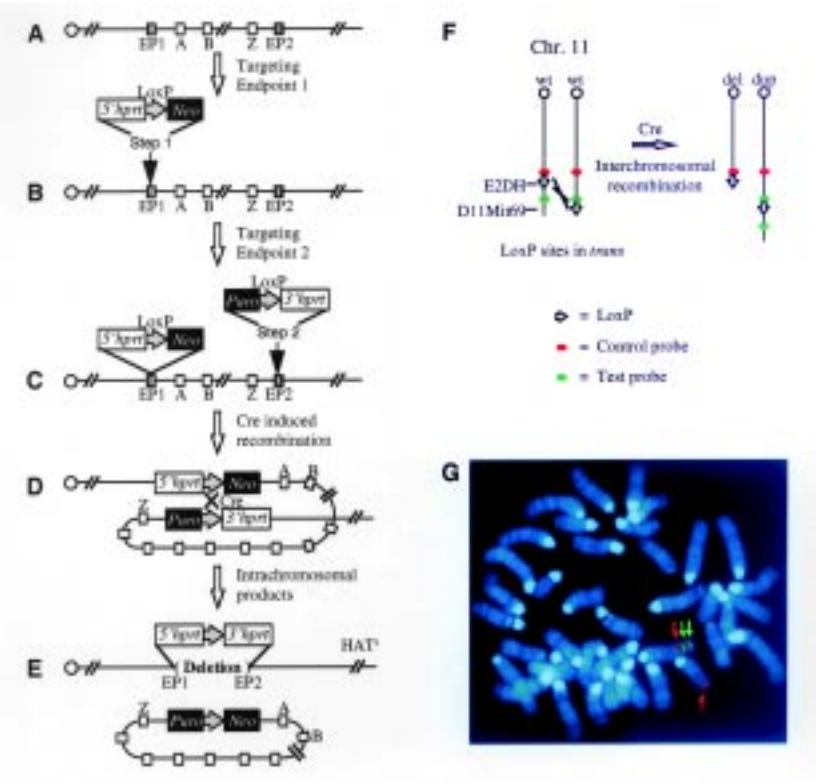

Fig. 8. Chromosome engineering in ES cells (Ramirez-Solis *et al.,* 1995). (A) *The unmodified chromosome containing all the genes between the deletion endpoint 1 (EP1) and the endpoint 2 (EP2) genes A-Z.* **(B)** *Targeting the loxP-deletion vector to EP1 using G418 selection and screening for targeted clones.* **(C)** *Targeting the second loxP-deletion endpoint vector to EP2 in cells carrying the first targeting event, using puromycin selection (only targeting in cis is shown for simplicity).* **(D)** *Cre expression in cells with the two endpoints targeted mediates recombination between the loxP sites at the deletion endpoints.* **(E)** *Cre recombination results in a deletion chromosome which is selected in HAT because the recombination reconstructs the Hprt gene from two non-functional parts and a ring chromosome which is lost during the normal process of cell division.* **(F)** *Cre-mediated recombination between deletion endpoints targeted to the two homologs also occurs. The recombination product is also selectable in HAT and is the deletion chromosome. However, the reciprocal product is not lost as a chromosome ring but is retained as a duplication on the homologous chromosome.* **(G)** *FISH confirms the generation of a duplication and a deletion chromosome from the trans recombination event.*

We elected to attempt to generate deletions using the *loxP*-Cre site specific recombinase system. Assuming that long range *loxP-Cre* recombination would probably be very inefficient a positive selection scheme was designed that would enable us to recover clones with the desired modification event. To do this, we divided an *Hprt* mini gene cassette into two overlapping but non-functional components where each piece included a *loxP* site. To generate deletions we had to construct a "pre-deletion chromosome" in which the cassettes with the *loxP* sites were targeted to the appropriate positions (Fig. 8). This was somewhat of a risk since we would not know if Cre would function over such large distances until the final step of the experiment namely, the expression of the Cre recombinase in the double targeted cell and the recovery of HAT resistant clones. In a series of experiments, we sequentially targeted the deletion endpoints on mouse chromosome 11 and tested the concept. Remarkably, we found that *Cre* recombinase would function over vast distances in the mouse genome at very high efficiencies. We could generate all classes of genomic rearrangements, deletions, duplications and inversions; chromosome engineering was born. (Ramirez-Solis *et al.*, 1995).

We have subsequently used this technology to engineer many different re-arrangements on mouse chromosome 11, including very large rearrangements of up to 70% of the chromosome, including balancer chromosomes tagged with recessive lethal mutations and coat color markers. There are many projects in the laboratory which are using these genetic reagents in screens with the goal of building a functional map of genetic elements from this region of the mouse genome.

Conclusion

The specificity with which either a gene or chromosomal region can be modified and the range of alterations that are now possible using the genome of ES cells as a surrogate mouse has made gene

targeting the dominant technology for manipulating the mouse genome for the last eight years. Making mutations by gene targeting is time consuming and for many laboratories rate limiting. In the genomics era it is desirable to obtain mutations without a customized approach for each gene. By solving the problem of identifying the integration site of the virus and implementing high throughput strategies, retroviral mutagenesis has re-emerged as a viable technology and is likely to establish itself as the dominant technology over the next few years. Homologous recombination will still have a place in the genomics era. Although *null* alleles generated by high throughput approaches are a good place to start the analysis of gene function, more detailed and specific questions based on knowledge of the gene product and gene structure are usually desirable and these can only be generated through customized approaches.

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