

Chapter Seventeen

Evolution of Genetic Manipulation of Laboratory Animals

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High quality research depends on the purity and consistency of reagents, including experimental animals, for efficient reproducibility of results. It is readily recognized that purity and consistency in experimental animals depends on both genetic homogeneity and controlled environments that avoid variation caused by nutritional, pathogenic, or other environmental effects. Evolution of genetic manipulation of laboratory animals for research use has been taking place for nearly a century, beginning with the creation of the first inbred strain started by C. C. Little around 1909 (1). The first 50 years of that evolution can be characterized by the achievement of genetic homogeneity through the creation of many inbred strains, primarily of mice. In the last 50 years that evolution is best characterized by discoveries of mutations and manipulations of the genome that have resulted in animal models of disease and specialized strains that have been used to answer very specific questions about genetics, nature of disease, and biological pathways. The story of the evolution of genetic manipulation of laboratory animals is primarily the story of the evolution of genetic manipulation in mice, and some of the milestones along that path will be described here.

Inbred Strains

The beginning milestone in controlling the genetic nature of mice for research purposes should be attributed to Clarence Cook Little. He began work with coat color mutants of mice believed to have come from Abbie E. C. Lathrop, who started a small "farm" in Granby, Mass. around 1900, raising mice for pets but eventually supplying mice to research institutions. Little was

an undergraduate at Harvard in 1907 under the mentorship of William E. Castle, who is considered the father of mammalian genetics. In 1908 the Bussey Institute of Harvard University was founded to study genetics of plants and animals with Castle and E. M. East as directors. Little became a graduate student in the Institute. In the beginning, not being long after the re-discovery of Mendel's postulations- there was much discussion within the Institute about a new theory of heredity and how genetic fixation, and therefore uniformity, could be brought about by inbreeding. Sewell Wright, who laid down much of the mathematical basis for inbreeding, was a fellow student and friend of Little. It does not take a stretch of the imagination to see how Little would have gained an insight and appreciation of the inbreeding process through him. Little began breeding the mice that were recessive for what are now known as the dilute, brown, and agouti coat color genes by mating brother by sister in each generation to maintain the three recessive genes. After continued sibling mating, the line became the DBA inbred strain and today two sublines, DBA/1 and DBA/2 are still widely used in research. The other family of inbred strains started by Little from Lathrop stock includes the C57BL strains, of which C57BL/6 is perhaps the most widely used inbred strain of mice in research today.



FIG. 1. Dr. Clarence Cook Little, founder of The Jackson Laboratory.

C.C. Little, although the first, was not the only one at the time to appreciate the value of using homogeneous animals for research. Other pioneers started to create inbred strains of mice. We are reminded of them today by names or subline designations associated with the strain they developed. Thus, we have C3H/St (Strong), AKR/Fu (Furth), BALB/c (Bagg's albino c stock), BALB/cAn (Andrevont), WC/Re (Russell), C57BL/10Sn (Snell), as well as many others. A fascinating accounting of the origins of inbred strains can be found in *Origins of Inbred Strains* edited by Herbert C. Morse III (2). Many of the chapters in this book were written by some of these pioneers themselves.

Morse later wrote an intriguing chapter on the origins of the use of mice in research that recounts early use of mice prior to the beginning of the evolution of genetic manipulation, as well as a thorough summary of the development of inbred strains (3). Initially homogeneous inbred mice were used to understand the basis of cancer. Some strains possessed unique susceptibility characteristics to various types of tumors. AKR mice were characterized by high leukemia incidence and C3H mice developed mammary tumors at an early age, while BALB/c mice had low incidence of mammary tumors, but were highly sensitive to mammary tumor agent-later identified as a mammary tumor virus.

Cancer susceptibility, while surmised to be influenced by genes in an inbred strain, did not; however, appear to follow

Mendelian principles. In 1914 C. C. Little postulated that multiple Mendelian factors could explain observations of tumor susceptibility in F1 and F2 crosses (4) and two years later carried out an experiment with E. E. Tyzzer to verify this explanation (5). It would be shown later that tumor growth is controlled by an immune response to cellular transplantation antigens (reviewed by Snell et al. (6)). George D. Snell, who also was a student at the Bussey Institute and recruited to the staff of The Roscoe B. Jackson Memorial Laboratory in 1935, called the genes responsible for these antigens *histocompatibility* or *H genes* (7). The next step, then, was to identify and isolate individual genes, and this led to the development of congenic strains.

Congenic Strains

A congenic strain is one where a gene from a stock is introduced into the genome of an inbred strain by crossing progeny carrying the selected gene of interest (differential gene) to mice of the inbred strain for a minimum of 10 backcross generations. In each backcross, half the introduced genes, not genetically linked to the differential gene, are lost as a result of genetic segregation. This backcrossing process results in the differential gene, and closely linked genes, being carried in the congenic strain.

Snell, who later received the Nobel Prize for his pioneering work in elucidating the histocompatibility system, was the first to develop congenic strains in mice. He selected for recessive tumor resistance genes by challenging individual progeny with tumors from the background strain. Because only homozygous recessive individuals would resist tumor growth and survive to be selected for breeding Snell intercrossed sib progeny after each backcross generation to produce homozygotes. A number of strains of several different combinations of donor and background strains were produced in this manner (8). By their use, Snell was able to study the separate H-genes that were responsible for both tumor and normal tissue transplantation.

Donald W. Bailey later developed a set of similar congenic strains by a slightly different and more direct approach (9). He used the fact that H-genes are co-dominant—that is the presence of a single “foreign” antigen determined by an H-gene in a skin graft would cause that graft to be rejected when placed onto a recipient lacking that antigen. By placing skin grafts from backcross progeny onto the inbred background strain recipients, graft rejection identified the graft donor as a carrier of a histocompatibility gene from the donor strain. Carrier progeny could then be directly identified in each backcross generation and intercrossing was not necessary. By simultaneously backcrossing a number of lines and selecting for differential histocompatibility genes in this manner, Bailey was able to develop a set of congenic strains, most carrying different H-genes (some lines carried the same H-gene by chance, since graft rejection does not necessarily distinguish different H-genes). These strains, carrying BALB/cAnBy differential H-genes on the C57BL/6By inbred background were then used to analyze individual H-genes. The number of H-genes by which the donor and background strain differed was estimated to be 32 (10).

Co-isogenic Strains

Inbred strains made it possible to discover many spontaneous mutations because the consistent phenotype of individuals of an

inbred strain made it easy to recognize deviations. The occurrence of a mutation and its subsequent fixation in an inbred strain results in a substrain carrying the mutation that differs from the inbred strain only by the single mutated gene. The new substrain is referred to as coisogenic in this case. This is to distinguish it from a congenic strain that differs from the parental or background inbred strain by a segment of donor strain chromosome carrying “passenger genes” in addition to the selected “differential” gene. In addition to spontaneous mutations, coisogenic strains can result from induced mutations, targeted mutations by homologous recombination, and transgenic inserts.

Spontaneous mutations discovered since the creation of inbred strains have been useful as models of human disease and have contributed substantially to the genetic mapping of the mouse genome. In 1955, midway in the period of the evolution, 70 genes



FIG. 2. The original building of The Roscoe B. Jackson Memorial Laboratory.

were genetically mapped primarily as a result of spontaneous mutations that were discovered. Now the number of mapped genes exceeds 6,000. The early history of gene mapping has been comprehensively reviewed by Eva M. Eicher (11), and a description of spontaneous mutations in the mouse can be found in *Genetic Variants and Strains of the Laboratory Mouse* (12).

While many of the spontaneous and induced mutations are maintained as coisogenic strains of the inbred background on which they occurred, many also have been placed onto other inbred backgrounds to produce congenic strains for study of the effects of genetic background on phenotype.

Recombinant Inbred Strains

D. W. Bailey used a very innovative approach to create a set of strains to aid in the analysis of the genetic location of the genes isolated in his congenic strains (13, 14). His idea was to create a set of inbred strains, which he referred to as recombinant inbred (RI) strains, each having a unique combination of genes from two different progenitor inbred strains. Such a set was produced by simultaneously inbreeding a number of lines stemming from different F2 pairs derived from the cross of the two progenitor strains. Each RI strain in the set would be different because of the random fixation of genes during the inbreeding process, but any gene would be represented by only one allele from one of the two progenitor strains. By typing each RI strain for a gene in which the two progenitor strains were known to differ, an “allelic distribution pattern” could be established. Any two different

genes having the same distribution pattern would most likely be “linked” or close to one another on the same chromosome, because the probability of having two genes with the same distribution pattern would be low. B. A. Taylor established quantitative methods for estimating genetic linkage from RI distribution data (15), and he and others created a number of additional sets using different inbred progenitor strains.

Transgenic Animals

The development of recombinant DNA technology, knowledge of mouse reproductive physiology and microinjection techniques provided the foundation for the ability to manipulate the mouse genome. The first transgenic strain was reported in 1980 when a functional foreign gene was introduced into the mouse genome by pronuclear injection (16). This initial report was quickly followed by others (17–20). We now know that the foreign DNA, or transgene, integrates randomly into the mouse genome, usually in tandem arrays at a single site (21). In general, high transgene copy number results in high expression, but there are poorly understood positional effects that may alter this (21). A recent report indicates that high copy number can actually have a repressive affect on expression (22). The stable germline transmission of the transgene permits the establishment of hemizygous or homozygous transgenic strains or stocks.

Transgenic strains were initially utilized to study the regulation of mammalian gene expression (i.e. (23)) and are now used in a wide variety of research areas. They provide models for a wide variety of human disease-including cancer, cardiovascular disease, development, immune dysfunction, neurological and neuromuscular disease, and others (for reviews see 24, 25). They have been used to generate human monoclonal antibodies and to investigate antisense mediated gene inhibition. They play an important role in the evolving field of conditional mutagenesis (see next section) where they are a necessary component of both the Cre-lox and tetracycline mutagenesis systems. Transgenic strains also provide valuable research tools such as ROSA26 strain (26) which carries a ubiquitously expressed LacZ transgene. The LacZ reporter transgene permits virtually any transplanted tissue or cell type from this strain to be identified by Xgal staining.

Transgene insertion may occur within a neutral site of the genome or within an endogenous gene (insertional mutagenesis). Disruption of an endogenous gene often results in a recessive mutation unrelated to transgene expression, which given an identifiable phenotype, may subsequently be mapped and cloned. This random mutagenesis has led to the development of gene-trap methodology where promoterless vectors carrying a LacZ reporter gene are retrovirally inserted into embryonic stem (ES) cells (27). Xgal straining is used to identify those ES clones that carry vectors which have inserted into an expressed gene. The ES cells may subsequently be used to generate a transgenic stock in order to characterize the mutation and clone the disrupted gene. This approach has now become an important commercial gene discovery tool (28).

Targeted Mutant Animals

Whereas transgenic technology makes it possible to randomly add genetic material to the mouse genome; gene targeting technology makes it possible to alter or remove the function of specific genes. The isolation and *in vitro* maintenance of pluripotent embryonic stem cell lines from mouse pre-implantation embryos, and the demonstration that these ES cells could recolonize blastocysts and be transmitted in the germline (30)

were major contributing factors leading to the development of gene targeting in the mouse (31, 32).

The first step in gene targeting is to design a targeting vector that will undergo homologous recombination with the endogenous target, rendering the target gene defective or inactive. This targeting vector is then introduced into ES cells where homologous recombination of the targeting vector with the endogenous gene results in the replacement of the endogenous functional gene with a non-functional, or altered, genetically engineered substitute. The targeting vector may also enter the host genome via a random insertional event (similar to generating a transgenic strain), as opposed to homologous recombination and in fact this is the most likely event to occur. A major factor influencing the ratio of random insertion to homologous recombination is the extent of homology between the targeting vector and the host genome. The use of isogenic



FIG. 3. Mouseroom of The Jackson Laboratory in 1953 showing wooden mouseboxes in use.

DNA in the targeting vector greatly increases the frequency of homologous recombination (33). Also, the use of positive negative selection (34) will greatly enhance the chance of recovering a correctly targeted ES cell clone. Correctly targeted ES cells are then microinjected into host blastocysts and implanted into recipient pseudopregnant females. Chimeric mice carrying the targeted allele are initially identified by their coat color, and are subsequently mated to establish that the mutation is transmissible in the germline. If transmissible, the stock is mated to determine whether homozygotes are recoverable. If transmissible, the stock is mated to determine whether homozygotes are recoverable, and if so, if they are viable and fertile. A homozygous or heterozygous colony carrying the targeted allele is then established.

The generation of mice carrying targeted mutations (primarily null alleles to date) has increased exponentially in the last few years. These strains provide experimental systems for understanding gene function and regulation. It may be argued that the definitive way to determine gene function in mammals is through the evaluation of null mutations. Predicting a phenotype from a gene targeting experiment is risky, as there are many examples of totally unexpected phenotypes. Examples of ‘no’ phenotype are numerous, especially in the field of immunology. In retrospect, it is not surprising that alternate pathways exist for so many genes; if they did not, there

would be little room for evolutionary experimentation.

Targeted mutant strains also provide the first truly specific models for human genetic diseases and permit studies that are inappropriate or impossible in human beings. These disease models are important for understanding the underlying cause of the disease and may be used for preclinical testing of therapeutic agents and developing new therapeutic interventions such as gene therapy. There now exist valuable mouse models for virtually every major category of human disease, with new strains being generated daily. Even for diseases that are not normally considered to be of genetic origin, for instance susceptibility to pathogens, these model systems are important for understanding the underlying disease process. In addition, the process has worked in the reverse direction; phenotypes presented in strains carrying targeted alleles have led to the discovery of the underlying defect in human disease syndromes.

Many null mutations present a developmentally lethal phenotype, preventing the study of adult gene function. This has led to the development of conditional mutagenesis, where a gene is expressed during embryogenesis, but is subsequently removed in the adult, often in a tissue specific manner. The most fully developed conditional mutagenesis system utilizes the patented (Du Pont) Cre-lox system (35). Cre recombinase will promote recombination at loxP sites resident on a mammalian chromosome. Two loxP sites are targeted within a gene such that gene activity is not lost until the section between them is excised. Such an animal may then be mated with another one carrying Cre recombinase under the control of a tissue specific promoter. Gene function is lost when Cre is expressed in the targeted tissue (36). Temporal control over this recombination event may also be obtained by the use of an inducible promoter (37).

By far the largest majority of ES cell lines currently used for gene targeting have been derived from one of the 129 substrains (38). In order to initially recover the strain on an inbred background, the chimera must be mated to a 129 substrain (preferably matched to the ES cell line [38]). The 129 lineage is most often not a desirable genetic background because of its poor breeding and behavioral characteristics (39). Many targeted mutant strains are initially evaluated on a mixed genetic background for this reason (most often a mix between C57BL/6 and 129), and later transferred to an inbred background (most often C57BL/6). Genetic background can have a dramatic affect on a mutant phenotype and the phenotype of such congenic strains must be thoroughly evaluated. When a phenotype is found to vary on different genetic backgrounds, it is possible to map the gene(s) responsible for this variation.

Issues Affecting Genetically Defined Animals

Widespread use of inbred strains of mice and their distribution to laboratories around the world have created new obstacles towards the goal of achieving homogeneity in animals for research. Genetic drift, the accumulation in time of mutations that occur and become fixed in inbred strains, proceeds in random directions in separate lineages of any particular inbred strain. That is, each subline will accumulate a unique set of new mutations. In time, separate sublines become increasingly diverse and mice from different sublines of an inbred strain are no longer genetically homogeneous (40, 41). Experiments using mice from different sublines may produce different results attributable to the genetic differences between the sublines.

Another problem that has occurred is referred to as genetic contamination, or the incorrect mating of individuals during

the propagation of an inbred strain. Such incorrect matings can result from human mistakes in identifying mice to be mated or by undetected, inadvertent mating (40). The latter can occur when a mouse from one cage escapes and mates with a mouse from another cage where, for example, cage lids have not been placed securely or have been accidentally knocked ajar.

Incorrect application or interpretation of nomenclature for a strain or subline can lead to erroneous assumptions about genetic homogeneity that, in turn, can affect interpretation of experimental results. Mice from two genetically different sublines of an inbred strain might be assumed to be identical if their nomenclature does not make a clear distinction and investigators are ignorant of the development history of the sublines. This has become most apparent with sublines of the 129 inbred strain (38). In addition to misleading nomenclature, genetic drift and genetic contamination have all plagued this strain, which is particularly unfortunate because of the large number of targeted mutant mice that have been developed from embryonic stem cell lines derived from different 129 sublines.

Genotype Preservation

Cryopreservation of germplasm from strains of experimental animals represents a different kind of milestone, but it has played an important role in the evolution of genetic manipulation of laboratory animals. Prior to germplasm cryopreservation there were certainly many strains of experimental animals lost because of the inability to justify the cost of their maintenance in the face of infrequent use or unrecognized potential future value. Cryopreservation offers a relatively economical means of keeping strains that otherwise would have been discarded, but most importantly it offers assurance against accidentally losing any strain. As a result of that assurance, management of large numbers of strains becomes practical by allowing any one strain to be maintained with a minimum number of breeding pairs, or terminated from conventional breeding altogether. Lastly, it permits a "freezing in time" of a given genotype so that one can go back to this point if there has been any genetic alteration whether deliberate or by accident.

Mammalian embryos were first successfully frozen in 1972 (42, 43) and preserved embryos have been the mainstay of cryopreservation programs for mouse strains ever since. Cryopreservation of sperm, while common in many mammalian species, has been problematic for mice until recently, but recent progress is making sperm cryopreservation increasingly possible (44–54). It is also possible to cryopreserve spermatogonia (55) and ovaries (56, 57). Recent reports describing the successful recovery of live offspring derived from freeze-dried mouse sperm (58), as well as from the transfer of somatic cell nuclei into enucleated oocytes (cloning) (59) open up new possibilities for preservation of important stocks. Factors that affect the choice of cryopreservation method are cost, genetic and reproductive requirements, and effort or time for live born recovery that is dictated by the manner in which germplasm was frozen.

Legal Issues

The right to commercialize intellectual property developed with federally sponsored research was conveyed to universities and other nonprofit institutions by the Bayh-Dole Act of 1980. Patent and license activity can have an inhibitory effect on both the free distribution of animal models and on the development of new models that potentially infringe on existing patent

rights. The development of transgenic and targeted mutant strains has seen considerable activity in both patenting and commercial licensing since the Bayh-Dole Act. Patents affecting transgenic and targeted mutant technology include: *genetic transformation of zygotes* (pronuclear microinjection), T. Wagner and P. Hoppe (Ohio University) inventors; *transgenic non-human mammals* (oncomouse), P. Leder and T. Stewart (DuPont) inventors; *gene targeting using positive negative selection*, Mario Capecchi (University of Utah), inventor; *gene targeting in animal cells using isogenic DNA constructs*, Anton Berns (Netherlands Cancer Institute), inventor; *site-specific recombination of DNA in eukaryotic cells* (Cre-lox), Brian Sauer (DuPont), inventor; *tetracycline-inducible transcriptional activator and tetracycline-regulated transcription units* (and others) H. Bujard, et. al. (BASF) inventors; and *FLP-mediated gene modification in mammalian cells, and compositions and cells useful therefor*, G. Wahl, et. al. (The Salk Institute for Biological Studies) inventors (60). In addition there have been patents awarded on specific strains; however, universities and nonprofit technology transfer offices generally have not sought patents on strains developed at their institutions. The expense of obtaining a patent and potential return on this investment has led these offices to seek licenses from commercial companies in place of patenting. In general, licenses are not required of academic or not-for-profit investigators performing government sponsored research.

Summary

The past 50 years have seen remarkable advances in biomedical science and technology and in the development of laboratory animals that contribute to furthering our knowledge of the human disease process. It is predicted that the entire genome of the mouse will be sequenced by the year 2005, which will potentially lead to the eventual cloning of all mouse genes and to the generation of targeted mutations at all loci. In the meantime there is much interest in utilizing ethylnitrosourea (ENU) to generate new mutants in order to map, clone, and characterize new genes. Patent rights notwithstanding, the net result will be the creation of an enormously large number of animal models available for biomedical study.

While the mouse has been the most studied mammal in research and is the most complete genetically defined mammal, other laboratory animal species may offer unique investigative opportunities. Certainly the rat provides important models in areas such as physiology, organ transplantation, neuroscience, and hypertension. The genetics of the rat are advancing rapidly (61, 62) and much of the early work promoting the use of genetically defined rats can be attributed to pioneering studies carried out at the Wistar Institute (63) and University of Pittsburgh (64). In laboratory animal species, the culture of functional ES cells has only been accomplished from mouse embryos, so the only animal models resulting from targeted homologous recombination, to date, have been mice. Transgenic models, on the other hand, have been produced in several other species, including the rat, and are providing important models for biomedical research. Cloning of individuals has been accomplished in domestic animals (65) and among laboratory animals has been achieved in mice (59), but undoubtedly this will also be extended to other species of laboratory animals. Cloning may provide a mechanism to produce targeted mutations in those laboratory animal species for which functional ES cell lines cannot be established.

The emphasis of biomedical research, currently on elucidation

of genome structure and sequence, will surely return to the whole animal for studies on the precise nature of gene expression and gene interaction as new strains with specific genetic alterations are developed. As a result our knowledge of human disease and normal biological processes is certain to advance at an unprecedented rate.

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