Leading Edge
Essay
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Gene Targeting in Embryonic Stem Cells Scores a Knockout in Stockholm

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The 2007 Nobel Prize in Physiology or Medicine has been awarded to Mario Capecchi, Martin Evans, and Oliver Smithies for developing specific gene modification techniques and mouse embryonic stem cell technology that, when combined, enable the creation of "knockout" mice. Analyses of these mutant animals have revolutionized the elucidation of gene functions, and these mice have proved to be valuable models of numerous human diseases.

Exactly a century ago, Clarence Cook Little, a graduate student in William Ernest Castle's laboratory at Harvard University, clung to the belief that the lowly mouse could one day become a model in which to study human physiology and disease. Little and Castle realized that, to achieve this goal, they had to develop mouse strains that were more genetically homogeneous. Backed by Castle's expertise as a respected authority on mammalian Mendelian genetics, Little commenced interbreeding wild mice in 1909. He hoped to obtain animals with a better defined genetic background that would simplify laboratory studies of mammalian traits. The success of this program marked the beginning of inbred mouse genetics.

This year, we celebrate a century of advances in a subject that has permeated every field of physiology and medicine: animal genetics. We rejoice that the Nobel Assembly at the Karolinska Institute in Sweden has awarded the 2007 Nobel Prize in Physiology or Medicine to three individuals who pioneered techniques of gene targeting in murine embryonic stem (ES) cells. The work of these researchers revolutionized the study of mouse genetics and has made it possible for scientists around the world to generate genetically defined mouse mutants for the study of functions of individual genes. The 2007 Nobel Prize winners are Mario Capecchi of the University of Utah, Martin Evans of Britain's Cardiff University, and Oliver Smithies of the University of North Carolina at Chapel Hill. The citation reads: "For their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells." This year's Nobel Prize nicely complements last year's award to Andrew Fire of Stanford University and Craig Mello of the University of Massachusetts at Worcester, who jointly received the 2006 Nobel Prize for Physiology or Medicine for their discovery of microRNAs. The principles by which microRNAs function have been exploited to develop interference RNAs that permit the silencing of gene functions at will and with relative ease.

Why Study Mice?

Scientists study biology to learn about physiology and investigate mammals to learn about human behavior, development, and pathophysiology. At the cellular and molecular levels, there are significant similarities between human cells and those of other multicellular or even unicellular organisms. However, at the organismal level, humans share extensive physiological characteristics only with other primates. Nevertheless, many features of human development and biology are closely analogous to those of fast-breeding and easily maintained rodents. Embryonic development, organogenesis, hematopoiesis, and immune responses are all strikingly comparable at the physiological level in humans and mice. Perhaps this should not be surprising, as 99% of the genes in these two species are shared. Thus, the use of the mouse as a model for studying human development and disease is an approach that can be readily justified.

The Early Days of Mouse Genetics

The early pioneers of mouse genetics were well aware of the possibilities of using rodents to learn more about human biology and genetics. Little, Leonell Strong, E. Carlton MacDowell, and others spent almost two decades systematically intercrossing mice captured from the wild and generating scores of inbred strains. A dozen of these lines are still commonly used in laboratories around the globe, including the Balb/c, B6, B10, C3H, CBA, and DBA strains. Comparative investigations of these multiple lines of mice derived from a mixture of forebears from relatively diverse geographic locations have allowed scientists to probe the extent of mammalian genetic diversity. Many of the mouse strains created by these researchers, as well as other rodent mutants, were eventually consolidated at the Jackson Laboratory in Maine, which Little founded in 1929. The Jackson Laboratory became and remains to this day one of the meccas of rodent genetics, devoted to the unearthing and housing

of interesting substrains and mutants. Back in the 1930s, investigations of the Jackson Laboratory's collection of mutants yielded major advances in our understanding of many aspects of mammalian bodily processes. For example, Peter Gorer and George Davis Snell, another student of Castle, joined the Jackson Laboratory and devoted 25 years almost exclusively to studies of mouse histocompatibility genes. In the course of these studies, Snell discovered the *H-2* complex (containing the MHC genes) that governs transplant rejection and immune responses. For this work, Snell shared the 1980 Nobel Prize in Physiology or Medicine with Jean Dausset and Baruj Benacerraf.

Little's trailblazing efforts were followed by the heroic labors of many who undertook the arduous task of creating and characterizing mouse models of human diseases. Although the initial objective was to study tumorigenesis in mice, the intercrossing of millions of animals over several decades also produced rare examples of mice exhibiting symptoms of anemia, immunodeficiency, fragile X syndrome, Alzheimer's dementia, or obesity (among others). The work of many dedicated individuals contributed to this cause, including the pioneering research of Elizabeth Russell, Sheldon Bernstein, and Jane Baker on mouse anemia; the landmark experiments of Douglas Coleman on obesity; and Donald Bailey's groundbreaking development of recombinant inbred strains that facilitate gene mapping. William and Lee Russell of the Oak Ridge National Laboratory in Tennessee, as well as Mary Lyon and Bruce Cattanach of the Atomic Energy Research Establishment in Hartwell in England, led research teams that formalized irradiation and other mutagenesis techniques that made it easier to study basic questions in mouse genetics. However, despite these prodigious efforts, elucidation of the genetic causes of physiological phenomena such as cancer and obesity proved to be exceedingly strenuous. One impediment to more rapid progress in these studies was the discovery that the mice in question were genetically more closely related than was ideal. Underneath their ostensible diversity, these rodents actually arose from a relatively small number of ancestors derived from a limited number of original sources. Even with the benefit of modern techniques of irradiation and chemical mutagenesis, the process of obtaining genetic variants via breeding programs remains lengthy, costly, and labor intensive.

Molecular Biology Revolutionizes Mouse Genetics

With the dawn of the molecular biology era in the mid-1970s, it became possible to identify the molecular bases of the physiological and pathophysiological variations observed in different mouse strains and their mutants. However, early attempts to delineate these mutations at the molecular level required an enormous struggle and consumed years of demanding experimentation. Even with today's technology, this type of "forward genetics" approach requires considerable effort to pinpoint the molecular changes occurring in randomly generated mutants. We now know, as a result of two massive DNA sequencing projects carried out by Fernando Pardo-Manuel de Villena at the University of North Carolina and his collaborators at the Jackson Laboratory, as well as by David Cox of Perlegen Sciences Inc., that the genomes of the commonly used laboratory strains and several lines of wild mice differ by only a few million base pairs (Callaway, 2007). This amount of variation, which is unevenly distributed in the mouse genome, is considerably less than expected. The creation of additional rodent strains from stocks of a more diverse genetic background are currently in progress.

While the enterprise of mouse genetics was steadily advancing from the 1930s to the 1980s, the study of the genes themselves was undergoing a revolution. By the late 1970s, the techniques of molecular biology were starting to deliver significant numbers of genes to biologists for study, and the need for a quick and easy means of generating defined mouse mutants became a priority. Solving this difficulty was never as dire in other species as it was in mammalian cells, for obvious reasons. Unicellular organisms such as bacteria and yeast, and even multicellular species like the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, reproduce at a much faster rate than mammals and are much more amenable to mutagenesis. Mammalian geneticists dreamt of refining the techniques of "forward genetics" used to generate mutations in flies and worms and applying them to mammalian cells. A technology that went a long way to satisfying the desire of biologists to study the functions of specific genes in whole animals was the independent development of transgenic mice in the early 1980s by Rudolf Jaenisch of the Massachusetts Institute of Technology, Frank Ruddle of Yale University, and Ralph Brinster of the University of Pennsylvania and Richard Palmiter of the University of Washington (among others) (Palmiter and Brinster, 1985). These mutant animals allowed researchers to assess a gene's function by examining the effects of its overexpression in either a whole animal or a specific tissue. Thousands of such mice were produced and studied and the application of this technology is still prevalent today. Nevertheless, despite the usefulness of transgenic mice, mammalian geneticists still sought a means of mutating a gene and observing the results of its loss of function in a whole animal. Thus, the goal was to generate somatic cell mutants or, better still, gene-targeted mice bearing specific alterations. The trick was to find a way to incorporate a defined mutation into the genome of a mammalian embryo that could then develop into a whole animal displaying the effects of that mutation.

From Multipotent Cell to Mutant Animal

The development of a whole animal from an embryo requires that the earliest embryonic cells be multipotent, that is, have the capacity to generate every cell type needed in the body. The multipotency of mammalian cells has fascinated embryologists and biologists for

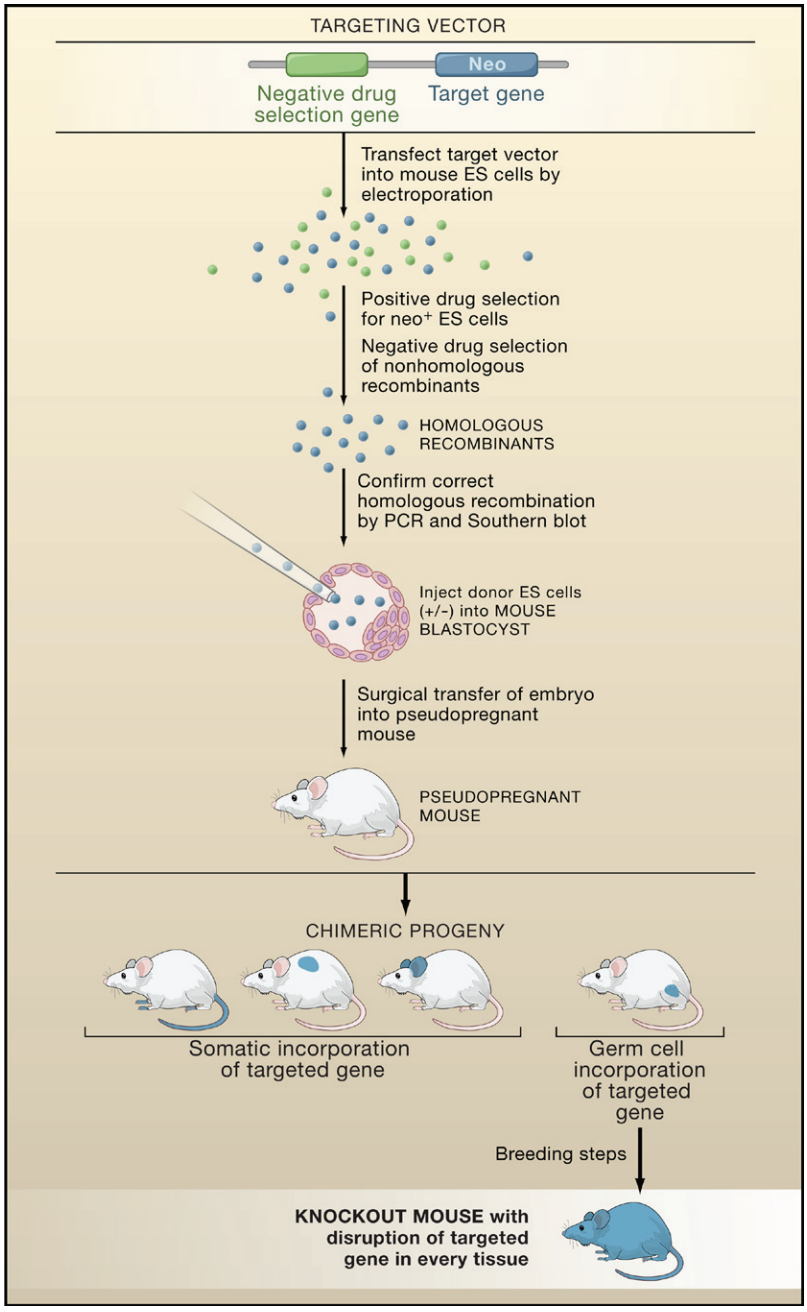

Figure 1. How to Make a Knockout Mouse

The first step in generating a knockout mouse is the construction of the targeting vector. The targeting vector generally contains a copy of the genomic murine gene disrupted by the insertion of a positive selection marker such as neomycin resistance (Neo). A negative drug selection gene is also often included. The targeting vector is then introduced into murine ES cells, usually by electroporation. Successive rounds of drug selection allow the isolation of ES cells that are homologous recombinants. Confirmation of the desired gene disruption is achieved by PCR and Southern blotting of the ES cell DNA. ES cells heterozygous for the gene disruption are then injected into mouse blastocysts to generate embryos that are transferred to pseudopregnant female mice. Chimeric progeny are born that show incorporation of the targeted gene into either somatic cells or the germline. Chimeric mice with germ cells bearing the targeted mutation are bred with wild-type mice to generate progeny heterozygous for the disruption. Intercrossing of these heterozygotes produces F1 progeny, one-quarter of which should be mutants homozygous for the targeted mutation: the classic knockout mouse. (Figure adapted from *The Immune Response: Basic and Clinical Principles* by Tak W. Mak and Mary E. Saunders, 2006, published by Elsevier Academic Press, London, UK).

decades. In 1961, Ernest A. McCulloch and James E. Till at the Ontario Cancer Institute in Toronto discovered that a single bone marrow precursor cell capable of forming a colony in an irradiated recipient mouse could give rise to multiple lineages of hematopoietic cells (Till and McCulloch, 1961). This result clearly demonstrated that at least some mammalian progenitor cells had the ability to differentiate into cell types of different lineages and suggested that it might be possible to manipulate the course of organogenesis. These experiments set the stage for the very ambitious dream (at the time) of regenerating an entire animal by injecting a small number of multipotent cells into an early embryo. The ability to propagate in culture, or isolate from animals, precursor cells having the capacity to give rise to an entire mammal would allow researchers to manipulate the genetics of a whole animal. The first step in this direction came from the work of Roy Stevens at the Jackson Laboratory, G. Barry Pierce of the University of Colorado, and Beatrice Mintz at the Fox Chase Institute in Philadelphia (among others). These researchers reported on studies in which teratocarcinoma cell lines were induced to differentiate into cells of various tissue types, yielding invaluable insights into the plasticity of multiple lineage commitment (Andrews, 2002). However, teratocarcinoma cells could not be used to regenerate an entire animal because these cells are intrinsically tumorigenic. The breakthrough came in 1981 when Evans and his colleagues and Gail Martin's laboratory independently succeeded in developing ES cell lines (Evans and Kaufman, 1981; Martin, 1981). These cells, which were derived from an early murine blastocyst, grew indefinitely in tissue culture and retained their multipotency as long as they were cultured on feeder layers. When injected into a new blastocyst, these ES cells contributed to the developing murine embryo, resulting in the creation of a genetic mosaic. The chimeric embryos were then brought to term by implantation in a pseudopregnant female mouse (see Figure 1). This seminal work provided researchers with the opportunity to manipulate the genetics of a mammal

at the embryonic stage. The Evans lab provided further proof of this principle by introducing the DNA of a retroviral provirus into ES cells and documenting the transmission of this foreign DNA through multiple generations of mice (Robertson et al., 1986).

Despite these triumphs, technical challenges remained in consistently sustaining ES cells in their multipotent state in culture. In 1988, Austin Smith and John Heath at Oxford University and their collaborators at the Genetics Institute in Boston, as well as Nicholas Gough of the European Molecular Biology Laboratory in Heidelberg, discovered that inclusion in the culture medium of the growth factor leukemia inhibitory factor (LIF) allowed ES cells to robustly retain their multipotency. With this difficulty conquered, ES cell technology became well defined and reproducible. The ability to reliably maintain ES cells in culture has allowed investigators to work out the principles and mechanisms of stem cell self-renewal and to identify critical regulators of tissue/cell differentiation. However, back in the 1980s, the more important observation was that ES cells offered an unprecedented opportunity to view the physiological consequences of specific genetic changes such as point mutations, insertions, and deletions. The next hurdle to overcome was finding a method of introducing such genetic alterations into a mammalian genome.

Homologous Recombination: Mutations to Order

It had long been known that bacteria and yeast could repair damage to their DNA through a process known as homologous recombination. Homologous recombination is a natural route by which a stretch of mutated DNA can be exchanged with a functional copy of this region of the genome if there is extensive nucleotide similarity between the two DNA sequences. By the early 1980s, studies of the somatic recombination of gene segments of the B and T cell receptor genes had made it clear that mammalian cells could also carry out this mechanism of DNA sequence exchange. However, it was not known whether this type of homologous recombination could be induced in mammalian cells through the introduction of naked foreign DNAs, or whether such introduction would be efficient enough to permit the creation of specific genetic changes. The laboratories of Capecchi and Smithies were the first to show that homologous recombination between plasmid DNAs could be detected when the plasmids were introduced into mammalian cells. These investigators also subsequently demonstrated that specific mutations could be incorporated into mammalian DNA via the introduction of a plasmid-based vector bearing foreign DNA. Surprisingly, a reasonably high degree of recombination was observed even when the vector and the intended genomic target sequence showed only a few kilobases of homology. In 1987, Capecchi's group succeeded in mutating the HPRT gene in ES cells by gene targeting (Thomas and Capecchi, 1987). Independently, the introduction of a vector bearing the wild-type HPRT gene sequence was used to target and functionally correct a mutated HPRT gene in an ES cell (Doetschman et al., 1987). These efforts formally proved that homologous recombination could be used to modify the mammalian genome in a predetermined manner, paving the way for the creation of gene-targeted "knockout" mice.

A Universe of Knockout Mice

The combination of the technologies of ES cell manipulation and homologous recombination provided the powerful "reverse genetics" approach that had been long sought to generate genetically defined rodents for the study of mammalian physiology and pathophysiology. Dozens of laboratories dived into the arena and discovered that, despite its complexity, the technology was relatively easy to master. Within a decade, over one thousand mutated mice were generated. In addition, the ability to manipulate homologous recombination in mammalian cells energized the study of the molecular mechanisms underlying this process. Today, a veritable cottage industry of hundreds of laboratories worldwide has produced an estimated 10,000 different types of genetically engineered mice. Furthermore, an international consortium has recently been formed with an eye to mutating all protein-encoding genes in the mouse genome using this technology. At this point, the bottleneck to progressing faster in defining gene functions lies in analyzing the phenotypes of these animals rather than creating them.

As the gene-targeting juggernaut gathered momentum in the mid-1990s, results were accumulating that had far-reaching consequences. It was soon discovered that null gene-targeted mutations, which disrupt gene function in every tissue of an animal, often precluded the study of a gene's function in adult tissues. Thus, other genetic modifications in rodents, including deletions, insertions, inversions, translocations, and point mutations, were devised to study the relationship between a gene's structure and its function. A further refinement of this approach was to conditionally delete or mutate genetic loci in an inducible fashion, such that specific genetic alterations could be turned "off" or "on" in a temporal or spatial manner. The technology to create these conditional mutations arose from an ingenious application of the bacteriophage enzyme Cre recombinase by the laboratory of Klaus Rajewsky, then at the University of Koln (Gu et al., 1994). By placing Cre recombinase under the control of a tissue-specific or stage-specific promoter and flanking the gene to be targeted with the loxP sites recognized by Cre, researchers could choose the timing and location of deletion of the gene of interest. These and other variations on gene-targeting techniques have led to the establishment of an estimated 500 mouse models of human diseases ranging from cancer, diabetes, and cardiovascular disease to neurological ailments. In addition to the basic research conducted on these mutants, pharmaceutical companies frequently use them to aid in drug discovery and testing.

From Knockout Mice to Healthier Humans

Like all outstanding work, the discovery of mouse ES cells and gene targeting has had an impact much greater than what might have been expected. Many researchers muse that parallel studies of human ES cells could perhaps move us closer to understanding true human traits. Such understanding could some day spur the use of this technology for the purposes of regenerative medicine. However, such research would entail the manipulation of human ES cells, a procedure that is considered unethical in many countries today. It is unlikely that this controversy will evaporate in the near future because gene-targeting techniques currently rely on ES cells, and human ES cells can only be derived from human embryos. However, several groups of investigators made a discovery last year that promises to provide a surrogate approach to studying human embryonic development and physiology without having to directly investigate human ES cells. Working in mice, teams led by Shinya Yamanaka of Kyoto University, Rudolph Jaenisch at the Massachusetts Institute of Technology, and Kathrin Plath of the University of California at Los Angeles and Konrad Hochedlinger of the Massachusetts General Hospital have found that a small percentage of murine skin fibroblasts can be transduced to revert to multipotent stem cells by the introduction of only four transcription factors (Rossant, 2007). These "reprogrammed" cells, which are termed "induced pluripotent stem cells," are quite similar in their properties to ES cells but are less tumorigenic than teratocarcinoma cells. Just three weeks ago, the laboratories of Shinya Yamanaka and James Thomson of the Genome Center of Wisconsin reported using a similar transduction approach to generate induced pluripotent stem cells from human somatic cells (Takahashi et al., 2007; Yu et al., 2007). These human ES-like cells express ES cell-surface markers, have normal karyotypes, express telomerase, and are capable of differentiating into cell types of all three germ layers. This exciting scientific advance means that it may soon be possible to provide patients with multipotent stem cells tailored for a given therapeutic purpose. Because these ES-like cells would have been generated from the patient's own fibroblasts, the chance of the patient's immune system rejecting them upon transplantation would be minimal. Even more satisfying may be the prospect that, using the homologous recombination procedure established by Capecchi and Smithies, ill effects from aberrations present in the germline of patients might be treated using tissue-specific ES-like cells. These possibilities show that the work of Evans, Capecchi, and Smithies has boundless implications for the scientific, medical, and ethical arenas.

As we move into the second century of studying mouse genetics, we eagerly await the next great discoveries in murine and human genetic manipulation that will further advance medical science. By awarding the 2007 Nobel Prize to the discoverers of knockout mice, the international community has vindicated Little's original faith in the power of small creatures to greatly benefit human health. Many believe that the 21st century will be the era in which studies of human genetics lead directly to regenerative medicine. If so, Little's conviction will have been validated beyond his fondest imaginings.

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