

# Gene-ablation and other genetic technique



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How has the use of gene-ablation and other genetic techniques in mice altered our approach to scientific problems in pharmacology and physiology?

280 pts (8-12 pages, 11pt Arial font, double spaced)

A gene is a region of DNA that controls a discrete hereditary characteristic, usually corresponding to a single protein or RNA<sup>1</sup>. Most scientists are interested in determining how genes, and the proteins they encode, function in the intact organism. One of the most direct ways to find out what a gene does is to see what happens to the organism when that gene is missing.

Because mutations can interrupt cellular processes, mutants often hold the key to understanding gene function. A well accepted strategy for evaluating gene function is to create and characterize a gene knockout mouse. Virtually any gene in the germ line can be mutated through gene targeting in totipotent embryonic stem (ES) cells or to use gene ablation technology to knock-out either a specific gene or a specific cell type in an intact animal<sup>2</sup>.

Both of these methods offer almost unlimited possibilities for addressing questions concerning the molecular and cellular biology of development, the relationship between genetics and biological function, unraveling the causes of disease, and enhance pharmaceutical research.

ES cells are a versatile source of cells for repair of damaged and defective tissues in the adult body<sup>2</sup>. Totipotent embryonic stem cells have been used *in vitro* to generate precursors for oligodendrocytes and astrocytes.

Transplantation of these ES cells into a model mouse of human myelin disease allowed the precursors to interact with host neurons and efficiently myelinate axons in brain and spinal cord. Further investigation of this mechanism could lead to pharmacological, physiological and therapeutic

treatments. ES cells also make possible the most precisely controlled forms of genetic modification, allowing animals to be created with virtually any desired alteration introduced into their genome<sup>2</sup>. One way to produce a transgenic (knockout) animal is through gene targeting in totipotent ES cells. By incorporating a null allele into one allele of murine ES cells, and injecting these cells into early mouse embryos, one can create chimeric mice (heterozygous for the knockout allele) containing tissues derived from both host cells and ES cells<sup>2</sup>. Mating the chimeric mice allows one to confirm that the null allele is incorporated into the germ line and can breed these heterozygous chimeric mice to homogeneity producing progeny that are homozygous knockout mice<sup>2</sup>.

A knockout mouse generated to carry a mutating apolipoprotein E (apoE) gene through inactivation by gene targeting in embryonic stem cells can be used as a model for atherosclerosis. apoE is a constituent of very low density lipoprotein (VLDL) synthesized by the liver and of a subclass of high density lipoproteins (HDLs) involved in cholesterol transport among cells. In humans a variant form of apoE, has been found to be defective in binding to the LDL receptor that is associated with familial type III hyperlipoproteinemia, a disease characterized by elevated plasma cholesterol and premature coronary heart disease. apoE deficient mice develop severe hypercholesterolemia due to a delayed clearance of large atherogenic particles from the circulation. A study was done to analyze the genesis of atherosclerotic lesions, including the nature of the cells involved, the sequence of cellular events and the anatomic location of specific lesion types over time on mice fed a normal or a high fat diet. They found that the lesions

were distributed throughout the arterial tree and contained many features of the specialized, chronic, inflammatory-fibroproliferative response characteristic of atherosclerosis with age. This mouse model is used to better understand the physiological role of apoE in lipid metabolism, atherogenesis, and nerve injury. The model can also be used as a way to test new pharmacological drugs in pre-clinical trials to determine drug interaction, new therapeutics or to develop variations of current drugs to better meet the needs of the vast array of genetic variability seen in the human population today.

Gene ablation involves the homologous genetic recombination of a gene in such a way that the gene has been made "null". Genetic ablation is a genetic engineering technique used to suppress selectively the growth of a specified cell line or cell type in an animal rather than suppress the activity of an individual gene<sup>2</sup>. Analyzing the *in vivo* function of cells can be accomplished through specific cell ablation. Using genetic ablation to produce a knockout mouse involves the injection of one or more transgenes (usually a DNA segment bearing its own promoter) into the pronucleus of a fertilized mouse oocyte, which, after reimplantation into a foster mother, gives birth to a transgenic mouse bearing one of several hundred copies of the transgene<sup>3</sup>. These mice again can be bred to produce a homozygous colony.

An overexpression Sod2 transgenic mouse was generated through the use of pronuclear injection of the Sod2 gene into fertilized eggs. These mice overexpressed superoxide dismutase which catalyzes the conversion of superoxide anion to hydrogen peroxide and oxygen. Superoxide anions are

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reactive oxygen species (ROS) generated in the respiratory chain as a byproduct of mitochondrial respiration. ROS cause oxidative damage to key mitochondrial components, such as protein, lipids and DNA. They are thought to lead to an overall decline in cellular function and ultimately determine the life span of an organism. Sod2 transgenic mice have been used to test the effects of overexpressing MnSOD on mitochondrial function, levels of oxidative stress or damage and live span in mice. They found that a two fold increase of MnSOD throughout the life of the organism resulted in a slight decrease in oxidative damage and enhanced resistance against oxidative stress. Oxidative damage is also associated with arthritis, heart disease, gastrointestinal disorders, multiple sclerosis, cancer and neurological diseases. This model can be used to physiologically improve ones understanding of oxidative stress and to develop pharmaceutical agents that can enhance ones ability to decrease or protect against oxidative damage.

Customized gene targeted animal models include constitutive knockouts or knock-ins, or conditional knockouts or knock-ins of known genes, a gene family, mutant genes or artificially created reporters and gene constructs. A constitutive knockout is a model that provides a broad overview of gene function, by the inactivation of the gene of interest at the genetic level, by random mutation or targeted insertion<sup>4</sup>. The advantage of using this model is that you get a total inactivation of the gene in any cell<sup>4</sup>. The disadvantage of this model is that the phenotypes can be complex since all organs are affected<sup>4</sup>. A conditional knockout is a model that provides a research model of high potential, by targeting tissue-specific inactivation of the gene of

interest, through the use of recombinase<sup>4</sup>. Recombinase is an enzyme that deletes the DNA fragment located between the two recombinase-specific sites<sup>4</sup>. This tissue-specific expression of the recombinase allows the inactivation of the gene of interest only in the tissue where the recombinase is expressed<sup>4</sup>. There are two advantages to using this model, the first is the inactivation is restricted to specific tissues and the second is that several tissue-specific models can be made in parallel<sup>4</sup>. The disadvantages of using this model include the efficiency of the excision and the inability to validate the recombinase system<sup>4</sup>.

There are a number of other strategies that can be used in place of, or to complement, conventional gene knockout technology. Two such examples are regulatable gene ablation and temporary gene ablation (gene knock downs). Ablation of a gene can be spatially or temporally regulated, that is only the organ of interest is affected or the mutation occurs only in one particular developmental state<sup>5</sup>. Through the use of a regulator, the promoter can be regulated to control gene expression in the organ or particular tissue of interest. A gene knockdown refers to the technique by which the expression of one or more of an organism's genes is reduced, either through genetic modification or by treatment with a reagent such as a short DNA or RNA oligonucleotide with a sequence complementary to either an mRNA transcript or a gene<sup>6</sup>. These approaches offer a rapid and economical method to examine the specific actions of a gene product at a particular time point. Genes can be transiently down-regulated by targeted blockade of gene expression, either systemically or within a specific tissue. Two such approaches are antisense technology and the use of short

interfering RNA (siRNA). Antisense technology is the use of short modified DNA sequences complementary to the mRNA of the gene of interest<sup>2</sup>. These sequences bind to the 5'UTR and the beginning of the coding sequence therefore preventing translation initiation and blocking ribosomal interaction with the AUG start codon<sup>2</sup>. Antisense oligonucleotide genetic-code blocking drugs might control disease by inhibiting deleterious or malfunctioning genes. siRNA on the other hand, utilizes small lengths of double stranded RNA designed against the gene of interest and is introduced into the cell where they interact with intracellular machinery to form RNA-induced silencing complexes (RISCs)<sup>2</sup>. These complexes unwind the siRNA strands and allow specific binding to the complementary mRNA sequence<sup>2</sup>. This binding cause's cleavage and destruction of the now double stranded mRNA, and subsequently prevents translation<sup>2</sup>. siRNA can be used to study gene function, manipulating gene expression, as well as drug development. siRNA compounds can be created and delivered into human cells were they are able to silence genes and viruses responsible for human diseases.

Two other genetic techniques that are used to approach gene investigation include suppressor analysis and reverse genetics. Suppressor analysis is used to identify genes that are functionally related to another gene of interest<sup>7</sup>. A suppressor mutation is a mutation that counteracts the effects of another mutation thereby reducing its phenotypic effect<sup>7</sup>. Suppressor mutations may be intragenic (in the same gene as the original mutation) or intergenic (in another gene)<sup>7</sup>. Intergenic suppressor mutations can be either information suppressors or function suppressors<sup>7</sup>. Of the two, function suppressors are most valuable for the genetic analysis of cellular processes<sup>7</sup>.

Reverse genetics is a process in which specific mutations are introduced into a cloned gene for the purpose of identifying functional domains<sup>8</sup>. One way to conduct reverse genetics is to do RNA interference (RNAi)<sup>8</sup>. RNA interference is an *in vivo* gene knock-down, which is used to insert a random shRNA construct into the mouse genome<sup>4</sup>. Here, double-stranded RNAs inhibit corresponding gene expression by inducing degradation of its mRNA<sup>4</sup>. RNAi can be used as part of the immune response to viruses and other genetic material; it can also be used to look at downregulation and upregulation of genes<sup>4</sup>. RNAi has been used to evaluate whether insulin-like growth factor binding protein-3 (IGFBP3) modulates gastrointestinal stromal tumor (GIST) cell response. Here, transfection of siRNA to knockdown IGFBP3 gene into GIST cells resulted in a significant loss of cell viability and therefore found that IGFBP3 gene is required for GIST cell survival. IGFBP3 gene expression is lost in many cancer cells and reintroduction of the protein often results in cell death. On the other hand IGFBP3 has been linked to renal cell carcinoma, breast cancer, and metastatic melanoma, which suggests that IGFBP3 may contribute to tumorigenesis or disease progression. By studying IGFBP3 and the IGF pathway one can explore IGF for prognostic and therapeutic value in GISTs and other cancers.

In order to develop new treatment and preventative strategies for a human disease, we must first understand the biology and pathogenesis of the human disease itself<sup>10</sup>. A way to study human gene function is by mouse gene humanization. Humanization is the replacement of the murine gene by its human counterpart<sup>4</sup>. Animal models of human disease have been crucial in elucidating normal organ biology, pathogenic mechanisms of the disease



and developing new therapeutic strategies<sup>10</sup>. Animal models are being used instead of human studies, because human studies are limited by a number of factors such as: the variability seen in genetic background and environmental influences, the inability to readily obtain human tissues for molecular analysis and the small numbers of patients who may have a particular genetic abnormality<sup>10</sup>.

Transgenic animals have already made valuable research contributions to studies involving regulation of gene expression, the function of the immune system, genetic diseases, viral diseases, cardiovascular disease, and the genes responsible for the development of cancer. Transgenic animal models are most effective if they have both construct validity and face validity.

Construct validity is the degree to which the model corresponds to the clinical state in humans. Face validity is the animal model responds in an appropriate manner to the effects of different therapeutic agents. An animal model that can recapitulate at least part of the neuropathology and the cognitive and behavioral impairment is the most effective representative of the disease. Several rodent models of Alzheimer's disease (AD) have been created. The first example of such a model has been created to mimic the cholinergic deficits of AD patients and to investigate the role of the central cholinergic system in the cognitive and behavioral deficits observed in AD<sup>9</sup>.

The second example of such a model has been created with acute or chronic injections of amyloid beta within the brain parenchyma of rodents, to overexpress AD-related mutated protein (amyloid deposits) in the central nervous system of rodents<sup>9</sup>. A third model of AD has been to create a transgenic mouse to express wild-type or mutant forms of the human APP

and/or PS genes. All three of these models provide many insights into AD pathogenesis and approached to new therapies. By exploring and understanding of the neuropathological processes underlying the cognitive deterioration leading to dementia and investigating of the effects of potential new treatments on both AD neuropathology and cognitive deficits we will be able to test drugs that could potentially alleviate symptoms of the disease.

Transgenic animals have also been used to develop animal strains that secrete important proteins in milk<sup>2</sup>. These animals are used to synthesize recoverable quantities of therapeutically useful proteins. These proteins can be harvested from the animal by simply milking the animal and then using separation techniques for protein purification<sup>2</sup>. For example cows have been used to purify collagen which can then be used to indicate burns and bone fracture<sup>2</sup>. Pigs have been used to purify human hemoglobin as a blood replacement for transfusion<sup>2</sup>. Goats have been used to make monoclonal antibodies for colon cancer<sup>2</sup>. In addition sheep have been used to make factor VIII and factor IX for hemophilia<sup>2</sup>.

Despite the large amount of knowledge that can be gained by knockout studies, there are some important considerations to be taken when investigating the role of a particular protein or gene of interest. These considerations include but are not limited to: redundancy, lethality, delineating systemic vs. local effects, subtle effects, and specific pathogen free conditions<sup>3</sup>. Gene redundancy is the existence of multiple copies of the same gene in the genome of an organism. This problem can be overcome to some extent by the use of multiple knockouts or in some situations where multiple ligands bind to the same receptor, a dominant negative receptor

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approach can knockout the effects of all ligands at once<sup>5</sup>. Lethality phenotypes severely compromise the ability of the organism to function. Some knockouts are developmentally lethal, which means that the genetically altered embryos cannot grow in adult mice. Knocking out a gene may also fail to produce an observable (phenotypic) change in a mouse or may even produce different characteristics from those observed in humans in which the same gene is inactivated. In some instances, a particular organ of interest can be transplanted into a healthy wild type host in order to conduct the analysis on that organ. Local effect refers to an adverse health effect that takes place at the point or area of contact, for example the respiratory tract<sup>11</sup>. Systemic effect refers to an adverse health effect that takes place at a location distant from the body's initial point of contact and assumes absorption has taken place, i. e. absorption into the blood stream<sup>11</sup>. Subtle effects are those that are so slight as to be difficult to detect or describe. For example, small changes in nutrient delivery to the fetus can program post-natal and adult metabolic status and lead to increased susceptibility to a range of adult onset disease, including stroke, hypertension and non-insulin dependent diabetes<sup>5</sup>. Specific pathogen free conditions refers to the fact that laboratory mice in research institution are generally housed in a specific pathogen free environment, and are therefore not challenged with the array of pathogens most mice and humans are exposed to.

When comparing transgenic models to humans a key difference could be in the initiation of the development or process in the mouse that may not have a similar mechanism as the human development or process it is thought to

represent. The presence of a specific mutation in the mouse from the time of its conception may enable a distinction between phenotypic changes due to the mutation itself and changes caused by adaptation and compensation for the mutation<sup>12</sup>. A gene can also be expressed in different tissues where it may have different functions; its alteration may induce unexpected consequences. Also, the function of two genes may overlap or a mutation in a single gene might not reveal an abnormal phenotype. There is also a possibility of random incorporation into genome, which may inadvertently disrupt other genes, or have a variable degree of gene silencing success. The gene may also induce gross morphologic or physiologic abnormalities that affect the gene directly. Note also, these animal models also only play a minor role in drug development, as relatively few human diseases are monogenetic<sup>12</sup>.

In most cases, gene ablation and other genetic techniques must address the basic question as to whether a particular gene, wild type, or mutant is involved in the pathogenesis of a particular disease. If not a particular disease, it may also address whether a gene product which plays a role in a specific signaling process contributes to disease in conjunction with other predisposing conditions. Animal models are crucial in providing a unified understanding of how drugs work, developing new therapeutic agents, and obtaining critical data needed to advance compounds into clinical trails<sup>12</sup>. Animal models are an important tool for scientist to investigate human diseases, especially in order to conduct time-course studies or when studying early disease. Transgenic and knockout animals are also used to determine the molecular mode of action of a gene product and the

downstream consequences of its misexpression on normal processes.

Assessing the role of a specific gene can now be accomplished through the use of transgenic animals whose genomes have been manipulated to under- or overexpress a target gene, or express it in a modified or defective, nonfunctional form<sup>12</sup>. Animal models represent an attempt to imitate the pathologies associated with human disease states in a preclinical setting<sup>12</sup>.

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Genes associate with drug responses can be grouped into three broad categories: those involved in pharmacokinetics (i. e. drug metabolism, transport), those encoding pharmacological targets (i. e. drug-target pathways) and those with physiological associations (i. e. homeostasis pathways)<sup>13</sup>. Pharmacokinetic pathways mediate drug absorption, distribution, metabolism and excretion<sup>13</sup>. Pharmacological pathways such as transcription factors and phosphorylation targets whose genes encode other proteins are also candidates for pharmacogenetic analysis<sup>13</sup>. Understanding the sensitivity of a particular drug and the genes that encode the drug metabolizing enzymes can help determine how the particular drug is transported and metabolized within the body. Understanding the gene variants and how they influence drug concentrations at the target site is a possible way to explain the differences in response to a particular drug dose among individuals.

Pharmacogenetics is the study of how an individuals genetic differences influence drug action, usage and dosing<sup>2</sup>. The molecular compositions of enzymes are currently being identified through the use of the Human

Genome Project. Scientists are able to study these enzymes and determine correlations between genotypic and phenotypic variability<sup>14</sup>. These current advances will allow scientists to use the molecular knowledge of enzymes to detect individuals who are likely to experience adverse reactions to medicines without having to use potentially dangerous methods of trial and error<sup>14</sup>.

An offshoot of pharmacogenetics is pharmacogenomics, which attempts to understand not only the molecular composition of genetic variants associated with drug response but also the behavior of those variants<sup>14</sup>. Pharmacogenomic knowledge can be used to spot the disease before it occurs in a patient, increase drug efficacy upon pharmacotherapy, and reduce drug toxicity<sup>2</sup>. It could also facilitate the drug development process to improve clinical development outcomes, reducing overall cost of drug development and lead to development of new diagnostic tests that impact on therapeutic decisions<sup>2</sup>. Pharmacogenomics can be used by doctors to identify the optimal dose and medicine for each individual patient with greater efficacy and safety. As the dosage would be based on factors such as age, weight, diet, lifestyle, liver and kidney function. By using a pharmacogenetic test a doctor can determine the right dose for individual patients. There are pharmacogenetic tests for cancer, HIV, depression, and cardiovascular disease<sup>15</sup>. These tests take in the factors which are most likely to affect the absorption and effectiveness of the drug.

There are many benefits that can come from pharmacogenetics and pharmacogenomics. Pharmaceutical companies can use information obtained to create more powerful medicines based on proteins, enzymes,  
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and RNA molecules associated with genes and diseases<sup>15</sup>. Again, doctors would be able to analyze a patient's genetic profile and prescribe the best available drug therapy from the beginning instead of the standard trial and error method of matching patient with the right drugs. A more appropriate drug dosage could be determined by not only taking in a person's weight and age but also accounting for their individual body processes, such as the time it takes for their body to metabolize the drug. Pharmacogenetics could also provide advance screening for disease, better vaccines, improvements in the drug discovery and approval process and decrease the overall cost of health care<sup>15</sup>.

Pharmacogenomics is still in the development stage in the research field. There are several factors that need to be overcome before many of the pharmacogenomic benefits can be realized. These include the complexity of finding gene variations that affect drug response, the limitation of current drug alternatives, disincentives for drug companies to make multiple pharmacogenomic products, and educating health care providers<sup>14</sup>. There are also ethical issues to consider, such as lack of data privacy, possibility that adverse reactions to drugs can occur during treatment, the cost of such designer drugs will be too expensive for everyone, and genetic testing can provide an array of diagnostic results (predispositional test) which may or may not be what the patient wants to know<sup>14</sup>.