MOLECULAR MEDICINE

KNOCKOUT MICE

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THE ability to remove or alter with precision a single one of the thousands of genes in the body and to transmit this mutation to all subsequent progeny was a science-fiction dream only a few years ago. But today this technique is part of a routine procedure for creating animal models that can be used to study the pathophysiology and therapy of diseases in humans.

In general, mutations that cause a gain of function produce disease even when they occur in only one of a gene's two alleles; for example, the oncogenic mutations that cause abnormal cell proliferation. In a recessive genetic disorder, by contrast, there must be mutations in both alleles for the disease to be produced, and the mutations cause a loss of function; for example, in patients with cystic fibrosis two copies of a recessive allele cause a loss of chloride-channel activity.

The methods needed to produce animal models of recessive genetic diseases differ from those used in studying autosomal dominant diseases. Integrating an oncogene that causes dominant disease, such as c-*myc,* into the genome of a fertilized mouse oocyte without altering the mouse's own genes creates a transgenic, cancer-prone mouse that transmits this trait to its offspring with a dominant pattern of inheritance. To create an animal model of an autosomal recessive disease, however, both alleles of the normal gene must be inactivated. The technique of gene "knockout" was developed for this purpose.

Several independent scientific advances have culminated in the ability to alter a single heritable gene in the DNA of a mouse with precision. Initially, chimeric mice with somatic cells from more than one genetic background were created, though inefficiently, by introducing embryonal carcinoma cells into normal mouse embryos early in gestation. In the early 1980s, the efficiency of the process used to generate chimeras improved markedly when methods were developed to culture totipotential cells from the inner cell mass of the blastocyst, the area destined to become the fetus. These pluripotential cells, termed embryonic stem cells, can be genetically altered and then microinjected into the cavity of an intact mouse blastocyst after 3.5 (of a total of 19.5) days of gestation. They can, from the blastocyst stage, populate all the tissues of the developing mouse. The contribution of the embryonic stem cells to the genetic makeup of the chimeric animal that develops from the injected blastocyst is most easily assessed by using embryonic stem cells and blastocysts whose genes for coat color differ. If the embryonic stem cells contribute to the germ cells (for example, the sperm) of the developing mouse embryo, their entire haploid genome can be passed on to subsequent generations.

The goal of the gene-targeting (knockout) method is to replace the specific gene of interest with one that is inactive, altered, or irrelevant. To increase the probability that such replacement will occur, rather than nonspecific random integration of the DNA, both ends of the replacement gene are flanked by long DNA sequences homologous to the sequences flanking the target gene (Fig. 1). Gene constructs of this type permit corresponding stretches of DNA to be exchanged (in what is termed "homologous recombination") when the DNA breaks and rejoins. The frequency of homologous recombination is low. Therefore, there must be a way of selecting the rare cells in which the target gene has been replaced by the constructed gene. Two selectable markers meet this purpose.

Figure 1 shows the use of selectable markers in an experiment using a genetically engineered knockout vector to inactivate the gene that encodes corticotropin-releasing hormone (CRH) in mice. The vector was created by replacing the gene encoding the CRH polypeptide with a gene that confers resistance to the antibiotic neomycin. This bacterial gene lies between the 5′ and the 3′ homologous flanking regions present in both the target and the replacement gene. The knockout vector also contains a gene that encodes viral thymidine kinase, which confers sensitivity to ganciclovir. This second selectable marker lies outside the 5′ and 3′ homologous flanking regions of the replacement gene. The genetically engineered DNA is introduced into embryonic stem cells that are then incubated with tissue-culture medium containing neomycin and ganciclo-

Figure 1. Homologous Recombination between a Cellular Gene and a Knockout Vector to Create Mice Lacking Corticotropin-Releasing Hormone (CRH), a Major Hypothalamic Regulator of the Stress Response.

Embryonic stem cells (upper left-hand panel) contain the *CRH* cellular gene (upper right-hand panel), which consists of exon 1 (olive green, a 5′ noncoding region), an intron, and exon 2 (red, a protein-coding region, and yellow, a 3′ noncoding region). A knockout vector, consisting of a collinear assembly of a DNA flanking segment 5′ to the cellular gene (blue), the phosphoglycerate kinase–bacterial neomycin gene (*pgk-neo,* violet), a 3′ segment of the cellular gene (yellow), a DNA flanking segment 3′ to the cellular gene (green), and the phosphoglycerate kinase–viral thymidine kinase gene (*pgk-tk,* orange), is created and introduced into the embryonic-stem-cell culture. Double recombination occurs between the cellular gene and the knockout vector in the 5′ homologous regions and the 3′ homologous regions (dashed lines), resulting in the incorporation of the inactive knockout vector, including *pgk-neo* but not *pgk-tk,* into the cellular genomic locus of the embryonic stem cell. The presence of *pgk-neo* and the absence of *pgk-tk* in these replaced genes will allow survival of these embryonic stem cells after positive–negative selection with neomycin and ganciclovir (see text).

In the bottom panel, the clone of mutant embryonic stem cells is injected into a host blastocyst, which is implanted into a pseudopregnant foster mother, and subsequently develops into a chimeric offspring. The contribution of the embryonic stem cells to the germ cells of the chimeric mouse results in germ-line transmission of the embryonic-stem-cell genome to offspring that are heterozygous for the mutated *CRH* allele. The heterozygotes are mated to produce mutant mice homozygous for CRH deficiency, with impaired hormonal responses to multiple stressors.

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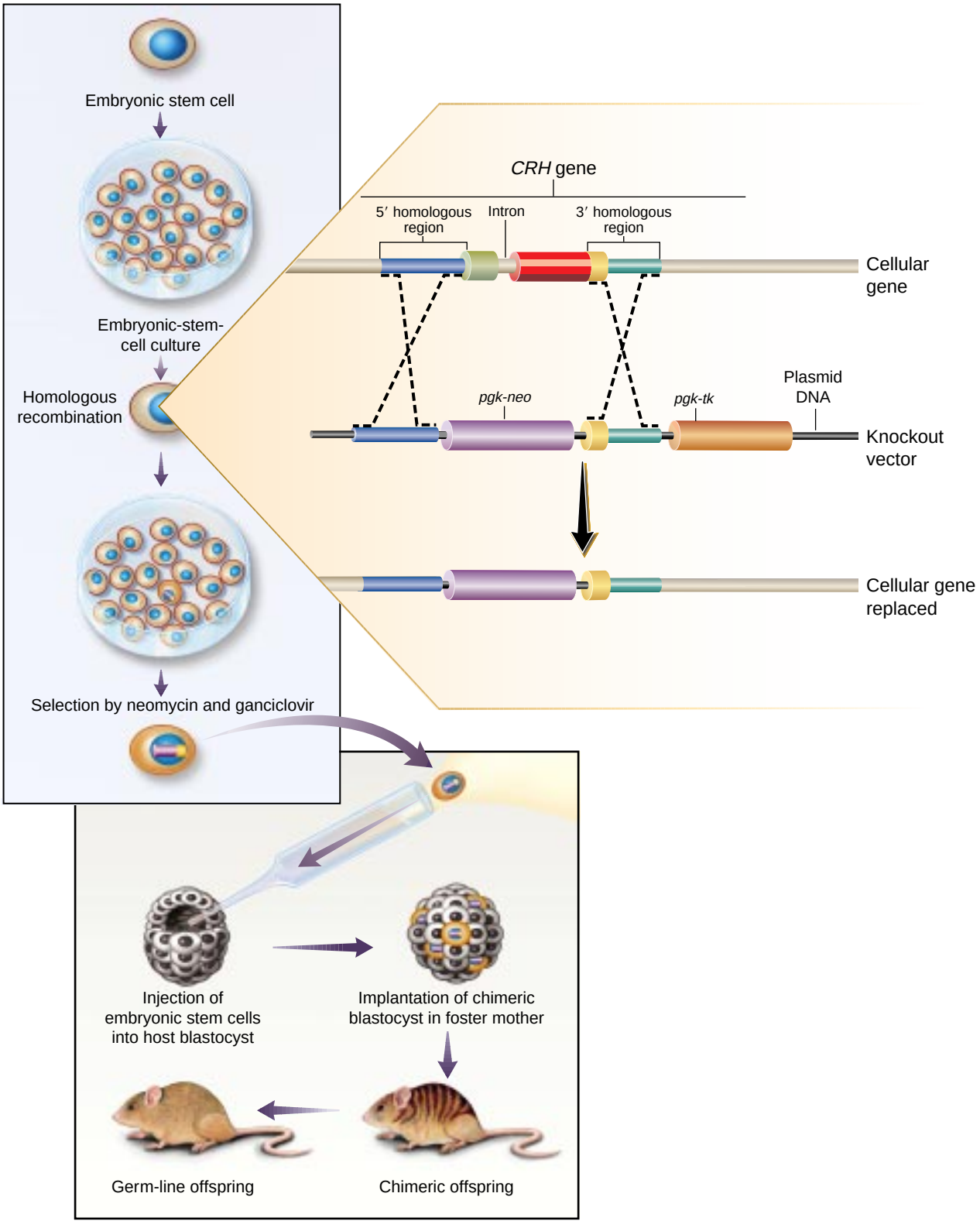
vir. Embryonic stem cells that have incorporated the new DNA by homologous recombination resist neomycin (positive selection). By contrast, cells that have nonspecifically taken up genetically engineered DNA, including the viral thymidine kinase, are killed by ganciclovir because of the gene for this kinase, which lies outside the region of specific homologous recombination (negative selection).

In embryonic stem cells that survived this "positive–negative" selection, the CRH knockout vector had correctly replaced one of the normal CRH alleles. These clones were then injected into blastocysts to initiate the



creation of chimeric mice (Fig. 1). Chimeras with germ cells derived from the altered embryonic stem cells transmitted the changed embryonic-stem-cell genome to their offspring, yielding mice heterozygous for CRH deficiency; the heterozygotes were then bred to each other to create mice homozygous for CRH deficiency.

The deficits present in a knockout mouse can reveal or clarify the function of the mutant gene. The analysis by targeted inactivation of the genes implicated in fetal development and organogenesis has been especially powerful. Gene knockout has shown that the gene *SF-1* (encoding steroidogenic factor 1) is essential for adrenal and gonadal organogenesis. Also essential are *WT-1* (the Wilms' tumor locus), for renal development, and the genes encoding myogenin (for skeletal-muscle formation), the glucocorticoid receptor (for glucocorticoid-mediated fetal development), and the estrogen receptor (for female sexual maturation). *RAG-2* (recombination-activating gene 2) and *GATA-1* (which recognizes a GATA-nucleotide motif) are critical to the development of lymphocytes and erythrocytes, respectively. Genetic deficiencies can be combined simply by cross-breeding mice with knockout of different genes. In this way it is possible to study overlapping genetic functions, such as those of the insulin-like growth factors I and II, during fetal development.

These experimental systems are also of great value in studies of the pathogenesis and treatment of disorders in all fields of medicine. Table 1 shows several examples. In many of these, the phenotype of the knockout gene was anticipated by prior knowledge of the gene's function. However, in several instances, such as mice in which inactivation of the interleukin-2 gene results in ulcerative colitis, an unexpected mutant phenotype provided important new clues about the disease mechanism. In addition, a growing number of disorders due to single-gene mutations (for example, Duchenne's muscular dystrophy, cystic fibrosis, and Huntington's disease) are being discovered entirely through the chromosomal location of their defects (positional cloning), with little or no prior knowledge of the pathogenesis of the diseases.

Consequently, a knockout mouse corresponding to a particular genetic disorder may help clarify the mechanism of the disease. For example, mice with homozygous knockout of the gene for Huntington's disease die as embryos instead of undergoing the clinically predicted postnatal degeneration of their brains. This result indicates that the abnormal expansion of nucleotides in the gene of patients with Huntington's disease causes a deleterious gain, rather than a loss, of function (as would be expected with an autosomal dominant disorder).

Pharmacologic manipulation of knockout animals will prove useful in screening therapeutic agents with potential for study in clinical trials. Therapy involving somatic-gene replacement can be tested in a disease model using knockout mice. An example of this is adenovirus-mediated gene transfer of the receptor for low-density lipoprotein (LDL) into LDL-receptor–deficient knockout mice, which results in partial amelioration of the hyperlipidemic phenotype.

Several considerations are important in evaluating the phenotype of a knockout mouse. The fact that a mutation has been present from the time of conception may make it difficult to distinguish phenotypic changes due to the mutation itself from changes caused by physiologic compensation for the mutation. If a gene is expressed in different tissues where it may have different functions, its inactivation may have multiple consequences. A gene mutation may cause embryonic death or death soon after birth, giving important information about the gene's role in development but preventing further study of its action. Conversely, the functions of two genes may overlap, in which case a mutation in only one gene may not reveal an abnormal phenotype.

Improvements in knockout technology would include the ability to inactivate a gene at a specific time after conception and in a specific tissue. These technical advances would circumvent embryonic death if gene inactivation occurred after a critical period in development. Time-restricted inactivation would also allow acute, as opposed to chronic, states of deficiency to be assessed, since the animals could be studied before compensatory pathways developed. New methods of tissue- and time-

Table1. Human Disorders Studied in Knockout Mice.

Disorder	Target Mouse Gene or Gene Product
Cardiology	
Atherosclerosis	Apolipoprotein E, low-density–lipoprotein receptor
Salt-sensitive hypertension	Atrial natriuretic peptide
Endocrinology	
Familial hypocalciuric hypercalcemia	Calcium receptor
Glycogen storage disease type 1	Glucose-6-phosphatase
Intrauterine growth retardation	Insulin-like growth factor II
Obesity	β_3-Adrenergic receptor
Gastroenterology	
Hirschsprung's disease	Endothelin receptor
Ulcerative colitis	Interleukin-2
Hematology	
α-Thalassemia	α-Globin
Hemophilia A	Factor VIII
Chronic granulomatous disease	Cytochrome b-245
Immunology	
Autoimmune lymphoproliferative syndrome	*FAS*
Bruton's agammaglobulinemia	Bruton's tyrosine kinase
Hyper-IgM syndrome	CD40 ligand
Severe combined immune deficiency	
Autosomal recessive	Janus kinase (JAK)-3
X-linked	Interleukin-2–receptor γ chain
Metabolism	
Gaucher's disease	β-Glucocerebrosidase
Homocysteinemia	Cystathionine β-synthase
Lesch–Nyhan syndrome	Hypoxanthine phosphoribosyltransferase
Niemann–Pick disease	Acid sphingomyelinase
Tay–Sachs disease	α-Hexosaminidase A
Neurology	
Short-term memory deficit	Calmodulin kinase II
Oncology	
Li–Fraumeni syndrome	p53
Neurofibromatosis	*NF-1*
Retinoblastoma	*RB*
Pulmonology	
Cystic fibrosis	Cystic fibrosis transmembrane conductance regulator

restricted gene inactivation use a bacteriophage site-directed recombination system (Cre-lox), consisting of a site-specific recombinase (Cre) and DNA recombination sites (lox). Together with standard knockout methods, this system shows promise but requires further work for gene inactivation to occur consistently. In addition, transgenic mice have been developed that express antisense RNA that inactivates a selected gene product by forming unstable duplexes of messenger RNA under the control of inducible, cell-specific gene promoters, but the degree of inactivation is variable.

Techniques of gene knockout represent major advances in biology. They have allowed molecular biology to be integrated with whole-animal physiology, creating an invaluable resource for understanding the molecular regulation of complex physiology and behavior. Gene knockout promises to provide links between genetics and the pathogenesis of disease that will lead to a better understanding of the treatment of human disorders.

Recommended Reading

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