A Knockouts Tale

Mice, zebra fish, nematodes, and cell lines transformed to lose genetic function hold hope for tailored drug discovery and understanding disease.

BY MARK S. LESNEY

Scan the pages of scientific advertisements and journals devoted to genetic analysis and you might imagine that modern biomedicine was becoming another remake of the movie *Willard*—with masses of knockout mice replacing the scurrying army of rats. Knockout technology is everywhere—applied to mice, zebra fish, *Drosophila*, and a host of other eukaryotic systems, including, most recently, human embryonic stem cells.

The reasons are straightforward. Sequence prediction for the proteins produced by discovered genes and subsequent protein modeling can often provide information on function, especially if the homologous protein is well known and understood. But for

ILLUSTRATOR: MICHELLE BARBERA

unknown proteins, or for genes with multiple effects, the best method for determining what a gene does in an organism requires tampering with its operation in a test animal—just to see what happens. One means of doing this is through the use of genetic "knockout" organisms—those in which a particular gene has been deliberately disabled.

Despite its obvious technological complexity and tremendous benefits, the knockout method of studying gene function is not a particularly sophisticated approach, at least conceptually. It is, instead, the genetic equivalent of trying to understand how a computer works by plucking a chip (random or selected) from a computer motherboard and seeing whether your favorite version of *Doom* still runs.

Surprisingly, though, we are still so new at the genome game that even this kind of rudimentary information can be a tremendous plus to the understanding of normal human physiology, much less disease. But with luck, knockout technology can provide much more. It has, in some cases, given researchers the basis for a whole new concept of inherited disorders and can be used to point to new potential targets for therapeutic intervention. Knockouts have also helped to invalidate previous (mis)conceptions of disease, and by their very failures have led to a new understanding of the complexity of genetic interactions.

Three rounds to a knockout

Although knockout technology requires considerable finesse, it is, at heart, an elegant adaptation of natural recombination such as occurs in crossover events. But rather than mutual exchange between natural homologous chromosomes, the transfer is between genetically engineered vector cassettes and the animal gene.

Round 1: Find and prep your gene. Knockout technology requires exact knowledge of the complete or a significant portion of the DNA sequence (especially in flanking regions of the exons) of the gene of interest. This means that it is not an exploratory genomic technique for finding genes involved in processes but rather a means of assigning process function to known genes with unknown, suspected, or poorly understood physiological roles.

Proceeding from known sequence information, researchers can tailor a knockout cassette to the gene of interest. The DNA cassette requires several functional components to be effective. First, the cassette must contain a dysfunctional insert flanked by DNA regions homologous to the two ends of the gene of interest. These homologous regions provide the bridge for binding and recombination. Recombinants are positively selected by inserting a marker gene for drug resistance between these homologous ends as well. Integration of this portion of the cassette thus confers drug resistance, and nontransformed cells die with the application of the appropriate compound. For example, the *neo* gene (encoding neomycin phosphotransferase) is often used to make cells resistant to the antibiotic G418.

To prevent random insertions from being selected, most researchers have adapted a technique first developed by S. L. Mansour and colleagues, called positive–negative selection (*www.*

the-scientist.com/yr2000/jul/profile_000724.html). By attaching a selection marker such as the thymidine kinase (TK) or hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene to the cassette outside the homologous DNA stretch, a negative selection can be performed for random inserts. Since these genes convey specific drug susceptibility, cells in which the entire cassette has been incorporated, rather than just the internal stretch between the homologous DNA regions, will be killed, whereas true homologous recombinations survive (see Figure 1).

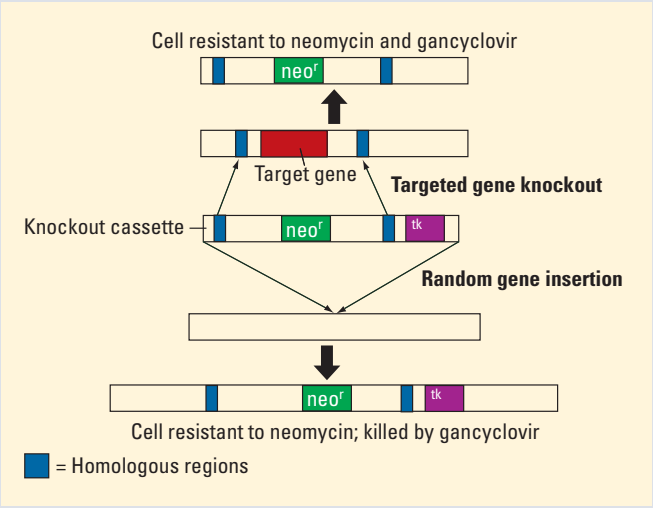

Figure 1. Typical positive–negative selection method of homologous recombination for generating gene knockouts. Cells with homologous recombination are resistant to both antibiotics; those with random insertion are killed by gancyclovir.

Round 2: Homologous recombination. Getting the knockout gene into place and growing out the transformed cell line is usually the most difficult part of the process. The DNA is most often transported into the cell using physicochemical rather than biological vectoring techniques. Among the chemical transfection reagents used are the cationic agent ExGen 500 (Fermentas, Hanover, MD) and the lipid agent FuGene-6 (Roche, Indianapolis, IN), both of which stimulate DNA uptake by cells. The most effective physical method of inducing knockout cassette uptake is electroporation.

Round 3: Best of breed. Although yeast has proved to be a valuable knockout organism for a wide variety of eukaryotic studies, especially on basic cellular growth and metabolism, mice have become the preferred model organism for examining human diseases, because of their more obvious complexities and similarities to human physiology. Some of the most significant mouse knockout lines to date include those with one or more of the known oncogenes affected, including those for skin and breast cancer, as well as other tumors.

Unlike yeast, knockout animals have to be generated through an embryo. In mice, this requires the use of embryonic stem cells that are transformed chemically or electroporetically with a knockout cassette. These transformed cells are injected into an early-stage (blastocyst) embryo to produce a chimera. (In some cases, mice with different coat colors have been used to make early identification of chimeras for further testing an easy process.) Interbreeding these chimeras can result in progeny that contain the desired knockouts in all of their cells. Traditional breeding methods can be used on heterozygous knockout populations to create homozygous animals that can act as a foundation for a "double knockout" population line. Heterozygous knockouts are the only viable option (literally and figuratively) when the gene product is critical to development.

Knock out and replace. In some cases, though, knockout technology is just the beginning. Often, when researchers study the effects of a particular human gene in an animal model, they encounter competition or interference from the animal version of the same gene. In this case, homologous recombination is used to simultaneously knock out the animal gene and replace it with the human variant. An example of this is Taconic's Cd4/CD4 Multiple Targeted Mutation/Microinjected Mice line (*www.taconic.com/anmodels/001175.htm*). Homologous recombination is also one of the options for producing humanized animals for disease studies or potential xenotransplantation.

This little piggy had a knockout . . .

Immerge BioTherapeutics (Columbia, MO) produced the first knockout pigs in the fall of 2001, and their research paper was presented only days after a PPL Therapeutics U.S. subsidiary announced the production of five knockout cloned piglets (produced using somatic nuclear transfer technology) named Noel, Angel, Star, Joy, and Mary, born on December 25, 2001. Both groups were driven by the intense desire to produce pigs that ultimately would be capable of producing organs for human transplants. The original research produced animals with a single deletion of *GGTA1,* which encodes an α-1,3-galactosyltransferase that synthesizes one of the key antigens that cause hyperacute rejection in organ transplants. In March 2003, David Ayares and his colleagues reported producing four seemingly healthy piglets with a double knockout of *GGTA1* that completely lacked the α-1,3-Gal epitopes (*6*).

The ultimate success of this approach will probably require eliminating other significant pig proteins that can participate in organ rejection, as well as inserting genes capable of inhibiting global human immune response behaviors at the site of the xenograft, including complement activation and clotting.

Winning by a knockout

The utility of knockout animals and cell lines to the study of biology and medicine is vast. Knockouts have shown themselves capable of playing a critical role in advancing research in many biological disciplines, from studying normal and pathological physiology to analyzing development and the response of cells and organisms to chemical (toxic or therapeutic) agents. Examples of these uses abound, especially in mice (*http://research.bmn.com/mkmd*).

Physiology. Analyzing the phenotypic effect of any particular knockout can be difficult, and determining the biochemical basis of it even more so. Subtle changes in protein synthesis, cell architecture, or receptors can be

next to impossible to tease out. Gross effects on organs, tissues, and development might be easy to discern but harder to explicate. But in cases in which the effect is both "natural" and detectable, knockout cells, and especially knockout organisms, can provide invaluable information on the behavior of genes in vivo. And knockouts can be used to study not only gene behavior but also animal behavior. In January, researchers at the Baylor College of Medicine and Case Western University reported that mice with knockouts of the *Pet-1* gene (involved in serotonin metabolism) became "superaggressive", and the mice are being studied as a new model of anxiety and aggression in humans (*1*).

Pathology. Although studying potential disease genes is an obvious desideratum of knockout models, environmental or unknown genetic background effects might mask or suppress the desired phenotype. And worse problems occur when studying genes that in humans have an observed or suspected outcome (such as a disease) that does not manifest in animal models. In such cases, human knockout cell lines might be required (see below). But for cases in which the disease model is similar, knockout animal models provide a wealth of information unlikely to be easily obtained through any other method. For example, researchers at the Wake Forest School of Medicine recently reported that ACAT2, an enzyme involved in cholesterol metabolism and found only in the liver and intestines, might be a vital link in the development of hardening of the arteries (*2*). Using an atherosclerosis-susceptible strain of mice, Lawrence Rudel and colleagues created ACAT2 knockouts. These mice showed a complete lack of atherosclerosis and a total cholesterol level 2.5 times lower than that of control mice. Whether this study will lead to the use of ACAT2 as a drug target in humans, who also contain the enzyme, remains to be seen. Similarly, knockout mice have been used to study the physiology of a wide variety of diseases, including many cancers, diabetes, and even narcolepsy.

Therapeutics. Many companies and institutions are banking on the potential benefits of knockout mice for drug discovery (see box, "Some knockout sources"). For example, Lexicon Genetics, Inc. (*www.lexgen.com*), uses patented gene-targeting technologies to alter specific DNA in mouse embryonic stem cells, which are then used to create their knockout mice. And DeltaOne knockout lines from Deltagen (*www.deltagen.com*) have targeted more than 500 genes. Recent studies have shown that knockout mice can be used to investigate the targeting of the 100 best-selling drugs currently on the market (*3*). According to Arthur T. Sands, president and CEO of Lexicon, "The top 100 selling drugs in 2001 are directed only to 29 drug targets. . . . Of these 29 targets, 23 have been knocked out, and in every case, the knockout mouse was highly predictive as to the on-target effects and side effects of the associated drug" (*www.currentdrugdiscovery.com*, August 2002, p 21). This facility of study has led most companies to work at developing knockouts to some of the most important drug targets, including the G-protein coupled receptors, ion channels, kinases, phosphatases, and proteases.

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Human cell knockouts

Since we are humans and not mice, there are genes that are not common to both species, many of which are important to disease formation. For this reason, it was important to adapt the knockout technology to *Homo sapiens*, or at least to our cell lines. Human cell knockouts have recently become available from a variety of sources. For example, PanGenex, a subsidiary of Cell Therapeutics, Inc., presented information on its gene knockout and transcription reporter technologies in a poster session at the International Conference on Molecular Cancer Targets in 2002 (*www.ctic seattle.com/investors_news.htm*).

In 2003, the laboratory that first produced human embryonic stem cells in the 1990s reported a significant new development in a method for producing human embryonic stem cell knockouts. Thomas P. Zwaka and James A. Thomson of the University of Wisconsin–Madison Medical School used

Knockout punched

It should be no surprise that knockout technology is a touchstone for animal rights activism and bioethics. This is especially true in the United States. Strong biomedical and agricultural lobbying kept laboratory mice in a gray category of regulation through the U.S. Department of Agriculture's successful campaign to keep them, as well as laboratory rats and birds, off the rolls of "experimental animals", in government parlance. Anything that stimulates increased use of animals for research or production purposes is seen as suspect. The rapidly expanding availability of such animals as displayed in marketing efforts that often seem to treat them like any other "reagent" has led to increased scrutiny. For example, according to Donald Bruce of the Society, Religion and Technology Project, Church of Scotland, "Knockout mice are ethically controversial, and the Church of Scotland has raised questions whether this has become too automatic a procedure because mice are still animals, not just items in a laboratory catalogue" (www.srtp.org.uk/clonin71.htm).

Beyond the issues of added animal exploitation (already at the heart of animal experimentation debates) and the morality of genetically engineering animals (which many see as an affront to a vision of "natural law"), knockout technology for making immune-competent organs in pigs for xenotransplantation has additional safety and bioethical implications, from fears of xenoviruses entering the human pathogen chain to religious objections against the combination of human and animal parts.

electroporation to force entry of knockout cassettes into human embryonic stem cells, allowing efficient homologous recombination similar to what occurs in mouse stem cells. Methods used with mice have proved less than optimal for the much larger human cells. In addition, unlike murine cell lines, viable human cell colonies do not easily arise from single cells (*4*).

Significantly, Zwaka and Thomson chose *HPRT1* as their target gene for proof of concept. In humans (but not in mice), deletion or dysfunction of the enzyme produced by *HPRT1* leads to a severe self-mutilation pathology known as Lesch-Nyhan syndrome. *HPRT1* is found on the human X chromosome; so in human XY cell lines, a single knockout event produces a "homozygous" culture. One of the benefits of choosing *HPRT1* is that the active gene product enzyme provides susceptibility to the compound 6-thioguanine, allowing it to become an added selection mechanism.

By varying the electroporation parameters, the researchers obtained transfection rates 100 times as high as those obtained with mouse cells, and by culturing the cells in clumps rather than singly, they were able to stimulate clonal growth of the transfected human embryonic stem cells. Selection was based on a dual gancyclovir and 6-TG resistance basis.

Although no one talks about producing knockout human individuals, the ability to produce knockout ES cell lines might prove valuable in developing clonal cell lines for a variety of therapeutic purposes, from gene therapy to organ regeneration. Not to mention the ability to study physiologically significant knockouts in cells capable of differentiating into many of the 220 cell types found in humans.

Not down for the count

Knockouts can never be the solution to all genetic analysis. For example, organisms that lack one or both functional copies of critical genes can fail to develop past the embryo stage. This outcome certainly demonstrates the importance of the gene product for development but severely limits the use of the knockouts in studying the function of the gene in whole organisms, especially as it relates to the development of human disease.

Perhaps even more significantly, the presence of a gene product can be just as important to causing disease as its absence—for example, in sickle cell anemia, an aberrant form of hemoglobin rather than a gene deletion causes the problem. And with knockout animals, there is always the possibility that multigene interactions with as-yet-unknown gene partners are not being addressed, especially if the model system does not contain the same gene partners as the human host.

For these reasons, researchers continue to use a variety of other genetic engineering techniques to study gene function. These include the use of standard transgenics that add, rather than delete, a gene or genes, providing positive expression of the protein of interest to allow researchers to determine its effects. But recently, it has become possible to "add" a knockout function positively rather than by deleting the gene. This significant spin on loss-of-function technology is the use of RNA interference (RNAi) to create a physiologically switchable means of inactivating genes that can be set to a chemical or environmental trigger.

A key benefit of RNAi technology is that it can knock out a function at a physiologically chosen time later in life without being constitutively expressed, thereby allowing the study of any number of life-cycle-dependent events and states such as disease. An interesting example of targeted gene activation using RNAi was developed recently for the nematode *Caenorhabditis elegans* (the most promising model of eukaryotic cellular development currently available). By feeding the worms bacteria genetically engineered to produce double-stranded RNA homologous to the genes the researchers wished to study, Julie Ahringer and colleagues demonstrated that they could inhibit the function of more than 85% of the 19,427 predicted genes in the *C. elegans* genome in a method nearly as elegant as the nematode's name (*5*).

But despite the development of these ancillary and alternative technologies, the utility of knockouts still holds such profound promise for turning genetic information into biological knowledge that it is likely to remain a core technique for disease analysis for the foreseeable future. And like a scene from *Willard*, or a musophobic's nightmare, knockout mice will continue to pour from the test tubes and breeding pens into drug discovery labs.

Some knockout sources

Deltagen
www.deltagen.com
Ingenotyping
www.ingenotyping.com
Lexicon
www.lexgen.com
Ozgene
www.ozgene.com
Taconic
www.taconic.com

A key benefit of RNAi technology is that it can knockout a function at a physiologically chosen time later in life without being constitutively expressed.

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