Scoring a Technical Knockout in Mice

Less is more, exult researchers, as mice with punched-out genes make their mark on fields such as immunology and developmental biology

Genetic engineering may be all the rage today, but back in 1980, when developmental geneticist Mario Capecchi submitted a grant proposal to the National Institutes of Health (NIH) suggesting an experiment to introduce foreign DNA into a specific place within a cell's chromosomes, he got a terse rejection: Forget the idea. The eminent scientists who had reviewed his proposal thought the experiment was impossible, recalls Capecchi, a Howard Hughes Medical Institute investigator at the University of Utah in Salt Lake City. Capecchi thought otherwise.

Hopeful that the evaluation was wrong, his group nervously diverted money from other grants to fund their speculative work. "We took a gamble," admits Capecchi, and it paid off. In 1984, with data to back up their ideas, the team submitted another grant request, expanding the scope of their earlier work. This time, NIH accepted the new proposal, and one comment from the grant reviewers read: "Glad you didn't follow our advice."

That sheepish note underscored that Capecchi's group, along with a few other teams such as one led by Oliver Smithies at the University of North Carolina in Chapel Hill, had laid the foundation for what has become an invaluable new research tool: the knockout mouse. Making use of a natural cell process known as homologous recombination, scientists can now almost routinely create strains of mice and other animals in which a selected gene is disrupted—"knocked-out" in the lab vernacular.

Gone is the kind of hit-or-miss random mutagenesis molecular geneticists previously depended on in their search for mutants that fit some particular bill. Want to study the importance of the immune regulator interleukin-2? Knock out the gene for it and watch what happens to the immune system. Need an animal model for a genetic disease like cystic fibrosis? Sucker-punch its gene—as a number of labs are now trying to do. Trying to find out how the homeobox genes control embryonic development? Disrupt one and see what happens to mouse embryos that lack it. "If you give me a gene, I could knock it out and tell you what its function is," says Capecchi, explaining the simple but powerful concept behind knockout research.

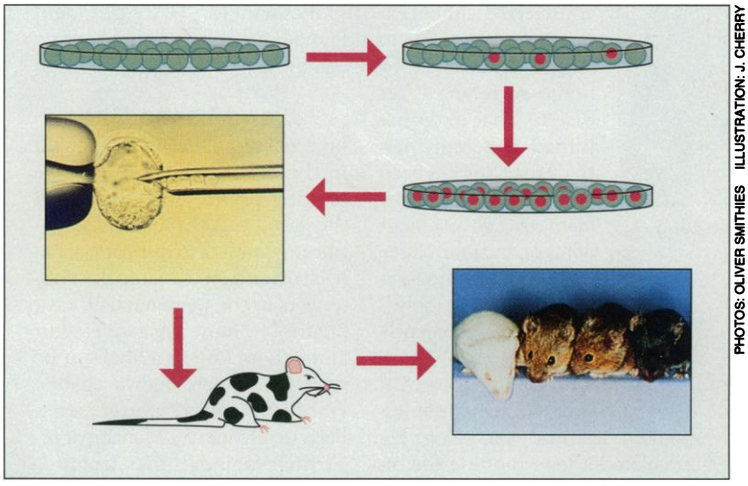
The fruits of this conceptual breakthrough have, in the last year or so, become increasingly obvious. Just a few years ago, data from the knockout mice Capecchi and others were producing entered the scientific literature in a trickle. But now the fields of immunology, cancer genetics, and developmental biology are blessed with a steady stream of results from these designer mutant mice. Indeed, the very success of the work has created a problem as the researchers grapple with how to make these popular animals available to others (see box).

Round One: The immune System

The field that has so far benefited the most from knockouts is immunology, where the technique is helping to clarify the functions of the myriad genes needed for the immune system's operations (*Science*, 24 April, p. 483). The reason is simple. When some genes are knocked out, the defects that result are so severe that the embryos die very early in development, before researchers can glean much useful information from them. But as molecular biologist Rudolf Jaenisch of the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts, explains: "The immune system is easy, because it's not essential for life." As long as mice are protected from infection, they can live with a knocked-out immune gene. Because of this, the immune system has been called the one "sure bet" for knockout research.

Take, for example, two groups that have recently seen their bets on knockout mice pay off handsomely. In the 6 March *Cell*, a team led by Frederick Alt of Columbia University's College of Physicians and Surgeons and another led by Susumu Tonegawa of the Howard Hughes Medical Institute at MIT and Virginia Papaioannou of Tufts University School of Medicine and Veterinary Medicine, report that they have knocked out the genes known as *RAG-1* and *RAG-2*, which make proteins needed for the assembly of the genes encoding the proteins used by mature B and T cells to recognize antigens and mount immune responses.

Both teams created their knockout mice using variations of the now standard approach pioneered by Capecchi and Smithies. The first step is to choose a cloned gene—here the *RAG-1* or *RAG-2* gene—and disable it. While other methods have emerged, most researchers do this by using recombinant DNA technology to insert an antibiotic resistance gene, known as *neo*, into the normal DNA sequence of the cloned gene, thereby disrupting it and preventing it from making an active protein product. The inactivated gene is then transferred into mouse embryonic stem cells, which are primitive cells capable of giving rise to all cell types during development. It's in these cells that homologous recombination takes place. The disrupted gene zeroes in on its functioning counterpart by matching nucleotide sequences. The two genes then either swap places, or the disrupted gene inserts itself into the cellular gene. Either way, the cellular gene is knocked out.



PHOTOS: OLIVER SMITHIES ILLUSTRATION: J. CHERRY

Route to a knockout. Researchers insert copies of a disrupted gene into mouse stem cells. The cells in which the transfer worked are identified and injected into mouse embryos, producing chimeric mice, which are bred to yield a strain in which all the cells contain the knocked-out gene (black mice).

Researchers next screen the pool of stem cells, looking for the ones that contain the inactivated gene. Those cells are then introduced into mouse embryos, where they and the natural stem cells develop together, producing chimeric mice that are a blend of both

Researchers Wrestle With Concerns Over Cost and Access

As novel knockout mice are produced with increasing frequency, delighted scientists are eager to get their hands on the animals to pursue their own particular interests, whether these be in immunology, developmental biology, or human genetic diseases (also see story on p. 1392). But beneath the excitement lurks a potentially troublesome issue: how to ensure that all the scientists who want access to these special mice get it.

Although the principle is not always honored, the general rule of thumb in science has been that data and materials are freely shared after publication. But knockout mice represent a major investment, both in time and money. One of the researchers who's making knockouts, cancer geneticist Tyler Jacks of the Massachusetts Institute of Technology (MIT), estimates that the laboratory bill for maintaining his animals will total $50,000 to $100,000 a year. And among those in the field, there is a growing concern that these financial and other considerations will make researchers reluctant to share the fruits of their labors.

"When someone feels they've put 2 years and lots of money [into creating a mouse], it's not easy to say, 'Here, take it—free of charge,'" explains Alexandra Joyner of Mount Sinai Hospital in Toronto, who has created a number of knockouts. "Next year, this will be an issue that comes to a head," she predicts, pointing out that many interesting mice, such as a strain containing a knockout of the cystic fibrosis gene, will be finished soon. "Not a lot of people are worrying about it yet, but it will become a problem," agrees MIT's Jacks.

Of course, a scientist could refuse to distribute the mice he or she has developed. This is not entirely unheard of, especially when requests come in from competing labs. But despite the difficulties that sharing their animals may entail, researchers working with knockout mice view the idea of prohibiting others from using them as a fundamental threat to good science. "I feel very strongly that these things should be shared and made available," says Joyner, echoing the feeling of all the investigators interviewed by *Science*.

But one method for making knockout mice available is already proving to be controversial. GenPharm International, a biotechnology company based in Mountain View, California, has entered into agreements with at least three universities to market their animals. One of these is the *p53* knockout recently made by Allan Bradley's team at Baylor College of Medicine in Houston. These cancer-prone, but otherwise healthy, mice should be in high demand by toxicologists and other researchers. As a result, says Jacks, the Baylor group faced a difficult question: "Do [they] want to spend a lot of money raising *p53* mice to give to other people?" The answer, the creators of that particular mouse decided, was no, and so they embarked on the agreement with GenPharm.

But GenPharm's marketing strategy has drawn a fierce reaction from some researchers. The company has put severe—and expensive—breeding restrictions on investigators buying its animals, says Derry Roopenian, a staff scientist at the Jackson Laboratory in Bar Harbor, Maine, a nonprofit, federally supported institution that raises and distributes special strains of mice and is a potential competitor of GenPharm on the knockouts. In effect, the restrictions mean that researchers cannot breed their own knockout colonies from the stock they purchase and must therefore pay the company for every mouse needed in an experiment.

Since GenPharm is charging from $80 to $120 a mouse—several times the $10 to $60 that the Jackson lab charges for a breeding pair of the mice it stocks—the cost of an experiment using knockouts can mount pretty quickly. "We can't afford to buy these mice," says Roopenian, calling the prices "unbelievably exorbitant." Joyner, who had not heard about the breeding restrictions until contacted by *Science*, called them "atrocious." If true, she said, "it's going to make life a real mess."

GenPharm's president, Jonathan MacQuitty, however, vigorously defends his company's practices. "We're taking very substantial losses in this area. People severely underestimate the difficulties of breeding these special mice," MacQuitty says, adding that knock out mice need special care because of their deficiencies. Nor is GenPharm unique, he says, in the way it markets genetically engineered mice. And while sympathizing with concerns over cost, MacQuitty says the high rates for mice arise, in large part, from the fees and royalties GenPharm must pay to the universities.

Some knockout researchers want to set up a more equitable means of distributing their animals. "We've been slow as a community to get our act together," says Harold Varmus, a Nobel Prize–winning virologist at the University of California, San Francisco. He hopes to help remedy that by holding an informal session to address the concerns over access to knockout mice at Cold Spring Harbor Laboratory's meeting on Mouse Molecular Genetics in August. Varmus himself suggests, for example, that a national repository be created for knockout and transgenic mice. Such a place, he says, could maintain frozen embryos for a wide variety of mice and guarantee their quality as well.

But that option may not be necessary, if the Jackson Laboratory is willing to take on the burden. Already considered the preeminent supplier of mouse mutants in the nation, Jackson Lab is the natural choice for a knockout library, agree most researchers. "If Jackson would take them, we would give them," says Joyner. And early indications are that Jackson will be open for business. It has recently accepted one knockout and is actively looking for more. The lab may be unable to take all knockout mice, says Roopenian, but "it's committed to making the important stocks available." And that should be good news for fans of knockout mice, although perhaps not for GenPharm.

–J.T.

normal cells and cells with the knocked-out gene. The chimeras are readily identifiable by their variegated coats, because the stem cells chosen for modification come from dark mice and they are put into embryos of mice with lighter coats.

At this point, an important step remains. To produce mice in which all the cells contain the knocked-out gene, the chimeras have to be bred in the hopes that the altered gene has entered the germline where it can be transmitted to the animals' progeny. Since getting the gene into the germline is strictly a matter of chance, a great many chimeras may have to be created and bred before the researcher strikes gold.

By using this technique to knock out the *RAG-1* and *RAG-2* genes, for example, the Alt and Tonegawa-Papaioannou groups confirmed the genes' importance in the immune system. When either gene was disrupted, the resulting mice were severely immunodeficient because most of their lymphocytes were immature cells unable to play an active role in the immune response.

Researchers have also successfully punched out the genes for soluble immune regulators, including several interleukins, as well as for some of the crucial proteins that mark the surface of various T cells and help mediate the immune cell interactions needed for initiating immune responses. On page 1448, for example, Karen Philpott of the Imperial Cancer Research Fund in London and her colleagues report that they have knocked out a gene needed to make one of two major receptors used by T cells in vertebrates to recognize antigens. "Sorting out which T cells do what has been an enormous

headache," says coauthor Adrian Hayday of Yale University, and these mice should help clear up the confusion.

Round Two: Developmental Biology

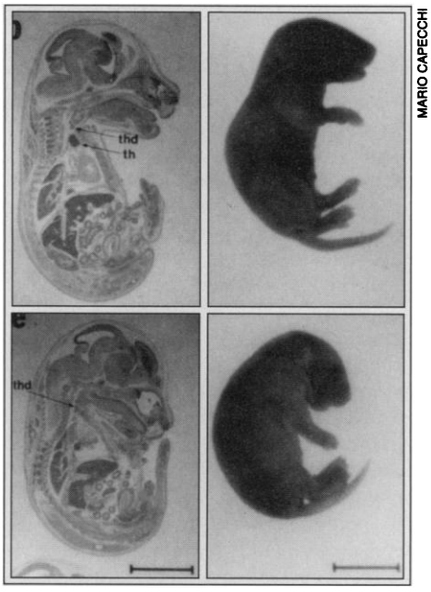
As the immune system begins to yield some of its secrets to knockout mice, developmental biologists are also turning to these mutant animals to help solve another fundamental problem in biology: how genes control early development. "For mammalian developmental genetics, this has really opened up the field. It's going to be used all over the place," says Alexandra Joyner of Mount Sinai Hospital in Toronto, whose own group just used a knock out to show that a gene called *N-myc* helps control the shaping of lungs in the embryo.

By far the biggest knockout effort in developmental biology focuses on the homeobox genes, so called because they contain an evolutionarily conserved sequence known as the homeobox. These genes, which were identified a few years ago as key players in the embryonic development of fruit flies, are also active in higher animals, including mice and other mammals. But before knockouts became available, researchers had to rely on indirect clues, such as where the gene was expressed during embryogenesis, as they sought to tease out the role of the homeobox genes in mouse development.

The emerging results from homeobox knockouts have been dramatic. In mice, as in fruit flies, the homeobox genes help specify body structures. Indeed, the majority of the homeobox knockouts die at birth, or soon after, due to structural defects that have occurred during the mouse's early development. Last year, for example, Capecchi and his colleague Osamu Chisaka found that disrupting the homeobox gene *hox-1.5* causes major abnormalities such as defects in the heart and arteries, a significantly reduced thyroid, and the absence of a thymus. In many cases, the throat area was so altered that the mice apparently "breathed" air into their stomachs and intestines.

In other knockouts, however, genes that were expected to play a crucial role in development did not. A prime example is a nonhomeobox gene known as *p53*, which is thought to be a key regulator of cell division. Because *p53* gene defects have been linked to the development of several types of cancer, including colon, lung, and breast cancer, it has drawn great interest from cancer researchers as well as from developmental biologists. They had placed such great importance on the gene that few thought that mice in which the *p53* gene had been knocked out would ever make it past birth. But, in what Allan Bradley, whose group at the Baylor College of Medicine in Houston created *p53* knockout mice, calls "a major surprise," these animals did more than survive birth: They came out normal-looking and apparently healthy.

While the researchers were not completely off base about *p53*'s function—the mice turned out to be extremely cancer-prone, with many developing tumors by the end of 6 months—this result highlights an important debate that knock outs have raised to the forefront: How redundant is the genome? Because some gene knockouts can result in dramatic changes while others do little or nothing at all, many molecular geneticists now argue that some genes are able to pick up the slack for their absent colleagues. "The big surprise to date is that so many individual genes, each of which had been thought important, have been found to be nonessential for development," says Robert Weinberg of the Whitehead Institute. Others dispute this view, however. "I don't believe in complete redundancy," says Capecchi. "If we knock out a gene and don't see something, we're not looking correctly."



MARIO CAPECCHI

Developmental woes. Abnormalities *(bottom in each pair)* caused by knocking out the homeobox gene *hox-1.5*.

Round Three: Genetic Disorders

So far, knockout technology's major contribution has been in basic research, helping researchers decipher the functions of individual genes, but it is beginning to open up a whole new avenue of more applied research: the creation of animal models of human genetic diseases. Cystic fibrosis, sickle-cell anemia, beta thalassemia, and Gaucher's disease are just a few of the genetic disorders that researchers are trying to recreate in mice with knockout technology. Even diseases that appear to involve contributions by multiple genes, like atherosclerosis and Alzheimer's, may reveal their secrets by combining two or more knockouts in the same animal, suggests Smithies. For instance, a group led by Nobuyo Maeda, who is Smithies' wife as well as his colleague at Chapel Hill, has two papers in press in the *Proceedings of the National Academy of Sciences* in which the researchers describe two new strains of knockout mice, each lacking a different gene needed for the body's normal handling of lipids. By combining these with knockouts of other genes involved in lipid metabolism, the researchers hope to gain a better genetic understanding of how atherosclerosis develops.

Although few complete models of human genetic diseases have yet emerged from the knockout work, the ones that do will be vital for the testing of new drug treatments and gene therapies (*Science*, 8 May, p. 772). In addition to testing potential new cures, such models should help improve the basic understanding of genetic disorders, since mice can be observed even before the onset of a disease, whereas humans are typically diagnosed later, after symptoms appear. "The type of analysis you can do in mice is vastly more in depth than we can ever have in humans," explains Capecchi.

But as valuable as knockout technology is proving to be, the researchers have encountered some problems. For one thing, for reasons that are only slowly being understood, certain genes are more reluctant than others to exchange places in stem cells. For another, the rate at which modified stem cells are successfully transferred into mouse embryos is low, although the chances of a "take" are improving as more efficient stem cell lines continue to be identified. Combine these obstacles with the need to maintain large populations of mice over a few years and knock out work can be both tedious and expensive. "It's time-consuming," says Jacks, "and that can be frustrating because you don't learn anything until you create a mouse. You have to be in it for the long haul."

But even if researchers are ready to face the technical, monetary, and temporal hurdles in pursuit of knockouts, there's a final trap awaiting them. It may take a year or two to create a particular knockout strain, but it can take even longer to understand the pathology of the mice. "The hard part now is to effectively analyze the mutant phenotypes," says Joyner. And the ultimate frustration, of course, is knocking out a gene and seeing no change at all.

Despite the problems, however, researchers are confident that knockouts will become easier. Experienced labs can now make a new knockout in less than a year. And some are even poised to take the next step: They are trying to do "cleaner" gene exchanges, sometimes called the "hit-and-run" method, in which the neo gene is not present in the final gene in the modified stem cells. This would open the way to recreating specific mutations in mice, not just knocking out a gene by disrupting its sequence. Meanwhile, most knock out researchers are ecstatic about the control they already have over the mouse genome. "It's opened up a completely new set of capabilities for understanding mammalian development and gene function," says Smithies.

–John Travis