Rudolf Jaenisch made the first transgenic mouse in 1974 and has pioneered the engineering of CRISPR mice.

MICE MADE EASY

The genome-editing tool CRISPR upends the vital business of creating mutant mice

By **Jon Cohen**

In the beginning of 2013, Michael Wiles sat down with high-level managers of the Jackson Laboratory in Bar Harbor, Maine, and told them about a novel way to cut DNA that had amazing power. The lab, called JAX for short, genetically engineers mice that it sells to researchers under a trademarked brand: JAX® Mice, it likes to boast, "are the highest quality and most-published mouse models in the world." Wiles evaluates and develops technologies for the lab, and he was convinced that this new tool, ingeniously adapted from an immune strategy that bacteria and archaea use to protect themselves from viruses, would revolutionize the way JAX engineered mice. "Of about a dozen people, nine were asleep," Wiles says. "No one had heard of CRISPR."

Now, most every mouse developer has. JAX and other labs making new mouse strains have long relied on a laborious multistep process that involves genetically altering mouse embryonic stem (ES) cells, injecting them into an embryo, and breeding multiple generations of animals. Even JAX's crack team took up to 2 years to engineer a mouse. CRISPR replaces all that with a molecular complex that can do targeted genetic surgery on a fertilized egg. It can produce a strain of transformed mice in 6 months. "It's night and day," Wiles says. "We had five or six people working with ES cells. They were close friends of mine and I said, 'You better look for a job.'"

Mice genetically modified to cripple or "knock out" genes or to add or "knock in" genetic information have become key research models for a wide array of human diseases, from cancer and atherosclerosis to Alzheimer's, osteoarthritis, muscular dystrophy, and Parkinson's. Knockout and knockin mice also offer a powerful tool for probing the functions of specific genes.

Most investigators get their engineered mice from colleagues or by purchasing them from commercial outfits like JAX or academic-based repositories. Popular engineered mice, such as JAX's immunodeficient NOD scid gamma strains, sell for as little as a few hundred dollars, but a custommade mutant could cost as much as $20,000. By making the engineering of mice far simpler and cheaper, CRISPR opens the way for more labs to do it themselves. "When you made knockout mice before, you needed some skills," says Rudolf Jaenisch at the Massachusetts Institute of Technology (MIT) in Cambridge. "Now, you don't need them anymore. Any idiot can do it."

If CRISPR's talent with rodents is shaking up individual labs, it is causing an earthquake in an international consortium to knock out all 21,000 mouse genes, one by one, in order to reveal their functions. The consortium, which includes JAX, has spent $350 million to date and is about a quarter of the way to its goal. Many investigators hope the speed and cost savings of CRISPR will accelerate progress. The National Institutes of Health (NIH), for one, is so impressed with CRISPR's ease and power that it no longer funds consortium investigators to use ES cells.

> ***"It's really changed the time and efficiency of getting these engineered animals."***
> **Tak Mak**, University of Toronto

But that's where some mouse engineers have second thoughts about the rush to CRISPR. Few doubt its potential, but the technique is still a work in progress, and its ability to alter genomes has one big gap. Although CRISPR knocks out genes with ease, it is less efficient at inserting, or knocking in, new DNA. That's important not just for giving an animal a novel function, but also for creating a knockin known as a "conditional" knockout, an animal model in which researchers can turn off a target gene at specific times of life or in specific tissues.

Because CRISPR is less adept at making conditional knockouts, William Skarnes, who led a team making mutant mouse ES cells at the Wellcome Trust Sanger Institute in Hinxton, U.K., worries that NIH is overemphasizing the new approach. "The decision to abandon the ES resource in favor of making simple knockouts is a mistake," Skarnes says. "You still want to make conditionals through the ES route."

CRISPR researchers are now refining the technique to do knockins with greater efficiency. But that entails tinkering with the mechanisms that cells use to repair broken DNA, which are critical to their health. "I'm cautious about overmanipulation of biology to increase efficiency," says Steve Murray, who helps run JAX's contribution to the Knockout Mouse Phenotyping Program (KOMP2), which is part of the international consortium. "We're waiting in the wings for the wizards in the field to help us with this."

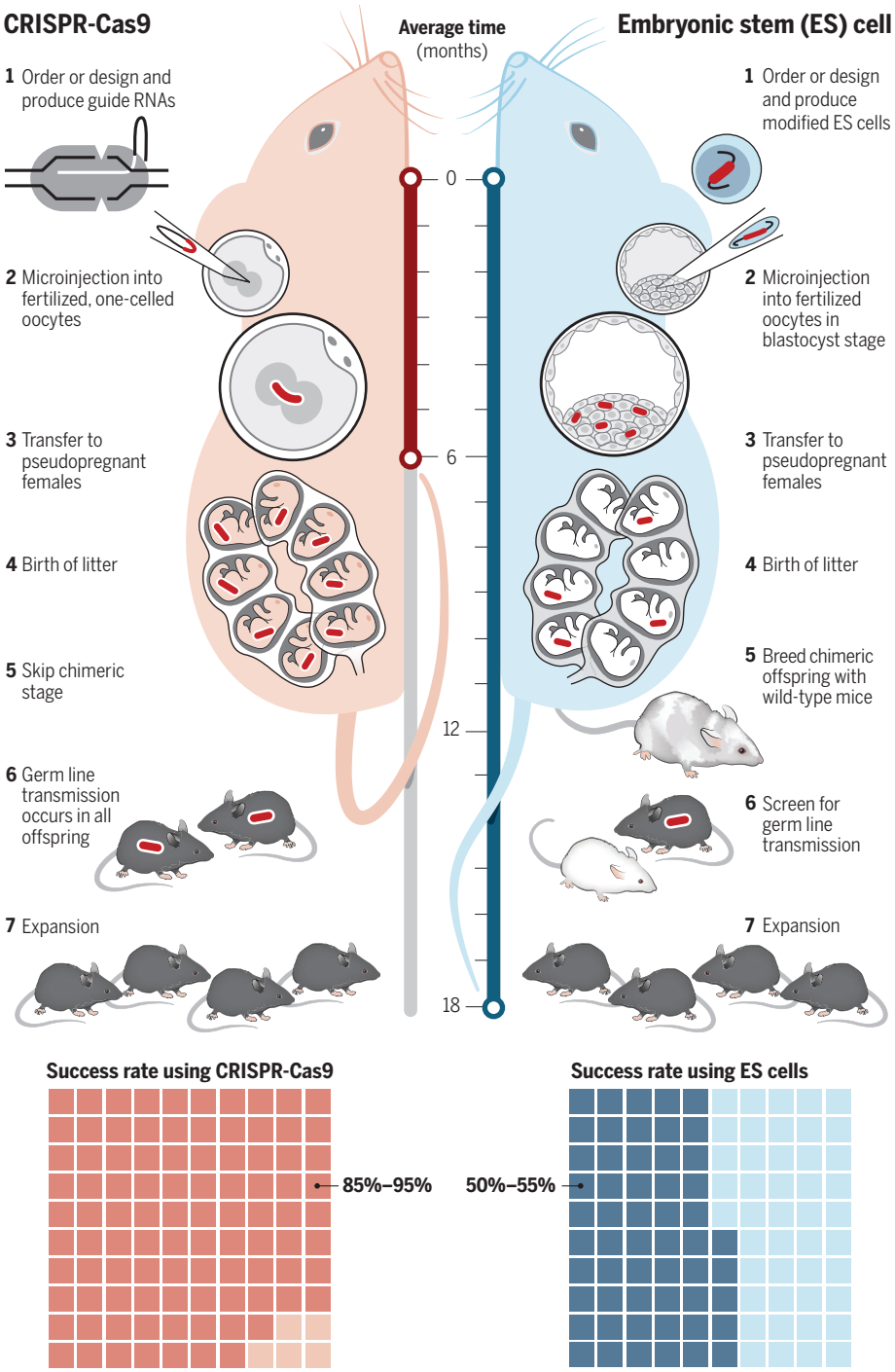
CRISPR STANDS FOR "clustered regularly interspaced short palindromic repeats," which is a description of the prokaryotic genetic material from which it was derived. It uses what's called a guide RNA to send biological scissors—usually the CRISPR-associated protein, Cas9—to a precise spot in a genome. Once Cas9 enzymatically makes the cut, the cell tries to heal the wounded DNA. One repair mechanism leads to knockouts, whereas the second leads to knockins. "All CRISPR does is cut the DNA," Wiles says. "Everything else is the cell repair system, and that's what we're hitching on to."

The cell's standard response is to try to paste the double-stranded DNA back together at the break points. This often requires eating away or adding a few bases—the As, Cs, Ts, and Gs that make up DNA—which leads to insertions or dele-

PHOTO: (OPPOSITE PAGE) KEN RICHARDSON

See how CRISPR runs

Using the genome-engineering tool to alter a fertilized egg is a quicker and more efficient way to engineer a mouse than the traditional route, which starts by modifying an embryonic stem cell.



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tions. In effect, the repair effort introduces typos into the DNA text, disabling the gene.

MIT's Jaenisch was the first to show the power of CRISPR for producing mouse knockouts. In a 2 May 2013 paper in *Cell* that appeared 5 months after researchers first showed CRISPR could work in mammalian cells, he and co-workers reported that the technique successfully disrupted five genes in a single set of mouse ES cells, something that was not possible before. More important, they showed that they could bypass ES cells altogether and simultaneously knock out two genes in single-celled mouse zygotes, or fertilized eggs. No longer would researchers have to modify ES cells and painstakingly breed several generations of mice to produce an animal that carried the mutant gene in its egg or sperm cells. And researchers who wanted mice with two mutations would no longer have to interbreed single mutants and go through a similarly time-consuming, cumbersome process to arrive at progeny with the altered germ line. As the title of Jaenisch's paper declared triumphantly, "One-step generation of mice carrying mutations in multiple genes."

Since then, more than 500 papers have detailed how CRISPR can both knock out and knock in genes in mice. "The impact it's had is enormous," says Jaenisch, who in 1974 created the first transgenic mouse. "It's really changed the time and efficiency of getting these engineered animals," adds biochemist Tak Mak of the University of Toronto in Canada, who was also a pioneer in the mouse-mutating business. Mak estimates it's about 30% cheaper to engineer a mouse with CRISPR than with ES cells, bringing his average cost down to about $100,000.

CRISPR's impact is measured in more than savings. The ease and speed of the technique makes it possible to engineer mice on the fly, to solve specific puzzles like one that C. C. Hui of the Hospital for Sick Children in Toronto recently confronted: a knockout in which the missing gene didn't have any observable effect. Hui realized that the knocked-out gene was linked to another gene that might be compensating for it. He took the problem to Lauryl Nutter, who oversees mouse making at the Centre for Phenogenomics in Toronto. She used CRISPR to mutate the offending gene in a zygote from the original knockout. "We got the zygote, injected it with CRISPR-Cas9, and 8 weeks later he had a double mutant on the ground," Nutter says. "That would have taken years with ES cells."

The revolution is not limited to making mice with germline mutations. CRISPR has allowed investigators to mutate several suspected cancer genes simultaneously in the somatic cells of adult mice, for example. CRISPR knockins have also corrected disease-causing gene defects in adult mice, such as the mutations that cause hemophilia and sickle cell anemia. And several groups plan to inject CRISPR into a developing mouse; the goal is to create mutations that act as barcodes and allow scientists to track cell lineages as they differentiate.

Mousemaking outfits like the Centre for Phenogenomics and JAX expect that CRISPR will vastly expand the range of mutants they produce. "Now I can take a really exotic mouse that has three genetic modifications and modify it again," JAX's Wiles says. "We couldn't do sequential modification with ES cells. We could breed a mouse

with two modifications with another that had two modifications and the alleles scattered like the wind. It would take years to get all four modifications."

Wiles says this likely won't affect JAX's bottom line. "Instead of shipping thousands of boxes with one variety, we will have hundreds of boxes with tens of varieties."

ON ONE FRONT, however, the CRISPR revolution is faltering. Three months after his lab's first CRISPR report, Jaenisch and co-workers published a second paper in *Cell* that suggested CRISPR could easily perform more complex genetic surgery, knocking in chunks of DNA rather than simply disabling genes. As a demonstration, they used CRISPR to knock fluorescent tags into mouse zygotes, which lighted up whenever a specific gene was turned on. They also created conditional mutants, which are key to many research efforts, including the knockout consortium.

CRISPR works in every strain of mouse, whereas ES-cell technology is mostly limited to one inbred strain.

Conditionals get around a barrier to making knockouts. About one-third of mouse genes are essential for embryonic growth; the mouse is never born if they are disabled from the start. So researchers working with ES cells cleverly designed a system called Cre-Lox recombination that knocks out genes only after the mouse has developed enough to survive their loss. It requires adding extra DNA: *Lox* sequences flanking the targeted gene plus a *Cre* gene, which can be turned on to produce an enzyme that modifies the DNA between the *Lox* sites. Using CRISPR to insert this same system into zygotes, Jaenisch's team reported making conditional mice with relatively "high efficiency"—about 16% of the zygotes led to mouse pups with the correct mutations.

Skarnes is one of many researchers bowled over by Jaenisch's initial reports, but he was disappointed when he tried to take the technique into his own lab. "It looked from his papers that this was going to be straightforward and I was quite confident this would make ES obsolete," Skarnes says. "What was disappointing is none of us could reproduce at the efficiencies reported by Jaenisch. … It works at 1% or 2% at JAX and a lot of projects are failing. It's really not proven to be a robust method."

There are several reasons why a CRISPR cut more readily leads to a knockout than a knockin. To create knockins with CRISPR, researchers introduce stretches of "donor" DNA—anything from a few bases to an entire gene—designed to integrate at the break points created by Cas9. Splicing in the donor DNA requires that its ends match, or are "homologous" with, the damaged DNA. A process of homologous-directed repair (HDR) then stitches the ends together.

The knockout repair mechanism, which is called nonhomologous end joining, can happen at any stage in the cell division cycle and occurs quickly. HDR, in contrast, mainly happens in one phase of the cell cycle and is far slower. Some genes, such as those Jaenisch selected for his initial knockin experiments, are also more conducive to HDR than others. "The paper reported what we found," Jaenisch says. "Now, we see there are issues."

To others, CRISPR's limitations raise questions about the knockout mouse consortium's decision to abandon ES cell technology. Launched in 2003, the project has created a repository of mutant ES cells, most of them conditionals, for nearly 18,000 genes. Any researcher can order a cell line and spend a year or more making a needed knockout mouse. It has also bred 5011 mutant mouse strains that have germ line transmission of the knockout. This summer, as part of KOMP2, NIH decided to extend the tally of live knockouts to 8000, funding JAX, the University of California, Davis, and Baylor College of Medicine in Houston, Texas, to do the work. But it specified that the knockouts should be made using CRISPR alone.

Colin Fletcher, a mouse geneticist at NIH's National Human Genome Research Institute in Rockville, Maryland, who oversees KOMP2, says advisers endorsed the switch to CRISPR. "You can't cling to the old technology," Fletcher says. "A lot of people have abandoned the ES cell repository and, on the other hand, a lot of people have come in to the field because of the new technology. People are voting with their feet. People are putting much more effort into making conditional alleles with CRISPR rather than making ES cells."

Skarnes, who has just moved from Wellcome to the JAX genomic medicine branch in Farmington, Connecticut, calls the shift premature. But he concedes that researchers will "eventually" figure out how to tweak CRISPR so that it makes conditional mutant mice with high efficiency. One route is to block an enzyme crucial to nonhomologous end joining. Another is to enhance a protein critical to the HDR process that makes knockins possible. Still other investigators have toyed with lengthening the cell-cycle phase that is most favorable to that repair process, zapping zygotes with electric pulses to aid the entry of the CRISPR-Cas9 construct, and creating mutant Cas9s called "nickases" that only break a single DNA strand and preferentially induce HDR.

Whatever CRISPR's shortcomings appear to be at this point, Wiles emphasizes that its potential for engineering mice should not be underestimated. "There is a massive number of things CRISPR can do that people are just beginning to grasp," he says. "We're really at the very, very early phases of development and the tool has infinite possibilities." ■

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