



Sigma Aldrich and the Medical College of Wisconsin

THE KNOCKOUT RAT PACK

The rat is the preferred model organism in many fields of biomedicine, such as toxicology testing. But when it comes to disease models, rats have been upstaged by mice, which traditionally were far easier to genetically engineer. **Elie Dolgin** follows the tale of the researchers who are bringing the rat back into the race.

On a chilly late autumnal day in November 2008, Aron Geurts marched into a windowless basement animal facility to check up on his rats. There, he found five newborns in the litter, clustered together and barely a week old. Three of the newborn rats were glowing bright green, but it was the two normal-looking newborns that intrigued Geurts. One of his first thoughts, he recalls, was “where’s the champagne?!”

A month earlier, Geurts had viewed these same newborns under a microscope at the stage when they were still one-celled embryos carrying a single copy of the green fluorescent protein (*GFP*) gene. He injected the rat embryos with a special construct designed to home in on this gene and disrupt its DNA, and then implanted them into a surrogate mother.

Geurts, at the time a postdoc in the lab of Howard Jacob, a geneticist at the Medical

College of Wisconsin (MCW) in Milwaukee, clipped off a bit of the newborn rats’ tails and analyzed the DNA. The two nonglowing rats still carried the *GFP* gene, but the sequence had been cut up and put back together again in such a way that it was rendered nonfunctional. To his surprise, Geurts had successfully created the world’s first targeted knockout rat in which a specific gene was deliberately mutated. “This blew me away, because nothing in the previous published work suggested it would work that well,” says Geurts, who now heads his own MCW lab.

Scientists consider rats as better models than mice for a number of human conditions, including cardiovascular disease, diabetes and behavioral disorders. Almost every drug tested out by pharmaceutical companies eventually passes through the rat at some point in preclinical development; so there are decades of

pharmacological data against which to compare the effects of new compounds. And the rat’s larger size makes it easier to conduct detailed physiological measurements of organs. “In a lot of cases, the rat turns out to be a superior model,” says the University of Wisconsin–Madison’s Michael Gould, who created the first nontargeted knockout rats with a toxic chemical that indiscriminately mutated the genome at random¹.

For example, two years ago, Gould and his colleague William Dove generated a rat knockout of the colon cancer–associated gene encoding adenomatous polyposis coli protein (*Apc*). Whereas mouse *Apc* knockouts mostly develop tumors in the small intestine, the rats develop colon cancer in the same place that humans suffering from familial colon cancer do².

Because of such biological and experimental

differences between the two rodents, researchers have long sought to disrupt the rat genome in a more systematic manner. Put simply, disrupting genes in knockouts would shed light on their function in regular rats—and, by extension, in humans. But a number of technological obstacles have made it very difficult to target specific genes in rats. By comparison, researchers have been swapping genes in and out of mice ever since researchers first discovered mouse embryonic stem (ES) cells in the 1980s.

Now, all of that has changed. Researchers are using Geurts's approach, which relies on 'zinc finger' molecules, as well as on manipulating stem cells with an eye to making targeted rat knockouts. Others are concerned less with disrupting specific genes and are instead attempting to create genome-wide transgenic rat libraries with the help of mutagenic chemicals and jumping genes. And all of this has become possible only because of recent technological innovations.

"New technology is rapidly filling the gap between what's been happening in the mouse for decades," says Edwin Cuppen, a rat geneticist at the Hubrecht Institute in the Netherlands. "The rat is catching up."

Finger on the pulse

To create the rat newborns that did not glow, Geurts used a new approach with constructs known as zinc finger nucleases (ZFNs). These ZFNs are generated by combining chains of amino acids that bind specific gene sequences with an enzyme that cleaves DNA. The particular three-dimensional conformation of a ZFN, which gets its name from the zinc that stabilizes the amino acid chains and the finger-like appearance of these loops, determines which sequence of DNA it will bind. Crucially, ZFNs can be engineered to bind any unique sequence in the genome, and, in this way, these constructs can be used to precisely alter the genetic blueprint of any higher organism, including the rat.

After struggling for decades to develop rat models of disease, in the past year alone various teams have used zinc fingers to switch off rat genes involved in cancer, drug transport, immunity and neurodegenerative diseases. "We've really gone in a year from rags to riches," says Jacob.

Jacob's lab first became involved in the zinc finger approach in January 2008 when Fyodor Urnov, a scientist at Richmond, California-based Sangamo Biosciences, approached him with a proposition—he wanted Jacob to try Sangamo's experimental knockout technology in rats. Sangamo had recently licensed its ZFN system to Sigma-Aldrich, a biotech out

of St. Louis, Missouri, and because it worked so well at targeting genes in mammalian cell cultures, the companies planned to give it a go in live animals. Mice might have seemed like the obvious choice, but targeting genes in mice was old hat, and Sigma wanted their unproven platform to open brand new research possibilities.

At the time, Sigma didn't have the appropriate animal facilities to handle live rats, so "the collaboration seemed like a natural one," says Edward Weinstein, director of Sigma Advanced Genetic Engineering (SAGE) labs. "We wanted to work with Howard [Jacob] because he's a leader in the rat field and he's been thinking about the genetic basis behind modified rats longer than anyone."

Jacob and Geurts set to work on the project and managed to breed live targeted knockouts within about three months. "It was insane," says Geurts. "I probably spent more time just trying to develop assays than I did trying to get the technology to work." Geurts had expected the zinc fingers to succeed, but not nearly as efficiently as they did. "Luckily, I was very wrong," he says.

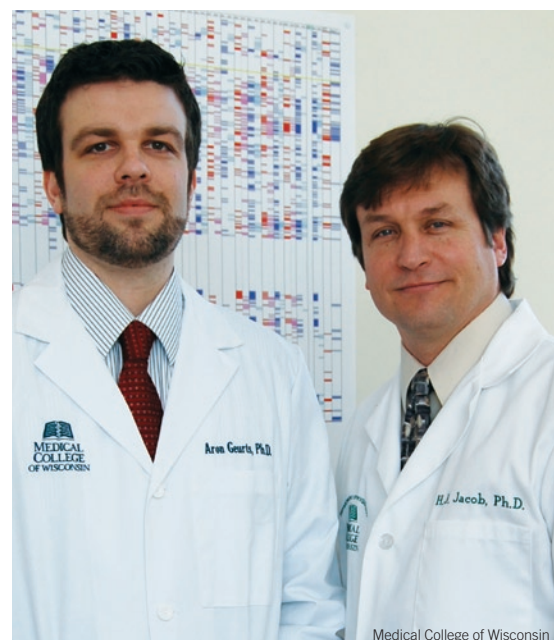
In September, a team led by Jacob and Geurts landed a \$6.6 million Grand Opportunity grant from the US National Heart, Lung and Blood Institute to create 100 rat models of hypertension and kidney disease with zinc fingers. Their strategy now is to disrupt genes implicated in human disease by genome-wide association studies and then test whether the knockout rat strains have altered blood pressure or renal function. So far, they have knocked out more than 25 genes, including the rat version of the gene encoding renin, an enzyme that helps stabilize blood volume.

Incredibly, in one week alone, the researchers even managed to knock out a record six genes. "That was a fun week," recalls Geurts.

Antibodies and beyond

At the same time that the MCW researchers were testing their ZFNs, Roland Buelow was also tinkering in rat cells with this technology. Buelow is founder and chief executive of Open Monoclonal Technology in Palo Alto, California, a company aiming to create transgenic rats with a human antibody repertoire, and he had arranged with Sigma and Sangamo to try the technology to disrupt the gene encoding a rat protein called immunoglobulin M (IgM), which is a large antibody that fights pathogens in the bloodstream.

All told, Buelow and his team injected close to 2,800 rats with varying concentrations of



On target: Aron Geurts and Howard Jacob.

the ZFNs into both the early nuclei and the cytoplasm of rat embryos, and, to Buelow's amazement, every technique succeeded in generating knockouts with completely inactivated rat antibody expression.

Buelow and the MCW researchers decided to team up to publish their results. Between the two groups, the researchers had managed to inject and breed 295 live rat pups, 35 of which harbored targeted mutations in three different genes—those encoding IgM, GFP and Rab38,

which is known to influence intercellular transport in hypertensive rats—as they reported last July³. "That [paper] changed the whole knockout field," says Birger Voigt, a rat geneticist at

Japan's Kyoto University.

With zinc fingers, researchers can now get targeted knockout rats in any strain in as little as four months. In contrast, knockout mice created by the traditional tack of introducing strings of DNA with the desired sequence into ES cells take more than a year to make. Plus, knockouts done with ES cells can only be created in the same strain as the rat used to derive the stem cells.

Since their paper, Buelow's team has bred its IgM-knockout rat with transgenic rats containing human immunoglobins. Knocking out the rat version of this protein was crucial for him, because his ultimate aim is to manufacture new human monoclonal antibodies in the rat for therapeutic use in people. Disabling the rats' IgM ensures that the rodents produce only the human version of these antibodies. The MCW

"The rat turns out to be a superior model."

—Michael Gould



Chang-Tong, University of Southern California

Runs in the family: A chimeric rat (right, agouti coat) and his stem cell–derived son (left, brown). The chimera only had one deficient copy of the gene for p53, and the son inherited the normal version.

researchers, meanwhile, keep plugging away at their list of 100 hypertension and kidney disease genes.

Other researchers have even started thinking beyond rodents. For example, Scott Fahrenkrug of the University of Minnesota–Twin Cities is testing out the ZFN technology in pigs, whereas Josef Platzer, scientific director of Roche Diagnostics in Penzberg, Germany, is taking a similar approach in rabbits. Using zinc fingers in rabbits “worked right away,” Platzer says of his unpublished results. “It was very simple compared to doing normal transgenesis.”

“The darn thing works so well, it’s like, ‘Geez, why not try it in everything?’” says Jacob.

Embryonic hopes

In December 2008, seven months before Jacob, Geurts and Buelow announced their knockout rats created with ZFNs, researchers led by Austin Smith of the University of Cambridge, UK, and Qi-Long Ying of the University of Southern California in Los Angeles reported another breakthrough for the rat.

For about three decades, scientists have manipulated mouse ES cells to create knockout mice by a technique called homologous recombination, in which a desired DNA sequence is swapped into the cells and thereby disrupts a selected gene. The ES cells are then injected into developing embryos, which grow up into mice with tissue from two cell sources. If the sperm and egg cells in these chimeric mice come from the manipulated stem cells, then you can breed

two siblings with each other to get a *bona fide* knockout.

For decades, however, this technique has failed to work in rats because no one has been able to manage to derive ES cells capable of forming rat chimeras. The problem: researchers were stuck on using the same cell culture medium that they had long relied on to obtain mouse ES cells.

By eliminating a cytokine called leukemia inhibitory factor and further tinkering with the medium, Smith and Ying obtained the first true rat ES cells that passed all of the tests of pluripotency, meaning the cells could be coaxed into becoming any type of cell in the body^{4,5}. When these cultured stem cells from one rat embryo were added to another developing embryo, it created a chimeric rat.

Ying has since used homologous recombination to knock out the tumor suppressor protein p53 in his stem cells. By injecting those cells into multicell embryos, he created 20 chimeras, but none of these rats has given birth to p53-deficient offspring.

In part, it seems that many of Ying’s ES cells had unusually high numbers of chromosomes, because he was selecting cells that attached to the substrate of the lab dish, as most mouse biologists do. Now, by picking only free-floating cells, he is finding that his cell lines are much more robust and genetically stable, which means they should have a better chance of contributing to the gonads of a chimera. Starting over, Ying

knocked out the gene encoding p53 in new ES cells, which he injected into developing embryos just last month. “This time we have a very big chance that we’ll make chimeras, and I think we’ll get germline transmission,” Ying says. And, if he succeeds, it should open the door to more sophisticated rat disease models, because multiple genetic manipulations should prove easier in a cell-based system than using ZFNs in live embryos, he adds.

Last year, two research groups also reprogrammed adult rat cells to create induced pluripotent stem (iPS) cells^{6,7}. Neither group has managed to make a knockout, but the team led by Sheng Ding of the Scripps Research Institute in La Jolla, California did generate chimeras from the reprogrammed cells. “The culture conditions are not stable enough to do long-term genetic manipulation in the rat iPS or rat ES cells,” says Ding, who is investigating better cell media than the one discovered by Ying’s team.

Although the use of homologous recombination in stem cells currently lags behind the use of zinc fingers for creating knockout rats, it might allow researchers to reliably introduce genes into specific locations in the genome to create what’s known as a knock-in rat. In fact, Ying has already introduced a *GFP* gene into ES cells at the *Rosa26* locus, a preferred site for genetic modification because it can be targeted with relative ease. He injected these cells into developing embryos in January, so it’ll be another month or so before he knows whether he has a true knock-in strain.

Although ZFNs have been used to knock-in genes in fruit flies, a mammal “is a very different beast,” says Gould. Silencing the DNA repair mechanism is necessary for knock-in technology to succeed. This works fine in cell cultures and flies, but mammalian embryos tend to die *in utero* if the repair system is shut off.

But Xiaoxia Cui, SAGE’s head of research, asserts that ZFNs will ultimately produce robust knock-in rats. Her team is still optimizing the protocols, but “we’re very optimistic about whether or not it’s going to work,” she says. “Our preliminary data looks very promising.”

Back to basics

Despite the excitement surrounding ZFNs, some researchers maintain that it might be cheaper to make rat disease models by mutating genes at random. They propose, for example, to use transposons, or ‘jumping genes,’ which insert themselves throughout the genome, disrupting genes all over the place. “At this point, the transposons could generate more mutants than these other technologies,” says Richard Behringer, a molecular geneticist at the MD Anderson Cancer Center in Houston.

“The darn thing works so well, it’s like, ‘Geez, why not try it in everything?’”

—Howard Jacob

Zsuzsanna Izsvák of the Max Delbrück Center for Molecular Medicine in Berlin and her colleagues have been using a transposon called sleeping beauty, which Izsvák discovered more than a decade ago⁸, to mutate rat sperm stem cells, which she then implants into the testes of sterile rats. So far, she has obtained dozens of knockouts, although she declined to name any specific genes. “Here you don’t need embryo manipulation, no chimeras, nothing, so it’s very, very simple,” she says.

What’s more, “the cost is incredibly cheap,” says Eric Ostertag, founder of Transposagen Biopharmaceuticals in Lexington, Kentucky. He estimates that his company could systematically knock out every gene in the genome with transposons for as little as \$100 per gene.

Transposons have the advantage that when they jump into a gene, they stay there to produce an easily identifiable tag, but they create only one type of mutation—insertions. If you want a point mutation that might more accurately reflect natural variation or disease, you need another technology. That’s why Cuppen is randomly mutating the sperm of male rats with a toxic chemical called *N*-ethyl-*N*-nitrosourea (ENU) and then sequencing hundreds of genes to find out which base pairs have changed. “It’s not about how you get there,” Cuppen says. “It’s what you have in the end.”

To search for genes that were successfully reached and disrupted by this chemical, Cuppen sequenced 250 genes coding for transmembrane proteins in 800 mutated rats. He found 12 genes that had been rendered completely nonfunctional. With the price of next-generation sequencing on the decline, Cuppen is now gearing up to sequence the entire coding portions of the genomes of 5,000 rats that he’s created after exposing sperm to ENU. Statistically, this should be sufficient to find knockouts for half of all rat genes. “It’s now the trick to scale from 300 to 30,000 genes,” he says. “These are not the things we can do nowadays, but they’re close by.”

Knockout markups

Researchers who want to get their hands on a knockout rat, but don’t want to go through the laborious process of making one themselves, now have a few options. Transposagen, which uses sleeping beauty or another transposon called piggyBac to genetically engineer rats, currently offers around 200 types of homozygous rat knockouts. Among these 200 varieties is the world’s first immune system-lacking severe combined immunodeficient rat, which the company made in collaboration with the MCW researchers. These rats cost about \$200 per rat for academic investigators

and come with a \$100 markup for commercial researchers.

Sigma, meanwhile, has started selling around a dozen types of zinc finger–derived knockouts. These go for \$300–400 each and cover a range of genes, including those encoding the proteins known as ‘disrupted in schizophrenia-1’ and ‘apolipoprotein E1’, which has a role in Alzheimer’s disease. Not every knockout is quite ready yet, though, because, after a pup with a ZFN-disrupted gene is born, it still takes another 22 weeks to breed a homozygous rat. Weinstein says that Sigma’s first two homozygous knockout strains were born in January—one for the gene encoding p53, the other for the gene encoding Mdr1a, a protein involved in drug transport through the blood-brain barrier.

Last summer, the Michael J. Fox Foundation (MJFF) in New York also gave Sigma a grant of more than \$230,000 to create five knockout rat models of Parkinson’s disease. “If these models can teach us anything about the biology underpinning any of the symptoms or pathological features, that just goes so much further to helping us determine disease mechanisms,” says Kirsten Carlson, MJFF’s associate director of research programs.

Sigma also sells custom-made ZFNs for \$35,000 to researchers with the capability to inject them on their own, or the company can do all the work and create an off-the-shelf knockout breeding pair for \$95,000. Gould opted to take the latter approach to obtain a rat missing a key gene implicated in breast cancer. “That’s good value for the money,” he says, because his lab is not set up to do targeted mutagenesis. “If we were only going after a single gene, it would probably cost us more going through the ENU approach.”

For many researchers, however, Sigma’s steep prices are a major deterrent. “I’ve talked to a couple of people and [they say the price is] very off-putting,” says Thom Saunders, director of the

University of Michigan–Ann Arbor’s Transgenic Animal Model Core. Jacob, who gets a bulk-rate discount for ordering so many ZFNs, scoffs at such criticisms. “Yes, it’s expensive,” he says. “But getting something that works and getting your animal in a few months is worth the cost!”

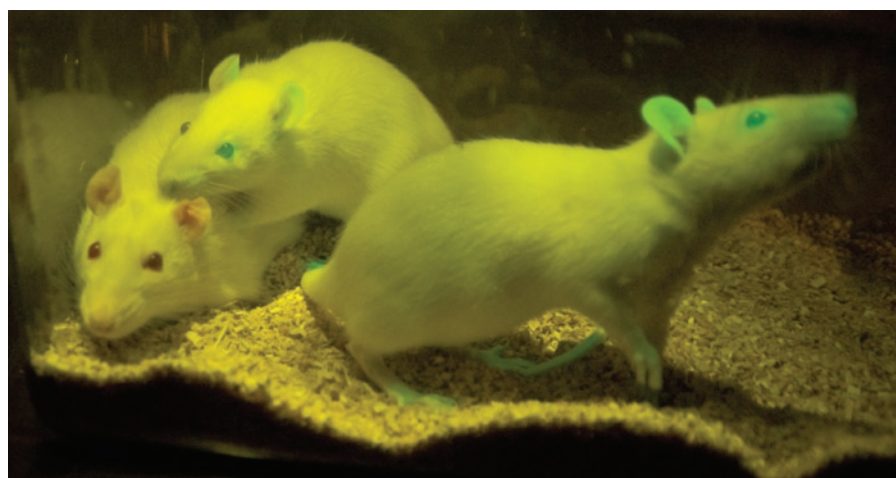
Even once stem cell technologies are up to scratch, ES- or iPS-derived knockouts will still cost in the same ballpark as ZFN knockouts, Saunders admits. But he and many others are quick to point out that with stem cells you can keep the intellectual property associated with your rat; with Sigma’s technology, the company retains the commercialization rights.

“A big problem is the major restrictions that Sigma puts on this technology, which make it almost impossible to use” for academic research, says Ron Korstanje, a research scientist at the Jackson Laboratory in Bar Harbor, Maine. For example, if researchers create their rats in house with the ZFNs, they can only breed 500 rats before having to apply to Sigma for an expanded license. If Sigma makes the knockout, researchers have unlimited breeding rights, but they cannot distribute the animals without a license, even to collaborators.

Nonetheless, the benefits of zinc fingers greatly outweigh the costs, argues Weinstein. ZFNs are quicker and more efficient and can be injected into any rat strain, he says. “We are really working with a technology that can change the face of research.”

Elie Dolgin is assistant news editor for Nature Medicine in New York.

1. Zan, Y. *et al. Nat. Biotech.* **21**, 645–651 (2003).
2. Amos-Landgraf, J.M. *et al. Proc. Natl. Acad. Sci. USA* **104**, 4036–4041 (2007).
3. Geurts, A.M. *et al. Science* **325**, 433 (2009).
4. Buehr, M. *et al., Cell* **135**, 1287–1298 (2008).
5. Li, P. *et al. Cell* **135**, 1299–1310 (2008).
6. Liao, J. *et al. Cell Stem Cell* **4**, 11–15 (2009).
7. Liu, H. *et al. Cell Stem Cell* **4**, 16–19 (2009).
8. Ivics, Z. *et al. Cell* **91**, 501–510 (1997).



She’s a knockout: The nonglowing rat (left) had her DNA disrupted

Sigma Aldrich and the Medical College of Wisconsin