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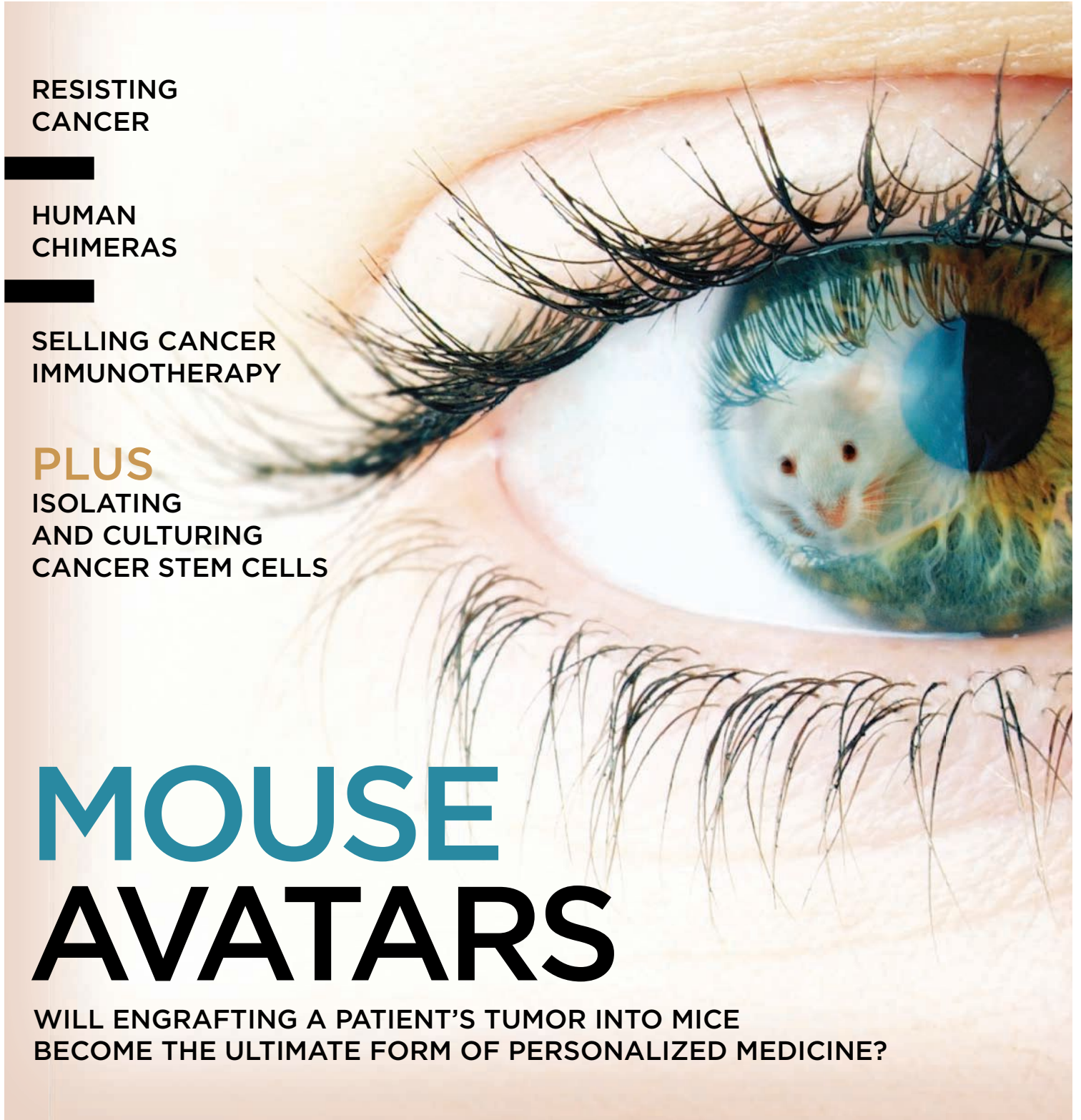
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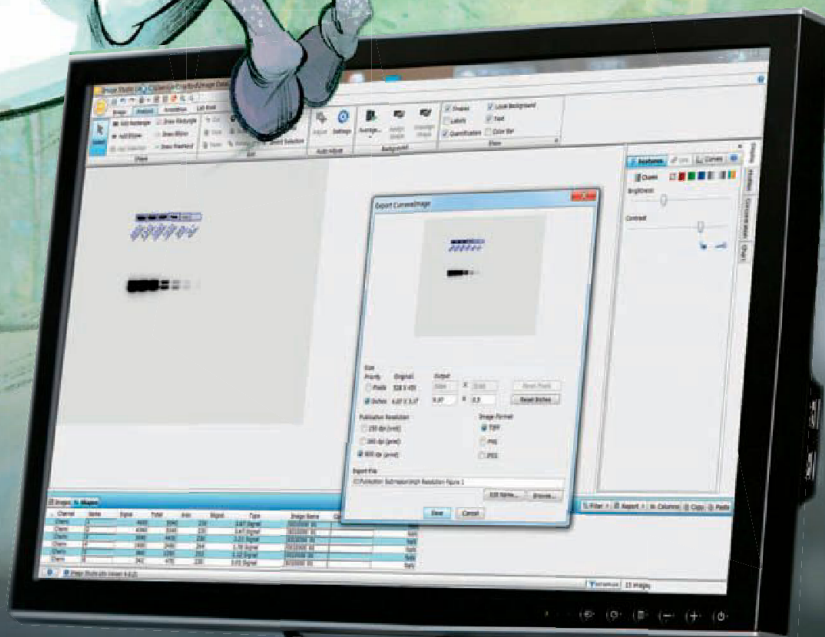


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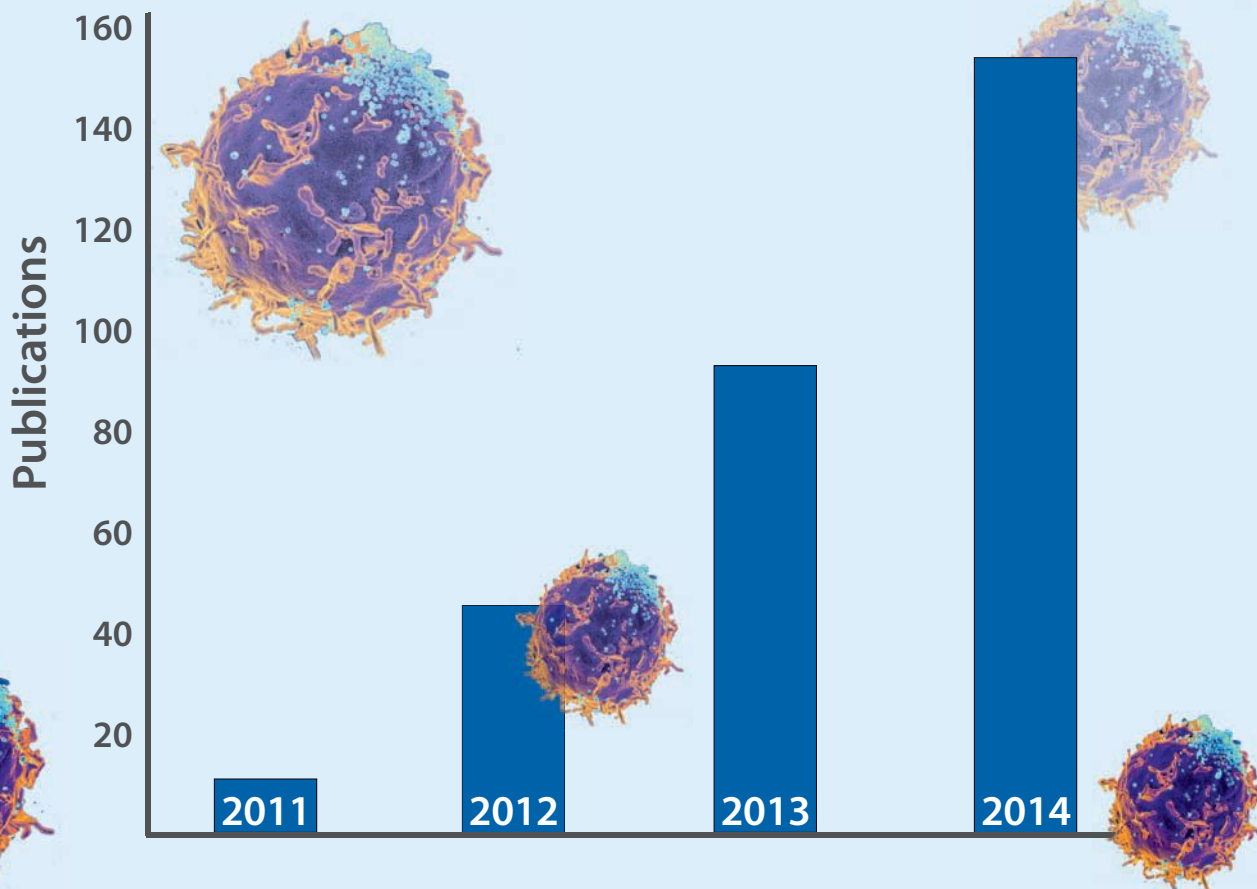
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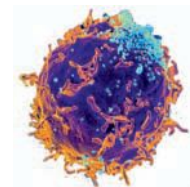


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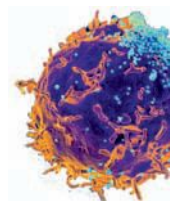
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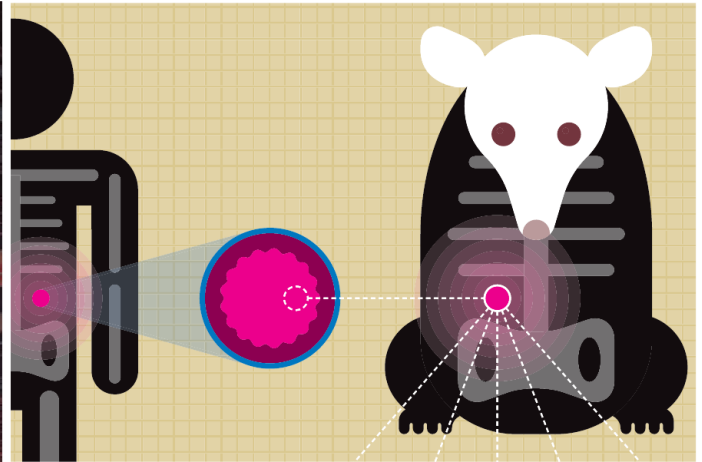
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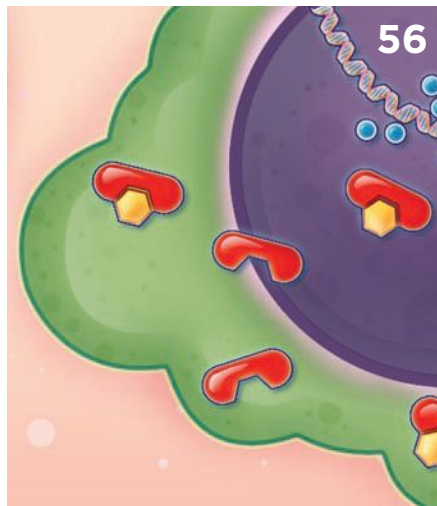
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CLARIFICATION:

An editorial change to "Nourishing the Aging Brain" (*The Scientist*, March 2015) may have given the incorrect impression that Morten Scheibye-Knudsen headed the lab whose work was described. He was actually research associate at that time. *The Scientist* regrets any misunderstanding.

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Disappearing Acts

See a panel of oncologists discuss cases of exceptional responders, cancer patients whose tumors disappear after a brief period of chemotherapy.

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A Jump Ahead

Watch Cornell researchers probe the brains of jumping spiders to gain insight into the arachnid's visual processing capabilities.

VIDEO

Biomarker Sleuth

Scientist to Watch Yvonne Saenger explains recent advances in using biomarkers to identify cancer patients who might benefit most from immunotherapy.

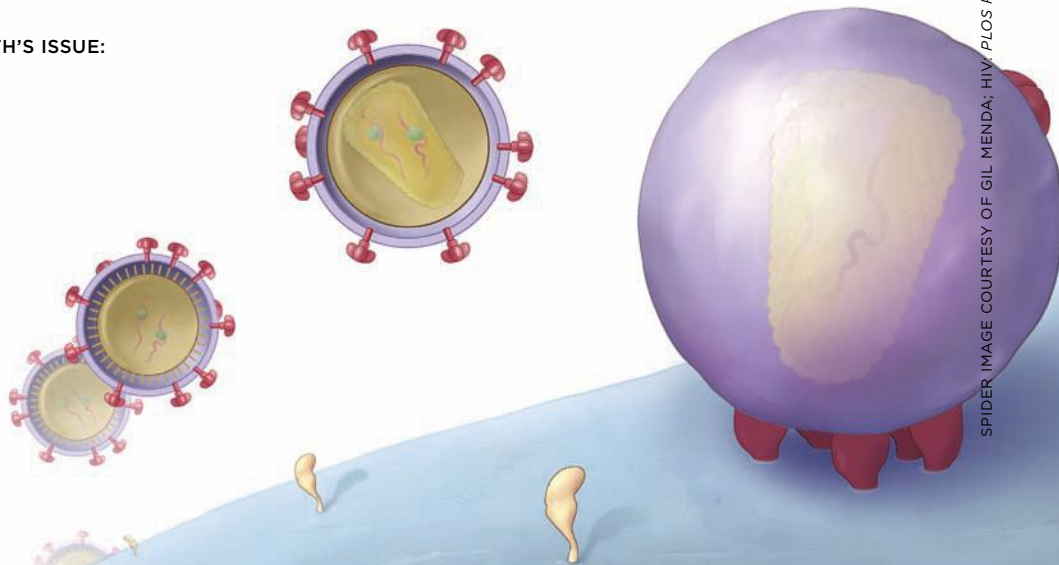
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- Cardiac regeneration using stem cells
- Going after private funding
- Improving Western blots

AND MUCH MORE



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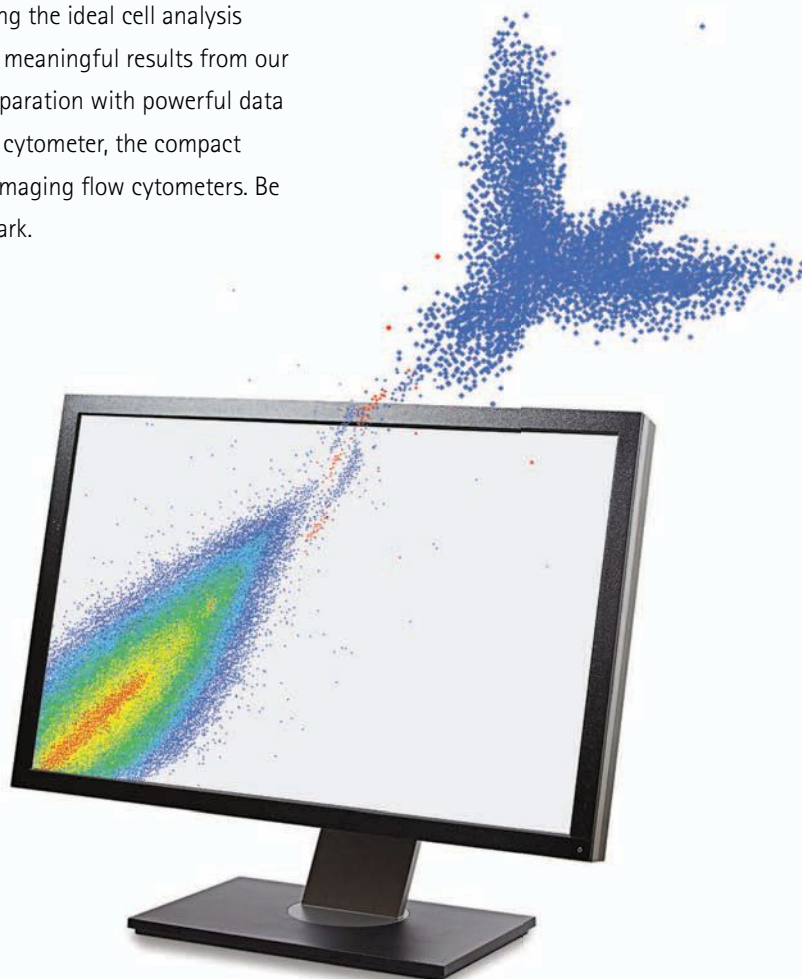
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Contributors



In the summer of 1947, **George Klein** was halfway through medical school in war-ravaged and increasingly dictatorial Hungary, his native country, when he met a young woman, Eva, who was, like him, a Jewish medical student who had survived the Holocaust. One week later, he received notice that against all odds his papers had been cleared for a student trip to Sweden. When Klein returned to Hungary in September, he and Eva married, and with much diplomatic wrangling they were both able to move to Sweden the following year. There, George completed his medical studies at the Karolinska Institut in Stockholm in 1951, and after Eva finished hers in 1955, they became partners not only in life but also in research. George headed Karolinska's Department of Tumor Biology from 1957 to 1993, and the couple investigated the heterogeneity of cell types in tumors and the role of viruses in cancer etiology, uncovering the connection between Epstein-Barr virus and Burkitt's lymphoma. After decades of shaping the field of tumor immunology, George's research has now taken a different turn. "I personally am most interested in the effect of stroma on tumor growth and not really immunology but more microenvironmental control of tumor growth," Klein said in a 2011 interview. "This is my particular interest at this last stage of my career."

George Klein explores the evidence for innate cancer resistance in the feature and the role of the tumor environment in a feature article, "Resisting Cancer" (page 38).



"I've always loved math. I love the fact that it's pure and perfect," **Elena E. Giorgi** says. "But after a while I realized that pure and perfect is not quite applicable to real life." When she moved from her home country Italy to pursue a master's degree in biostatistics at the University of Southern California, Giorgi took her passion for mathematics and applied it to a newfound interest in genetics. "I was absolutely fascinated by how cells work," she recalls. "It wasn't easy, but I really enjoyed learning the literature on my own." After completing her master's degree, Giorgi moved with her husband, a theoretical physicist, to the Los Alamos National Laboratory, where she completed a PhD in applied mathematics, studying HIV vaccine design under the guidance of Bette Korber. Now a computational biologist at Los Alamos, Giorgi is also a novelist. She has written short stories off and on for many years, but her interest in biology inspired her first in a series of detective novels, *Chimeras*, published in 2014. "It's really the science and the genetics that got me going with all these ideas," she says. Her research into chimeras, in particular, has changed the way she thinks about identity: "Who are we if we carry cells from somebody else?" she asks.

Elena Giorgi's feature, "From Many, One" (page 50) examines the weird and surprising science of genetic mosaicism.



As a newly minted MIT PhD, **Jenny Rood** decided to enroll in that institution's science writing master's program. It was not the first time the Colorado-raised biochemist had taken leave of research. Years earlier, after finishing her undergrad degree at Harvard University, Rood packed up for Germany to spend five months as an International Parliament Fellow, interning in the office of a member of parliament. As the daughter of a linguist and a scholar of German-language literature, Rood already spoke German and became a valuable translator of documents. Although she returned to the bench after Berlin, her second exit from the lab would prove permanent. "It's been almost three years since I've had a pipette in my hands, and I don't miss it at all," Rood says. As an intern for *The Scientist*, Rood wields a pen rather than a pipette, contributing articles on topics as varied as the discovery of thirst neurons in mice to an art installation based on bacteria. "The sheer variety of [science writing] is something that appeals to me," says Rood. In her reporting on page 88 about the discovery of leukemia, Rood stumbled upon a parallel history of the use of micrographs in communicating science discoveries.

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To Each His Own

Cancer treatment becomes more and more personal.

BY MARY BETH ABERLIN

For the past four years, the April issue of *The Scientist* has focused on cutting-edge cancer research. A look at the four covers gives a taste of each year's hottest topics. In 2011, molecularly guided melanoma therapies claimed the cover; in 2012, that spot went to cancer stem cells; 2013 featured zebrafish embryos as a new model for the study of cancer dynamics and drug screening; and last year's April cover was devoted to cancer immunotherapy.

This year's cover story on mouse avatars, and much of the issue's other content, focuses on the remarkable progress made in designing cancer therapies that embody the ideals of precision, or personalized, medicine—increasingly patient- and tumor-specific, and often less toxic to normal cells. Both next-generation sequencing data and advances in basic science continue to rewrite the narrative of cancer research. There is now palpable excitement about how new insights into cancer biology will soon drive ever more precise tumor targeting, even though treating each patient's disease as unique still faces many hurdles, which Adam Marcus outlines in a Critic at Large essay on page 31.

How personalized can such targeting get when cancer is such a mess of things gone awry? In “My Mighty Mouse” (page 44), Megan Scudellari reports on the use of mice engrafted with a piece of a patient's tumor to home in on the best drug or drug combo to knock out that person's disease. Several drawbacks still keep this from becoming standard practice, among them the technique's very high cost and a lack of definitive data illustrating its benefits over current treatment decision making. On the plus side, however, academic and pharmaceutical researchers are using the method with donated material from a wide variety of cancers to test highly targeted, but not exactly individualized, new therapies. And some researchers are devising a fruit-fly model that could be personalized and would be much, much cheaper.

Despite the advances in precision drug targeting, the fact that tumors develop resistance to chemotherapeutics continues to be a major problem. Profilee Charles Sawyers (page 58), who co-developed imatinib (Gleevec), one of the first molecularly targeted anticancer drugs, talks about this problem and the later development of a second-generation

drug to treat imatinib-resistant cancers. Next-gen sequencing has allowed researchers to look at changes in the genes of entire signaling pathways to better understand cancer drug resistance (page 57). To further fine-tune therapeutic regimens, the National Cancer Institute's newly launched Exceptional Responders Initiative will sequence the tumors of patients who exhibited remarkable remissions after treatment (page 25).

Tumor heterogeneity and the influence of the surrounding tissue microenvironment are two other areas drawing heaps of attention. Cancer stem cells contribute to tumor heterogeneity and seem to resist radiation and chemotherapy. “In Custody” (page 65) offers tips for isolating and culturing cancer stem cells in order to devise better strategies to take them out. Further evidence of heterogeneity abounds in the study of microRNAs, which are abnormal in all tumors. But findings on miRNAs conflict and seem to depend on the tumor's environment, as described in “Two-Faced RNAs” (page 21). Single-cell sequencing may be needed to more completely understand the implications of tumor heterogeneity.

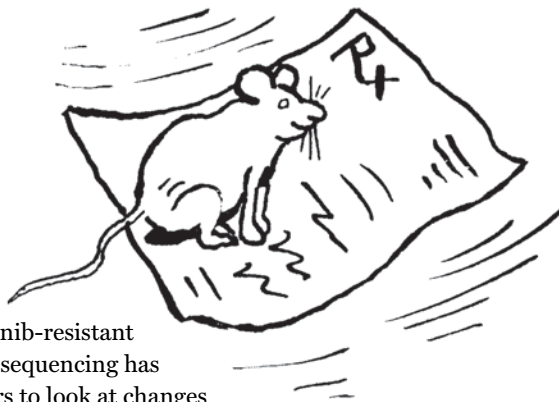
Even though cancer cells are nefarious warriors, our bodies go to great lengths to stop their forays. Tissues seem to have an evolutionarily programmed arsenal to keep their environment normal. In “Resisting Cancer” (page 38), George Klein gives an overview of the various mechanisms we and other animals use to keep cells from dividing uncontrollably and escaping their normal confines.

Last year's cover story on cancer immunotherapy research is updated in this issue in a Bio Business article, “The CAR T-Cell Race,” which documents the veritable explosion of clinical trials and business investment in chimeric antigen receptor (CAR) T-cell therapies.

At this rate, we should have no trouble picking next year's cover. ■



Editor-in-Chief
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Speaking of Science

A world without intelligence is a primitive place, Doctor, not an enchanted place. Intelligence is part of the advancing scheme of evolution. Without it, nature reaches a plateau very quickly and does not progress beyond raw survival. The full flavor of possibility goes unsavored. And that... is a true shame.

—Mr. Spock, science officer aboard the starship *Enterprise*, first portrayed by Leonard Nimoy, who died on February 27
(*First Frontier*, Star Trek, Book 75, 1995)

My efforts to cope with budgetary limits have been guided by Lord Rutherford's appeal to his British laboratory group during a period of fiscal restraint a century ago: "... we've run out of money, it is time to start thinking."

—Outgoing National Cancer Institute Director, Harold Varmus, in his resignation letter (March 4)

We knew smoking was bad, but we now have direct, independent evidence that confirms the disturbing findings that have been emerging internationally.

—Australian National University researcher Emily Banks, lead author on a *BMC Medicine* paper reporting that 67 percent of smokers died from smoking-related illness and that the habit increased the risk for at least 13 types of cancer (*The Washington Post*, February 26)

Handshaking may functionally serve active yet subliminal social chemosignaling, which likely plays a large role in ongoing human behavior.

—Idan Frumin et al., researchers at Israel's Weizmann Institute of Science, in a recently published *eLife* paper that suggests that people unconsciously smell their fingers after shaking hands with someone of the same sex (March 3)



Why us? Why? Is it something in our water? Is it genetic? Why both of us in such a short time frame? The doctor said it's not the water, it's not the environment, it's just a freak act of nature.

—Missy Shatley, 38-year-old Prairie Farm, Wisconsin, mother, speaking about how she and her 14-year-old daughter Brooke were diagnosed with cervical cancer this past December, just 13 days apart (*ABC News*, March 5)

Unnecessary quarantines do not save lives; they lose lives.

—Amy Gutmann, chair of the Presidential Commission for the Study of Bioethical Issues, on the quarantining of US health-care workers who had returned home from treating Ebola patients in West Africa last year (February 26)



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Notebook

APRIL 2015



Through a Spider's Eyes

Gil Menda was bored. It was 2012, and his research on facial recognition in wasps was going nowhere. The Cornell University graduate student turned to his advisor, neurophysiologist Ron Hoy, as the professor was running out the door to teach a class. There were jumping spiders in the lab already, so Menda asked for permission to attempt the impossible: to tap into the central nervous system of an arachnid that was far more liable to depressurize and die than sit still for brain surgery. Hoy assented.

It was a problem that had vexed biologists for decades, says Paul Shamble, an

arachnologist who was then a fellow Cornell graduate student. The jumping spider is unusual among arachnids, most of which have relatively poor vision compared to insects, even though arachnids have four pairs of eyes. While most spiders build webs and wait for their prey to come to them, the jumping spider stalks and pounces much like a cat, displaying remarkable visual acuity, despite having a brain no larger than a poppy seed.

By the time Hoy returned from teaching his class, Menda had succeeded in his efforts. Using an ultrathin metal wire, he'd gently poked a hole in the spider's cuticle that was small enough to self-heal. A glass-insulated tungsten extracellular electrode implanted within range of six neurons

DON'T CALL ME FOUR-EYES: Jumping spiders have four pairs of eyes that work in concert to help them sense and capture prey.

in the spider's brain registered data in the form of voltage spikes.

Shamble then helped Menda design and 3-D print harnesses for the male and female spiders. Refrigeration and a drop of wax immobilized them for study. Now that jumping spider vision was no longer a hypothetical research topic, Cornell grad students outside the Hoy lab took note. Soon, James Golden, a computational neuroscientist, and Eyal Nitzany, a biological statistician, joined the team. The foursome collaborated on experimental design and obtained the first recordings from the visual processing centers of

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the spiders' tiny brains (*Current Biology*, 24:2580-85, 2014).

Menda recalls one day of that project in particular. He'd come into the lab early to take out the spiders and start recording neuron activity while using the visual stimuli Nitzany had developed. He showed an immobilized spider potential mates and its natural prey, flies, on a screen.

You could put a hundred jumping spiders along the frame of your glasses. If you could turn those eyes onto what your eyes were doing, you would have a gangbusters eye tracker.

—Ron Hoy, Cornell University

"I saw that [the spider] concentrated on only one from the variety I was showing him," he explains. It was the prey stimulus that held the spider's attention. The longer the spider focused, the more spikes Menda recorded. "I was like, 'Whoa! This is recognition of the object. This is really interesting.'" Immediately, he sent out a group text, imploring fellow grad students and Hoy to drop what they were doing and come to the lab. They did—and they stayed all day, watching the live recording, developing new stimuli to test, and building computer programs that could sort and analyze the incoming data right then and there.

"I've had a lot of graduate students in my time," Hoy says. "It's usually one student, one problem, and everyone is working at their own cubicle. But in this case, it was kind of like mission control!"

The Cornell team discovered that it's not just that the jumping spider has eight incredible eyes—it's that it uses them together. When Golden's algorithms sorted the thousands of recorded neural spikes into individual classes by height and shape, the researchers saw that the neurons interacted differently depending on the stimuli the spider was shown. Jumping spiders, which are roughly the size of a pencil eraser, are able to process

visual information gathered by any of their eight eyes, decide what action they need to take depending on what the stimulus is, and then alter their body position and behavior accordingly.

Collaboration was key to the project's success. "To really make a bigger impact, to see a problem from multiple perspectives, this kind of research is critical to arrive at novel insight and novel solutions," says John Wen, a roboticist at Rensselaer Polytechnic Institute in Troy, New York, who was not involved in the study.

Meanwhile, the Cornell team's ongoing work—using the same microelectrode to record neuron activity in other spiders, wasps, dragonflies, bumblebees, and monkeys—has important implications for Wen's field. As technology becomes increasingly focused on high performance in small packages, the team's research offers a visual processing solution on a precise nano- and microscale. Hoy says that for scientists studying dyslexia and autism, "to be able to monitor eye movements in miniature would be fantastic."

The current commercial eye tracker market is dominated by slim, Nintendo Wii-like tracking bars and Google Glass-style wearables. These devices typically max out at five or six fingertip-size cameras, and can be intrusive and limited in their ability to capture data points as the subject's head moves, which makes them difficult to use successfully when studying children and young adults with developmental disorders (*J Vis Exp*, doi 10.3791/3675, 2012).

But studying the jumping spider's tiny visual processing system could lend insights that pave the way for eight cameras and a communication network far smaller than a pinky. "You could put a hundred jumping spiders along the frame of your glasses," Hoy says. "If you could turn those eyes onto what your eyes were doing, you would have a gangbusters eye tracker."

Nitzany offers a more macro application: subway surveillance cameras. "You have thousands of people going through the subway, and you need to know which one you need to focus on," Nitzany explains. "You don't want to focus on everyone. You have to know how to integrate all those signals so you only focus

on the most important one. This is very much the same task as the jumping spider [does].”

It’s all part of the charisma of the jumping spider, whose specialness Hoy finds impossible to overstate. “It’s like they’ve been cast from web heaven to earth, where they have to find their own food and find mates,” he says. “People had pretty much given up on recording from them, but with good luck, a good set of hands, and a great team, we managed to crack it.” —Brittany Taylor

Two-Faced RNAs

In the early 2000s, scientists were first starting to appreciate the idea that microRNAs—small, noncoding RNAs that interfere with protein translation—could have something to do with cancer. One clue came from developmental biology, when Frank Slack, then at Yale University, and colleagues found that mutating a microRNA gene called *let-7* led to increased cell divisions. Then in 2002, Carlo Croce’s team, then at Thomas Jefferson University in Philadelphia, mapped genetic deletions common in chronic lymphocytic leukemia to two microRNA loci, *miR15* and *miR16*.

Now, a little more than a decade later, “we know that microRNA levels are essentially abnormal in every type of tumor that’s been examined,” says Joshua Mendell, a microRNA researcher at UT Southwestern Medical Center. In some tumors, a microRNA may be overexpressed, while in others it may be missing altogether.

Some experimental studies have also shown that manipulating a microRNA associated with a cancer—say, deleting one that’s overexpressed or boosting one that’s in low abundance—can knock back tumors. Such is the case with *miR15* and *miR16*, for instance. After Croce and colleagues found that these genetic loci are missing or downregulated in more than half of B-cell chronic lymphocytic leukemias (CLL), another group demonstrated that experimentally deleting this region in mice causes symptoms similar to those in humans with CLL. “That’s great evidence

that the loss of that microRNA is causal in that disease,” says Slack, who is now director of the Institute for RNA Medicine at Beth Israel Deaconess Medical Center.

But as Mendell, Slack, Croce, and others have discovered, to call a microRNA a tumor suppressor or an oncogene is far too simplistic.

In 2009, Mendell was working with a microRNA called *miR-26*, levels of which are depleted in liver cancer. “We were hopeful that, based on earlier findings, if we could restore expression of *miR-26* this might be therapeutically beneficial,” he says. So he and colleagues boosted *miR-26* levels in transgenic mice with an overactive cancer pathway, and, lo and behold, tumor growth stopped. “That was very exciting to us.”

But just as his paper was published, another group published a study suggesting the opposite role for *miR-26*: in a mouse model of brain cancer, *miR-26* downregulated a tumor suppressor called *PTEN*. In doing so, the microRNA appeared to be acting as an oncogene.

The conflicting results were confusing. Mendell says there wasn’t a lot of evidence showing that microRNA targets could be tissue-specific. “That’s why we decided to do another series of experiments in which we could more precisely control levels of *miR-26* in any tissue of the body.”

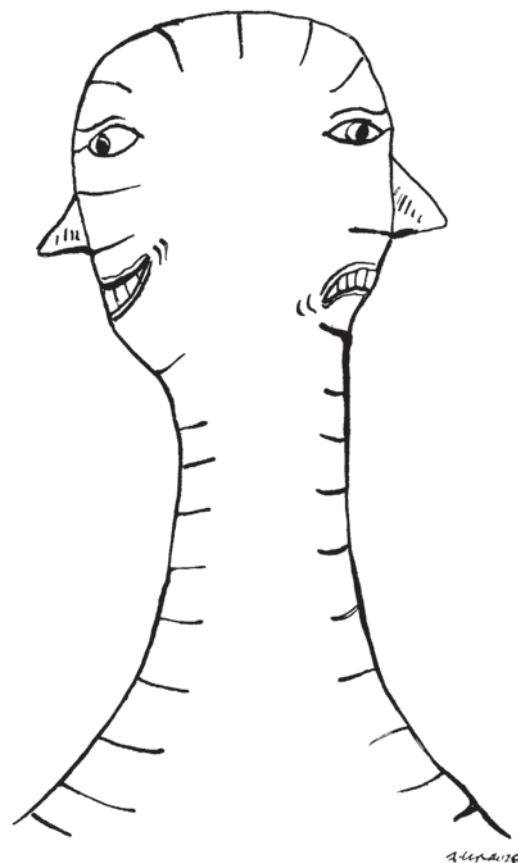
What he found was that *miR-26* could act in both tumor suppressive and oncogenic ways. Although Mendell’s team confirmed the earlier finding that *miR-26* inhibits the tumor suppressor *PTEN*, “the phenotype we saw when you overexpress *miR-26* was not a copy of what you would see when you lower *PTEN*,” he says. In mice with an overabundance of *miR-26*, cancer didn’t pop up all over the place, and in fact, intestinal tumor growth was blocked (*Genes Dev*, 28:2585-90, 2014).

The explanation for the paradox lies in *miR-26*’s other targets. Although the microRNA inhibits the tumor suppressor *PTEN*, *miR-26* also puts a lid on a broad suite of pro-proliferative genes, making its overall effect tumor suppressive. “When looking at the function of a microRNA, you need to look at the overall set of tar-

gets,” says Mendell. “If you pull out one, it doesn’t give you the full picture.”

miR-26 is not the only example. “We found cases of microRNAs that behave like an oncogene and a tumor suppressor gene depending on the cell,” says Croce. “The cellular environment is critical for the action of microRNAs.” For example, *miR-221/222* acts as an oncogene in the liver and other tissues and as a tumor suppressor in other contexts, such as tongue squamous cell carcinoma. Croce says it all depends on which targets of the microRNA are present.

Christopher Park, who studies blood disorders at Memorial Sloan Kettering Cancer Center, adds that there is yet another layer of complexity to the behavior of microRNAs in cancer. Scientists must also consider the stage of the cancer: are they manipulating a microRNA in an already established tumor, or before an animal develops cancer? Take, for example, *miR-29a*. Croce’s group has found that overexpressing the microRNA’s



gene in leukemia cell lines slows growth, suggesting the miR-29a acts as a tumor suppressor. Yet Park's team showed that increasing circulating levels of miR-29a in mice initiates the development of leukemia. "The role of the gene can vary during the cancer process," says Park.

The first microRNA cancer treatments are now in early human clinical trials. Croce says he's not too concerned about the risk that a therapy against one type of cancer might promote another, primarily because of the time frame. As he's seen preclinically, microRNA-targeting drugs act on cells quickly, and he hasn't witnessed any tumorigenic side effects. Slack says the ultimate answer will come out of the clinical trials. "I think the clinicians and myself have our fingers crossed and hope there's not going to be some weirdness in these patients because of the complexity of the system."

Continuing knockout and overexpression studies in mouse models will

help identify microRNAs' global roles in tumorigenesis and tumor progression. "We think in our lab it's very important to continue to look at microRNA functions in their natural context, in whole animals," says Mendell. "When we do that we're often surprised; microRNAs don't seem to do exactly what we thought they did in cell lines."

—Kerry Grens

Cancer Kismet

Marine invertebrates may be unassuming creatures, but at the turn of the 20th century they found themselves at the heart of a scientific controversy. In 1891, the German biologist Hans Driesch claimed that each of the four cells in an early-stage sea urchin embryo, if separated, could give rise to an entire larva. Four years earlier, however, Laurent Chabry's experiments had shown that each cell from an equally early-stage invertebrate—a sea squirt, or tuni-

cate, which has a chordate larva—could only make a partial embryo. To resolve the conflict, Edwin Conklin spent the summer of 1904 hunched over a light microscope in Woods Hole, Massachusetts, observing the development of one of these tunicates, *Styela partita*. His detailed drawings of the embryos' bright yellow crescent and four clear compartments, which he published the following year, traced the sea squirt's development from its single-cell beginnings to the 112-cell stage. By inventing a technique now known as fate mapping, Conklin put the debate to rest by demonstrating that Chabry had been right all along.

Over the next century, researchers fine-tuned the technique by adding dye to embryos that weren't naturally colored, as were those of sea squirts. Walter Vogt injected dye into frog embryos in 1929, and by the 1990s researchers had developed rudimentary genetic dyeing techniques. These days, researchers can insert the label directly into a mouse

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cancer metabolism research

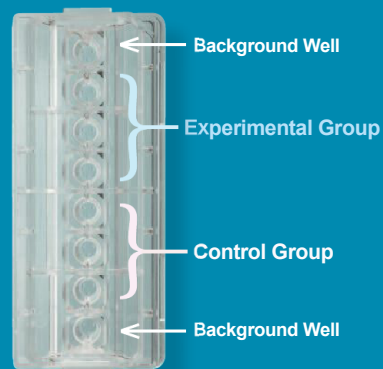
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— Kacey Caradonna PhD,
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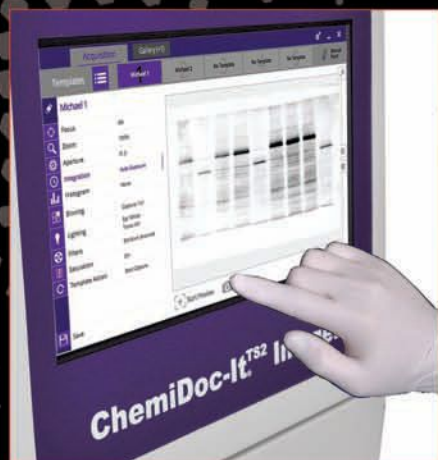
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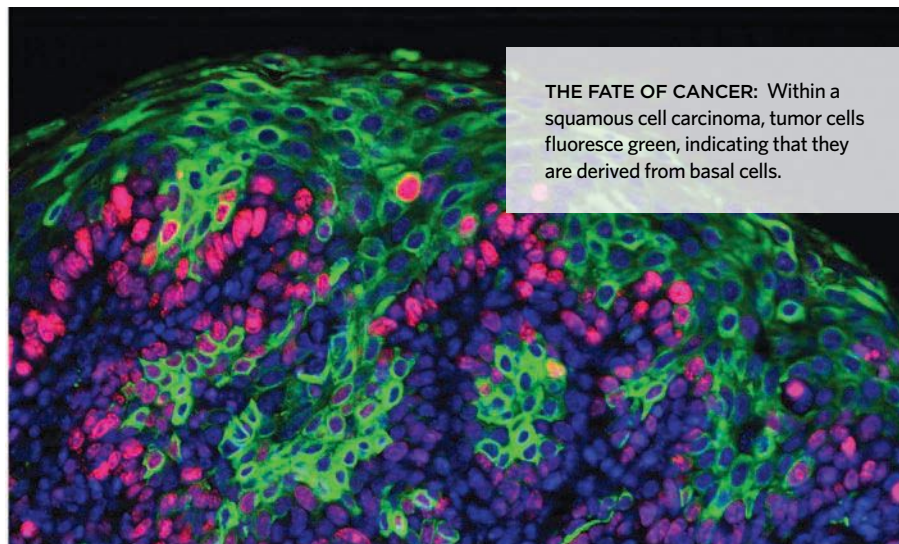


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THE FATE OF CANCER: Within a squamous cell carcinoma, tumor cells fluoresce green, indicating that they are derived from basal cells.

genome using two transgenes. One gene encodes a site-specific Cre recombinase under the control of a cell type-specific promoter, and the other encodes a fluorescent reporter, separated from a constitutive promoter by a stop codon that is flanked by the sites recognized by Cre. Targeted cells with both genes express the fluorescent label, as do all of the cells' future daughters, since the stop codon in front of the fluorescent protein gene has been permanently disabled.

Developmental biologist Alexandra Joyner's lab at Memorial Sloan Kettering Cancer Center, one of the first groups to use the technique to map the mouse mid-brain and anterior hindbrain (cerebellum), soon noticed that there was a problem with the system. "To be really sure that you're following the fate of cells, you need to know you just marked them at one time, and that you could never mark them later," Joyner says. To avoid this issue, researchers developed an inducible system, where the Cre reporter will only become active at the moment that the mouse is treated with the drug tamoxifen. This was how Cathy Mendelsohn and her group at Columbia University traced the origin of new urothelium, the inner bladder lining that regrows to replace tissue damaged by a urinary tract infection. Most scientists assumed that the bladder lining would be similar to our skin,

where stem cells lurk in a basal layer and give rise to all the cell types needed. But Mendelsohn and her colleagues used fate mapping to determine that the cells of the urothelium are instead "just another population that can self-renew but not generate other cell types," she says.

Having used fate mapping to learn about normal recovery from injury, Mendelsohn wondered what might be happening in bladder cancer. "It's really important that you have a way of tagging, or you could never find the daughters otherwise," Mendelsohn says. Tumor cells in the bladder look nothing like the cells from which they arose, she explains. So Mendelsohn decided to apply fate-mapping techniques to help answer a contentious question in the field: "Do different types of bladder cancer arise from different cell types?"

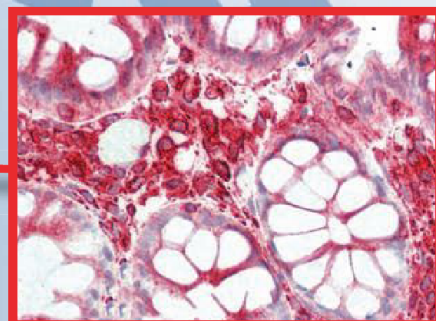
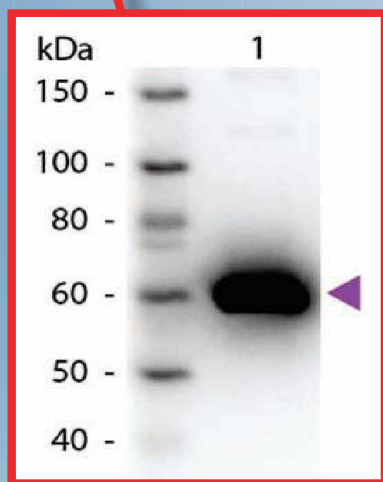
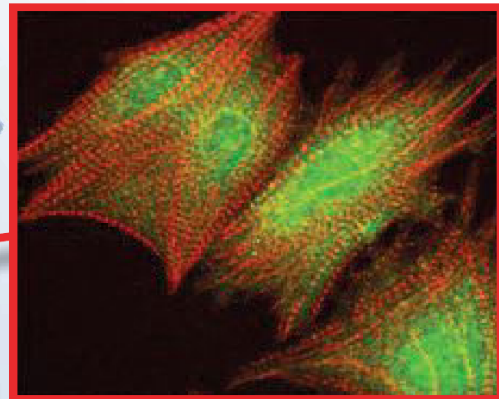
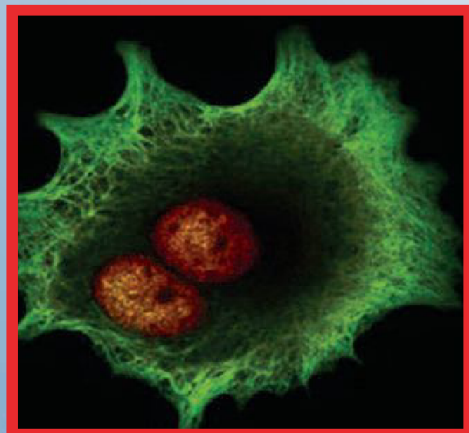
Mendelsohn and her colleagues searched for the cell types that give rise to preinvasive carcinoma in situ, which sits in the bladder's lining, and the presumably noninvasive papillary carcinomas that she describes as looking like "a hand that grows up out of the urothelium into the bladder lumen." Her team's lineage-tracing experiments revealed that carcinoma in situ arises from basal cells, while papillary lesions come from a different cell type called intermediate cells.

Mendelsohn is not the only researcher interested in tracing cancers back to the cell type of origin. Columbia colleague Michael



Choosing the best secondary antibody for your research

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Secondary antibodies play an important role in many research applications, including immunofluorescence (IF), immunohistochemistry (IHC), Western blot (WB), enzyme-linked immunosorbent assay (ELISA), flow cytometry (FC), gel shift assay or electrophoretic mobility shift assay (EMSA), immunoprecipitation (IP), and chromatin immunoprecipitation (ChIP). Choosing the right secondary antibody will improve your results by increasing signal detection while reducing background and nonspecific staining.

Important selection tips:

1. Match the host of the primary antibody

If your primary antibody is raised in rabbit, for example, use an α -rabbit secondary antibody.

2. Match the class/subclass of the primary antibody

Polyclonal antibodies are usually IgG isotype; therefore, use an α -IgG antibody that recognizes either the heavy chain or the heavy and light chains of the primary antibody (α -IgG Fc or α -IgG H+L).

Monoclonal antibodies are subclass specific; therefore, use a secondary antibody directed against that specific subclass: e.g., if the primary antibody is mouse IgM, choose a secondary antibody that reacts with mouse IgM (α -mouse IgM); if the primary antibody is mouse IgG1, choose a secondary antibody that recognizes all IgG subclasses, or, when working in multiplex assays, use a subclass-specific antibody (α -mouse IgG1).

3. Select the correct reporter for your application

Fluorescence: secondary antibody is conjugated to a fluorophore, e.g., FITC, Texas Red, Alexa Fluor®, Cy™, or DyLight™. A light source excites the fluorophore, and its brightness is measured.

Chemiluminescence: secondary antibody is enzyme linked, e.g., HRP (horseradish peroxidase). An enzymatic reaction produces light when chemiluminescent substrate is applied.

Chromogenic: secondary antibody is enzyme linked, e.g., alkaline phosphatase or HRP. An enzymatic reaction converts a chemical substrate to a colored precipitate.

Did you know?

Pre-adsorbed antibodies improve specificity by eliminating cross-reactivity with immunoglobulins of undesired species, antibody fragments, or cell tissue samples.

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Western Blotting

Gel electrophoresis



Transfer



Block membrane

Reduce nonspecific secondary antibody binding → use appropriate blocking agent (BSA, nonfat dry milk, casein or FBS)

Try several blocking agents if necessary

For interference with the reporter system or auto-fluorescence → change blocking agents

For chromogenic detection → block endogenous enzyme activity



Primary antibody incubation

Control: omit primary antibody to detect nonspecific binding of the secondary antibody



Wash

Remove residual primary antibody → agitate appropriately

Reduce nonspecific bands → use a mild detergent in wash steps



Secondary antibody incubation

Reduce nonspecific antibody binding → titrate your secondary antibody

Block binding of secondary antibody to Fc receptor → add normal serum to antibody

Reduce cross-reactivity → use pre-adsorbed secondary antibodies

Reduce nonspecific binding in fluorescent WB → add 0.01% SDS to secondary antibody



Wash → Detect

For single protein detection → use HRP or alkaline phosphatase-conjugated secondary antibody

Chemiluminescence detection → use X-ray films or imaging systems

Avoid protein loss due to stripping → multiprotein detection in a single blot (multiplex)

Save time from multiple primary antibody incubations → multiplex WB

Multiplex WB → important to detect each antigen using primary antibodies raised in different hosts, each matched with a specific secondary antibody conjugated to fluorescent dyes that can be imaged at different wavelengths

For simultaneous detection of modified (phosphorylation or methylation) and unmodified protein → multiplex WB

Do not use sodium azide with HRP-conjugated secondary antibodies.

The background on a WB can be improved by using the right antibody dilution and incubation time.

Blot C with high background was incubated overnight at 4°C at 1:1,000 dilution. Blot D was incubated for 1 hr at room temperature with a primary antibody dilution of 1:2,000.

IHC

Chromogenic
Fluorescence

ELISA

Chromogenic
Chemiluminescence
Fluorescence

FC

Fluorescence

WB

Chromogenic
Chemiluminescence
Fluorescence

EMSA

Chromogenic
Chemiluminescence
Fluorescence

IF

Fluorescence



IP and ChIP rely on biotin-labeled, enzyme-conjugated, epitope-tagged secondary antibodies or IgG-binding proteins to detect the protein complex or protein/DNA complex.

Immunofluorescence

Sample Preparation

Tissue autofluorescence can be impacted by the fixation method

Compare fixation methods for samples with high autofluorescence → sample treatment with autofluorescence reducing chemicals (sodium borohydride)¹

Wash

Improve membrane permeabilization for antibody staining → add detergents

For membrane proteins → avoid detergent usage²

Block

Reduce high background → block with serum from the same host species as the secondary antibody

Primary antibody incubation

Control: omit primary antibody to block nonspecific binding of the secondary antibody. Alternatively, use an isotype control that corresponds to the isotype of the primary antibody.

Secondary antibody incubation

Minimize nonspecific background → use pre-adsorbed secondary antibody

Reduce nonspecific binding → titrate the secondary antibody

Perform the secondary antibody incubation in the dark

Wash

Wash

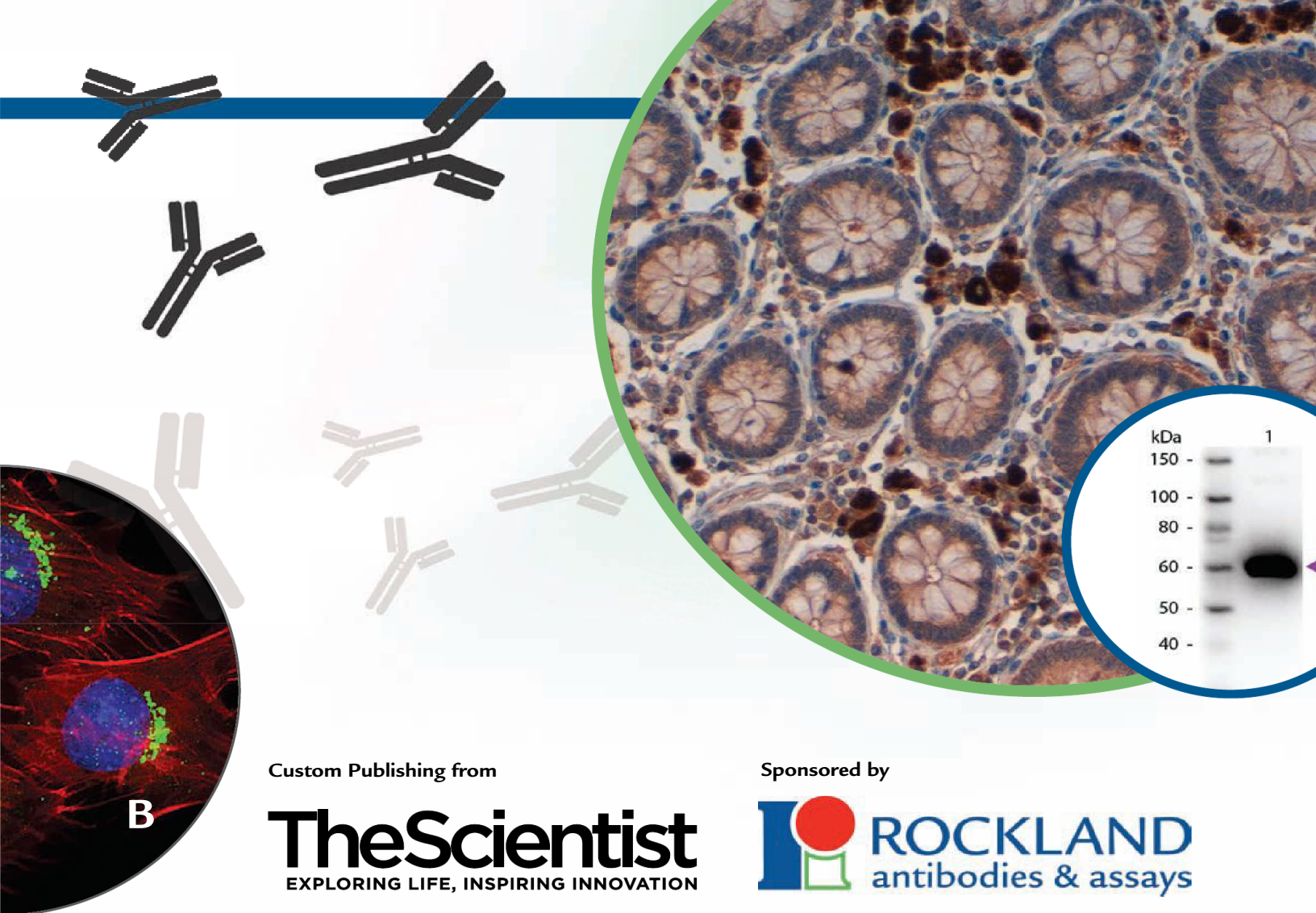
Mount

Use antifade mounting medium to preserve the fluorescent signal

Multiplex fluorescent WB of GST spiked albumin-depleted human serum samples. Simultaneous detection of transferrin (green), α -trypsin (red) and GST (blue). Here, α -GST (mouse host), α -transferrin (rabbit host), and α -trypsin (goat host) were detected using secondary antibodies: α -mouse IgG DyLight™ 488, α -rabbit IgG DyLight™ 549 and α -goat IgG DyLight™ 649 (all raised in donkey).

Using too much primary or secondary antibody can cause nonspecific staining. Cells were stained with Tubulin (red), Akt (green), and DAPI (blue). Image A used a high concentration of primary and secondary antibodies, resulting in nonspecific staining. Titrating the antibodies reduces nonspecific staining (Image B) so that localization of Akt in the Golgi is visible.

IP Tip: Use of a secondary antibody that recognizes the native, nonreduced form of IgG can increase specificity by avoiding interference from denatured antibody fragments and nonspecific binding to contaminants.



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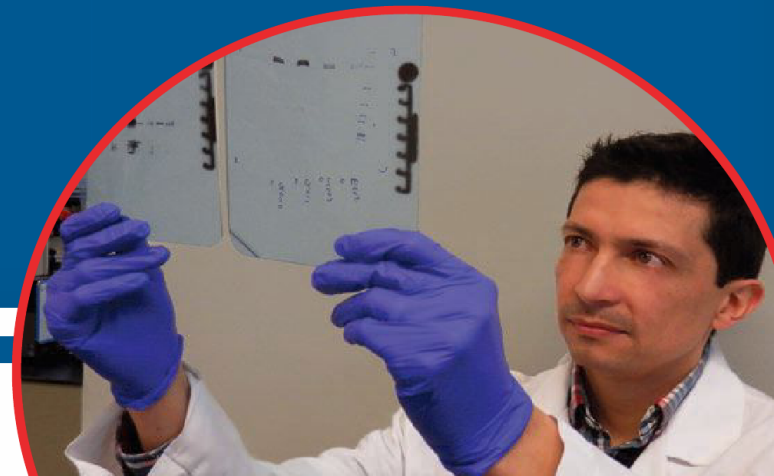
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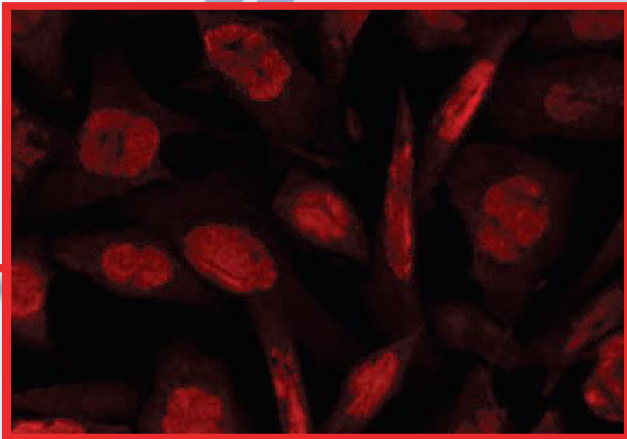
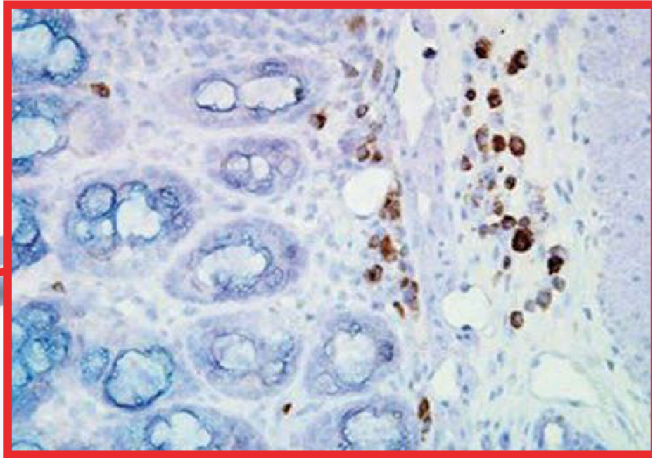
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2. Goldenthal, K.L., Hedman, K., Chen, J.W., August, J.T., & Willingham, M.C. (1985). Postfixation detergent treatment for immunofluorescence suppresses localization of some integral membrane proteins. *J Histochem Cytochem*, 33(8):813–20.
3. All images provided by Rockland Immunochemicals, Inc.

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Dif-tor heh smusma,



James Fendrick
President & CEO

Shen and his group have recently done similar work in a prostate cancer model. “The prostate is another nice example of how they’ve used [fate mapping] to hit the basal cells and luminal cells,” Joyner says. Shen adds that the technique has stretched far beyond understanding tumors of the bladder and prostate. “You name a cancer, there are people trying to fate-map it,” he says.

And the power of fate mapping to trace the origins of tumors is far from being fully realized. “We could also look at the evolution of the lesion, from it being inside the urothelium to invading the tissue below,” Mendelsohn says. Dan Theodorescu, a urologic oncologist and bladder cancer researcher at the University of Colorado Denver, is particularly interested in using fate mapping to determine if cells originating in two different layers of the bladder result in two different tumor behaviors. “What you want to do is show that cancers coming from the basal are invasive, and cancers coming from the nonbasal cells are nonmuscle invasive,” he says. “And nobody has done that. That’s the key.”

There’s also an opportunity to use fate mapping to further fine-tune the cells’ origins. “You would like to be able to define specific subtypes within the basal or luminal compartments that are potential cells of origin,” Shen says. Researchers may be onto a way to do just that, by combining two different recombinases to “get a higher resolution of what cell type you’re marking,” Joyner says. “There are all kinds of new applications now combining . . . recombinases to allow us to ask even more interesting questions.”

“Fate mapping is really the only way to look at what a cell will do after a certain time,” Mendelsohn says. “I don’t think it would be possible to do this without . . . mouse models where you have genetic lineage tracing.”

—Jenny Rood

Mining the Outliers

Last year, Harvard’s Jochen Lorch and colleagues took a leap of faith by enrolling a handful of patients with a deadly form of

thyroid cancer in a clinical trial involving patients with a much more tractable form of the disease. The study was designed to test Novartis’s Afinitor (everolimus) on patients with differentiated thyroid cancer, typically considered a curable disease. Lorch and his colleagues didn’t have high hopes for the anaplastic thyroid cancer patients, who tend to survive less than five months after diagnosis, but with few treatment options, they figured it was worth a shot. Amazingly, the second anaplastic thyroid cancer patient they enrolled responded to the drug: the mass in her chest began to shrink almost immediately.

For about 18 months, the patient’s tumor continued to get smaller. But then it started to grow again. Searching for clues regarding her initial drug response and her cancer’s acquired resistance to everolimus, the researchers biopsied the mass and sequenced it, along with a sample from the original tumor that had been surgically removed from her neck prior to the trial. In both specimens, the team identified a mutation in the tuberous sclerosis 2 (TSC2) gene, a tumor suppressor and negative regulator of mTOR. Immediately, this result made sense with regard to the patient’s initial response to everolimus. While this was the first documentation of a TSC2 mutation in anaplastic thyroid cancer, abnormalities in TSC2 cause tuberous sclerosis complex, a

disorder usually manifest in childhood that involves abnormal skin growths and benign tumors in many organs, including the brain. But these tumors can be treated with everolimus. And a second mutation, found only in the tumor that shrank and then regrew during the trial, seemed to explain the acquired resistance to the drug, by altering the exact site where everolimus binds (*NEJM*, 371:1426-33, 2014). “The tumor had found a way to modify mTOR so it was still biologically active, but the drug could no longer bind,” says Lorch, who is now partnering with Millennium Pharmaceuticals to test a second-generation mTOR inhibitor.

This case serves as an example of how sequencing the tumors of so-called “exceptional responders” could inform future clinical study. Most oncologists have stories about unusual cases of remission—a drug that didn’t work in the majority of patients but for whatever reason significantly helped one or a few patients. For years, such outliers have made for good news stories, but not powerful data. Indeed, in the context of clinical trials, these therapies aren’t likely to make it to

TREATABLE: Exceptional responders are patients whose therapeutic experience diverges from that of the majority of clinical trial participants.



market, despite the fact that they may have helped a small number of patients.

“When a clinical trial [fails] or you don’t see broad responses, because there are so many drugs to test and so many diseases in which to test them, we typically move on,” says Barry Taylor, associate director of the Center for Molecular Oncology at Memorial Sloan Kettering Cancer Center (MSKCC). “And yet these phenomenal cases stick out in the memories of treating physicians. . . . It wasn’t until recently that we really were able to capture them to explore them in extreme molecular and genetic detail.”

A couple of years earlier, Taylor, along with MSKCC colleague David Solit and other collaborators, had a similar experience in a trial testing everolimus in bladder cancer patients. While the drug had not elicited positive responses in the majority of patients, one woman had undergone complete remission, with

no signs of recurrence even a couple of years after the trial concluded. Sequencing her tumor’s genome had revealed mutations in the related gene *TSC1*, and sequencing the tumors of eight additional patients who had shown minor responses also revealed mutations in this gene. The difference between the exceptional responder and those with minor responses appeared to be a second mutation in another gene involved in the same pathway, found only in the woman who had undergone remission (*Science*, 338:221, 2012). Researchers in Boston are now initiating a clinical trial that will enroll patients on the basis of the presence of these and nearby mutations.

Recognizing the value in studying these remarkable responses, the National Cancer Institute (NCI) last September launched the Exceptional Responders Initiative. “The whole idea of exceptional responders rings true for clinicians,

These phenomenal cases stick out in the memories of treating physicians. It wasn’t until recently that we really were able to capture them to explore them in extreme molecular and genetic detail.

—Barry Taylor,
Memorial Sloan Kettering Cancer Center

because they have a minority of patients—whether they’re treated with investigational agents or treated with standard treatments—that do exceptionally better than other patients treated that way,” says Barbara Conley, associate director of NCI’s Cancer Diagnosis Program.

After identifying some 100 cases of exceptional responders among trial results from the NCI’s Cancer Therapy Evaluation

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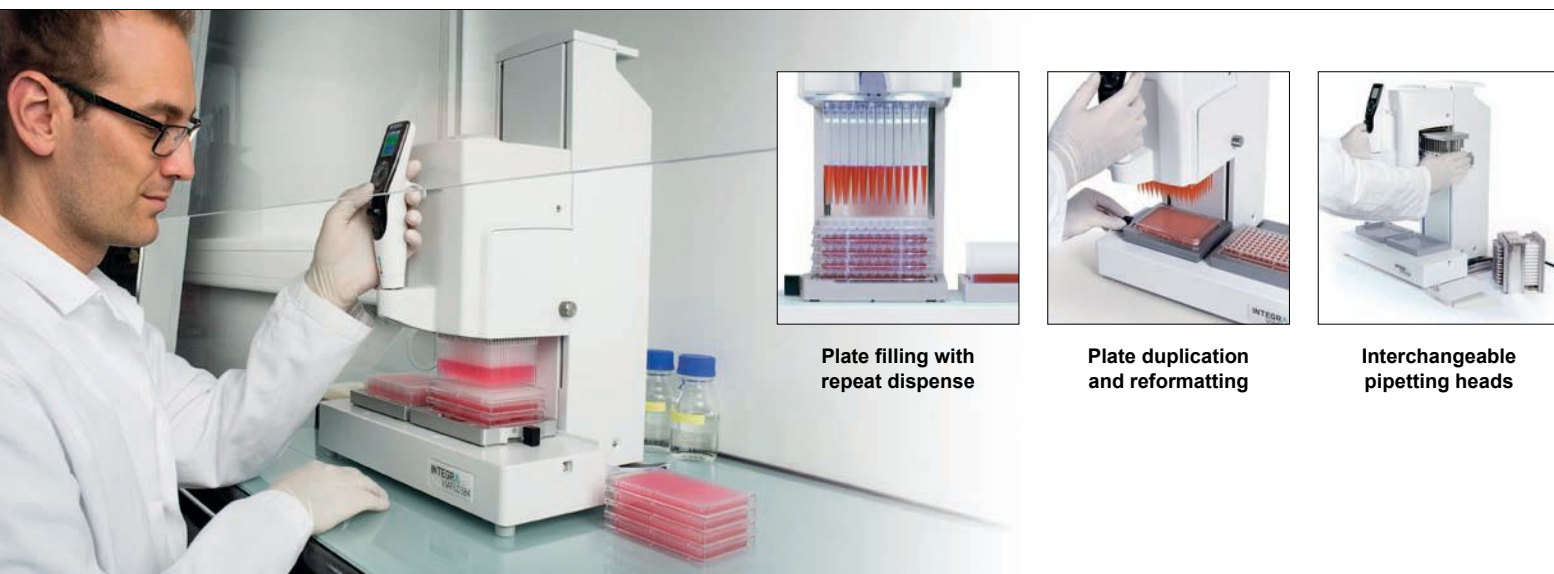


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Program (CTEP), the agency felt it could find sufficient numbers of these patients to warrant the establishment of a program dedicated to understanding their responses. The program is now inviting case submissions via e-mail for any patient who underwent a durable remission following treatment with a drug—experimental or already approved—that fewer than 10 percent of patients responded to. The initiative has so far received about 120 submissions. NCI researchers are look-

ing into each case to see which qualify as exceptional responders and have sufficient tumor samples for analysis, and soon, with the help of collaborators, they will begin analyzing the genomes and transcriptomes of biopsies to identify patterns that may explain the patients' exceptional responses to their anticancer therapies.

"The NCI initiative [serves to] catalyze a much larger-scale exploration of these [exceptional responders]," Taylor says. "It's critically important because it

means we can explore this in a greater number of patients for a greater number of therapies with a greater number of potential mutations."

"We can't afford to keep giving drugs that don't work," says oncologist and cancer researcher Andrew Biankin of the University of Glasgow. "If we can measure these [genetic] differences [between responders and nonresponders], we can start to impart that knowledge across the broader communities." —Jef Akst

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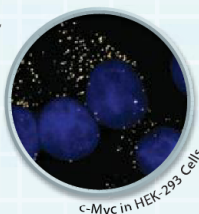
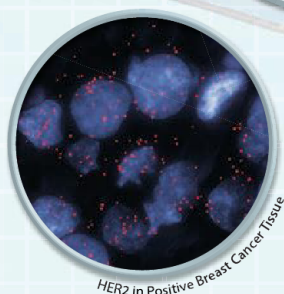
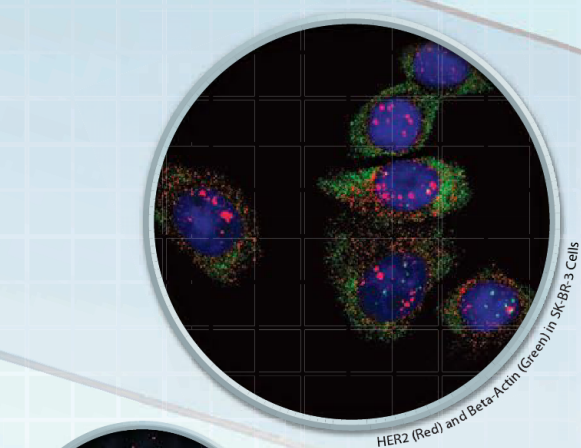
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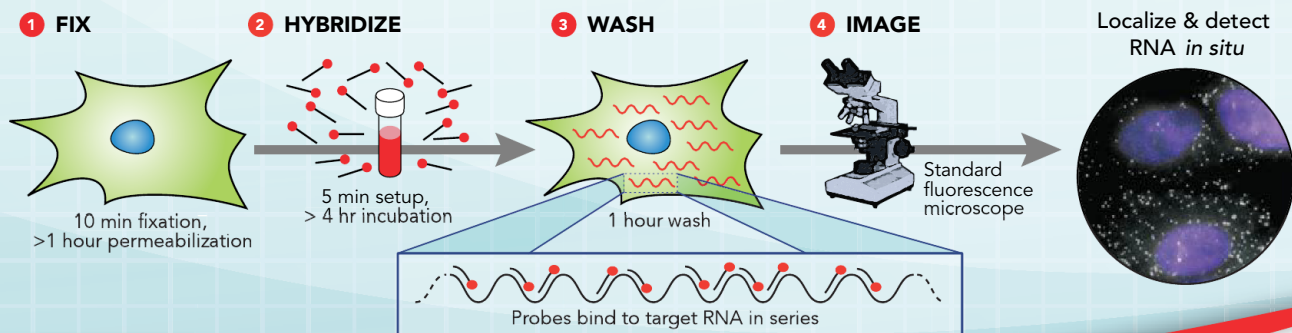
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The Challenges of Precision

Researchers face roadblocks to treating an individual patient's cancer as a unique disease.

BY ADAM MARCUS

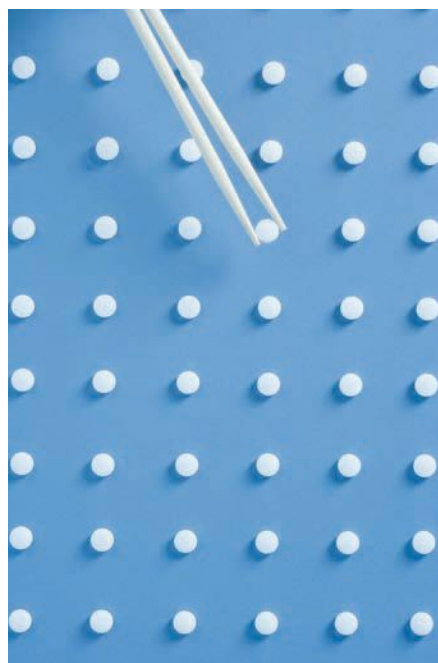
The amount of genetic variation between cancer patients is astounding. Two people diagnosed with cancer in the same organ may, in fact, have two very different diseases. Consequently, we are now in the middle of a transition: we no longer classify cancer based solely on its tissue of origin, but also on the key mutations driving growth and spread of a patient's tumor.

This concept of diagnosing and eventually treating based upon the mutations in the tumor is well known within the cancer research community, and is termed personalized or precision oncology. Precision oncology—and, in broader terms, personalized medicine—has received tremendous attention recently. President Obama proposed a \$215 million investment for the National Institutes of Health focused on precision medicine, of which \$70 million would be allocated to the National Cancer Institute. This type of effort hopefully signifies a new era of research funding that should lead to advances, not just in the understanding and treatment of cancer, but of other diseases as well.

Despite the influx of funding and increased research effort around the world, precision oncology still faces significant challenges. First and foremost, we must understand the biology behind the driving mutations. I have heard arguments along the lines of “if it works, who cares?” In my opinion, this is not the right approach; without understanding the biology and making intellectually informed decisions based on the science of the disease, we will never effectively match the treatment to the disease, which, in essence, is what precision oncology is all about. Strictly employing genomic technologies will not lead to a complete understanding of the biology, and we should therefore not forget

our roots in traditional molecular biology, cell biology, and biochemistry.

Another, related challenge is to use this biology in conjunction with the growing genomic data to develop treatments that target driver mutations. While we know that not every mutation is actionable, we do have excellent success stories such as Gleevec in chronic myelogenous leukemia, ALK inhibitors in lung cancer, and mutant B-RAF inhibitors in melanoma. But mutations still evade us. *Ras* mutations, for example, are widely observed across multiple tumor types, but a successful *Ras* inhibitor has not yet made it to the clinic, despite some promising recent data toward targeting and inhibiting the protein (*Nature*, 503:548-51, 2013). Clearly, we are still dealing with a large gap between discoveries at the lab bench and treatments at the bedside.



A third challenge may in fact be the most ominous: tumor heterogeneity within a single patient. One recent study of lung cancer patients, for example, showed that different masses within a patient could be classified as genetically different tumor types (*Science*, 346:251-56, 2014). This directly impacts treatment, as it indicates that a therapy designed for a driver mutation found in one area would be unlikely to impact those in other regions. The authors state, “The regionally separated driver mutations, coupled with the relentless and heterogeneous nature of the genome instability processes, are likely to confound treatment success in NSCLC [non-small cell lung cancer].”

Nevertheless, genomic approaches are revealing a tremendous amount of information and leading to a new conceptual framework for our understanding of cancer signaling, diagnosis, and treatment. Such methods are also directly impacting patient care, with precision oncology tumor boards composed of genomicists, ethicists, bench scientists, oncologists, and surgeons meeting to discuss the best course of action for a patient given his or her tumor genotype. While these concepts are still in their infancy, they represent a new paradigm for cancer treatment. Ultimately, it is our collective understanding of the science and our ability to communicate this knowledge to our peers and patients that will support the success of truly personalized medicine. ■

Adam Marcus is an associate professor in the Department of Hematology and Medical Oncology at the Winship Cancer Institute of Emory University in Atlanta. Marcus's laboratory studies how cancers invade and metastasize, with the goal of developing treatments that target metastasis.

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Control ALT, Delete Cancer

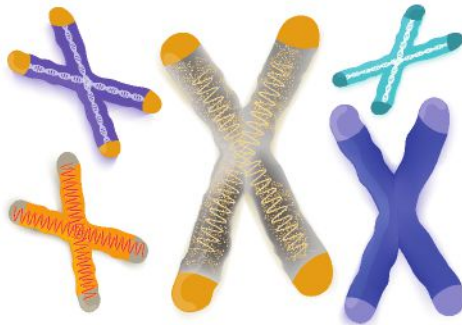
Treating cancer by shutting down the alternative lengthening of telomeres (ALT) pathway

BY HAROLDO SILVA, DAVID HALVORSEN, AND JEREMY D. HENSON

Because age is the largest risk factor for cancer, as the life expectancy of the world's population continues to increase, cancer incidence is projected to rise dramatically. A 2011 report on Global Health and Aging released by the National Institutes of Health and World Health Organization predicts a tripling of the number of people aged 65 or older to 1.5 billion by 2050, and the annual number of new cancer cases is projected to reach 27 million by 2030. Undoubtedly, alleviating the diseases and disabilities associated with an aging global population will require the development of new anticancer approaches to avoid economic and humanitarian calamities.

Cellular immortality is a hallmark of cancers that distinguishes them from normal tissue. Every time a normal somatic cell divides, the DNA at the ends of its chromosomes, called the telomeres, gets shorter. When the telomeres shorten too much, an alarm signal is generated, and the cell permanently stops dividing and enters senescence or undergoes apoptosis. Telomere shortening thus acts as a biological mechanism for limiting cellular life span. Cancer cells, on the other hand, can become immortalized by activating a telomere maintenance mechanism (TMM) that counteracts telomere shortening by synthesizing new telomeric DNA from either an RNA template using the enzyme telomerase or a DNA template using a mechanism called alternative lengthening of telomeres (ALT).

Because the presence of a TMM is an almost universal characteristic of cancer cells, and experimentally repressing these mechanisms results in cancer cell senescence or death, TMMs may be useful targets in treating cancer. Indeed, several therapies targeting the well-described



telomerase-based pathway are in the advanced stages of clinical development. There are currently no ALT-targeted therapeutics, however, largely because this process is less well understood.

In contrast to telomerase-driven telomere lengthening, which does occur in the stem cells of healthy tissues and organs, ALT activity is not found in normal human postnatal tissues—a fact that would allow for more-effective dosing with minimal side effects. And based on the conservative estimate that 10 percent of cancers employ an ALT strategy to achieve cellular immortality, there are about 1.4 million new cases and 820,000 deaths globally due to ALT cancers every year. These include some of the most clinically challenging cancers to treat, such as pediatric and adult brain cancers, soft tissue sarcoma, osteosarcoma, and lung cancers. Clearly, targeting ALT is a very attractive strategy in the development of novel cancer therapies.

Moreover, as with any cancer therapy, there is the potential for development of resistance against telomerase-based strategies to defeat cancer. Several in vitro studies report that human cancer cell lines can overcome loss of telomerase by activating ALT. Inhibition of telomerase in mice has also been shown to cause existing tumors to activate the

ALT pathway as telomeres shorten with treatment. Importantly, some human tumors have both ALT and telomerase activity, although not necessarily simultaneously within the same cell. It is therefore plausible that telomerase-dependent cancer treatments will introduce selective pressure in tumors to activate the ALT pathway and/or to select for cells already using ALT within the tumor, which makes development of ALT-specific therapies imperative for the success of anticancer approaches targeting TMMs.

ALTering cancer

Telomeres consist of repetitive hexameric DNA sequences (5'-TTAGGG-3') intertwined around a group of proteins collectively known as the shelterin complex. These structures form a protective cap at the ends of every chromosome. Because all telomeres contain the same repetitive DNA, new telomeric DNA can be generated by copying another molecule that contains the telomeric sequence via homologous recombination.

One of the hallmarks of ALT cancer cells is that they contain large amounts of telomeric repeat DNA that are dissociated from chromosomes. These extrachromosomal sequences, which can be linear or circular, may serve as the template for ALT-mediated telomere elongation. The template can also be another telomere, such as the end of a sister chromatid, or even the same telomere looping back on itself.

ALT activation is associated with loss of important chromatin remodeling proteins at the telomeres, possibly allowing the DNA damage response and homologous recombination machinery to initiate ALT activity. ALT activation is also

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accompanied by abnormal association of some nuclear proteins with telomeres, as well as recapitulation of some recombination pathways used in meiosis. ALT and ALT-like mechanisms seem to be evolutionarily conserved from yeast to worms to humans. In fact, some yeast strains contain mitochondria with linear genomes and telomeric-like repeat sequences at their termini. These mitochondrial genomes maintain their telomeres via a recombination-based mechanism resembling the one found in human cells employing ALT. This raises the intriguing possibility that ALT evolved out of necessity to maintain stability in linear genomes after they transitioned from circular form, before the emergence of telomerase as a TMM.

Studies in mice suggest that the recombination-mediated ALT mechanism is the main driver behind the telomere lengthening observed in the early stages of embryonic development. Telomere lengthening by ALT may also play a role in the reprogramming of murine somatic cells into induced pluripotent stem cells and the generation of embryonic stem cells by somatic cell nuclear transfer. So cancers that activate the ALT mechanism may be tapping into a key biological phenomenon to achieve immortalization and cause harm. Therefore, a better mechanistic understanding of the ALT pathway could lead not only to more efficacious cancer therapies, but also to a better grasp of genomic biological evolution.

The development of ALT-targeted therapies is quite challenging, however. Unlike the telomerase pathway, the ALT mechanism has no known specific enzyme activity, and all the enzymes identified to date that play a role in ALT are also essential to normal cellular pathways. The presence of ALT activity has often been inferred from detecting telomere-related phenotypes, such as long and heterogeneous telomere length distributions or ALT-associated promyelocytic leukemia nuclear bodies (APB), which indicate the abnormal presence of telomeres inside

There are about 1.4 million new cases and 820,000 deaths globally due to ALT cancers every year.

a complex formed from otherwise ubiquitous nuclear proteins. (See "Going Long," *The Scientist*, April 2014.) These markers are not entirely satisfactory, as they can sometimes yield inaccurate results and are not practical for high-throughput applications or clinical laboratories.

A key step towards the development of ALT-targeted cancer therapeutics and diagnostics was the discovery of the first ALT-specific molecule, the telomeric C-circle (*Nat Biotechnol*, 27:1181-85, 2009). C-circles are an unusual type of circular DNAs that are created from telomeres. The level of C-circles in cancer cells accurately reflects the level of ALT activity, and this biomarker can be found in the blood of patients who have bone cancers positive for ALT activity. The development of the C-circle assay as well as improvements to the APB assay could, in the near future, make it feasible to perform robust high-throughput screenings to search for modulators of the ALT pathway, which will greatly speed the pace of discovery in this field. Further research will no doubt lead to a more complete mechanistic understanding of this phenomenon and to the first ALT-specific therapies against cancer. Controlling ALT could very well help delete the burden of cancer from society. ■

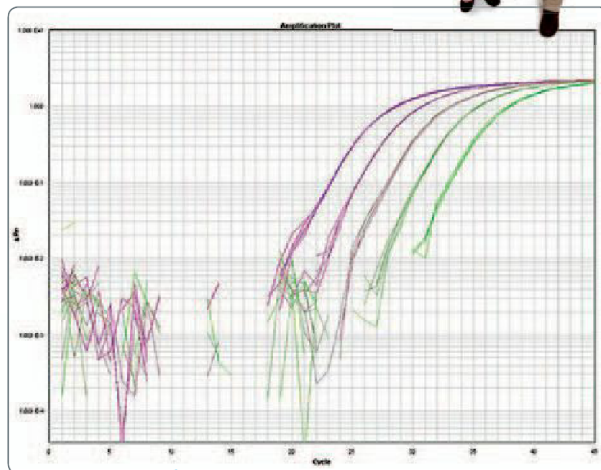
Haroldo Silva is lead scientist in the oncology research group, OncoSENS, in which David Halvorsen is a research associate, at the SENS Research Foundation. Jeremy Henson is a practicing clinician and the head of the Cancer Cell Immortality Group at the Prince of Wales Clinical School at the University of NSW in Australia. Henson discovered the C-circle biomarker and invented the C-circle assay when he was in the Red-dell laboratory at the Children's Medical Research Institute in Sydney, Australia.



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Bursting Cancer’s Bubble

Scientists make oxygen-filled microbubbles designed to increase tumor sensitivity to radiation.

BY RUTH WILLIAMS

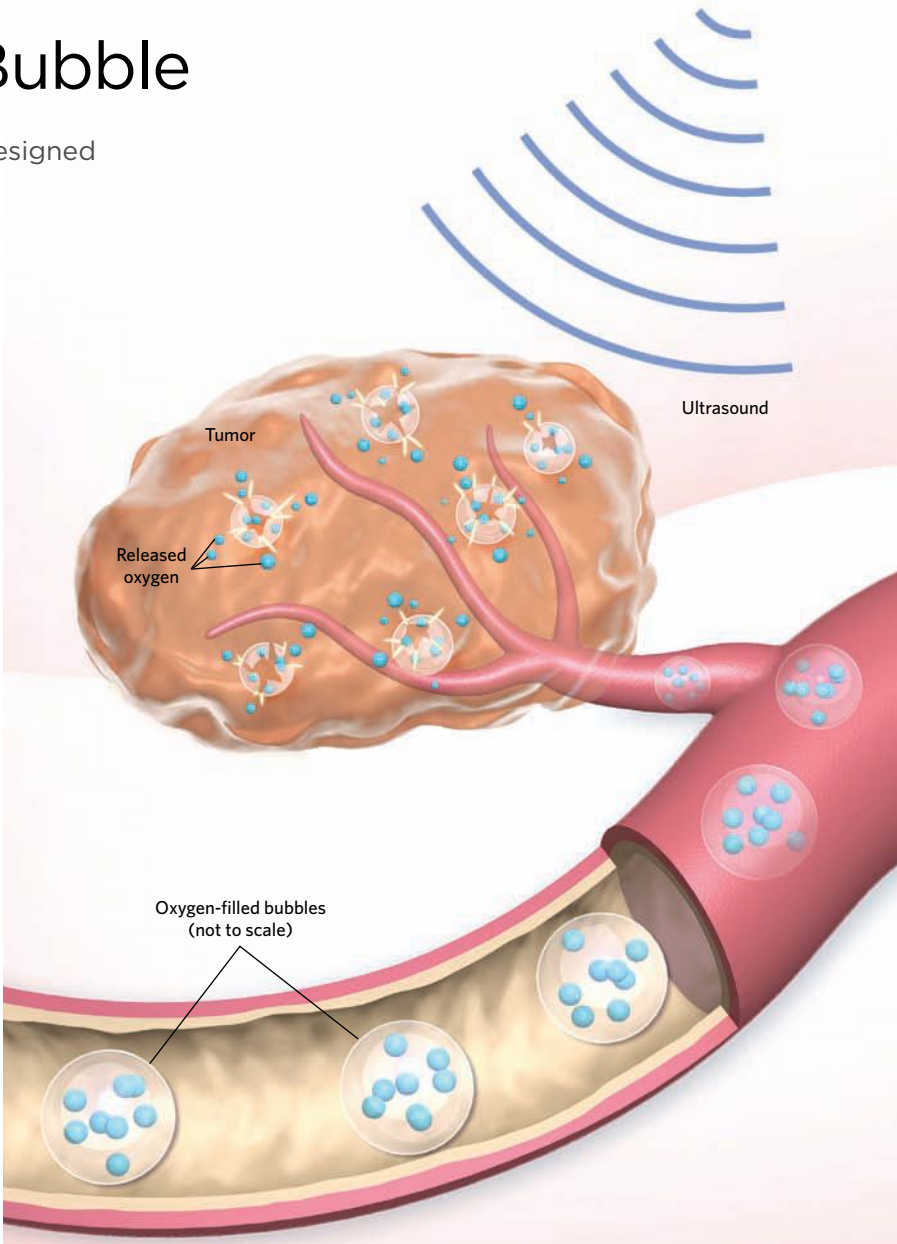
The rapid growth and high metabolic activity of a tumor can cause its cells to become hypoxic due to an insufficient blood supply. Somewhat counterintuitively, however, these sickly, oxygen-starved cells are actually harder to kill with radiation treatment than healthy tissue. Researchers are thus investigating ways to boost oxygen levels in tumors so the cells can be nuked more effectively.

One idea has been to inject tiny oxygen-filled bubbles into a patient’s bloodstream. The bubbles would then enter the tumor—where blood vessel walls tend to be leaky—and burst to locally release the oxygen, explains John Eisenbrey of Thomas Jefferson University in Philadelphia.

Such bubble-based treatment is not as strange as it might sound. Indeed, the ultrasound community has been using gas-filled microbubbles as a contrast agent to improve imaging for some time. And local rupturing of these bubbles by high-intensity ultrasound is being investigated as a drug-delivery method.

With ultrasound, “you can see where these [microbubbles] go and then you can disrupt them at the site you want,” explains Steve Feinstein of Rush University Medical Center in Chicago.

Eisenbrey and others are thus co-opting the technique for oxygen delivery to tumors. In a recent experiment on two mice with breast tumors, Eisenbrey showed that oxygen-containing microbubbles were visible in the tumors and, upon rupture, were capable of increasing detectable oxygen by up to 30 mmHg. That’s a significant amount, explains Eisenbrey, because “if you can just deliver between 10 and 15 mmHg of oxygen to cells, you can make them twice as sensitive to radiation.” Eisenbrey’s next step, he says, will be to see whether the oxygen boost does indeed improve the effectiveness of radiation therapy. (*Int J Pharm*, 478:361-67, 2015)



TINY BUBBLES: Oxygen-filled bubbles—3 μm in diameter—are delivered to a tumor via blood vessels. Sonication at the site of the tumor causes the bubbles to explode, releasing oxygen. Enriching tumors with oxygen is thought to enhance radiation treatment.

AT A GLANCE

O ₂ DELIVERY	DIAMETER	SHELL MATERIAL	OXYGEN RELEASE IN SOLUTION	OXYGEN INCREASE IN TUMORS UPON SONICATION
Nanobubbles*	Approximately 500 nm	Dextran	Rapid release prior to sonic rupture. Twofold increase after.	Not tested
Microbubbles	Approximately 3 μm	A mixture of two surfactants: sorbitan monoester and water-soluble vitamin E	Negligible release prior to sonic rupture. Approximately threefold increase after. Localized release can thus be tightly controlled.	Approximately 20-30 mmHg above hypoxic baseline

*(*Int J Pharm*, 381:160-65, 2009)

Resisting Cancer

If one out of three people develops cancer, that means two others don't. Understanding why could lead to insights relevant to prevention and treatment.

BY GEORGE KLEIN

Cancer is a scourge of humanity, and one that will affect more and more people as life spans increase. Approximately one in three people is struck by neoplastic disease in his or her lifetime. But, the other side of that coin is that two out of three people remain unaffected. Even the majority of heavy smokers, who bombard their lungs with carcinogens and tumor promoters over many years, remain cancer free. Naturally, the suffering of cancer patients and their families has inspired researchers to study the cellular changes unique to cancer and the genetics of cancer susceptibility. The genetics of cancer resistance, as a topic in its own right, has remained largely unexplored.

Pathologists have shown that virtually all men age 60 or older have microscopic prostate cancer when examined at autopsy. Most of these microtumors

Metazoan evolution has led to many adaptations that protect species across the animal kingdom from outlaw cells.

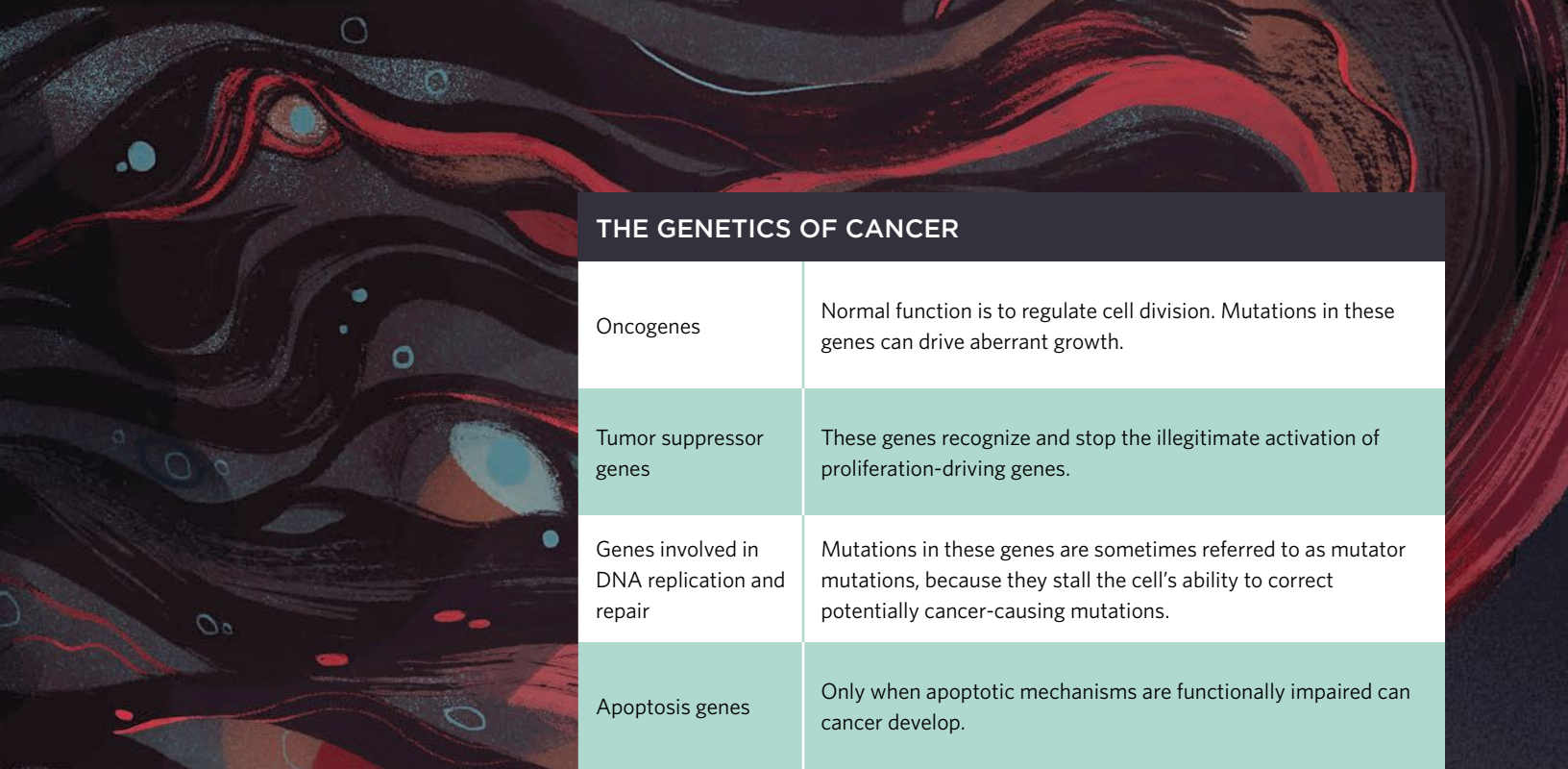
never develop into cancer, however. It is also known that disseminated cancer cells are present throughout the body in most cancer patients, but only a small minority of these cells develop into secondary tumors. The rest are kept under control by the body.

Indeed, metazoan evolution has led to many adaptations that protect species across the animal kingdom from outlaw cells. Immune surveillance plays a major role in the defense against virus-associated tumors, where the virally encoded transforming proteins provide readily

recognizable foreign targets. But nonviral tumors, which are composed of aberrant host cells, do not provide such targets, and the immune response is suppressed by defenses against autoimmune reactions. Rather, we now know that the main safeguards against cancer are not immunological at all.

Several cancer-resistance mechanisms appear to have evolved to maintain cellular or genomic integrity. For example, normal stroma, the connective material that supports the cells of a tissue, appears to inhibit cancer growth. Other resistance mechanisms include DNA repair, suppression of oncogene activation, tumor-suppressor genes, epigenetic stabilization of chromatin structure, and apoptosis. These mechanisms are well-studied, and each provides a potential road map for prevention and treatment.





THE GENETICS OF CANCER

Oncogenes	Normal function is to regulate cell division. Mutations in these genes can drive aberrant growth.
Tumor suppressor genes	These genes recognize and stop the illegitimate activation of proliferation-driving genes.
Genes involved in DNA replication and repair	Mutations in these genes are sometimes referred to as mutator mutations, because they stall the cell's ability to correct potentially cancer-causing mutations.
Apoptosis genes	Only when apoptotic mechanisms are functionally impaired can cancer develop.

Genetic oversight

Abnormal cell division is a hallmark of tumor cells. The mutated genes responsible for this proliferation are called cancer genes, or oncogenes, but this is a misno-

function is to snip out molecular DNA lesions caused by exposure of skin epithelium to ultraviolet light. Patients with xeroderma pigmentosum must protect themselves from sunlight all their lives,

the body, the B cell producing it will be stimulated to expand clonally. But those B lymphocytes whose product does not bind a corresponding antigen must die to avoid clogging the system. Potential cancer cells that upset tissue integrity also elicit signals that initiate apoptosis. Cancers cannot develop unless the apoptotic mechanism is functionally impaired.

The genes that contribute to cancer development by mutation, illegitimate activation, or—conversely—inactivation or loss are practically identical in all mammals.

mer. Their normal function is not to cause cancer but to participate in the regulation of normal cell division. Meanwhile, more-appropriately named tumor suppressor genes recognize the illegitimate activation of proliferation-driving genes and hit the brakes. Once again, mutations that disrupt this function can lead to cancer.

Tumor risk can also be influenced by mutations in genes that control the fidelity of DNA replication, the efficacy of DNA repair, and the checkpoint controls involved in DNA synthesis and chromosomal mechanics. Xeroderma pigmentosum, for example, stems from a specific DNA repair deficiency. The predominant form of this condition is caused by a recessive mutation in one of the essential components of the nucleotide excision repair (NER) system, whose main

but they nevertheless develop multiple skin carcinomas, and in about one-third of cases, also progressive neurological abnormalities. This highlights the paramount importance of DNA repair as a frontline surveillance mechanism.

Yet another group of genes associated with cancer formation regulates programmed cell death, or apoptosis. The normal function of the proteins expressed by these genes is to recognize superfluous, damaged, aged, or aberrant cells that must be eliminated. The importance of apoptosis is exemplified by the vertebrate adaptive immune system, in which B lymphocytes, as they differentiate from their precursor cells, rearrange the DNA of immunoglobulin genes to generate a vast number of potential antibodies. If an antibody happens to recognize an antigen in

The genes that contribute to cancer development by mutation, illegitimate activation, or—conversely—inactivation or loss are practically identical in all mammals. Many of them are even conserved among distant groups, such as *Drosophila*, *C. elegans*, and yeast. Moreover, these cancer-associated genes mutate at similar frequencies across species. Therefore, it might have been expected that the blue whale, which has 10^{17} cells, would develop cancer more often than the mouse, with 10^9 cells, or humans, with 10^{13} . This, however, is not so. In fact, blue whales rarely get cancer, and when they do, they seem able to live a full life span with it. The fact that the frequency of tumors does not increase with the total number of cells that make up an animal suggests that some sort of systemic control is preventing cancer development. Even typical laboratory animals vary in their cancer-proneness. Mice, rats, and hamsters are relatively cancer-

prone, whereas rabbits and guinea pigs are relatively resistant. At the far end of the spectrum, two species of mole rat have been found to be completely cancer free, despite living relatively long lives. (See “Cancer-Free for Life” at right.)

Is there any genetic variation among humans with regard to cancer resistance? There is no proof of this, but it is highly likely that it occurs. In genealogically well-mapped Icelandic families, there are several cases of six or more long-lived siblings with no cancer incidence and whose parents did not die of the disease. It is not possible, however, to distinguish between the existence of genetically resistant families and the occurrence of cancer-free families purely by chance.

Interestingly, the sharp increase in human tumor incidence with age is followed by a decrease around 80 to 85 years. Centenarians rarely develop new cancers. The most probable reason is that cancer-susceptible individuals in the population have already died off. Freedom from cancer may be a necessary, but in itself insufficient, precondition to reach very old age.

People with a type of dwarfism called Laron syndrome represent another documented example of cancer-resistant humans. Found in an Ecuadorian population with frequent cousin marriages, Laron patients have no problem with skeleton, cartilage, or joint development and, in contrast to other dwarfs, have very high levels of growth hormone. However, their cells lack a receptor required for the functional activity of the hormone.¹ An unusual mutation in the growth hormone receptor gene appears to be responsible for cancer resistance in this case.

There thus appear to be diverse mechanisms that protect against cancer. In addition to driving the proliferation of cancer cells, mutations or epigenetic changes may influence the ability of the cells to invade bordering tissues, to give rise to systemic metastasis, or to resist treatment. Notably,

CANCER-FREE FOR LIFE

Two species of mole rat serve as exceptional examples of cancer resistance. The blind mole rat (BMR) and the naked mole rat (NMR), which live up to 20 and 30 years, respectively, never develop the disease. Understanding the molecular mechanisms that afford these animals such powerful protection against cancer could inform the fight against human cancers.

The NMR, a eusocial species that lives in highly organized matriarchal societies, has to force its way through narrow and often sinuous underground tunnels. The connective tissue in its skin contains a high-molecular-weight form of hyaluronic acid (HA) that makes the animal's skin malleable like model clay. The corresponding HA in mice and humans has less than one-fifth the molecular weight. The heavy form of HA that occurs in the NMR prevents the transformation of normal cells into cancer cells; only after it has been removed can the NMR's cells be transformed into cancer cells (*Nature*, 499:346-49, 2013).

Moreover, NMR fibroblasts exhibit an unusual form of contact inhibition. Mouse and human fibroblasts inhibit their own continued proliferation when they bump up against other cells. NMR fibroblasts do this much earlier, when cells barely touch one another. When the cells come into contact, p16, a protein involved in causing growth arrest, is upregulated. This is not seen in the late contact inhibition of human and mouse fibroblasts, where another growth-arrest protein, p27, is upregulated.

In contrast to the NMR, the BMR is a solitary, nonsocial, and aggressive species. And its cancer resistance mechanisms differ from the NMR's. The BMR does not have heavy HA or early contact inhibition, but when its fibroblasts grow to confluence they

commit collective suicide by releasing a type of interferon (*PNAS*, 109:9392-96, 2012). Once again, evolution appears to have devised diverse solutions to keep rogue cells in line, and in these two cancer-free species, those mechanisms may be key to their remarkable longevity.



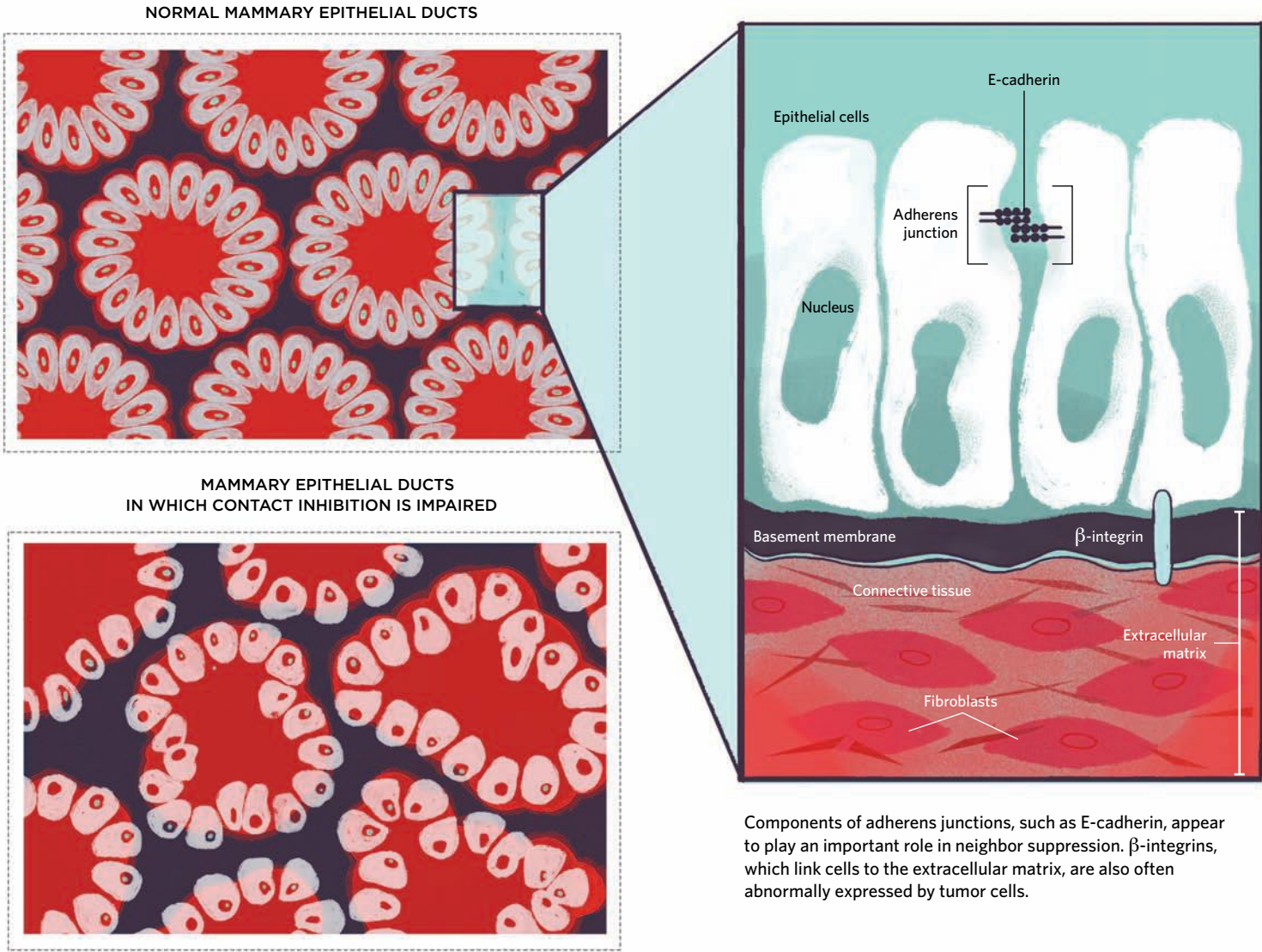
metastasis-favoring mutations may occur at a very early stage of tumor development, long before there are sufficient numbers of tumor cells to allow the manifestation of this property.

Neighbor suppression
In the 1960s, British cancer biologist Michael Stoker made an unexpected observation: when he explanted a certain number of tumor cells into a dish with

appropriate nutrients, he obtained about 100 small, growing tumor-cell colonies. But when he mixed the same number of tumor cells with normal fibroblasts, the number of growing colonies was reduced

HOW NEIGHBORS FIGHT CANCER

Resisting overproliferation of rogue cells in the body involves a complicated interplay between cancer-related genes, the microenvironment, and the tendency of tissues to build normal structures. Among the body’s defense mechanisms is a phenomenon called contact inhibition or neighbor suppression, in which cells sense the density of their population and stop growing when a critical level is reached.



Components of adherens junctions, such as E-cadherin, appear to play an important role in neighbor suppression. β-integrins, which link cells to the extracellular matrix, are also often abnormally expressed by tumor cells.

When contact inhibition is impaired, cells lose their ability to properly sense their population density and stop dividing when critical levels are reached. This can lead to the overproliferation of cells and formation of cancerous masses.

by 99 percent. It seemed as though normal neighbor cells had suppressed the ability of the tumor cells to make colonies, a phenomenon later termed contact inhibition or neighbor suppression. (See illustration on opposite page.)

Adherens junctions, protein complexes that occur at cell-cell points of contact in epithelial and endothelial tissues, appear to play an important role in this phenomenon. E-cadherin, an essential mediator of adhesion in these junctions, is downregulated in most epithelial tumors, usually by methylation of the gene's promoter region. Reexpression

used to grow in size and make proteins, they could not divide. Fibroblasts taken from cancerous prostates, on the other hand, hardly inhibited cell proliferation at all.⁵ In fact, these so-called cancer-associated fibroblasts (CAFs) may even support the growth and survival of adjacent tumor cells. Looking more closely at gene expression in these fibroblasts, we identified genes activated by inflammation or wound healing, a finding in line with the established fact that inflammation can favor the growth of cancer cells that have been inactive, often for long periods of time.

Interestingly, the sharp increase in human tumor incidence with age is followed by a decrease around 80 to 85 years. Centenarians rarely develop new cancers.

of E-cadherin appears to suppress the transformed phenotype in isolated cancer cells, whereas blocking E-cadherin function enhances invasion.²

Other structural components of the cell membrane may be similarly involved in microenvironmental inhibition of tumor growth. β -integrins, subunits of transmembrane receptors that link cells to the extracellular matrix and to each other, and that also play key signaling roles in tissue organization, are often abnormally expressed by tumor cells. Targeting tumor-associated β -integrins with antibodies inhibits tumor growth.³ Components of the Notch signaling pathway, which regulates cell differentiation and proliferation, are another example. Deletion of Notch receptors or their ligands from the basal layer of mouse epidermis can lead to epidermal hyperplasia and skin tumors.⁴

A few years ago, we reexamined the role of neighbor suppression in cancer resistance by labeling human prostatic carcinoma cells with green fluorescent protein and plating the cells alongside normal fibroblasts from different tissues. Fibroblasts from skin strongly inhibited tumor growth. While the tumor cells were not killed, and contin-

Resetting cancer

Another defense against cancer was discovered in the 1970s by American biologist Beatrice Mintz. Working with a highly malignant line of teratoma cells, Mintz found that adult mice subcutaneously inoculated with the cells all succumbed to teratomas. When the teratoma cells were introduced into very early embryos, however, no malignancies developed. The introduced cells simply became part of the organism, participating in the normal development of the mouse and contributing to many tissues of the embryo. The normalized tumor cells even contributed to the production of sperm in the recipient mouse.⁶

This normalization of embryonic tumor cells in the early developmental environment is the most extreme example of the differentiation of malignant into normal cells. There are many other, less spectacular examples in the later literature. Highly malignant murine leukemia cells can be induced to differentiate into nonmalignant cells by natural or artificial substances. One form of human leukemia, acute promyelocytic leukemia, is routinely cured in this way: the combination of a naturally occurring signal receptor and an empirically identified chemical

compound, genistein, induces the cancerous cells to differentiate from a granulocyte precursor into a mature myelocyte that can no longer divide.⁷ Although similar examples are still relatively few, they suggest that most and perhaps all tumors could be induced to differentiate and stop growing if one could find the right combination of inducing substances.

Evolution has provided us and other animals with multiple mechanisms that stop normal cells from escaping into uncontrolled division. If they nevertheless escape, the cell mobilizes multiple mechanisms to stop the outlaw cell and preserve the normal organization of tissues. These mechanisms can nip life-threatening malignant growths in the bud. Only after the normal tissue neighborhood has been corrupted by the tumor cells so that it no longer inhibits, but rather stimulates, malignant growth can the cancer sail forth uninhibited. ■

George Klein studies Epstein-Barr virus, oncogenes, and tumor-suppressor genes in the Department of Microbiology, Tumor and Cell Biology at the Karolinska Institute in Sweden.

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MY MIGHTY MOUSE

Personal drug regimens based on xenograft mice harboring a single patient's tumor still need to prove their true utility in medicine.

BY MEGAN SCUDELLARI



o two cancers are alike. But tumors, across the myriad permutations of the disease, share one characteristic: unpredictability. That unpredictability

includes how the tumor will react to treatment. Because of the toxicity of chemotherapy, no patient wants to find out by trial and error how his or her particular tumor will respond to a given drug. So doctors have long sought ways to identify which therapies will be most beneficial—before actually treating the patient.

In the early 1980s, researchers commonly tested drug efficacy against patient tumor cells in a petri dish, but the method often failed to predict treatment success. At the University of Freiburg in Germany, oncologist Heinz-Herbert Fiebig had a different idea. Fiebig had been implanting pieces of human tumors into mice with compromised immune systems, which do not reject foreign tissue. He surmised that human tumor cells grown in a living, breathing mouse, instead of a dish, would be more likely to predict the drug response of an actual human tumor.

Over eight years, Fiebig tested his idea by transplanting fragments of more than 400 human tumors, each about the size of the head of a pushpin, just below the skin of immunocompromised mice. Using 80 of the successful tumor xenografts—from the Greek “xenos” meaning “foreign”—he compared how the mouse’s tumor responded to a drug or drug combination with the treatment response of the corresponding human patient. Of 21 patients who responded to a particular drug or drug combination, their xenograft mice correctly predicted the out-

come 90 percent of the time. Similarly, of 59 patients who did not respond to treatment, the mice correctly reflected such tumor resistance in 96 percent of cases.¹

“We found the system was very predictable and very useful for screening drugs,” says Fiebig. But the idea of building mouse models for individual patients to guide treatment was impractical, he found. The xenografts often grew too slowly to provide timely results; more than half of the 80 patients needed treatment before their mice yielded actionable data. Sometimes the grafts did not grow at all; Fiebig obtained successful engraft-

Some see mouse avatars as the epitome of personalized medicine. Others argue that they are far from ready.

ments only 50 percent of the time. Simply making and maintaining the mice was also prohibitively expensive.

In a 1988 paper summarizing his findings, Fiebig concluded that xenograft mice were wonderful models for broadly testing new drugs against human tumors, but they “cannot be used as a clinical routine method” for predicting patient treatment.¹ The idea of using xenograft mice as personal avatars for cancer patients was discarded. But not forgotten.

More than two decades later, avatars are making a comeback, driven largely by New Jersey-based Champions Oncology. The company will develop a personalized mouse model for any cancer patient who can afford the service, which is not covered by insurance.

“The idea of really trying to match the perfect drug to the perfect patient is something we’re all really trying to invest more

effort into,” says Keren Paz, chief scientific officer of Champions, which has ongoing collaborations with numerous universities and hospitals.

These days, mice grafted with human tumors, known as patient-derived xenograft (PDX) mice, are common in cancer research laboratories. In academia, researchers often create the mice as live mammalian models of whatever type of cancer they are studying. Additionally, organizations such as Freiburg-based Oncotest, a company founded and directed by Fiebig, and the Jackson Laboratory in Bar Harbor, Maine, provide access to a wide range of PDX mice made from donated tumor tissue. After growing the donated tissue in mice, they cryopreserve some of it for future use, and offer drug-testing services to researchers and pharmaceutical companies. Oncotest, for example, provides drug-testing services to 16 of the 20 largest pharmaceutical companies, using a library of more than 350 PDX mouse models.

But while most PDX mice are used as general models of human cancer in the laboratory, others seek to use them as Fiebig originally hoped—as avatars to guide customized patient care. Indeed, some see mouse avatars as the epitome of personalized medicine and point to the handful of success stories in which such mice have revealed new treatment options for patients who’d failed to respond to traditional therapies. Others, however, argue that PDX mice are far from ready to be used as a routine part of clinical care.

“No one has really shown that [using PDX mice to evaluate drug responses] actually changes and improves outcome,” says Judy Boughey, an oncologist and researcher at the Mayo Clinic in Minnesota, who is using PDX mice in a breast cancer study. “[The technique] requires

further evaluation and validation before it's ready for everyone to just go and have their tumor xenografted." Indeed, PDX mice have yet to undergo a controlled, prospective clinical trial to compare avatar-based predictions with physician recommendations, she notes.

"This platform has tremendous potential to advance precision oncology, where we can use a patient's tumor DNA to really tailor therapy, but we have to be really rigorous about the science of developing the platform," agrees Carol Bult, director of the PDX/Cancer Avatar Program at the Jackson Laboratory.

If you made a prediction from a human and it works in a mouse avatar, now the chances of it working back in a human are much, much higher.

—Andrea Califano, Columbia University

Corralling cancer

Fueled in part by interest generated by Champions, more and more scientists now use PDX mice to study the potential cancer drug responses of individual patients. Their hope is that these personalized cancer models might allow doctors to more effectively treat cancer based on the specific molecular makeup of a patient's tumor, achieving the ideal of precision medicine, as touted by President Obama in this year's State of the Union address. (See "The Challenges of Precision" on page 31.)

At Columbia University in New York City, systems biologist Andrea Califano and pediatric oncologist Andrew Kung are using patient-specific PDX mice to assess breast, neuroendocrine, and brain cancers. First, the team sequences DNA and RNA from tumor biopsies and inputs that information into a computer model that identifies a handful of dysregulated proteins necessary for survival of the tumor cell, what Califano calls the "master regulators." The computer then predicts three drugs and three drug combinations likely to target those master regulators. Instead of testing the drugs in cultured cells as he might have done in the past, Califano tests them in PDX mice, developed individually

for each patient in the trial. "If you made a prediction from a human *and* it works in a mouse avatar, now the chances of it working back in a human are much, much higher," says Califano.

The goal, for now, is to prove that the drug predictions from the computer model successfully treat their corresponding mouse tumors. So far, they do: the team identifies a drug or combination therapy that works in the mice almost 100 percent of the time, says Califano. The next step will be to use the computer model results and PDX mice in a clinical trial to guide patient care by sharing the results with physicians.

Researchers at the Mayo Clinic are taking a similar approach, using PDX mice to match genetic characteristics of an individual's tumor to specific drugs. From 2012 to 2014, the Breast Cancer Genome-Guided Therapy (BEAUTY) study enrolled 140 women with high-risk breast cancer. Each of the patients underwent tumor biopsies before, during, and after a 20-week regimen of chemotherapy. A team led by Matthew Goetz and Boughey used the biopsies for sequencing and to create PDX mice for each participant. The researchers are now linking the genetic information with the human drug responses and exploring drug options in the chemotherapy-resistant PDX mice. Once again, the next step will be to see if the results beneficially impact patient care, Goetz says.

Before that happens, however, there are still a handful of challenges that must be overcome. As Fiebig found in his early work, tumor samples do not always successfully graft onto the mice. Boughey and her colleagues achieved only about a 40 percent graft rate, she notes. Not surprisingly, the more aggressive a tumor, the more likely it is to engraft—probably because those cancer cells are particularly adept at growing and spreading.

Mayo's Prostate Cancer Medically Optimized Genome-Enhanced Therapy (PROMOTE) study, inspired by BEAUTY, has had even more difficulty getting grafts of prostate cancer to take hold in mice. Since the study began in June 2013, only 6 of 80 total tumor grafts, or about 7 percent, have been successful.

"There is some talk of going into 3-D cell models rather than xenografts, because it is so difficult and challenging to grow [the transplanted tumors]," says Manish Kohli, the Mayo Clinic oncologist leading the PROMOTE study. "We're probably going to have to make a call by midsummer."

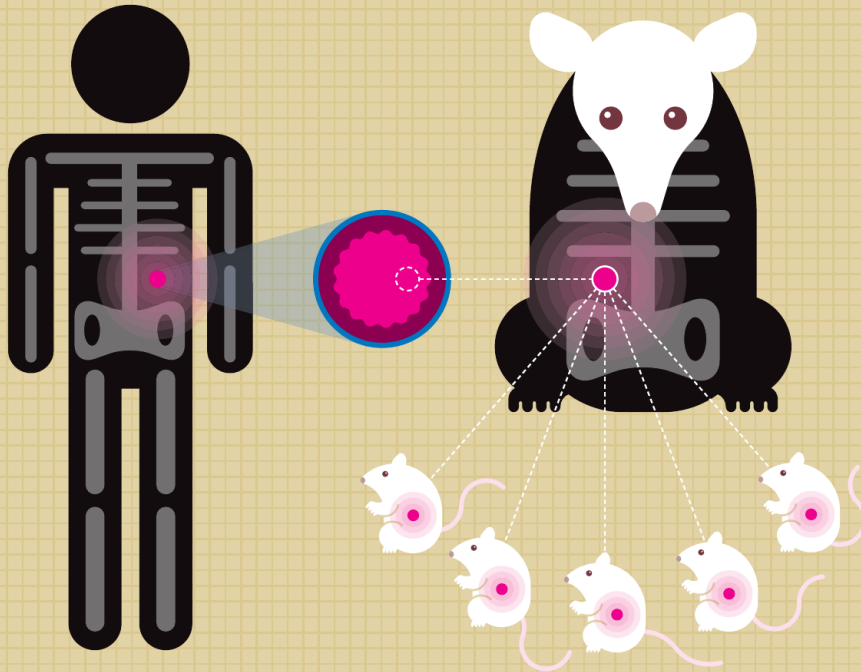
Even if the grafting challenge is overcome, the cost of developing individualized PDX mice still stands as the reigning barrier to their widespread use. First, a single xenograft mouse is not enough. To make a model system, a piece of a human tumor is implanted and grown in several immunocompromised mice, then harvested, fragmented, and implanted again in a larger set of mice that are treated with a variety of drugs and drug combinations. The cost of creating and maintaining the mice, plus doses of expensive drugs, can put the price tag of a PDX experiment at tens of thousands of dollars—for just a single patient's tumor.

Even using PDX mice in government-funded basic research can be cost-prohibitive. Bult, of the Jackson Laboratory, recognizes that mice are not always the right option. The Jackson Laboratory maintains a resource of more than 300 PDX mice developed from a range of cancers. The mice are used in numerous studies at the laboratory as a preclinical platform to test novel therapeutics against specific tumor types, but in some cases the expense is not appropriate for a study. "We are looking at cell-based and 3-D culture-based methods that would allow us to get to the same endpoint for some drugs faster and cheaper," says Bult.

But while 3-D cell cultures are good at predicting a tumor's drug resistance, notes Fiebig, whose company, Oncotest, offers such testing in addition to xenograft mice, they are not as sensitive to detecting what

CANCER AVATARS

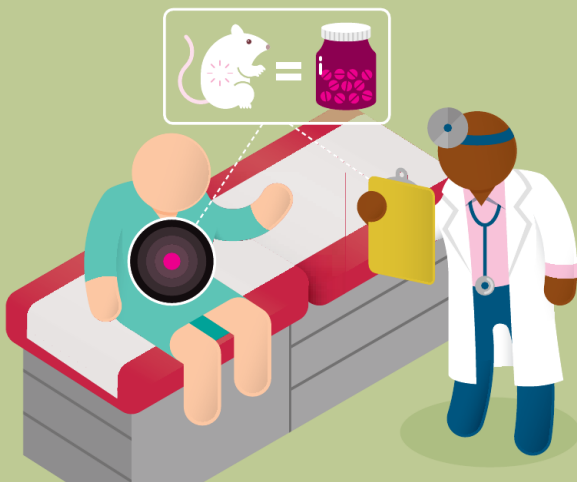
Patient-derived xenograft (PDX) mice provide arguably the closest model to human cancer available without using humans themselves. PDX mice are derived from donated human tumor tissue and may be used for biomarker-driven cancer research, preclinical drug testing, or to predict the drug responses of a specific patient's tumor.



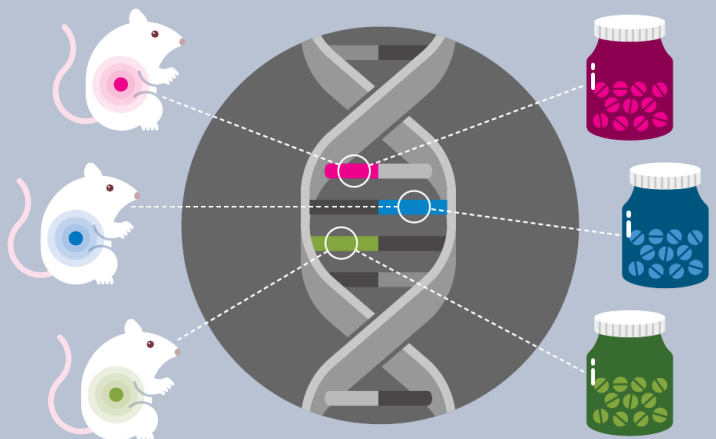
To develop a PDX mouse model, a researcher grafts a bit of a tumor into a handful of mice. Tumor sections from a successful graft expand, then are harvested, divided, and regrafted into many more mice, providing a large number of animals on which to test a variety of drugs or drug combinations.



For individualized mouse avatars sold by Champions Oncology directly to patients, treatments that show success in shrinking the mice's tumor may then be attempted in the patient, with the guidance of an oncologist. This strategy will soon be tested in clinical trials to see if drug predictions from the mice have a positive impact on patient outcomes.



More commonly, academic and pharmaceutical researchers use donated tumor tissue to create PDX mice with a variety of cancers for preclinical drug screening. In this case, researchers analyze donated tumor tissue using whole genome or exome sequencing, then test a drug in question against mice engrafted with that tissue. This research is helping to guide early-stage drug development for specific subtypes of cancer based on genetic markers.



drugs will kill a tumor in the first place; 3-D assays are around 60 to 70 percent accurate for predicting responsiveness, as compared with 90 percent for PDX mice.

The patient's Champions

For those who can afford them, PDX mice are commercially available from Champions Oncology, which boasts a much higher graft-acceptance rate than academic labs—ranging from 25 percent for less aggressive cancers up to 90 percent for highly metastatic cancers, according to the company. Johns Hopkins University's David Sidransky, chairman of the company's board of directors, attributes that success to optimization of the grafting process, including restricting the amount of time between surgery and implantation in the mouse. The company has labs around the world, from London to Israel to Singapore, to reduce the amount of time a given tumor sample spends on a plane, for example.

The growing process takes three to six months, says CSO Paz, and the xenografts, which the company has dubbed

"TumorGrafts," are routinely analyzed using exome and RNA sequencing to confirm that they match the genetic profile of the original tumor. "We see they are nearly identical," says Paz. "It definitely keeps a high level of fidelity; . . . you can trust them to reflect sensitivity to drugs."

In a handful of anecdotal successes, the TumorGrafts have demonstrated their value. Fifty-year-old Evan Rose thought he had beat cancer in 1985, when he was successfully treated for metastatic testicular cancer. But 22 years later, ear pain led his doctors to discover a large mass in his neck. He was eventually diagnosed with a rare sarcoma called ossifying fibromyxoid tumor. Only about 200 cases have ever been recorded.

With such a rare cancer, there was no standard drug therapy for Rose's condition. "I don't know how many different chemos I went on. We'd start one, a couple months later do a CT scan, see it wasn't working, and start another," recalls Rose, an urban designer and architect living in Brooklyn with his wife and young son. "For almost three years—you name it, I tried it."

None of the drugs shrank his tumors, and Rose was constantly ill due to toxins coursing through his body.

Tired of being a guinea pig in the search for remission, in August 2012 Rose sent his excised larynx tumor to Champions. "Fortunately, we had the resources to be able to do that," he says, estimating the mice initially cost between \$30,000 and \$40,000, plus another \$20,000 for a second round.

After a seven-month wait, Rose got results from his TumorGraft mice in February 2013. By that time, he had become very sick, and his doctor didn't expect him to make it through the year without a successful therapy. The TumorGraft results pointed to one promising combination, which Rose began in March. By April, a scan showed an unprecedented image—his tumor had shrunk. "My doctors were literally jumping up and down," says Rose.

That drug combination worked for about a year before Rose's cancer became resistant in April 2014. He moved onto a second regimen predicted by his mice, but this time, the drugs did not work. Rose is currently awaiting results from a new round of mice in development using newer tumor cells. "We'd like results from Champions again," says Rose. "We're not out of options yet."

Champions has recently been working to prove the utility of their method outside of anecdotal stories like Rose's. In a study published last April, Sidransky and colleagues created TumorGrafts for 29 patients with advanced metastatic sarcoma. Of the 22 patients whose tumors successfully grafted, six died before data from the mice were available, but in 13 of the remaining 16 cases, there was a positive correlation between mouse and human results.² In a second study, performed in collaboration with Manuel Hidalgo of the Spanish National Can-



MINI-ME MOUSE: Champions Oncology sells mouse avatars, in which a sample of a patient's tumor is grafted under the skin of immunocompromised mice. These patient-specific rodent models are then used to test treatments that might work on the patient's cancer.

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A FLY ALTERNATIVE

The two greatest barriers to widespread use of mouse avatars are the time and expense required to breed and maintain mice engrafted with human tumor tissue. An obvious way to surmount those obstacles is to use a cheaper, faster-breeding organism.

Enter the humble fruit fly. Ross Cagan's laboratory at Mount Sinai Hospital in New York City has developed a method to recreate human tumors in *Drosophila*, which is believed to have functional homologs for some 75 percent of human disease-causing genes. First, the researchers analyze sequence data from an individual's tumor and identify four to 10 genes altered in the cells. Next, they genetically modify a fruit fly to dial up or down the activity of those genes, and, if possible, do so in the location where the original human tumor was found. For example, to mimic a colorectal cancer, the team alters genes in cells of the fruit-fly gut. The result is a fly tumor: cells that proliferate, invade nearby tissues, and even metastasize to other parts of the body.

The tumor-riddled flies are then used in a unique high-throughput drug-screening process. A total of 960 flies are grown in a plate with 96 wells. Ten embryos are hatched per well, each of which contains food laced with a different drug or drug combination. The young cancerous flies are very sick, but begin eating the food. The flies that survive are those treated with drugs that were effective against the tumor but not so toxic as to kill the fly. "We set up the model so the flies are [almost] dead, if you will, and screen for drugs that bring them back to life. Anything moving—you're in," says Cagan.

The team creates and screens about 100,000 fly avatars, as Cagan fondly calls them, per patient in about six months, and for a fraction of the money it takes to make a PDX mouse. And while personalized fly models are not yet available to cancer patients, the process has proven useful to human disease. In 2005, Cagan's team created a general fly model of a human thyroid tumor caused by mutations in the Ret receptor tyrosine kinase gene, then screened a panel of drugs including a kinase inhibitor called vandetanib that suppressed the tumor (*Cancer Res*, 65:3538-41, 2005). Based on data that included those from the fly, AstraZeneca took the drug into human clinical trials, and in 2011, vandetanib became the first drug approved for late-stage medullary thyroid cancer.

But fly avatars won't work for every type of cancer, Cagan notes, as fruit flies do not share hormones similar to ours, so hormone-dependent diseases, such as prostate cancer, cannot be recapitulated. But we may be seeing more of these flies soon. Cagan's lab recently received approval from the US Food and Drug Administration to begin a clinical trial in colorectal and medullary thyroid carcinoma to see if fly avatars can successfully help guide patient care. More than a dozen cancer patients have already applied to participate in the trial.

researchers and physicians. Today, doctors have clear guidelines to treat tumors with specific drugs, and they are unlikely to deviate from those established procedures unless all else fails, says Fiebig.

Rose acknowledges that the mice aren't right for everyone, but for him, they made a difference. "So far it bought me at least two years of life," says Rose. "It seems like money well spent."

Unfortunately, stories like Rose's are few and far between. While PDX mice used for basic research have helped identify drug candidates, personalized mouse avatars have yet to demonstrate clear changes in the course of a person's disease, says Edward Sausville, an oncologist at the University of Maryland School of Medicine. Because of that, he says, "I would categorically never recommend it to my patients."

"This could be a fruitful area in the future, but we have to show there is value," agrees Goetz.

In the end, many oncologists believe that PDX mice are a powerful means to an end—achieving successful, individualized treatment plans for cancer patients based on the genetic makeup of their tumor—but not the end itself, as developing PDX mice for every cancer patient is simply not practical. If researchers can use the mice to gather information about specific tumor responses to specific drugs, then they can bank that information to be used in the general population.

"We're looking to jump over this need to have an individual patient have an avatar," says Bult. "We want to make this approach of tailored cancer therapy faster and cheaper for everyone." ■

cer Research Center, the team found that 6 of 13 patients with advanced solid tumors who were treated based on results from personalized PDX mice had partial tumor remissions, even in cases where genetic sequencing of the tumor showed no actionable mutations.³

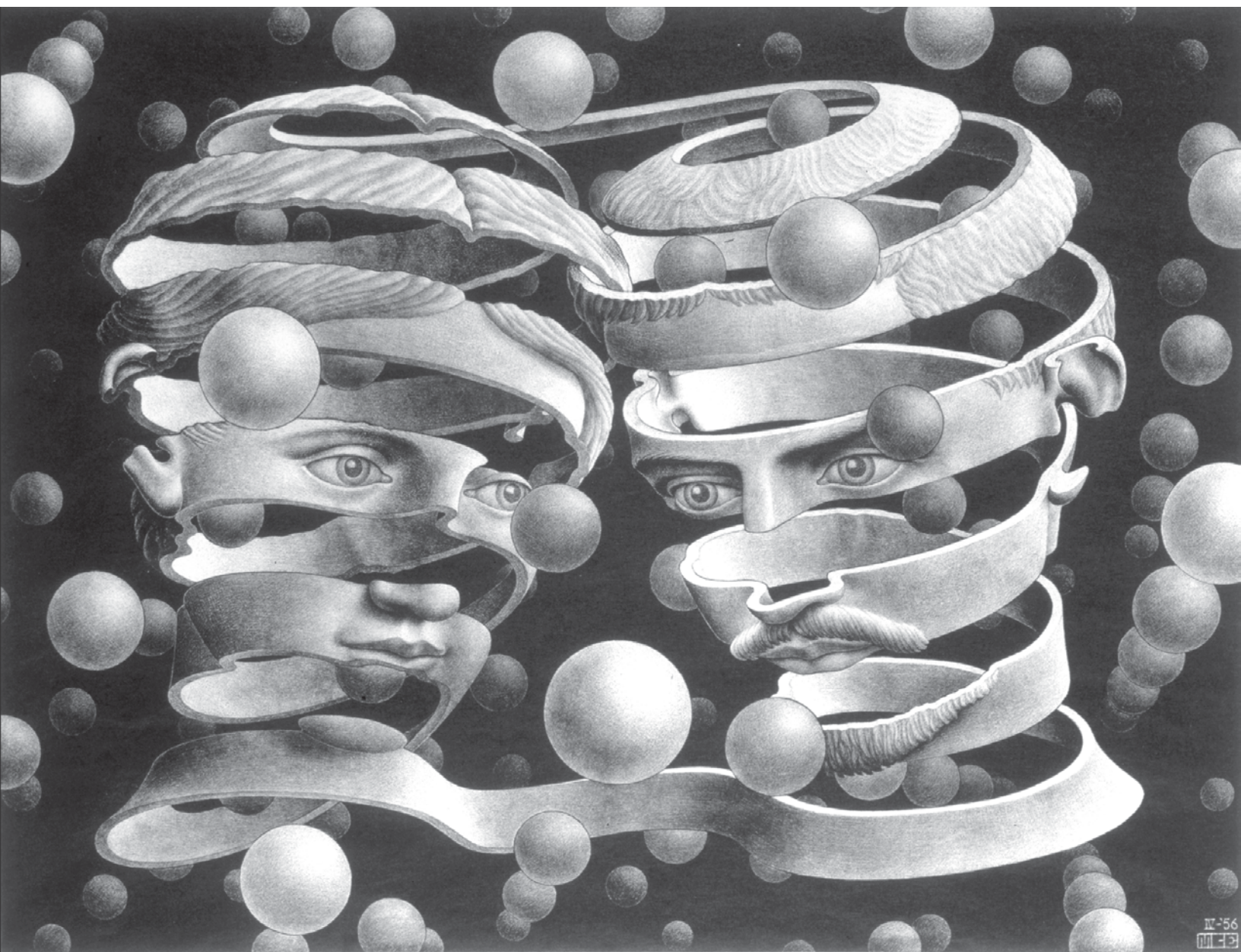
Champions would like to conduct a prospective controlled study in which phy-

sician recommendations will be compared side by side with the recommendations derived from mouse avatars, says Paz. A head-to-head comparison would go a long way toward showing that PDX mice are not just a nice model for human cancer, but can actually improve patient care.

Such data will be needed to convince a largely skeptical community of cancer

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From Many, One

Diverse mammals, including humans, have been found to carry distinct genomes in their cells. What does such genetic chimerism mean for health and disease?

BY ELENA E. GIORGI

In 1976, researchers from the Lister Institute of Preventive Medicine in London published the puzzling case of a woman who had two different blood types: 93 percent of her red cells were type O, while the remaining 7 percent were type A1, the most common type A subgroup.¹ A few years later, Winifred Watkins of the MRC Clinical Research Centre and colleagues came across another blood donor with two distinct red blood cell types, and further investigation led to an even more astonishing finding: a phenotypically normal man, with presumably XY cells in his testes and most of his body, was found to carry XX cells in his skin and other tissues.²

These case studies heralded a new appreciation for the phenomenon of genetic chimerism—when an individual carries two or more genetically distinct cell lines in different parts of her body. Until the advent of techniques for blood typing and karyotyping cells, genetic chimeras were thought to be very rare. They only came to light when the phenotypes associated with the two distinct genomes were so discordant that the resulting individual

While most fictional works portray chimeras as an amalgam of two individuals, the truth is that the individuality of the distinct cell lines is lost as the two combine.

was clearly exceptional, with patches of distinct skin coloration throughout the body, for example, or hermaphroditic genitals. In reality, genetic chimeras may be quite common, disguised in perfectly normal bodies harboring genetically distinct cell lineages.

In all likelihood, most chimeras are not even aware of their condition. Boston resident Karen Keegan, for example, would have never discovered her mixed genetic makeup if in 1998, at age 52, she hadn't needed a kidney transplant. When doctors tested the human leukocyte antigen (HLA) type of her three children to see if any of them could donate a kidney, they were surprised to find that two of the children could not have been hers at all: while all three carried one HLA copy that matched their father's, only one child carried a second copy that matched Karen's. Of course, having given birth to these children, she knew they were hers. Sure enough, while Karen had one cell line only in her blood, the doctors eventually found the "missing" HLA type in a second cell line in her skin, hair, bladder, mouth mucosa, and thyroid.³

For years the concept of a genetic chimera has sparked the imagination of writers, from Stephen King to Michael Crichton, presenting endless fodder for science fiction, horror, and even murder mysteries. But while most fictional works portray chimeras as an amalgam of two individuals, the truth is that the individuality of the distinct cell lines is lost as the two combine. Indeed,

genetic chimerism is subtle, and may still often be overlooked if the second cell line exists at concentrations too low for our current technology to detect.

Since the first cases were published in the 1970s and '80s, genetic chimeras have been the focus of much research, leading to the advent of new blood testing techniques to identify distinct cells in donors that may cause complications in transfusion recipients. Chimerism in donors could have consequences for organ transplants, too. Furthermore, some types of chimerism have been associated with an increased risk of certain autoimmune diseases, while the stem-like qualities of cells transferred from fetus to mother appear to have implications for tissue repair. Understanding how chimeras form and, in particular, what mechanisms allow the immune system to accept genetically distinct lineages within one body could lead to new therapies for cancer and autoimmune disorders.

Types of chimeras

The best-known form of chimerism happens at conception, when two fertilized eggs join to form a single morula, resulting in one organism with two genetically distinct cell populations—a dispermic chimera. Because each fertilized egg carries its own genome, the resulting chimera has distinct DNA in different tissues—a liver arising from one cell line and kidneys from the other, for example. If the two genomes result in markedly different phenotypes, the individual can be a hermaphrodite or present different hair or skin coloration in different parts of the body. (See photograph on page 54.)

But not all chimeras result from the aggregating of two distinct zygotes. Rather, during embryonic development, mutational events can result in the emergence of two or more genetically distinct cell lines within an individual. Errors in chromosome segregation during cell division, for example, can lead to daughter cells with different chromosome numbers. When this type of error, called chromosome nondisjunction, happens early in fetal development, it gives rise to two different cell lines that become randomly distributed throughout the body as the embryo develops—a type of chimerism called mosaicism.

Meiotic nondisjunction usually causes an individual conceived with an abnormal sperm or egg to have the chromosome error in every somatic cell of the body: for example, a female child with only one X chromosome (Turner syndrome) or a male child with an extra X chromosome, XXY (Klinefelter syndrome). But there are also less-common mosaic forms of these syndromes in which the error occurs after fertilization and affects only some of an individual's cells. Pallister-Killian syndrome is a rare form of mosaicism where meiotic nondisjunction results in an abnormal copy of chromosome 12, which persists in some cells but is eliminated from others during development.

Somatic mosaicism can also happen later in life. Recent studies have found evidence of mosaicism and aneuploidy—an abnormal number of chromosomes—in healthy brain tissue, introduced by errors in chromosomal segregation during cell division or by the activity of mobile genetic elements called retrotransposons, bits of DNA that can replicate and reinsert themselves in different regions of the DNA.⁴ This is likely to confer upon the brain the plasticity it needs to constantly adapt to the environment, but it may also impact the development of many diseases.

Indeed, while somatic mosaicism has been observed in healthy brain tissue, there is evidence that it could also play a role in diseases such as Alzheimer's. A group of researchers from the Scripps Research Institute analyzed the nuclei of neurons harvested from the prefrontal cortex and cerebellum of 134 postmortem brains, 47 of which came from subjects with Alzheimer's, and found that the gene *APP*, which encodes for an amyloid precursor protein, was mosaically amplified in brains affected by the disease. Some neurons had up to 12 copies of the gene.⁵

Each one of us is made of trillions of cells, and some of those cells could very well belong to our siblings, our mother, or our child.

Sometimes the second cell line is present in very low concentrations, a form of chimerism called microchimerism, which may be much more widespread than realized. Not surprisingly, this type of chimerism has been detected in recipients of organ transplants. However, a study conducted by researchers at the Leiden University Medical Center in the Netherlands found that microchimerism unrelated to transplants is also quite common. Among autopsy samples from 75 women, the researchers found male cells in 13 kidneys, 10 livers, and 4 hearts of 23 of the women. Notably, none of these women showed signs of organ failure, thus demonstrating that perfectly healthy organs can harbor small numbers of chimeric cells.⁶

One common cause of such microchimerism is maternal-fetal trafficking of cells during pregnancy. The placenta is not an unbreachable barrier. Evidence of two-way cell transport across the placenta was reported as early as the 1950s and '60s. While the mother's immune system gets rid of most of her baby's cells shortly after delivery, small numbers of fetal cells have been observed in mothers decades after they have given birth. In fact, because even spontaneous abortions cause fetal cells to be released into the mother's body, women who become pregnant but never give birth can also display this form of microchimerism.⁷ Conversely, maternal cells have been found in the liver, lung, heart, thymus, spleen, adrenal, kidney, pancreas, brain, and gonads of healthy adults.⁸ Microchimerism could also originate from siblings' cells, transferred from the mother during successive pregnancies. Regardless of the direction of transfer, the exogenous cells can migrate to a certain tissue, where they differentiate and proliferate, acting as if they were engrafted.

Likely critical to this integration of fetal cells in a mother's tissues are the immune changes a woman undergoes during pregnancy to allow for tolerance of the fetus, which is genetically distinct from the mother and therefore potentially subject to immune attack. These changes could also explain why numerous autoimmune disorders seem to be associated with pregnancy. For example, the symptoms of rheumatoid arthritis sometimes improve during gestation but tend to return within three months of delivery. But could microchimerism also play a role in the etiology of these and other disorders?

Microchimerism and disease

Because it's easier to test for a male's DNA in a mother by looking for evidence of the Y chromosome, many studies have focused on detecting microchimerism in mothers of boys. In a 2012 study, William Chan of the Fred Hutchinson Cancer Research Center in Seattle and colleagues looked at the autopsied brains of 59 women, 33 of whom had Alzheimer's, while 26 exhibited no evidence of neurological disease. They found male DNA—detected by the Y-chromosome marker *DYS14*—in diverse brain regions of 63 percent of the women, and, notably, the prevalence and concentration of microchimerism were significantly lower in the women affected by Alzheimer's.⁹

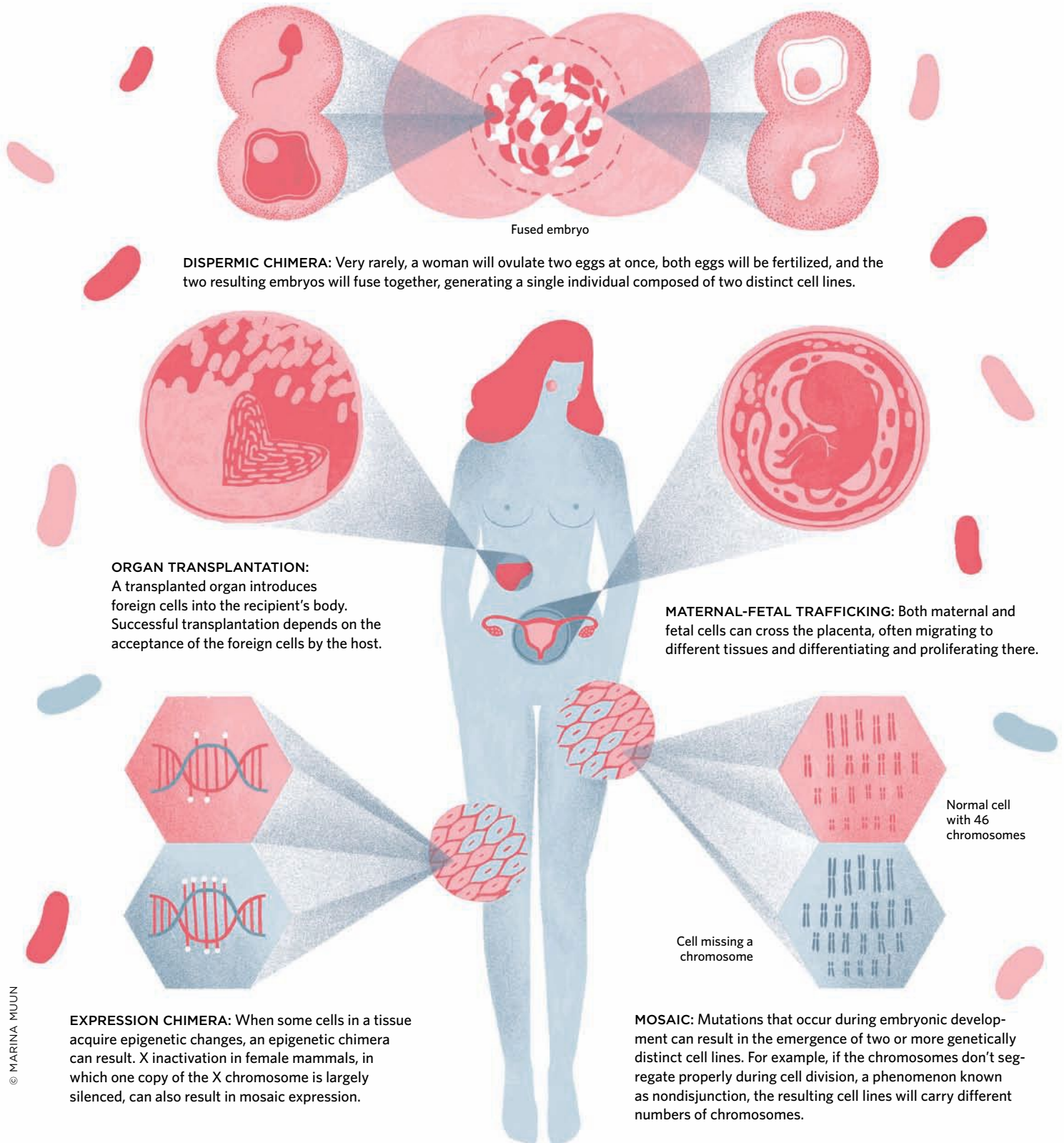
Conversely, a number of studies have found significantly higher levels of fetal microchimerisms in mothers affected by systemic sclerosis, an autoimmune disorder characterized by the hardening and scarring of the skin. Similarly, both animal and human studies have found higher concentrations of microchimerism in females with some form of thyroiditis,⁶ an autoimmune disorder in which the immune system perceives the thyroid and its hormones as threats and starts producing antibodies against the gland. Interestingly, thyroid disorders of this kind are much more common in women than men, with females being at particularly higher risk shortly after giving birth.

Microchimerism has also been reported in several cancer studies. For example, Valentina Cirello of Fondazione Policlinico IRCCS in Milan, Italy, and colleagues found lower circulating levels of fetal DNA in the blood of female patients with thyroid cancer than in the blood of healthy controls, though they pinpointed higher concentrations near or around tumor tissue.¹⁰ These findings have fueled speculation that fetal cells could be recruited from the blood to the tumor site in an attempt to repair the tissue.¹¹ Researchers have reported similar findings for breast cancer, where fetal microchimerism also occurs at lower concentrations in the blood of cancer patients, compared with controls.^{12,13} It is well known that multiparous women have a lower risk of developing breast cancer compared with women who have no children, yet why this happens is not entirely known. These latest findings on fetal chimerism could perhaps explain part of the puzzle.

The opposite trend has surfaced in colon cancer, however, with higher blood concentrations of fetal DNA detected in cancer patients.¹⁴ Rather than having a salutary effect, fetal cells

CELLULAR PATCHWORK

Chimerism—the existence of genetically distinct cell lines within an individual—is probably much more common than once believed. It can result from a variety of sources, from organ transplantation, in which genetically distinct tissues are implanted into the body, to biological mutation that spawns cells with different chromosome numbers. Oftentimes, the concentration of a distinct cell line is so low that it is overlooked entirely. But even this low-level microchimerism, such as the presence of fetal cells in a mother's body, may have consequences for health and disease.



could possibly contribute to cancer pathology by triggering inflammatory responses that lead to tissue damage, similar to what occurs during transplant rejection. Alternatively, the chimeric cells may be completely neutral to the host, and their presence or absence in damaged tissues could be due to side effects of the disease.

The reverse form of mother-fetus chimerism—the presence of maternal chimeric cells in her children’s tissues—has also been studied extensively in both infants and adults. This type of microchimerism has been associated with diseases such as diabetes¹⁵ and biliary atresia, a congenital defect in which the bile duct between the liver and the small intestine is blocked or absent.¹⁶ But again, results gathered thus far seem to be inconclusive as to whether the presence of the maternal DNA is a cause or result of the disease.

“It is such a new area of work and there is so little knowledge,” fetal microchimerism specialist J. Lee Nelson of the University of Washington told me in an e-mail. “Overall we expect microchimerism to have the potential for both beneficial and/or detrimental effects, whether it is of fetal origin or maternal, or other sources of microchimerism.”

Diverse factors can complicate the influence of microchimerism on the health of host tissues. The compatibility of the HLA genes of the mother with those of the fetus could play an important role in the way they interact, for example. Normally, cells exchanged between mother and fetus are rapidly cleared by the host immune system, but matching HLA types could increase the chance that the exchanged cells behave like grafts and persist in the host long after arrival. Certain HLA combinations may also translate to a higher chance of developing autoimmune diseases.

The origin of the microchimerism could also be a key factor, whether it’s from the mother, a full-term pregnancy, a sibling, or a spontaneous abortion. As miscarriages are often caused by genetic defects, this could lead to defective cells being transferred, which could be potentially harmful.

Finally, the gestational age of the fetal cells that are transferred to the mother could also influence their effects on her physiology, because the cellular composition of the fetus changes as it develops.¹⁷ For example, fetal T cells are formed around week 13 of pregnancy, and couldn’t be transferred prior to that. Transfer of cells from the immune system could be either beneficial or detrimental depending on HLA compatibility. Conversely, the age of the maternal cells transferred to the fetus could affect whether or not maternal microchimerism is beneficial, as older cells may have accumulated DNA damage or other signs of aging. While a transfer of cells from the maternal immune system could bring beneficial acquired immunity, some cells could have also accumulated precancerous mutations. In fact, although cancer during pregnancy is uncommon, there have been reports of maternal tumors that metastasized to the placenta and, in rare instances, to the fetus.

HUMAN CHIMERA: When two fertilized embryos fuse, the resulting individual carries two distinct genomes. If the cell lines carry genes that code for significantly different skin colors, this form of chimerism can manifest in variegated skin patterns known as lines of Blaschko.



Beyond DNA: Expression chimerism

During embryonic development, the genomes of different tissues acquire signature epigenetic patterns in the form of DNA methylation marks. But within one cell population, the assumption is that the epigenome is largely uniform. When some cells out of the group acquire a different set of methylation marks, creating a chimeric mixture within the tissue, the result is an epigenetic chimera. Changes in epigenetic patterns may happen throughout our lifetime as a result of environmental changes such as stress and trauma, but early in embryonic development, such changes can be fatal. (See “Pushing the Limits,” *The Scientist*, February 2015.)

One of the genes involved in early epigenetic regulation is *TRIM28*. This gene mediates transcriptional control in certain regions of the chromatin and is thought to play a fundamental role in resetting the epigenome during embryonic development. Researchers in Singapore, at the Jackson Laboratory in Maine, and at Stanford University created epigenetic chimeras by disrupting the *TRIM28* gene in mouse mothers.¹⁸ The scientists showed that not all cells in the resulting embryos had their epigenomes reset, as is expected following genomic reprogramming, but instead ranged from normal to completely unmethylated. This effectively created a chimeric mixture—with a disastrous outcome. Mouse embryos produced by *TRIM28*-deficient mothers did not survive more than five days postfertilization.

Many cancers can also be considered chimeric, resulting from genetic or epigenetic alterations that activate onco-

genes capable of promoting cell growth or, conversely, silencing tumor-suppressor genes that inhibit cell growth. Understanding epigenetic chimerism, as well as the mechanisms that disrupt the epigenetic markers and how to fix such disruptions, could help provide new targets for fighting cancer. Indeed, while genetic mutations that lead to cancer are irreversible, the same is not true of epigenetic changes, and restoring normal methyl-

Understanding how chimeras form and what mechanisms allow the immune system to accept genetically distinct lineages within one body could lead to new therapies for cancer and autoimmune disorders.

ation patterns could reverse the disease phenotypes. When the research team from Singapore and the U.S. transferred wild-type pronuclei into the mutant mouse embryos, for example, 25 percent survived.

Another form of mosaic expression stems from the phenomenon of X inactivation. During early embryonic development of female mammals, one of the two copies of the X chromosome is largely silenced in each cell, and it may be the maternal X that is silenced in some cells and the paternal X in others. In this sense, all female mammals are mosaics, since the X they inherit from the mother is not identical to the one they inherit from the father. Tortoiseshell cats are an example of this type of mosaic, with a heterozygous X-linked gene that regulates coat coloration leading to the patchwork pattern.

This kind of mosaicism gives females an advantage when it comes to X-linked genetic diseases: cells that express the “healthy” X gene will often compensate for those expressing the mutant variety. In heterozygous women carrying the allele for the X-linked lysosomal disorder called Fabry disease, for example, cells that produce the healthy enzyme can make enough to at least partially compensate for the lack of production from those carrying the mutated gene. Another X-linked disorder, called Lesch-Nyhan, is characterized by the lack of production of another important enzyme, leading to a build-up of uric acid in the body. While boys born with this defect face severe mental and physical problems, in heterozygous females, cells expressing the defective gene usually receive the missing enzyme from normal cells in at least some body tissues and are gradually eliminated from tissues where they do not. In the latter case, the cells that express the normal allele have a growth advantage and outgrow those expressing the mutant gene.¹⁹

One or many?

We consider ourselves to be single individuals, but when you stop and think about it, who are we, really? Each one of us is made of trillions of cells, and some of those cells could very well belong to our siblings, our mother, or our child. Add the fact that we harbor

millions of bacteria and other microorganisms that can indeed impact our phenotype depending on what genes they express, and you get the picture of an organism made of many entities rather than a single individual. A new school of thought in biology claims that we are not individuals, but rather communities of symbionts—distinct organisms living with one another.

In this light, chimeras are no longer a genetic paradox—rather, just another path in an incredibly variegated evolutionary landscape. ■

Elena E. Giorgi is a computational biologist at the Los Alamos National Laboratory. Although she normally works on HIV research, she learned a great deal about chimeras while writing her debut novel Chimeras.

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The Literature

CELL & MOLECULAR BIOLOGY

Toggling Between Life and Death

THE PAPER

J.L. Schwartz-Roberts et al., “Interferon regulatory factor-1 signaling regulates the switch between autophagy and apoptosis to determine breast cancer cell fate,” *Cancer Res*, 75:783-91, 2015.

About 70 percent of breast cancers express estrogen receptor- α (ER- α). In these cancers, the receptor binds estrogen then translocates to the nucleus, where it upregulates genes leading to cancer growth. Antiestrogen drugs, which compete with endogenous estrogen, can prevent this from happening. When they work, antiestrogen therapies lead breast cancer cells to undergo apoptosis, often through a signaling pathway involving the transcription factor interferon regulatory factor-1 (IRF1). Unfortunately, most breast cancers eventually become resistant to such drugs.

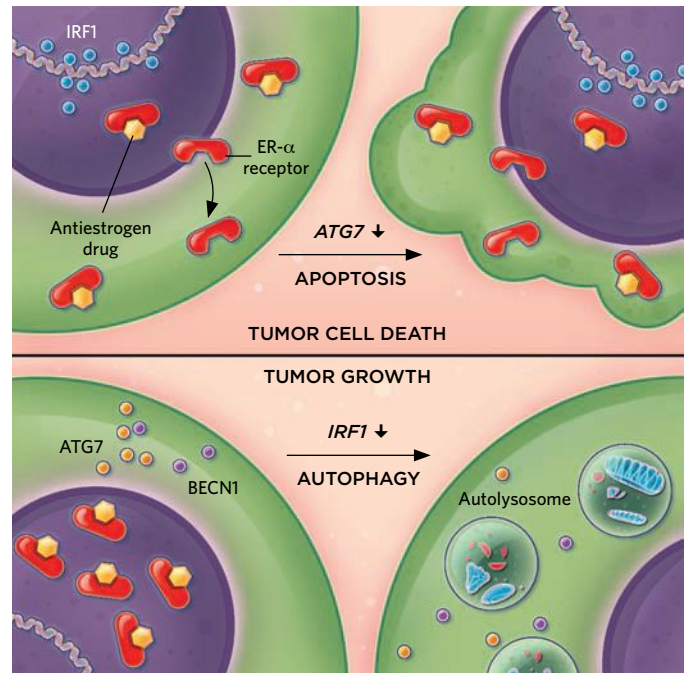
Previous studies showed that *IRF1* was downregulated in antiestrogen-resistant breast cancer cell lines, suggesting that it was the loss of IRF1's ability to induce apoptosis that led to resistance. Additionally, it was known that autophagy, a process in which a cell recycles its own cytoplasmic materials, increased during antiestrogen therapies and helped cancers survive. But what promoted autophagy and tamped down apoptosis was unknown.

Georgetown University's Robert Clarke and colleagues now report that the genes influencing apoptosis and autophagy seem to keep each other in check. Mammary tumors from mice with only one copy of the autophagy gene *ATG7* had higher nuclear levels of IRF1 than tumors from control mice. This inverse relationship held up in ER- α -positive human breast cancer tissue, where levels of *ATG7* were inversely correlated with levels of nuclear IRF1.

To see if this association reflected causality, the researchers knocked down autophagy genes in human breast cancer cell lines using RNA interference and observed an uptick of both nuclear IRF1 and apoptosis. Conversely, knocking down *IRF1* led to an increase in autophagy. They also found that as nuclear IRF1 levels increased, the estrogen receptor “gets popped out of the nucleus so that it can't . . . regulate transcription in a pro-survival mechanism,” says Clarke. “So you get sort of a double hit” on the cancer cell. Apoptosis is induced, while cell growth is blocked.

“At the same time, the silencing of *ATG7* and [another autophagy gene] *BECN1* blocks the autophagy piece,” adds Clarke. “There are lots of things that happen all at once.”

“What they showed that was new is that . . . IRF1 is important for not only apoptosis but also it's able to switch between autophagy



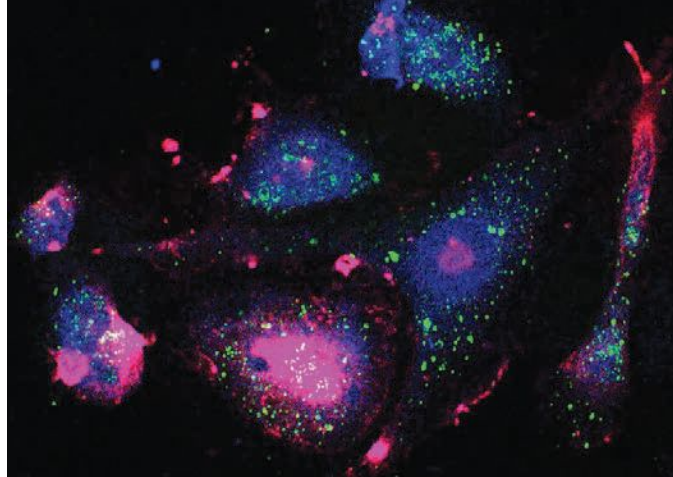
LIVE OR DIE: In breast cancer cells expressing estrogen receptor- α (ER- α), the inhibition of the autophagy gene *ATG7* (top panel) allows an apoptosis transcription factor, IRF1, to lead the cancer cell to die upon antiestrogen therapy, which outcompetes estrogen for binding the receptor. When *IRF1* is knocked down (lower panel), antiestrogens are less potent as the activity of *ATG7* and another autophagy factor, *BECN1*, aids in cell survival by supporting autophagy.

agy and apoptosis,” says Victoria Seewaldt, who studies breast cancer at the Duke University School of Medicine.

In the presence of an antiestrogen, knocking down *IRF1* made cells less sensitive to the drug, while knocking down *ATG7* had the opposite effect. Stabilizing *IRF1* expression might be a way to treat antiestrogen-resistant breast cancers, Clarke says.

However, knocking down both *IRF1* and *ATG7*, an experiment that mathematical modeling had suggested they try, restored antiestrogen sensitivity, which led the authors to conclude that there must be another pathway, independent of *IRF1*, that can lead to apoptosis when cells are treated with antiestrogens. Solving this apparent paradox is the Clarke group's next ambition.

—Ashley P. Taylor



PATHWAYS PROBED: Researchers search for molecular pathways that allow tumor cells (shown here) to outlast anticancer drugs.

CELL & MOLECULAR BIOLOGY

Signaling Resistance

THE PAPER

C.A. Martz et al., "Systematic identification of signaling pathways with potential to confer anticancer drug resistance," *Science Signaling*, 7:ra121, 2014.

THE DEBATE

Researchers have two theories about how tumor cells develop resistance to anticancer drug treatments: either through an uptick in pro-growth signals or via dedifferentiation pathways that return them to a stem-like state. Single-gene studies have provided evidence for both strategies.

THE SCREEN

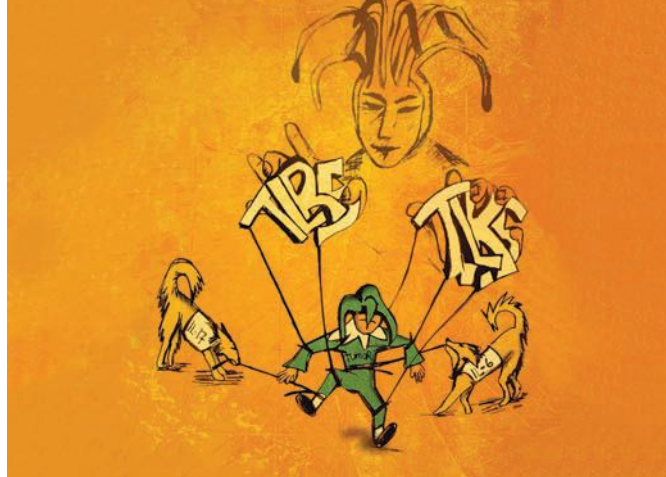
To get a more comprehensive view of what's happening, researchers from MIT and Duke University looked at signaling pathways, instead of individual genes. They used a library of 40 mutant cDNAs to activate or inactivate 17 cancer-related pathways in melanoma and breast cancer cell lines, then assayed the cells' responses to 13 common targeted anticancer treatments. In addition to confirming the resistance-establishing role of pathways that promote growth, they also found that the Notch1 pathway played a previously undetected role in conferring drug resistance to numerous cancer cell types, mouse models, and human tumor samples.

THE RECONCILIATION

Notch1 is thought to play a role in stem cell renewal, making dedifferentiation a "plausible biological mechanism" for drug resistance, says Charles Sawyers of Memorial Sloan Kettering Cancer Center who was not involved in the work. In the study, activated Notch1 and pro-growth pathways seemed to be equally effective at establishing resistance, supporting the idea that "both of these schools of thought are correct," says study coauthor Kris Wood of Duke.

THE POTENTIAL

"Directly screening signaling pathways instead of individual genes . . . can potentially accelerate the identification of the pathways that drive drug resistance," Wood says. He hopes to expand the library to 200 constructs to assay more pathways and perhaps to study other cancer characteristics such as metastasis and recurrence. —Jenny Rood



CANCER COMMAND: The microbiome (as puppeteer) affects the immune system's influence on tumor growth, in tandem with inflammatory cytokines (dogs).

CELL & MOLECULAR BIOLOGY

Manipulative Microbiomes

THE PAPER

M.R. Rutkowski et al., "Microbially driven TLR5-dependent signaling governs distal malignant progression through tumor-promoting inflammation," *Cancer Cell*, 27:27-40, 2015.

THE POLYMORPHISM

The gut microbiome influences nonintestinal cancer progression, but the role of toll-like receptor 5 (TLR5), an immune system protein that recognizes commensal bacteria, was a mystery. As more than seven percent of people have nonfunctional TLR5, José Conejo-Garcia of the Wistar Institute in Philadelphia and colleagues explored how TLR5 impacts tumor growth.

THE CONTROLLER

In a mouse model of sarcoma, the researchers found that tumors grew much faster in wild-type mice than in TLR5-deficient ones. Wiping out the mice's microbiomes diminished the disparity, indicating that TLR5's tumor-promoting effects are driven by commensal bacteria. TLR5-dependent signaling triggered the production of inflammatory interleukin 6 (IL-6), which in turn activated an immunosuppressive cascade—not switched on in TLR5-deficient animals—that promoted tumor growth.

THE TWIST

In human breast cancers or mouse mammary carcinomas, where IL-6 levels are much lower, TLR5 had the opposite effect. Tumors in TLR5-deficient mice grew better, due to the upregulation of IL-17, a different inflammatory cytokine. As in the sarcoma model, any differences between TLR5-deficient and wild-type mice disappeared upon antibiotic treatment. "Cancer is a systemic disease that is very dramatically influenced by commensal bacteria," Conejo-Garcia says.

THE MANIPULATION

"It's very exciting to see this kind of progress," says Susan Erdman of MIT. She says looking into the effects of human polymorphisms such as TLR5 "really contributes to the concept of personalized medicine in cancer therapy." As a first step toward future treatments, study coauthor Melanie Rutkowski is exploring how manipulating the microbiome could inhibit tumor growth. —Jenny Rood

Professional Marksman

Charles Sawyers, who began his research career just as the genetic details of human oncogenes were emerging, codeveloped Gleevec, the quintessential targeted cancer therapy.

BY ANNA AZVOLINSKY

Even before beginning a clinical fellowship at the University of California, Los Angeles (UCLA), in 1988, Charles Sawyers already had an intense interest in chronic myeloid leukemia (CML). In the late '80s, allogeneic bone-marrow transplants were a cutting-edge therapy that gave CML patients a chance at a cure. Sawyers had cared for a patient with chronic-phase CML. "He was my own age, a fireman," recalls Sawyers. After the transplant, the patient developed graft-versus-host disease and died. "That had a profound impact on me—to watch someone with a lethal disease, who was feeling fine and probably would have survived for 5 years with standard therapy, make the decision to undergo a potentially curative therapy and die from its toxicity. 'There has to be a better way,' I thought."

Sawyers had moved to UCLA so he could work in Owen Witte's laboratory on the molecular determinants of CML. Witte was the first to clone the BCR-ABL oncogene, a fusion gene created by a translocation that gives rise to the Philadelphia chromosome, now known to be responsible for CML. "It was incredibly exciting to now have demonstrations that cancer can be caused by a single genetic event," says Sawyers, who received both his clinical and research training at a turning point in oncology—when laboratory studies on the molecular biology of cancer began to merge with clinical care. "The whole cancer field was asking whether these oncogenes were really capable of causing cancer in an experimental model."

Here, the clinician-scientist talks about his experience in the operating room right after high school, how he migrated toward laboratory research, and his role in developing two highly successful targeted cancer therapies.

SAWYERS SETS OUT

A medical family. Sawyers grew up in Nashville, Tennessee, surrounded by physicians. His mother was an anesthesiologist, and his father and two uncles were surgeons. He did well in high school and thought about going into medicine. "But at that point, I just wanted to go to college and get out of Nashville."

Surgeon's assistant. For two consecutive summers starting after his senior high school year, Sawyers worked as a scrub technician at the hospital where his mother was on staff. "That was an important experience," he says. "It made me not want to be a surgeon! The techniques were fascinating and this affirmed my interest in human biology, but—and this is going to sound terrible to surgeons—I didn't find it intellectually inspiring."

Barefoot medicine. At Princeton University, Sawyers majored in history, focusing on the history of science. He wrote his senior thesis on how medical education changed in China in the 20th century, from the pre-Communist era, when there was investment in Western-style medicine, to a new generation of health-care workers, known as "barefoot doctors," who delivered basic medical care in rural areas after Mao Zedong came to power. In 1980, after his junior year, Sawyers experienced the phenomenon firsthand when he traveled to China with his father, who had been invited to give lectures and meet with clinicians because of his expertise in stomach surgery. "I remember seeing very traditional medicine practiced. It was really eye-opening," he says.

"I would like to see every patient's tumor genotyped to guide treatment decision making . . . even if there are not currently therapies to target certain mutations."

SAWYERS SHAPES UP

Playing catch-up. After college, in 1981, Sawyers entered medical school at Johns Hopkins University in Baltimore. "I was not the typical premed biochemistry major who had worked in a lab in college. As soon as medical school started, I realized I was way behind in knowledge of fundamental biology compared to my classmates. I thought, 'Wow, everyone around me has already had this material.' That first year of medical school was miserable."

A new sense of self. By his second year, Sawyers had caught up academically. "I began to rethink my worldview of medical science and became much more interested in research." Sawyers says he was also inspired by physician lecturers who approached a clinical issue by dissecting it down to the molecular level. "The hemoglobin gene mutation that causes sickle cell anemia had been defined, and a hematologist who taught us had collaborated with a structural biologist to solve the crystal structure of the protein that explained the disease. That was mind-blowing—to understand the medical disease at that level of detail."

"I knew I was a late bloomer scientifically, but I wanted to give it a real solid try to become a physician-scientist."

Going west. After medical school, Sawyers went to the University of California, San Francisco (UCSF) for a residency in inter-



CHARLES SAWYERS

Chair, Human Oncology and Pathogenesis Program
Memorial Sloan Kettering Cancer Center
Researcher, Howard Hughes Medical Institute

Greatest Hits

- Demonstrated that PCR could be used to detect levels of BCR-ABL messenger RNA in blood or bone marrow, allowing its use as a biomarker of chronic myelogenous leukemia (CML)
- Showed that the normal cellular function of the ABL gene is to curb cell growth
- Codeveloped the oral targeted CML therapy imatinib (Gleevec), which inhibits the BCR-ABL oncogenic tyrosine kinase, one of the first targeted cancer therapeutics; and dasatinib (Sprycel), a second generation BCR-ABL inhibitor
- Identified resistance mutations to imatinib within the BCR-ABL gene
- Demonstrated that prostate tumors continue to rely on androgen signaling despite being refractory to hormonal therapy
- Codiscovered enzalutamide (Xtandi), an oral androgen-receptor antagonist approved by the US Food and Drug Administration for the treatment of castration-resistant metastatic prostate cancer

nal medicine. “Molecular technologies had allowed people to ask disease-focused questions in a new way. This was a powerful tool to dig deeper, and I saw that happening in all kinds of ways on the UCSF campus.”

Leukemia calling. “I knew very early on that oncology was what I wanted to do because of the combination of science that was breaking, especially the concept of oncogenes. At UCSF, one of my early rotations was on the leukemia service. Everyone said this was the worst rotation because you work your butt off and the patients are dying, which is, of course, depressing. But I found it to be exhilarating. It was super intensive medicine, and many of the patients I took care of were my own age. Emotionally, it’s extreme and draws you in.”

Resident to researcher. While Sawyers was at UCSF, Witte published his study on the Philadelphia chromosome. Witte had also been to medical school, where he had caught the research bug early and never pursued clinical training. “Witte told me that he usually didn’t take MDs but that he would be interested in speaking to me. After the interview, he offered me a spot in the lab,” says Sawyers, who ended up spending five years there. “It was more than enough time to get me where I needed to be, scientifically. After two years, I felt I was on a postdoc level, and the final year prepared me to start my own lab.”

Molecular detection. While in Witte’s lab, Sawyers demonstrated that you could molecularly detect the BCR-ABL fusion transcript in the blood of CML patients who have relapsed after a bone marrow transplant, and that this molecular marker appears before cytogenetic signs of relapse, the standard way pathologists measured tumor growth. “The PCR technique had just come out when I joined the lab. I was at the right place at the right time to optimize the PCR conditions and use clinical samples to analyze the levels of BCR-ABL mRNA.” Sawyers also showed that the MYC transcription factor together with BCR-ABL can transform a normal cell into a malignant one. He also showed that the primary function of the normal ABL protein, a tyrosine kinase located primarily in the nucleus, is to negatively regulate cell growth.

A lab of his own. “At this point I knew I had the right stuff for a lab-based career. I had no hesitation; I was totally immersed.” In 1993, Sawyers accepted an assistant professorship at UCLA, moving just 50 yards from his postdoctoral lab into UCLA’s new

cancer center. “When I first joined the Witte lab, the question was whether this oncogene causes cancer. Then, the question had become ‘How?’ We were working on the signal-transduction pathways of what happens in the cell downstream of BCR-ABL.” Sawyers’s laboratory identified two independent signaling pathways activated by the BCR-ABL oncogene and showed that the Jun kinase, activated by BCR-ABL, is required for transformation by the oncogene. Although busy with research, Sawyers kept one foot in the clinic, spending 20 percent of his time as an attending CML physician.

SAWYERS STICKS TO IT

A drug for CML. During a 1995 visit by Sawyers to Brian Druker’s Oregon Health and Science University lab, Druker showed him data on a tyrosine kinase inhibitor, imatinib, that selectively killed leukemia cells expressing BCR-ABL. Druker and Sawyers worked to convince Switzerland-based pharmaceutical company Ciba-Geigy, which had discovered the compound, to test it in CML patients. Sawyers, Druker, and Moshe Talpaz, a hematologist then at MD Anderson Cancer Center in Houston, were the investigators responsible for the first human clinical trial of imatinib, now approved by the US Food and Drug Administration (FDA) as Gleevec. The trial began in 1998, two years after Ciba-Geigy had merged with Sandoz to become Novartis. The team used the PCR assay Sawyers had developed to detect BCR-ABL in patient samples. Other than bone marrow transplantation, which was only available to a minority of patients, therapies for CML had infrequently led to a reduction in the percentage of CML cells in the bone marrow, and complete remissions were rare.

“One of my patients was the first to have a complete remission, and others soon followed. That’s when we knew this was a game changer,” says Sawyers.

From relapse to response. Sawyers and colleagues tested imatinib not just on chronic-phase CML patients, but on very advanced blast-crisis CML patients who had only a few months of life expectancy. “We thought, ethically, we should offer this to these patients, and amazingly, even some blast-crisis patients went into remission, which was incredible. These patients are often in wheelchairs and can require oxygen. Within a week, some were like new people.”

But the responses were short-lived. Finding out why patients relapsed after therapy became the most important question in the world for him, says Sawyers. Using patients’ samples, his lab showed that resistance was due to new mutations within the BCR-ABL kinase domain that interfered with drug binding. Sawyers’ work benefited from research by John Kuriyan’s group at Rockefeller University identifying the crystal structure of the BCR-ABL protein. “This was one of the most exciting periods of my life, realizing we can totally understand how this drug is causing resistance.” Sawyers proposed a way to overcome resistance, describing a theoretical new compound at the 2002 American Association for Cancer Research annual meeting. A few days later, he received a phone call from a scientist at Bristol-Myers

Squibb (BMS) who had heard his talk. It turned out that BMS had a compound with the characteristics Sawyers had described. Sawyers’s laboratory showed that this compound, which eventually became dasatinib (Sprycel)—approved by the FDA for treatment of CML in 2006—can overcome resistance to imatinib. And Sawyers again partnered with Talpaz to lead the clinical trial that showed dasatinib worked in imatinib-resistant patients.

A new direction. In 2005, Sawyers received an invitation from Harold Varmus, then president of Memorial Sloan Kettering Cancer Center, to head a new research program that would capitalize on using genomics data to analyze tumors and identify new cancer drug targets. “I thought it would be an amazing opportunity. With my experience with Gleevec, I had a sense of where the field could go. Seeing how you could get a tumor to shrink well, even though you are poking it in just one spot, was really eye-opening, and generated great optimism in my mind to go after complex solid tumors. I knew there were Achilles heels that had to be there, because I had seen it firsthand.”

Beyond CML. While still at UCLA, Sawyers had begun to work on prostate cancer. “I wanted to branch out and began thinking that hormone therapy for prostate cancer was a similar question to what we were asking with CML,” he says. From seven different mouse models, Sawyers’s lab got the same answer—that prostate tumors resistant to hormonal therapy expressed much higher levels of the androgen receptor. Knocking down the androgen receptor using a genetic trick called RNA interference restored sensitivity to hormone therapy. “It was quite parallel to what we had seen with the kinase inhibitors—that despite genetic complexity, tumors tend to fall back on the original driving oncogenic event to escape targeted therapy.” Sawyers collaborated with synthetic chemist Michael Jung to develop a new antiandrogen therapy for prostate cancer. The compound, enzalutamide (Xtandi), was licensed by biopharmaceutical company Medivation and is now approved by the FDA for treatment of advanced prostate cancer.

SAWYERS SPEAKS OUT

Genomic sequencing. “I would like to see every patient’s tumor genotyped to guide treatment decision making. I think that information is going to be useful, even if there are not currently therapies to target certain mutations. It’s such a compelling question that the cancer centers with the resources are just doing it themselves.”

Cancer drug costs. “I don’t think it’s justified to charge some of the amounts being charged for incremental benefit, but for really dramatic benefit, I do think it can be justified. I don’t know what the right price is, but companies need incentives to recover their research and development expenses. There is an inability of the US Medicare system to negotiate drug prices with pharmaceutical companies, whereas in other countries there are intense negotiations to prove the value of a new drug in economic terms. I think it’s clear that the tide is pushing in that direction.” ■

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Yvonne Saenger: Immunotherapy Pioneer

Assistant Professor, Department of Medicine, Columbia University. Age: 41

BY JEF AKST

Yvonne Saenger majored in Russian at Harvard University, but, realizing that “medicine would allow me to have an impact on people’s lives in a way the humanities wouldn’t,” she also took a full suite of premed courses, then headed to Columbia University for medical school. After her younger sister was diagnosed with cutaneous T-cell lymphoma, she homed in on oncology. (Her sister is in remission and doing just fine now, Saenger notes.)

At med school, Saenger spent some time in the lab of Leonard Chess, where she worked on regulatory T cells in the context of treating autoimmune disease. It was interesting work, Saenger says, but “I was fascinated by the flip side of it: you could also activate your cells to kill off the lymphoma.”

During her fourth year of med school, she again returned to the lab, this time working with Alan Houghton and Jedd Wolchok at the Memorial Sloan Kettering Cancer Center (MSKCC) on assays to measure T-cell activation. “[Saenger] was very, very motivated; very, very passionate about making the most of her time in the laboratory,” Wolchok says. He recalls one occasion when Saenger came into the lab at night, after everyone else had gone home, and ended up getting questioned by security. “[She took] the risk of appearing like she was a trespasser to actually get some research done.”

Saenger stayed at Columbia for her residency, but she returned to MSKCC to do a fellowship with Houghton and Wolchok. Saenger examined the effects of combining tumor-targeting monoclonal antibodies with vaccines designed to activate T cells. “We wanted to show that activating both arms of the immune system, the B cells and T cells together, would be a more effective strategy against the tumor,” she says. And in mice, at least, it was.¹

At the same time, Saenger became involved with some of the first patients to receive ipilimumab (Yervoy), “the first med-

icine ever to improve overall survival in patients with melanoma,” says Wolchok, who led early-stage trials of the drug.

But the improvements the researchers saw tended to occur later than expected, sometimes months after the patients were first treated, Wolchok adds.² “The types of responses we were seeing didn’t follow the same sort of kinetics that you’d expect from, say, a chemotherapy or targeted therapy. It influenced the endpoints for the pivotal Phase 3 trial.”

In August 2009, Saenger started her own lab at Icahn School of Medicine at Mount Sinai, where she began investigating the expression of immune genes in tumors. After developing an immune biomarker for melanoma, Saenger and her team “found that a higher expression of certain immune genes predicts better outcomes in melanoma,” she says.³ The results could help tailor immunotherapies in the future, Saenger notes, with patients showing severely depleted immune responses requiring a greater therapeutic boost, for example.

In August 2014, she headed back to Columbia, where she continues to identify biomarkers of immune response and to examine the tumor microenvironment, both pre- and post-treatment. Saenger’s also exploring combining oncolytic viruses with checkpoint blockade therapies. “I’m trying to do research and also tie it back into the clinic,” she says. “It’s challenging because you have to be comfortable in both worlds.”

“Yvonne is a very, very intelligent, motivated, and compassionate physician. She’s an astute researcher,” says Wolchok.

“She’s a very lively, enthusiastic person,” adds Nina Bhardwaj, director of immunotherapy at Mount Sinai who collaborated with Saenger on the biomarker work. Between seeing patients, doing research in the lab, and conducting clinical trials, “she’s one of those people who’s doing everything at the moment. . . . I think she’s going to do very well.” ■

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In Custody

Expert tips for isolating and culturing cancer stem cells

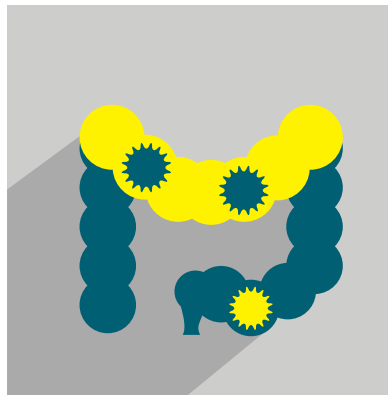
BY WUDAN YAN

For years, cancer researchers have sought to understand why tumors grow back in patients who have successfully undergone chemotherapy and/or radiation. One explanation, proposed in the late 1970s, was the cancer stem cell (CSC) hypothesis, which posits that a small fraction of cancer cells can self-renew indefinitely and give rise to the different cell types in a tumor. The CSC hypothesis can also explain why many cancers are resistant to standard courses of therapy: the drugs don't affect the stem cell population. If that's true, then drugs that eradicate CSCs could in theory cure cancer. By purifying CSCs from patient samples, scientists can study these cells in the lab to understand why they persist after cancer treatment.

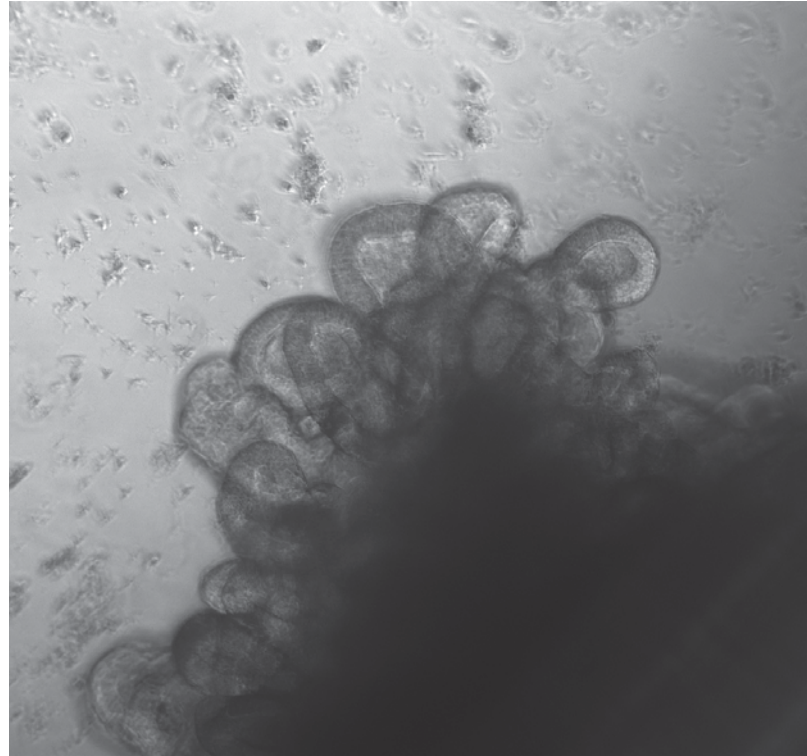
Researchers definitively identified CSCs in blood tumors beginning with acute myeloid leukemia (AML) in 1997, but the presence of CSCs in solid tumors was hotly debated because experiments using cell-surface markers to demonstrate their presence in solid tumors often yielded inconsistent results. In recent years, however, cells with self-renewing and tumor-initiating properties have been found in all tumor types: glioblastoma, colorectal, breast, pancreatic, and lung cancers. The debate no longer centers around "Do cancer stem cells exist?" but rather, "How many different types of cancer stem cells are there?"

One big barrier to understanding the biology of CSCs is easy access to patient samples; many hospitals freeze tumor sections, but it's much harder to obtain large numbers of viable cells from thawed tissues than from fresh ones. Another challenge lies in isolating pure populations of cells based on cell-surface markers. *The Scientist* sought out some expert advice on isolating and culturing stem cells from blood cancers and solid tumors for scientists who are eager to try their hand at working with cancer stem cells.

HANDLING PRIMARY PATIENT SAMPLES



John Dick, a stem cell researcher at the University of Toronto who first isolated cancer stem cells associated with AML, maintains that securing fresh tissue from clinicians is crucial to successful isolation of CSCs. Even if that proves difficult, however, pipetting or sectioning patient spec-



CANCER STEM CELLS IN A DISH: Cells isolated from a patient with metastatic colon cancer grown in organoid (3-D) culture. The organoid shown here recapitulates the crypt-like structures typical of those in the colon tissue of humans.

imens gently and keeping them on ice will maximize the number of viable cells that can be isolated.

The first step in isolating CSCs from primary patient samples is to obtain a single-cell suspension of the tumor. The benefit of working with blood cancers is that tumor cells are already in a single-cell suspension, and they can be separated from red blood cells and platelets using the well-established Ficoll separation protocol. To prevent the Ficoll solution from splashing, Christopher Dove, a graduate student in Ravindra Majeti's stem cell lab at Stanford University, advises placing a glass Pasteur pipet to the very bottom of the test tube, and pouring the Ficoll solution down through the pipet. Zero contamination of the lymphocytes is essential: if you think red blood cells have snuck into the test tube, Dove suggests redoing the Ficoll separation.

When handling frozen blood samples, Dove advises transferring the thawed sample into media containing DNase to degrade any DNA that is exposed when cells die during a rapid

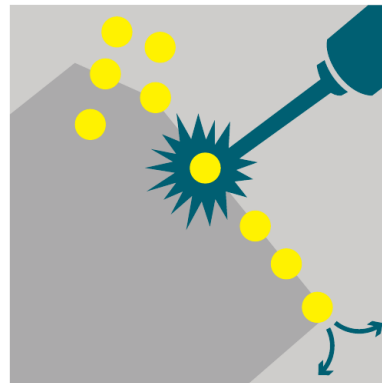
freeze-thaw. The exposed DNA is ‘sticky,’ and can clump cells to one another by acting like a strip of Velcro. Adding DNase to the media helps maintain a single-cell suspension. “If you’re thawing 40 million cells, but count 3 million and wonder where the other 37 million went, try using DNase,” he suggests.

Solid tumors, on the other hand, need to be mechanically and chemically digested to yield a solution of single cells. This process is often traumatic for the cells, as they must be enzymatically disaggregated from each other. Researchers need to be mindful of the enzymes they use. For example, trypsin, commonly used to passage cells in tissue culture, should be avoided because it works by cleaving cell-surface markers. You’ll need those later for detecting your stem cells.

Catherine O’Brien’s research lab at the University of Toronto and Hans Clevers’s group at the Hubrecht Institute in Utrecht, The Netherlands, use TrypLE Express, a gentler yet equally robust enzyme for the dissociation step. Collagenase can also be used as an alternative to trypsin for colorectal cancers, and usually, a 1 cm³ block of tumor is sufficient for good yield. Piero Dalerba, a professor of pathology and cell biology at Columbia University, advises tinkering around with the dissociation protocol, given that different cocktails of enzymes can work better for different tumor types. Because colorectal tumor cells grow in a single layer, it is easier to disaggregate the cells from patient tissue than it is from tougher, more fibrotic tumors. For these

more difficult tumors, Clevers suggests incubating the primary tissues overnight with collagenase.

SORTING STEM CELLS BY FLOW CYTOMETRY



The next step is to separate CSCs from the single-cell suspension using flow cytometry. CSCs are most commonly recognized by markers expressed on the cell surface and are stained with fluorophore-conjugated antibodies, with different colors of fluorescence designed to target the

different markers. The cytometer detects these fluorophores to separate the CSCs from the rest of the cells in the suspension. Although many staining protocols use monoclonal antibodies, the cautious researcher can incorporate a blocking step to prevent the antibodies from recognizing anything that they shouldn’t.

Irving Weissman, a pioneer in the stem cell field and director of the Stanford Institute for Stem Cell Biology and Regenerative Medicine, says it is not good enough to isolate AML stem cells, for instance, using just one marker. The steps through which a normal hematopoietic stem cell progresses to AML are clearly defined, so it is important to go “all the way” by including the most specific set of markers possible. Weissman recommends isolating the CD34⁺, CD38⁻, CD90⁺, and Lin⁻ cell fractions to robustly enrich for an AML stem cell population.

For solid tumors, Clevers advises researchers to be very familiar with the stem cell literature for the type of cancer under study. Although Clevers discovered that Lgr5 marks colon cancer stem cells, Lgr5 expression on the cell surface is generally low. Other markers such as EpCAM, CD44, and CD166 have been validated by multiple research groups to enrich for colon cancer stem cells from patient samples (*PNAS*, 104:10158-63, 2007; *J Clin Pathol*, 65:140-45, 2012; *Oncol Lett*, 7:1544-48, 2014).

Michael Clarke, a clinician and stem cell researcher at Stanford University who was the first to isolate cancer stem cells from solid tumors, cautions that while cell-surface markers are useful for enrichment, their presence doesn’t guarantee that the sorted cells will give rise to a full tumor—the crucial test that you have isolated a CSC population. Because markers for CSCs in solid tumors can vary greatly, researchers should run all the cell types sorted from a single tumor through a transplantation assay, described below.

“The trickiest thing for most people new to working with stem cells is the sorting,” Dove says. “Flow machines are big, expensive sorters that require a considerable amount of exper-

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tise to operate in a reproducible fashion.” Researchers who have not had significant experience with a flow cytometer should consult an expert at their institution’s flow core to set up the proper gates, calibrations, and parameters for obtaining the purest population of cells. In particular, Dalerba notes that using many antibodies to isolate the CSCs can lead to confusion because of overlap of emission spectra. Researchers should carefully titrate each of the monoclonal antibodies in their analyses and compare different fluorophore-antibody combinations. In other words, take the time to experiment and optimize a tissue-specific staining protocol.

INTO THE MOUSE



The “gold standard” proof that cells isolated by flow cytometry are indeed cancer stem cells involves transplanting them into immunocompromised mice to see if they can give rise to an entire tumor. This process normally consists of diluting the total number of cells and

injecting different dilutions into mice. If the number of cells in a tumor is proportional to the number of cells initially injected, you can be fairly sure that your cells were CSCs.

For blood cancers such as AML, cells are transplanted via tail-vein injections. AMLs typically take around two weeks to engraft.

For solid tumors, cells are most commonly transplanted subcutaneously near the organ in which the tumor typically forms. The diluted cell fractions are mixed with Matrigel, a gelatinous protein matrix that provides a support for the cells to latch on to. Colorectal tumors take, on average, 100 days to engraft following subcutaneous injection, but it can take anywhere from eight weeks to six months for a tumor to grow in a mouse.

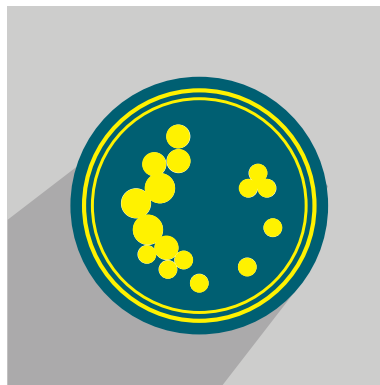
For breast cancer, gender of the mice and the method of cell delivery matters. According to Clarke, breast CSCs should be injected into the mammary fat pad of female mice along with an estrogen pellet, as estrogen is often involved in the maintenance of breast cancers. Triple-negative breast cancers can take 2–6 months to engraft, and ER⁺ tumors up to a year, says Clarke.

One major caveat of the transplantation assay is that not all isolated CSCs engraft. The reason for this is unknown, but one possibility is that additional immune cell types are needed for the cancer cells to thrive, particularly in solid tumors.

The time lag between getting cells from a patient and injecting them into mice is also important. “The faster you can get the tumors into mice, the better the chance of engraftment,” says Clarke. John Dick’s research group in Toronto has devel-

oped such a close collaboration with clinicians that they are often able to get AML patient samples into mice in as little as two hours.

IN A DISH



In vitro assays can help confirm that you have indeed isolated CSCs, and they can also aid in studying CSC biology. CSCs seem to grow best as spheres that form in the presence of a 3-D matrix such as Matrigel. Because stem cells are thought to form spheres in culture, researchers often use sphere-form-

ing assays to test these cells’ self-renewing properties.

A related in vitro approach is the organoid culture system—essentially, a 3-D culture to which growth factors are added in order to coax cells into forming miniature organ buds. The Clevers lab developed such a system in 2009 to grow structures of the internal small intestine and colon called intestinal crypts; later, they expanded the technique to cancer cells (*Gastroenterology*, 141:1762–72, 2011). The organoid system ensures that the phenotype of the cells taken from patients does not change. Cells typically take a week to form small organoids in culture. One important consideration is that different tumors may require different growth factors: many solid tumors require EGF and FGF; breast cancers can require estrogen; and prostate cancers, testosterone.

These in vitro experiments should not replace the in vivo transplantation assay to validate the true potential of the CSCs. CSCs that do not form tumors in mice can grow in culture, and the converse can be true as well. The lesson? Do both in vitro and in vivo assays.

One further caveat is that culture conditions for blood cancers such as AML have yet to be established. Unlike solid tumors, they do not need a surface to latch onto in order to grow. To work around this limitation, researchers typically expand populations of stem cells derived from AML using mice.

Once you are relatively sure you have indeed isolated CSCs, cells can be expanded in mice or by using the organoid culture technique. These CSCs can also be used for drug screens, and for other in vitro and in vivo assays. Having as close to a pure population of cells as possible is critical, particularly for sequencing and gene-expression profiles. “If we don’t have a pure population, we’re not going to have a clean answer coming out,” says Weissman. The hope is that by mastering the methods of working with stem cells derived from patient tumors, researchers can use these populations to better understand tumor biology. ■

Getting Your Sugar Fix

A guide to glycan microarrays

BY KATE YANDELL

Lipids and proteins on the surfaces of cells are bedecked with sugar chains, which determine how cells develop, adhere to one another, and communicate. Bacteria and virusesglom onto these complex linear or branched oligosaccharides, called glycans, to infect cells. The immune system fights back, learning to recognize microbes' sugar coatings and mounting both an innate and an adaptive defense.

Despite glycans' fundamental importance to biology, they remain poorly understood compared to DNA and proteins. Because of their structural complexity, glycans are arduous to manufacture. And unlike DNA, they are impossible to clone and amplify, so quantities are limited.

But one technology initially developed for understanding genetic material has proven a perfect fit for glycobiology: the microarray. The first glycan microarrays came onto the scene in 2002, just seven years after the advent of microarrays to study gene expression.

Glycan microarrays consist of small quantities of a variety of natural or synthetic oligosaccharides affixed to a surface. Researchers use the arrays to identify proteins, cells, and microbes that bind to the sugars. Because printed microarrays require minuscule quantities of sugars, they made it possible, for the first time, to broadly screen proteins' glycan affinities.

These days, more than a dozen labs make their own glycan microarrays, and researchers say they will only improve as more glycans are isolated and synthesized. The largest and most widely available arrays are produced by the Consortium for Functional Glycomics (CFG), an international collaboration among glycomics researchers, and by Imperial College London's Glycosciences Laboratory. Other labs make smaller arrays targeted for use on more-specific projects.

"At this point I don't think there is a single presentation or single platform that is a clear winner," says Jeffrey Gildersleeve, head of the Chemical Glycobiology Section at the National Cancer Institute's Center for Cancer Research in Frederick, Maryland.

And while DNA microarrays' glory days may be fading as RNA sequencing prices fall, the future of glycan microarrays appears sunny. "I think that [glycan microarrays are] really just about to, in the next five years, come into their own in terms of commercial use, industrial applications, and clinical use," says Richard Cummings, professor of biochemistry, director of the National Center for Functional Glycomics (NCFG) at the Emory University School of Medicine, and current chair of the CFG.

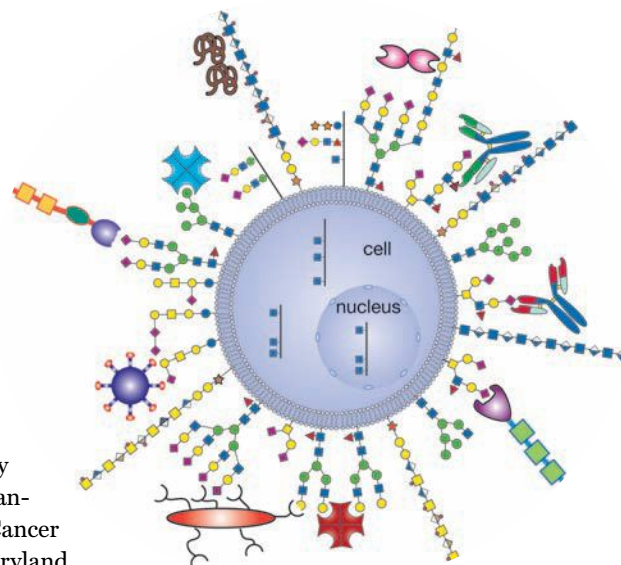
Here, *The Scientist* profiles a selection of today's leading glycan microarrays and some of the discoveries they have enabled.

OUR GLYCANS, OURSELVES

The CFG was first funded in 2001 by the National Institute of General Medical Sciences (NIGMS) to study protein-carbohydrate interactions. An early CFG project resulted in an ELISA-based glycan array to be shared with the entire glycomics community. By 2004, the CFG had switched to a printed microarray including 200 glycans (*PNAS*, 101:17033-38). Currently, the array consists of 610 mammalian glycans covalently attached to slides via amide linkages.

ADVANTAGES

The CFG has proven to be an invaluable resource to researchers who don't have voluminous glycan libraries of their



own, says CFG chair Cummings. "Most of the glycans of interest are not commercially available," he says. And even if they were, one would have to purchase them in large quantities.

The CFG's mammalian glycan array has contributed to discoveries ranging from the receptor affinity of flu viruses, to the lectins bacteria use to invade cells, to the cancer-specific antibodies that bind to glycans present on the surface of tumor cells.

Most recently, Stephan von Gunten of the University of Bern's Institute of Pharmacology in Switzerland and colleagues used the array to determine glycan binding of immunoglobulins isolated from the blood of healthy donors (*Sci Transl Med*, 7:269ra1, 2015). They discovered that some immunoglobulins bind to mammalian glycans that bacteria and viruses coopt to invade cells. The researchers hypothesize that human antibodies block these adhesion sites in order to outcompete binding by the pathogens.

Cummings and his colleagues also developed a method called shotgun glycomics, in which they harvest and purify glycans from an array of sources, ranging from breast milk to pig lungs (*Nat Meth*, 8:85-90, 2011). The researchers then observe binding patterns of known glycan-binding proteins to the arrays and sequence the glycans that they bind. Alternatively, researchers can use the shotgun

arrays to identify the glycans to which particular microbes attach.

ACCESS

Although the original NIGMS funding ended in 2011, screening using the CFG array remains available via Emory's NCFG. Researchers hoping to test the glycan-binding properties of their own samples can apply on the CFG website to send them to Emory for screening, which costs \$300 per slide. Any results produced by the CFG array can be accessed at www.functionalglycomics.org. Researchers hoping to use shotgun glycan arrays or other specialized arrays offered by the NCFG can pay to access them. The NCFG will also print custom arrays for a fee.

BENDY PRESENTATION

ARRAY

Researchers at the Glycosciences Laboratory at Imperial College London produced one of the earliest glycan microarrays (*Nat Biotechnol*, 20:1011-17, 2002). The group's microarray system has since evolved to include 830 glycan probes. Each synthetic glycan is affixed to a lipid tag, a combination called a neoglycolipid (NGL), and then printed onto a glass slide coated with nitrocellulose. The microarray also includes naturally occurring glycolipids.

ADVANTAGES

Imperial College's NGL-based microarrays stand out from other microarrays because

the lipids and the noncovalent bonds linking the sugars to the slide are slightly flexible, allowing the glycans to wiggle around. "We believe that element of mobility is really important," says Ten Feizi, director of the Glycosciences Laboratory and one of the original creators of the microarray.

The strength of bonds between individual glycans and proteins is weak. Because of this, groups of glycans often interact with proteins or groups of proteins with multiple glycan binding sites on their surfaces. These bonds between proteins and groups of glycans are called multivalent interactions.

All glycan microarrays allow for multivalent bonding, as multiple sugar chains are printed side by side on the array. However, the density of the glycans and their orientation influence whether they are able to slot into their allotted protein binding sites. Feizi says that the NGL-based microarray is more forgiving, as the non-rigid linkers allow the glycans to adjust to the multivalent binding sites on proteins, much as glycans on a fluid cell membrane might adjust during binding.

NGL-based microarrays have in recent years helped researchers understand viruses, innate immunity, and cancer. Feizi recalls that her group's microarrays really shined in studies of sugar-coated receptors used by the 2009 H1N1 swine flu to infect cells. The researchers employed a small NGL-based array to show that the virus bound not only to a

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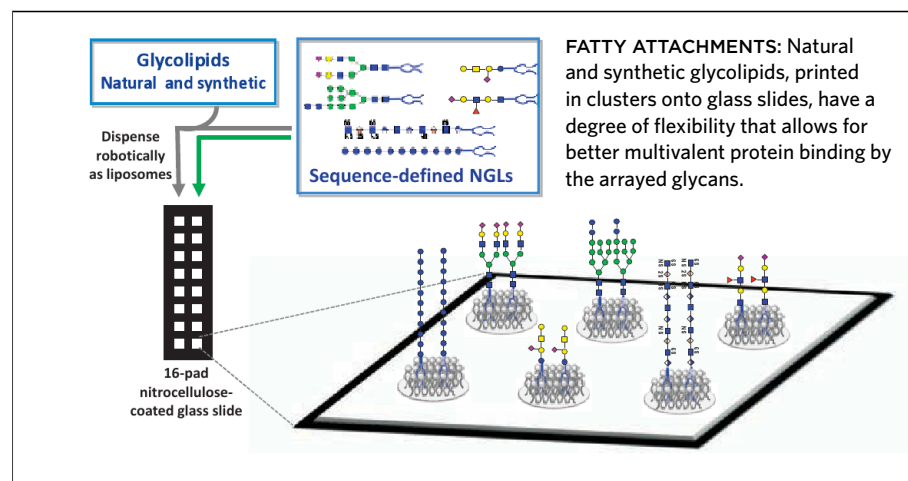
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sugar found on the surface of cells in the upper respiratory tract, but also to a different glycan found deep in the lungs (*Nat Biotechnol*, 27:797-99, 2009). Other glycan microarrays failed to detect the relatively weak binding to the alternate lung receptor. Feizi and her colleagues subsequently showed that a mutated version of the virus had a heightened affinity for the lung receptor, and that this mutant tended to cause more-lethal infections (*J Virol*, 84:12069-74, 2010).

Last year, the researchers used their microarrays to determine the identity of a glycan structure, F77, bound by an antibody known to stick to prostate cancer cells (*J Biol Chem*, 289:16462-77, 2014). They first assessed the antibody's binding against NGLs and glycolipids on their broad screening array. But the array only generally revealed the carbohydrate structures the antibody targeted. After discovering that the antibody also binds heavily glycosylated proteins called mucins, the researchers released glycans from naturally occurring mucins to create "designer arrays," allowing them to more precisely establish the sugar chain structure the antibody binds.

ACCESS

Researchers hoping to use NGL-based arrays from the Glycosciences Laboratory can apply online at www1.imperial.ac.uk/glycosciences/request/. Glycosciences Laboratory personnel will analyze one to three samples using the large screening array for £200 per sample. The Wellcome Trust currently funds the facility.

CANCER QUESTIONS ARRAY

Over the past decade, the National Cancer Institute's Jeffrey Gildersleeve has developed a glycan microarray consisting of 503 natural glycoproteins, synthetic "neoglycoproteins," peptides, and controls. On the surfaces of the neoglycoproteins the researchers vary the arrangements and densities of glycans. This variation allows users to understand how altering the presentation of glycans affects binding of proteins.

ADVANTAGES

Gildersleeve developed his array to screen vast quantities of serum from people with cancer. By presenting glycans in multiple configurations and densities on the surfaces of proteins, he encourages multivalent bonding. Being able to control presentation allows him to understand which configurations of glycans bind which proteins, and allows him to replicate arrangements that work.

"We're trying to identify unique subpopulations of antibodies in serum that are relevant to cancer care," he explains. These antibody subfamilies may only be distinguishable by which presentations of glycans they bind.

The Gildersleeve group is probably best known for its success in characterizing antibodies in prostate cancer patients to help predict and then assess how they respond to the Phase 3 cancer vaccine PROSTVAC-VF, in development by Bavarian Nordic.

"The big challenge for most cancer therapies, and especially cancer vaccines and other immunotherapies, is that they can have really, really good results in some patients . . . but lots of patients really see no benefit at all," Gildersleeve says. Discovering biomarkers to predict how patients will do on therapies aids doctors in choosing the right ones for their patients.

Using their microarrays, the group analyzed the serum of patients prior to their participation in Phase 2 clinical trials of the vaccine and then several months after beginning the therapy. The researchers found that patients who showed elevated immunoglobulin binding to a blood group A antigen before the trial were likely to survive longer than average (*Clin Cancer Res*, 19:1290-99, 2013). The researchers also found that patients who received the vaccine and then did well tended to show increases in antibodies to the Forssman antigen, a glycan present on viral vectors used to deliver the vaccines (*PNAS*, 111:E1749-58, 2014).

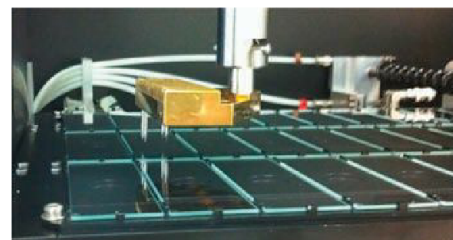
ACCESS

The Gildersleeve lab is willing to collaborate with other research groups on projects using their microarray. The group is particularly interested in work related to cancer or HIV.

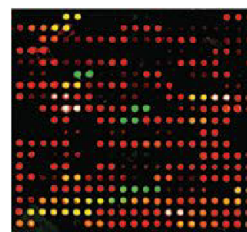
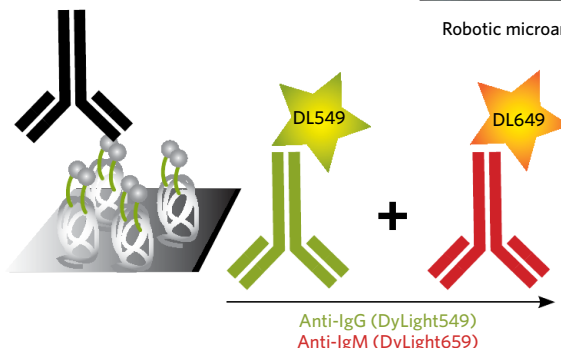
ANCIENT IMMUNITY ARRAY

While most glycan microarrays focus on the mammalian glycome, some researchers are starting to pay attention to the sugars decorating microbes. Last year, researchers led by Cummings and his frequent collaborator

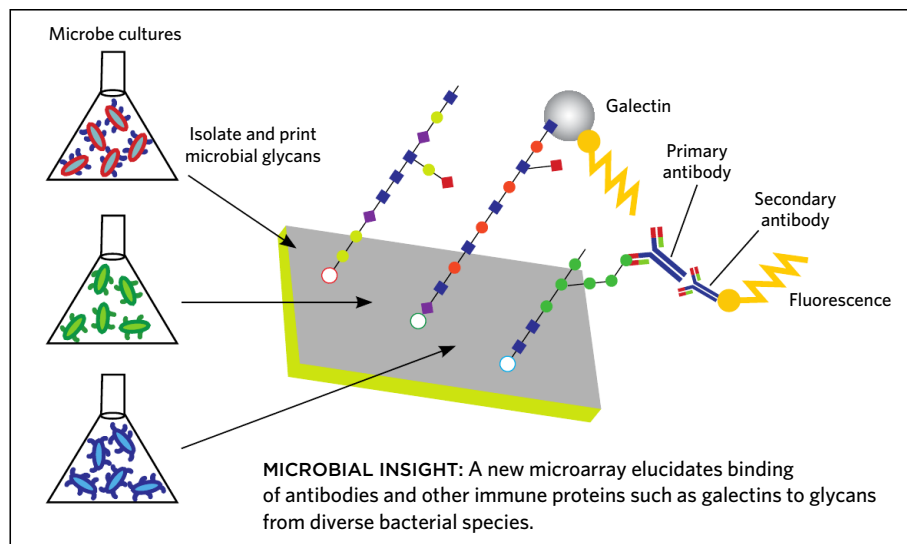
PROTEIN FOUNDATION: Jeffrey Gildersleeve's microarray of natural and synthetic glycoproteins can be used to assess the binding of antibodies present in the sera of cancer patients. Fluorescent secondary antibodies (shown in green and red) are used to label bound antibodies (black), revealing which glycan probes they bind to on the array.



Robotic microarrayer prints ~500 array components



GenePix scanner



Jim Paulson, professor of cell and molecular biology at the Scripps Research Institute in La Jolla, introduced an array that now includes approximately 350 microbial glycans derived from diverse species.

ADVANTAGES

The CFG began to construct a microbial glycan microarray (MGM) around a decade ago, but halted the project after their funder, the NIGMS, decided the array did not fit into the scope of the consortium's mission, according to Paulson, who was then CFG director.

Paulson and his colleagues recently began work on the array again and introduced a new and improved version in June 2014 (*Nat Chem Biol* 10:470-76). Paulson and his colleagues demonstrated that the microarray shows different binding patterns when presented with human versus other mammalian sera. They also showed that rabbit sera's reactivity to glycans changes after the animals have been exposed to specific microbial strains. These results indicate that the adaptive immune system quickly reacts to the presence of specific microbes, and also that the new microarray, while small, is sufficiently sensitive to pick up these changes.

The researchers further investigated the binding of innate immune glycan-binding proteins called galectins to the array, finding that several of them bind a probe isolated from a strain of the gut bacterium *Providencia alcalifaciens*.

Further investigation showed that binding by some galectins kills this strain and that galectins also bind and kill an assortment of other gram-negative and gram-positive bacterial strains carrying similar glycan antigens.

This type of antigen mimics mammalian glycans, possibly as a way for bacteria to evade the immune system. Indeed, the researchers showed that the same galectins bind analogous antigens on the CFG mammalian array. However, the galectins do not harm mammalian cells when they bind to this antigen. Thus, the MGM was able to uncover an entirely new innate immune mechanism for fighting off diverse bacteria that try to mimic mammals' own cells.

Many in the glycomics community see the MGM as long overdue, as microbial and mammalian glycans are generally quite distinct. "I think this is a part of the immune system we haven't mined," says Laura Kiessling, director of the Keck Center for Chemical Genomics at the University of Wisconsin-Madison. "It's been opaque to us because we haven't had any way of looking at it." Kiessling is currently using the MGM to further explore innate immunity.

ACCESS

Those wishing to use the array can obtain it through the CFG at cost. ■

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The CAR T-Cell Race

Tumor-targeting T-cell therapies are generating remarkable remissions in hard-to-beat cancers—and attracting millions of dollars of investment along the way.

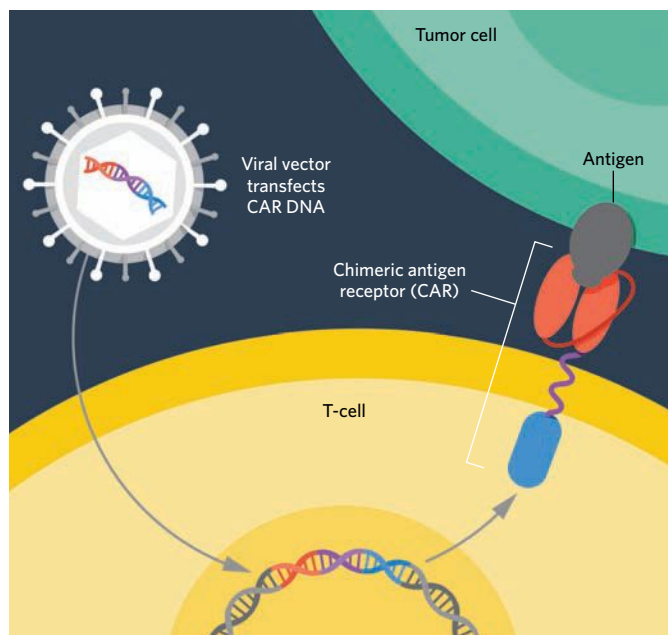
BY VICKI BROWER

Last December, scientists at Juno Therapeutics reported at the American Society of Hematology (ASH) meeting that, in an ongoing Phase 1 trial, its chimeric antigen receptor (CAR) T-cell therapy, JCAR015, put 24 of 27 adults with refractive acute lymphoblastic leukemia (ALL) into remission, with six patients remaining disease free for more than a year (ASH 2014, Abstract 382, 2014). This disease is extremely hard to treat and progresses rapidly when it becomes refractory; most patients die within a few months. “This response rate is unprecedented for patients who had stopped responding to all other treatments,” says Michel Sadelain, a founding director of Memorial Sloan Kettering’s Center for Cell Engineering and a cofounder of Juno.

Founded just a year earlier, the Seattle-based company now has four CD19-targeting CAR T-cell therapies in trials. The premise is simple: extract a patient’s T cells from blood and train them to recognize and kill cancer by modifying them with a viral vector to express an artificial, or chimeric, receptor specific for a particular cancer-associated antigen—in this case, CD19, an antigen expressed in B-cell-related blood cancers—then reinfuse the cells back into the patient. (See illustration at right.) The engineered cells recognize and kill cancerous cells, while reactivating other immune players that have been dampened by cancer’s inhibitory signals. “CAR therapy is at the same time cell therapy, gene therapy, and immunotherapy,” says Sadelain. “It represents a radical departure from all forms of medicine in existence until now.” Promising preclinical results have moved Juno’s CD19 therapies into trials for ALL, non-Hodgkin’s lymphoma, and chronic lymphocytic leukemia (CLL), and the company has three more CAR T-cell immunotherapies for a number of solid cancers close behind.

A few weeks after the ASH meeting, Juno went public for a whopping \$264.6 million, the largest biotech initial public offering (IPO) of 2014. Within a month, the company’s valuation rose from \$2 billion to \$4.7 billion, the largest among biotechs in a decade. By the end of 2016, the company plans to have 10 drug trials for six diseases up and running using CAR T cells produced in a brand-new manufacturing facility.

And Juno is not alone. This relatively new sector is experiencing a frenzy of scientific activity, corporate partnering, and financing that took off in late 2013, continued throughout 2014, and moved straight into the new year with no sign of letting up. By now, most major pharmaceutical companies have jumped into the CAR T-cell arena. In the past two years alone, at least half a dozen companies have made deals worth hundreds of millions of dollars up front, with much more expected in the future as prod-



BUILDING A BETTER T CELL: By modifying T cells to express chimeric antigen receptors (CARs) that recognize cancer-specific antigens, researchers can prime the cells to recognize and kill tumor cells that would otherwise escape immune detection. The process involves extracting a patient’s T cells, transfecting them with a gene for a CAR, then reinfusing the transfected cells into the patient.

ucts move through the pipeline. (See chart on page 75.) This influx of funding is now supporting dozens of clinical trials.

While most of these studies are currently aimed at late-stage disease for which other therapeutic options have failed, researchers in the field anticipate that these immunotherapies could replace standard cancer treatments in the future. “While we are evaluating these therapies in advanced cancer now, we absolutely believe that they have the potential to become frontline therapies,” Sadelain says.

Long time coming

CAR T-cell therapy has had a lengthy run-up to what may appear to be overnight success. The first CAR T cells were developed at the Weizmann Institute of Science in Israel in the late 1980s by chemist and immunologist Zelig Eshhar. In 1990, Eshhar took a year-long sabbatical to join Steven Rosenberg at the National Institutes of Health, where they prepared CARs that targeted human melanoma. “We designed CAR T cells to overcome a number of

problems in getting T cells to attack cancer,” says Eshhar. These problems included a tumor’s ability to escape immune recognition by silencing the major histocompatibility complex molecules and the generally immunosuppressive tumor microenvironment.

Eshhar and Rosenberg constructed the CAR T cells with a modular design that included a specific cancer-targeting antibody, and later added a costimulatory signaling domain that amplifies the activation of the cells, giving them a stronger signal to multiply and kill cancer cells. Since that early work, researchers in both academia and industry have developed and tweaked each section of the modular design. (See illustration below.) “Ultimately, we needed 20 years to learn how to supercharge these cells to deliver anticancer activity,” says Arie Belldegrun, president and CEO of Kite Pharma in Santa Monica, California, which is assessing CAR T cells in six trials for B cell leukemia and lymphomas, and glioblastoma. Eshhar, a member of Kite Pharma’s scientific advisory board, continues to collaborate with Rosenberg, who serves as a special advisor to the company.

Juno is now working on two second-generation CAR technologies that incorporate mechanisms to further amplify T-cell activation or to dampen it, in the case of adverse reactions. (See “Safety concerns” on page 76.) These so-called “armored” chimeric antigen receptors are designed to combat the inhibitory tumor microenvironment by incorporating a signaling protein such as IL-12, which stimulates T-cell activation and recruitment. Juno believes “armored CAR” technology will be especially useful for solid tumors, whose microenvironment and potent immunosuppressive mechanisms can make raising antitumor responses more challenging.

Like Juno, Houston, Texas-based Bellicum Pharmaceuticals is working on refinements for next-generation CAR T-cell treat-

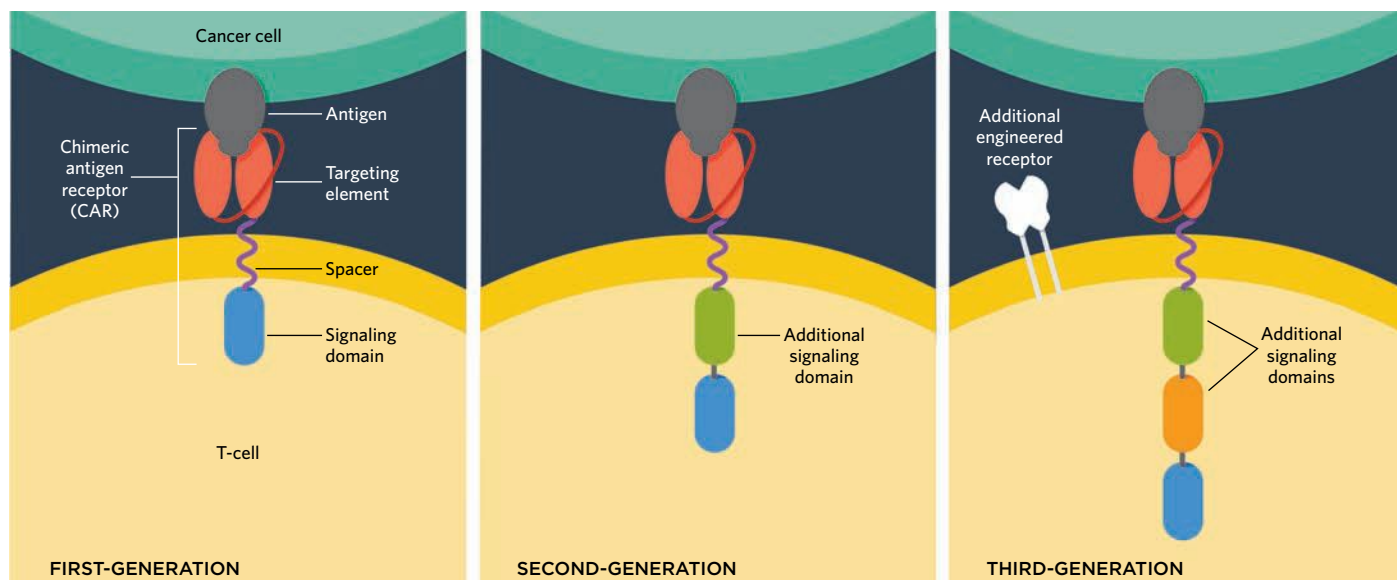
ments. To better control antigen activation by its CAR T cells, for example, Bellicum is separating its dual costimulatory domain from the antigen-recognition domain, moving it onto a separate molecular switch that can be controlled by the small-molecule drug rimiducid. These T cells, known as GoCAR-Ts, can only be fully activated when they are exposed to both cancer cells and the drug.

In addition to altering the components of the CAR T cells themselves, researchers are also experimenting with different methods to introduce the receptors into the patients’ cells. At MD Anderson Cancer Center in Houston, Laurence Cooper and his colleagues are using a nonviral system called “Sleeping Beauty,” licensed from the University of Minnesota’s Perry Hackett, that relies on a transposon derived from fish to paste any desired gene into the genome. “This system employs electroporation [an electric current] to introduce elements of the Sleeping Beauty system into T cells,” says Cooper, who hopes the system will be less complex and cheaper to use than viral vectors.

While CAR T cells are being tested first as monotherapies, researchers are also giving thought to how best to use CAR T cells with other immunotherapies in the future. “We are excited about combining checkpoint inhibitors such as PD-1 [programmed death-1] inhibitors and anti-CTLA4 [anti-cytotoxic T-lymphocyte antigen 4] drugs with CAR T cells,” Eshhar says.

A frenzy of deal making

Over the past few years, the industry has been a hive of activity, with a half dozen companies forging deals valued at more than a half billion dollars in total. In addition to the perceived financial potential of these therapies, the feeding frenzy may in part be attribut-



NEW AND IMPROVED CARs: Zelig Eshhar and Steven Rosenberg constructed the first CAR T cells using a modular design, including a specific cancer-targeting antibody outside the cell, a transmembrane component, and an intracellular costimulatory signaling domain that amplifies the activation of the CAR T cells. Second- and third-generation CAR T-cell technologies have added additional costimulatory domains within the cells, as well as additional receptors to improve targeting of the T-cell attack and minimize side effects.

CAR T-CELL DEALS

Institution/Company	Date	Partner	Terms
University of Pennsylvania	August 2012	Novartis	Undisclosed
Celgene	March 2013	Bluebird Bio, Baylor College of Medicine	Unspecified upfront payment plus up to \$225 million per product in option fees and milestone payments
Collectis	June 2014	Pfizer	\$80 million upfront plus up to \$185 million per product and royalties
Collectis	January 2015	Ohio State University	Undisclosed
Kite Pharma	January 2015	Amgen	\$60 million upfront and up to \$525 million per product in milestone payments, plus royalties on sales and IP licensing
MD Anderson	January 2015	Ziopharm, Intrexon	\$100 million in stock and \$15–20 million/year for 3 years

able to the fact that regulatory authorities are giving CAR T-cell treatments priority review for filling unmet medical needs. Many of these therapies are receiving orphan or breakthrough status from the US Food and Drug Administration (FDA), bringing expedited regulatory review, which translates into earlier realization of financial benefits from more rapid market entry. In November 2014, for example, the FDA granted orphan status to Juno's JCAR015. Kite's KTE-C19 for refractory aggressive non-Hodgkin's lymphoma also recently received the designation from both the FDA and the European Medicines Agency. And the University of Pennsylvania / Novartis's CTL019 for ALL received breakthrough status last July.

A new report by EP Vantage, a financial analysis company, notes that while investor enthusiasm for this sector is unlikely to diminish anytime soon, "there may be hidden dangers" for those in it to make a big return. "CAR T therapy looks like it's becoming little short of a revolution in the treatment of some cancer types, but numerous risks are being lost in the hype," writes report author Jacob Plieth, a biochemist by training. "It is important to appreciate the risks as well as the opportunities to have a clear understanding of the market potential of these therapies and their developers."

Clinical results spark hope

In 2011, the Penn group described the results of an early trial of its CTL019 CAR T-cell treatment in three advanced chronic lymphocytic leukemia (CLL) patients (*Sci Transl Med*, 3:95ra73, 2011). The findings—including two patients who have now remained in remission 4.5 years after their treatment—served as an early demonstration that CAR T cells can successfully treat patients with late-stage disease. The team has now tested CAR T-cell therapies in about 125 people, with six different trials underway for pediatric and adult ALL, CLL, multiple myeloma, and non-Hodgkin's lymphoma. Other CAR T-cell therapies are in trials for solid tumors, including ovarian, breast, and pancreatic cancers, and mesothelioma and glioblastoma.

"One of the exciting things about these cells is that they expand to high numbers and maintain long-term functional persistence," says David Porter, a leukemia specialist and director of blood and marrow transplantation at Penn.

CAR T-CELL BIOTECH IPOs

Company	Date	Value
Kite Pharma	June 2014	\$134.1 million
Bellicum	December 2014	\$160 million
Juno	December 2014	\$264.6 million
Collectis	February 2015	\$115 million

In a recent Penn study of 30 children and adults with relapsed or refractory ALL who received CTL019, 90 percent achieved total remission, and 78 percent were still living at the end of the study two years later (*NEJM*, 371:1507-17, 2014). Moreover, "very few patients—three—got a bone marrow transplant after CTL019, suggesting that this could be a replacement for bone marrow transplant and not just a bridge to transplant," added senior author Stephan Grupp, director of translational research for the Center for Childhood Cancer Research at the Children's Hospital of Philadelphia.

At ASH, Grupp discussed a follow-up study, including 39 pediatric patients, which showed a 92 percent complete remission rate following CTL019 treatment. Of those, 76 percent remain in complete remission after six months (ASH 2014, Abstract 380, 2014). "The first ALL patient treated is still in remission nearly three years later," says Grupp. In September, Novartis pledged \$20 million to build a Center for Advanced Cellular Therapeutics to manufacture CAR T cells on the Penn campus, to be completed next year. Penn is now conducting pilot trials aimed at solid tumors, including mesothelioma; ovarian, breast, and pancreatic cancers; and glioblastoma.

A team at the National Cancer Institute, including Rosenberg, has also reported successes with CAR T cell therapy, focusing on patients with refractory diffuse large B-cell lymphoma, an aggressive disease for which survival without treatment is measured in months. Following treatment with CD19-targeting T cells, 22 of 27 patients had either complete or partial remissions; 10 have remained cancer free for up to 37 months (ASH 2014, Abstract 550, 2014). These and other trials have demonstrated that CAR



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BIO BUSINESS

T-cell therapies can successfully treat leukemias and lymphomas in some patients for whom there are no other treatments.

There are currently many other CAR T-cell trials underway in leukemia and lymphoma, and more beginning in the near future. Scientists in this arena are energized by these and other results, and many see CAR T-cell therapies as the future of cancer treatment. "I believe these trials indicate that chemotherapy may be on its way out," says MD Anderson's Cooper. ■

Vicki Brower is a freelance science writer living in New York City.

SAFETY CONCERNS

Despite the growing number and length of remissions using CAR T-cell therapy to treat leukemias and lymphomas, key challenges remain—first and foremost, safety. There have been a half-dozen treatment-related deaths in the University of Pennsylvania and Juno trials in the past few years that involve a major side-effect of CAR T-cell therapy called cytokine-release syndrome (CRS). T-cell activation causes the release of inflammatory cytokines, producing symptoms including high fevers, aches, hypotension, and, more rarely, pulmonary edema and neurologic effects such as delirium.

Researchers tie the severity of what they call a "cytokine storm" to tumor burden—a patient's total mass of cancer tissue or quantity of malignant cells. One hypothesis for this is that higher tumor burden seems to incite a stronger immune reaction. Moreover, the deaths have all occurred in adults, some of whom had serious underlying medical issues, and others who had undiagnosed infections. Interestingly, children seem relatively resistant to severe CRS and, when they get it, are more easily managed, says Michel Sadelain of Memorial Sloan Kettering and Juno. "Adults do not tolerate the treatment as well as children, in whom the cells differ in speed of action and persistence," Sadelain says. Treatment with an anti-IL6 antibody, or in severe cases, corticosteroids, can mitigate a cytokine storm's severity, as can dosing with lower numbers of CAR T cells.

"In our trial [on diffuse large B-cell lymphoma], we saw that toxicity was reduced in patients who received low-dose chemotherapy rather than high-dose [prior to CAR T-cell treatment], and lower numbers of engineered T cells [than given previously]," says James Kochenderfer of the National Cancer Institute (NCI).

Bellicum is partnering with the University of Leiden in the Netherlands and the NCI, among others, to develop "suicide switches," or safety on-and-off switches that are incorporated into CAR T-cell candidates to control T-cell activation and proliferation. And Juno's second-generation "armored" CAR technologies include mechanisms to dampen T-cell activation. "It will be important to find new ways to overcome toxicity of CAR T cells," says the Weizmann Institute's Zelig Eshhar.

Setbacks and Great Leaps

The tale of *p53*, a widely studied tumor suppressor gene, illustrates the inventiveness of researchers who turn mishaps into discoveries.

BY SUE ARMSTRONG

In 2002, Larry Donehower of Baylor College of Medicine in Houston, Texas, was creating a mouse model to explore the workings of the tumor suppressor gene *p53* when he made a surprisingly fruitful mistake. Donehower had used an unfamiliar technique to create a mouse with *p53* knocked out, and so, instead, he ended up with mice in which the gene was not only still present, but hyperactive. Predictably, his super-*p53* critters proved highly resistant to developing tumors. But what no one expected to see was that they aged exceptionally fast: within months their fur was bedraggled and gray, their backs hunched, and they died prematurely, losing about 30 percent of their normal life span.

That aging and cancer were related was common knowledge, since the risk of cancer increases with age. But few suspected they might be two sides of the same coin, sharing a mechanism through which the scales could be tipped either way.

As I researched and wrote *p53: The Gene That Cracked the Cancer Code*, I became intrigued by how often apparent experimental failures have provided vital clues to unraveling the mysteries of this particular gene.

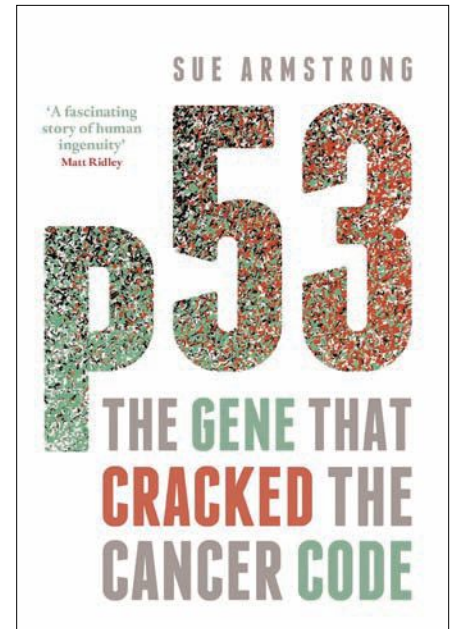
Even the discovery of *p53*, in 1979, was arguably the result of failure. By coincidence, four different labs, working independently and unaware of each other's quests, discovered *p53* simultaneously. Three were working with the oncogenic monkey virus SV40, trying to isolate the specific viral gene and its protein product responsible for causing tumors. But no matter how hard they tried, none of the groups was able to separate the viral protein from one produced by the host cell—a protein with a molecular weight of 53 kilodaltons, which seemed to piggyback on the viral protein. Fellow scientists were apt to dismiss the pesky cell protein as a

contaminant. Fortunately, the researchers—David Lane in London, Arnie Levine in Princeton, and Pierre May in Paris—recognized something significant, though they had little clue as to just how significant it would turn out to be. They published their results and turned their attention to figuring it all out.

The first step was to clone the gene coding for the mysterious piggybacking host protein in order to obtain endless copies for research. But those early clones turned out to be mutants, which led everyone up a blind alley. They suggested *p53* was an oncogene, a tumor driver, rather than a tumor suppressor. It was only when Levine's clone failed repeatedly to reproduce everyone else's results that the light bulb went on: his was the only normal clone, and it didn't cause cancer. Clearly, normal *p53* was not an oncogene.

Not long after researchers recognized *p53* as a tumor suppressor, experiments showed that the gene product's modus operandi is to ensure faithful copying of DNA when cells divide. If DNA is damaged during mitosis, *p53* stops the cell cycle in its tracks, and sends in the repair team before allowing the process to proceed. Once again, it was a failed experiment that revealed yet another, more potent ploy in the gene's anticancer repertoire: *p53*'s product can induce senescence and even suicide in cells that are beyond repair.

At Israel's Weizmann Institute in 1990, Moshe Oren was moving his small lab to another room. Unbeknownst to him and his team, the thermostat malfunctioned on one of two cabinets containing rat embryo fibroblast cultures with identical *p53* mutants plus the oncogene *ras*. In the affected cabinet, where the temperature was lower, the cells' transformation was inhibited and their growth



Bloomsbury USA, February 2015

was arrested, while they continued to transform and proliferate in the neighboring cabinet under what the researchers believed were the exact same conditions. It took time and repeated failure of the experiment in the faulty cabinet for Oren to grasp what was happening, and to realize that they had stumbled across an invaluable tool in molecular biology: a *p53* mutant that was temperature-sensitive, behaving like wild type below 32 °C and a mutant at 37 °C. But most importantly, working with this new tool they soon made the heady discovery that the wild-type gene can force cancer cells to kill themselves by apoptosis.

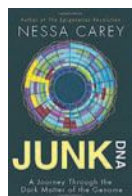
Many other apparent failures pepper the study of *p53*, and most suggest a universally relevant lesson: never be too ready to dismiss as scientific failures things that don't go the way we expect, for setbacks may prove fruitful after all. ■

Sue Armstrong is a science writer and foreign correspondent. Her previous book, A Matter of Life and Death: Inside the Hidden World of the Pathologist, was published in 2010. Read an excerpt of p53: The Gene That Cracked the Cancer Code at www.the-scientist.com.

Junk DNA: A Journey Through the Dark Matter of the Genome

Nessa Carey

Columbia University Press, March 2015



Can we please stop calling non-protein-coding DNA “junk?” We’ve had more than a decade—since researchers first sequenced the human genome in

2001—to come to grips with the fact that 98 percent of our DNA doesn’t get translated directly into proteins. And in the intervening years, what was initially considered perplexingly abundant detritus has evolved into a wondrous genomic landscape that hasn’t disappointed intrepid explorers. Imperial College London molecular biologist and author Nessa Carey details this transformation and the progress science has made in her latest book, *Junk DNA*.

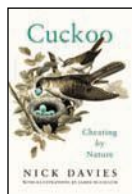
Carey examines the importance of noncoding DNA, from its role in controlling gene expression to the ways in which it influences RNA behavior, disease progression, and sex determination. Although we can now be sure that 98 percent of the human genome is anything but junk, researchers are only just beginning to fully appreciate the complex influence of all that once-maligned genomic material.

Junk DNA serves as a nice primer for the still-uninitiated, but here’s to hoping that continued research, and the emerging view that genomes behave like networks rather than a string of isolated genes, soon relegates the J-word to the junk pile of science history.

Cuckoo: Cheating by Nature

Nick Davies

Bloomsbury, April 2015



Cuckoos are masters of the art of deception. The medium-size, grayish birds have evolved a reproductive strategy called brood parasitism, in which they lay their eggs

in the nests of other bird species, duping those hapless parents into rearing cuckoo

young. Although there are other brood parasites among birds, fish, and insects, behavioral ecologist Nick Davies has made the cuckoo the lens through which he views the fascinating adaptation.

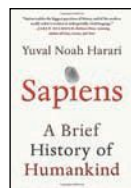
In *Cuckoo*, he relays the history of how science has viewed the behavior—from early observers, who struggled to fathom why the Creator made an animal with so little affection for its own offspring, to Charles Darwin, who reasoned that the interplay between cuckoo and host bird species reflected the same evolutionary arms race he’d noted between predator and prey.

Davies shares insights into his years of research on the specifics of how the cuckoo evolved brood parasitism. His obvious passion for observing cuckoos, their host birds, and the behavioral and physical traits that make them nature’s perfect cheat is liable to spark similar stirrings in aspiring naturalists who read this book.

Sapiens: A Brief History of Humankind

Yuval Noah Harari

Harper, February 2015



Human beings are the most destructive force this planet has ever seen. We change the climate, extirpate entire species, and transform ecosystems. But just how has our species come to domi-

nate the Earth? Such is the weighty question tackled by historian Yuval Harari in his latest book, the international best-seller *Sapiens: A Brief History of Humankind*. “The most likely answer is the very thing that makes the debate possible,” he writes. “*Homo sapiens* conquered the world thanks above all to its unique language.”

And with language, *Sapiens* (as Harari refers to the species in the book) has created powerful myths about gods, money, politics, and human rights. These myths, when codified and shared, can draw together multitudes of individuals into cohesive groups. And in numbers, there is strength.

Harari recounts the meteoric rise of our species in light of the advantages

bestowed upon us by our supple and descriptive language. But the attribute that made us so powerful could be our undoing. The author decries the seeming aimlessness with which *Sapiens* wields its unique skill set. “We are more powerful than ever before, but have very little idea what to do with all that power,” he writes. “We are consequently wreaking havoc on our fellow animals and on the surrounding ecosystem, seeking little more than our own comfort and amusement, yet never finding satisfaction.”

Cool: How the Brain’s Hidden Quest for Cool Drives Our Economy and Shapes Our World

Steven Quartz and Anette Asp

Farrar, Straus and Giroux, April 2015



Fads may come and go, trends fluctuate, but cool is always in style. Even the word, likely co-opted to mean something hip or au courant around the late 19th

century, retains its meaning among toddlers and tweens, the middle-aged and octogenarians. But why are we so universally attracted to things that are cool? The answer may lie deep in our brains, argue cognitive scientist Steven Quartz and political scientist Anette Asp in *Cool*.

The authors suggest that the brain’s cool circuits, which probably involve the medial prefrontal cortex and the striatum, likely drive the consumerism that so dominates myriad human cultures. Cool, in fact, is intimately related to our desire to possess, Quartz and Asp write. “Cool turns out to be a strange kind of economic value that our brains see in products that enhance our social image.”

The evidence for their model comes from studies involving functional magnetic resonance imaging (fMRI)—not the most rigorous of neuroscientific techniques—but they make a compelling case, in a series of consumer scenarios, that our modern-day spending habits reach back deep into our evolutionarily enhanced drive to be socially accepted.

—Bob Grant

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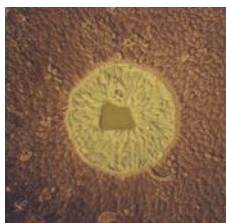
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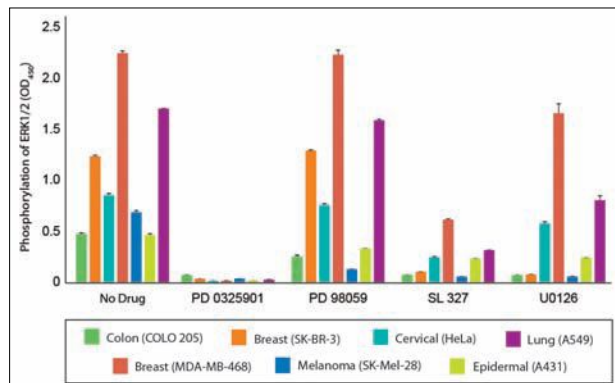


Figure: The Effectiveness of Four MEK Inhibitors in Seven Cancer Cell Lines was Assessed using R&D Systems ELISAs.

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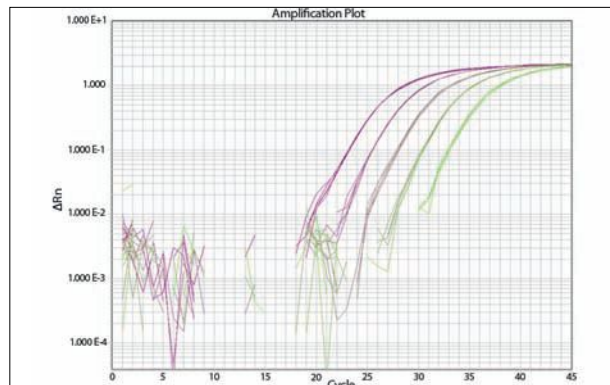


Figure: Standard cDNA samples serially diluted 5-fold and amplified in 5 replicates.

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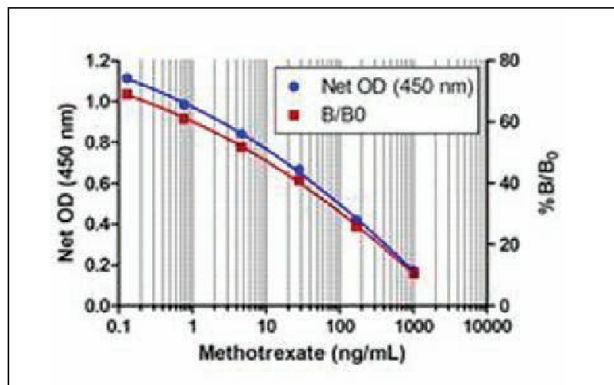
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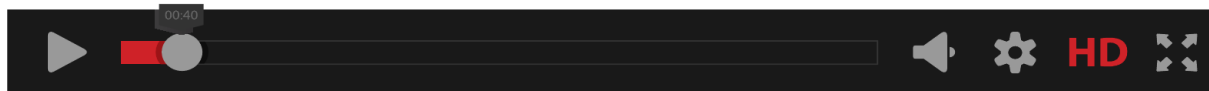
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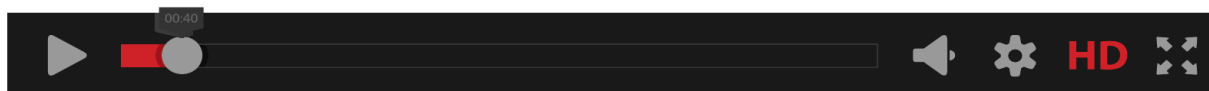
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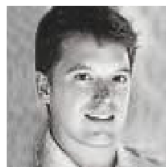
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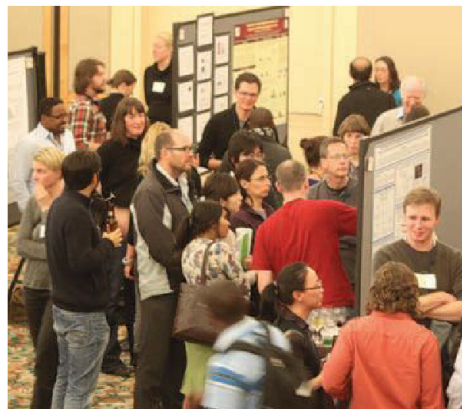
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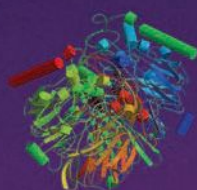


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Leukemia Under the Lens, 1845

BY JENNY ROOD

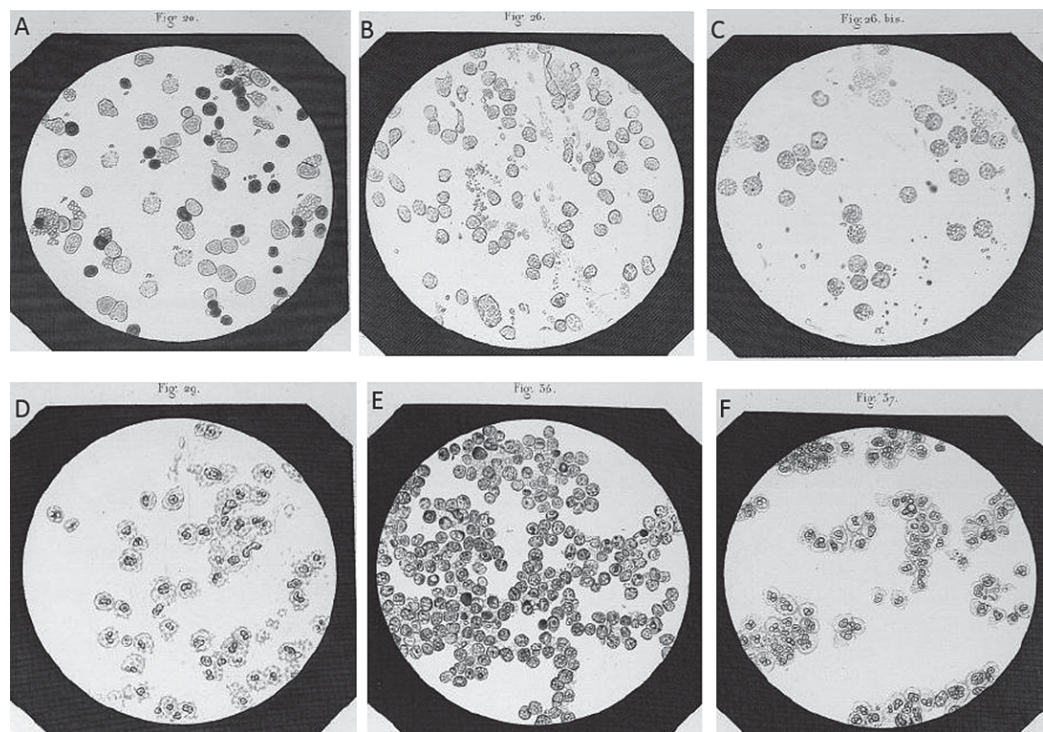
In 1837, over the objections of skeptical colleagues, Alfred François Donné set up 20 microscopes at his own expense in the lecture hall of the Medical Faculty of Paris. There, he provided practical microscopy lessons to students. His goal was to make microscopy a standard part of medical practice, an aim he had already championed with the invention of a foldable pocket microscope.

When Louis Daguerre presented his brand-new photographic technique in 1839, Donné leapt at the chance to modify it to capture his microscopic images to spice up his lectures to ever-growing audiences. Donné's adoption of such cutting-edge technology established him as a pioneer in the use of photographs—rather than hand-drawn sketches—to communicate scientific discoveries.

One of Donné's students, John Hughes Bennett, only briefly attended the Frenchman's microscopy course in 1841, but four years later would credit Donné's enthusiasm for inspiring his own microscopic investigation of a patient with an enlarged spleen and liver. In the patient's blood, Bennett found a large number of "colorless corpuscles," or white blood cells. He named the disease leucocythemia, a condition we now know as leukemia.

A case describing leukemia-like symptoms was first published in 1811, but the few early reports suggested that patients' thick and milky-white blood was not the result of an overabundance of living white blood cells, but rather due to the blood's deterioration into pus, which contains dead leukocytes. "When we get samples from patients, we always see the white layer, and for us, it's normal," says Kim Kampen, a leukemia researcher at the University Medical Center Groningen in the Netherlands who has written a history of the disease's discovery (*Leukemia Research*, 36:6-13, 2012). "But [back then] they didn't know what it was."

Bennett was the first physician to recognize that leukemia was a systemic dis-



ease rather than a localized infection, but he was not the first to produce microscopic images of diseased blood. That distinction instead belongs to Donné. In 1845, Donné published 80 engravings based on his daguerreotypes of crystals, cells, milk, pus, blood, and more, most at 400× magnification. Some of the samples pictured in the supplement to his 1844 book, *Cours de Microscopie Complémentaire des Études Médicales*, were sent to him by colleagues in 1839, and were taken from a woman who died of an abdominal tumor (see image). At first, Donné also thought that the blood might contain pus, "but in the end, I was able to observe a clear-cut difference between [pus] cells, and the white cells," he wrote. "What you see here is a representation of what you still see when you extract the bone marrow from a leukemic patient," Kampen says.

Today, the credit for the discovery of leukemia often goes to Rudolf Virchow,

OF BLOOD AND PUS: Alfred Donné's microscopic views of bodily fluids, published in the supplement to *Cours de Microscopie* in 1845, allowed for a clearer distinction between white blood cells and pus, which were often confused by Donné's contemporaries. Such investigations provided a clue to the origins of leukemia as a disease. A) Red and white blood cells from a leukemic patient; B) and C) "mucous globules," or white blood cells, from the same patient; D) mucous globules treated with acetic acid to visualize nuclei; E) recently secreted pus globules from a different patient; F) pus globules treated with acetic acid to visualize nuclei.

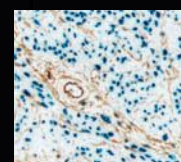
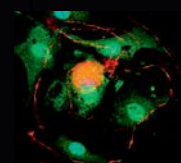
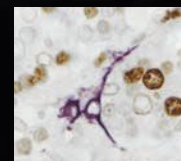
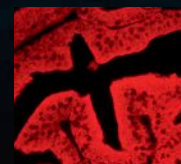
who gave the disease the name we now know it by in 1847. But the contributions of Donné, Bennett, and others were critical, Kampen says. "They talked to each other and learned from each other's knowledge. . . . You really accomplish much more if you collaborate, and I think that's still the case."

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CANADA

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