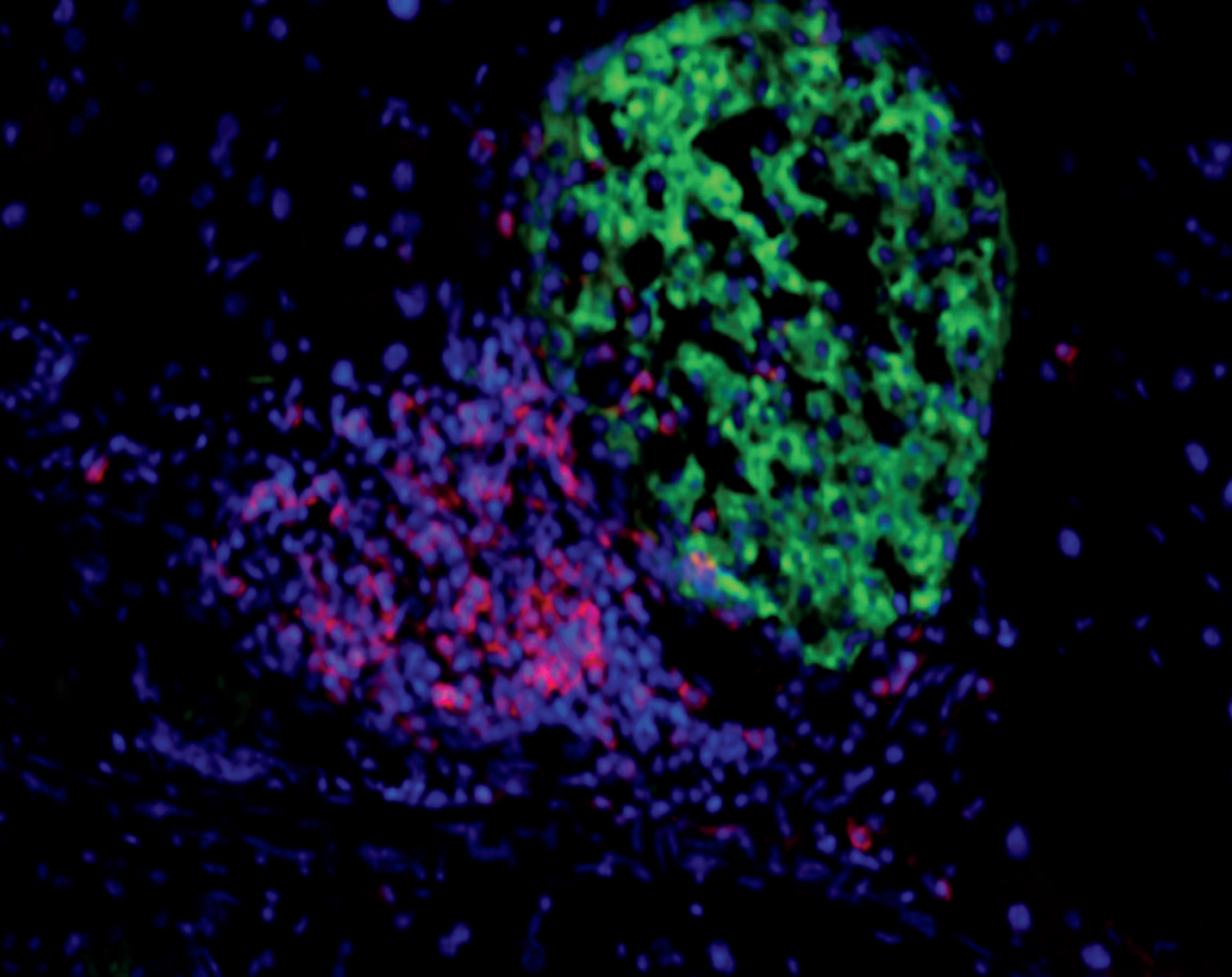


AUTOIMMUNITY'S



THE OVERLOOKED THYMUS

BY MELINDA WENNER MOYER

TRUE NORTH?

Ask a handful of biomedical scientists which organ plays the leading role in type 1 diabetes, and most will name the pancreas. After all, the disease, which afflicts some three million Americans, develops when the body erroneously attacks the beta cells of the pancreas—leaving them unable to produce insulin, the essential hormone that removes sugar from the blood. Because the pancreas is where the dirty work of diabetes unfolds, it is, as would be expected, where most researchers focus.

This cannot be said of Yong Fan, a research assistant professor in the University of Pittsburgh's Department of Pediatrics, and his mentor Massimo Trucco, Hillman Professor of Pediatric Immunology and an MD professor of pediatrics, pathology, human genetics, and epidemiology at Pitt who heads the Division of Immunogenetics at Children's Hospital of Pittsburgh of UPMC. But then, one might not call them typical diabetes researchers. Fan, who hails from China, has a background in developmental biology, which few diabetes researchers do. And Trucco, who is Italian, wears the same uniform every day (black jeans and a black shirt), has a guttural laugh that slips out frequently, and exudes a keen wisdom: He seems to know that where the human body is concerned, things are not always how they first appear.

A young researcher demonstrates the importance of the thymus in treating type 1 (juvenile) diabetes. Here we see what happens when Pitt's Yong Fan engineers mice so that the thymus doesn't recognize important insulin-producing cells. Immune cells (red) attack the pancreatic cells.

IMAGE COURTESY FAN AND TRUCCO LAB

Fan and Trucco are marching in the vanguard of what might be called a diabetes revolution. It is their belief that many of the disease's unsolved mysteries can be rectified not by looking at the pancreas but at the thymus, a small immune organ found in the center of the upper chest "that generally, in clinic, nobody cares about," Trucco says. In a study they published in September 2009 in *The EMBO Journal*, Fan and Trucco showed that mice that are genetically resistant to type 1 diabetes nevertheless develop the disease in only three weeks—about nine times faster than usual—if they are born without the ability to make insulin in the thymus, even if their pancreatic beta cells can produce plenty of the hormone.

It is an astonishing finding—"against prevailing dogma," according to a 2010 commentary published about it in *Pediatric Diabetes*—and it illustrates something that Trucco says he has come to understand about diabetes over the years: "The more we study the disease, the more we understand that it is more complicated than we wanted it to be," he says. Fan and Trucco's findings suggest why a diabetes cure has been so difficult to find—researchers may have been looking in the wrong place. But now they may have identified diabetes' true north.

Fan hasn't always been passionate about the thymus. Prior to 2002, he, too, was focusing on the pancreas—more specifically, he was trying to coax stem cells to develop into pancreatic beta cells in hopes of using them as replacement cells in diabetes patients. But he was starting to grow wary of his approach, because he knew that "no matter how many [beta cells] you make, once you put them in the patient, the body will destroy them," he explains. "I realized the more fundamental question was unresolved: We needed to crack open what actually was causing this autoimmunity," meaning the tendency for the body's immune cells to attack its own tissues.

One day at around the same time, Trucco invited Constantin Polychronakos, a pediatric endocrinologist at McGill University, to give a talk to his lab members. In his presentation, Polychronakos mentioned his own discovery that low levels of insulin were produced in the thymus. This "was a total surprise to me," Fan says. The thymus, Fan knew, was the immune organ that produced T cells, specialized white blood cells responsible for fighting infections. (They get their name because they come from the thymus.) Polychronakos went on to sug-

gest that insulin might be a kind of classroom tool used by the thymus to "train T cells to be useful," Fan recalls.

It made sense. T cells mercilessly fight intruders, but how do they learn what belongs in the body and what does not? Somehow, the immune system must explain to developing T cells that the proteins and hormones produced by the body should not be destroyed. Low levels of many of the body's proteins are produced in the thymus, and it was Polychronakos' belief that T cells were being introduced to these proteins—such as insulin—in a biological meet-and-greet designed to show them that the proteins were friends, not foes. If any rogue T cells did not get the drift—if they attacked the friendly proteins they met in the thymus—they would be killed before they left the thymus and did serious damage.

The idea immediately piqued Fan's interest. "I said, 'Wow, this is interesting,'" he recalls.

If insulin is being produced in the thymus in order to train T cells not to attack insulin-producing cells—i.e., pancreatic beta cells—then perhaps type 1 diabetes develops in people who are not producing enough insulin in the thymus. In 1997, Polychronakos had shown that people who produce less bodywide insulin as a result of genetic abnormalities are more likely than others to develop type 1 diabetes. Perhaps, Fan thought, too little insulin production in the thymus was actually the culprit. Without it, T cells aren't primed to leave insulin alone. Then, when they are released into circulation and "pass by the pancreas and find cells producing insulin, they kill those cells because they believe that they are foreign," Trucco explains.

To find out whether he was right, Fan set out to engineer mice that could not produce insulin in the thymus. First, he bred mice so that the predominant insulin gene in all of their cells was flanked by two short genetic sequences called loxP sites. Then he engineered a second group of mice so that they contained a gene called *CRE* that excises any gene found in between two loxP sites, effectively removing it from the genome so that it cannot be expressed. In front of the *CRE* gene, he placed a master controller gene that switches *CRE* on only in the insulin-producing cells in the thymus.

In his final step, Fan bred the two groups of mice together, producing baby mice that contained both the *CRE* gene and the two loxP sites flanking the insulin gene. The end result:

CRE cut the insulin gene out of the mouse genome, but only in the insulin-producing cells in the thymus, leaving insulin production everywhere else in the body—including the pancreas—untouched. In effect, Fan had engineered mice that could not produce thymic insulin, but who could produce insulin elsewhere.

Then Fan and Trucco watched what happened. Typically, 80 to 90 percent of female mice with a genetic predisposition to diabetes develop the disease within 20 weeks, with progression in males being slower. But in this case, within three weeks, every single one of the male and female mice who could not produce insulin in the thymus developed severe, fatal diabetes. Fan had shown that the thymus was arguably as important for diabetes development as the pancreas.

For the many researchers who study diabetes, Fan and Trucco's findings are a wake-up call. For one thing, they shed light on the importance of central tolerance—the schooling of T cells in the thymus to prevent them from attacking self-made proteins—and reveal at least one important way it can go awry. "It opens avenues to explore how T cells escape central tolerance, resulting in autoimmunity," says Anil Bhushan, a cell and developmental biologist at UCLA.

And if the disease starts in the thymus, then the organ may be a far better target for a cure than the pancreas. The game, as Trucco puts it, then becomes trying to find a way to help people whose thymuses don't express enough insulin to start expressing it.

That said, if the problem is only discovered after rogue T cells have already attacked the pancreatic beta cells, then additional treatments may be necessary to replace them, since beta cells don't naturally regenerate. That problem may also be solved at Pitt: Earlier this year, Andrew Stewart, chief of the Division of Endocrinology and Metabolism in the School of Medicine, showed that when human beta cells are engineered to produce high levels of certain regulatory molecules, they can regenerate continuously for four weeks when transplanted into diabetic mice.

Trucco and Fan's findings also help explain something that has perplexed scientists for years: Some children develop type 1 diabetes at a very young age despite not having a known genetic predisposition to the disease. Perhaps their problems can be traced to the thymus.

Fan and Trucco's discovery could shed light on the root causes of other autoimmune diseases. After all, thymus cells don't just express insulin—"Twenty to 30 percent of the genes in our [bodies] are expressed in this cell population," Fan explains. Like insulin, these other proteins teach developing T cells that they are not enemies. It's possible that if these other proteins aren't produced in large enough quantities, different autoimmune diseases—such as rheumatoid

arthritis, multiple sclerosis, and lupus—might develop. It makes sense, especially considering that people with type 1 diabetes often have other autoimmune diseases, too. Perhaps they suffer from a central thymus defect that could be treated by increasing thymic gene expression on a global level. Trucco and Fan are now engineering mice so they are unable to produce other proteins in the thymus, to see whether they develop other autoimmune diseases.

For Trucco, the discovery is gratifying on an even deeper level. He has long believed that although biology is full of surprises, everything happens for a reason. But try as he might, he could not find an explanation for why the T cells of patients with diabetes suddenly started attacking the body instead of defending it. Now he finally has one.

"I knew there must be some sort of logic," he says, then paraphrases Shakespeare. "There must be reason in this madness." ■

FATTY ACIDS AMPED

In biology, free radicals have the reputation of a no-good street gang. They sneak around the body, brimming with incessant energy, doing damage here and there for seemingly no reason at all. When too many of them troll around for too long, bad things can happen. But Bruce Freeman, UPMC Irwin Fridovich Professor and Chair of the Department of Pharmacology and Chemical Biology in the School of Medicine, has seen unique potential in these historic hoodlums. Now, he thinks free-radical reaction products might even have what it takes to beat type 2 diabetes.

Freeman didn't always have a soft spot for free radicals. In 1990, while at the University of Alabama at Birmingham, he published a seminal paper—among the most frequently cited papers in biology—revealing the major pathway by which free radicals cause tissue injury. But when he started testing the effects of these free radicals in animal models, he found that one free radical in particular, nitric oxide, didn't always cause damage. When it interacted in the body with fatty acids like oleic acid—the major component of olive oil—the free radical actually *reduced* inflammation rather than causing it.

"It was heretical," Freeman says of the discovery. Since then, he has published paper upon paper showing that these nitro-fatty acids, as they are called, can in fact limit and even heal tissue injury. The nitro-fatty acids are what Freeman refers to as electrophiles, molecules that interact with electron-rich molecules and thereby initiate signaling cascades and gene expression profiles that attenuate stress in the body.

"There are about 300 to 500 major genes whose expression is modified by electrophile levels," Freeman explains.

Recently, Freeman's team uncovered a link between these nitro-fatty acids and type 2 diabetes. The acids, the researchers discovered, bind to a cell receptor called PPAR-gamma that is well-known in the diabetes world. Diabetes drugs like Avandia work by binding to PPAR-gamma in a way that activates a host of biochemical cascades modulating insulin resistance and sugar metabolism. Problem is, some of these cascades also spark water retention and fat production, causing serious weight gain, a common diabetes drug side effect. But when Freeman studied how nitro-fatty acids interact with PPAR-gamma, he discovered that they bind dif-

ferently, via a covalent bond. He wondered: *Was it possible that by binding differently, the nitro-fatty acids might turn on all the good cascades—improving insulin sensitivity and glucose metabolism, and thus treating type 2 diabetes—without eliciting the unwanted side effects?*

To find out, the researchers synthesized nitro-fatty acid in the lab and administered it to obese, insulin-resistant rats for four weeks. Within just four days of starting, the treatment normalized their blood-sugar levels and significantly reduced their insulin levels, a change that did not occur in mice that had been given naturally produced fatty acids, such as those found in olive oil. Most importantly, though, the mice he treated did not gain any weight. Compared to existing diabetes drugs, the nitro-fatty acids seem to cause "less stimulation of fat metabolism, less weight gain, and less fluid retention," he says. Freeman and his colleagues, who include Pitt's Francisco Schopfer and Chiara Cipollina, published their results in April 2010 in *The Journal of Biological Chemistry*.

Certainly, some fatty acids—such as omega-3 fatty acids—have anti-inflammatory effects of their own, which is why we're told to eat lots of fish. But his work suggests that when these fatty acids interact with nitric oxide (or other oxygen-centered free radicals), they become even further activated. "What's really impressed us is if we modify the fatty acids chemically to make them electrophilic, there's a very significant increase in their anti-inflammatory capability," he says.

These molecules don't just have implications for type 2 diabetes, either. Since 2009, Freeman has shown in rodent models that nitro-fatty acids attenuate atherosclerosis, inflammatory bowel disease, hypertension, and the damaging inflammation that develops after heart attacks and cardiac arrest. Now he is turning his attention to heart failure: "We're predicting we'll have a significant impact," he says.

Ultimately, Freeman hopes to manufacture large quantities of these molecules using synthetic, inexpensive strategies. He has started a biotechnology company, Complexa, to commercialize the concept and is currently testing the compound in different animals for safety. "What we'll be doing is increasing the concentrations of the activated form of unsaturated fatty acids to levels greater than we would otherwise achieve through dietary strategies," he says. —MWM