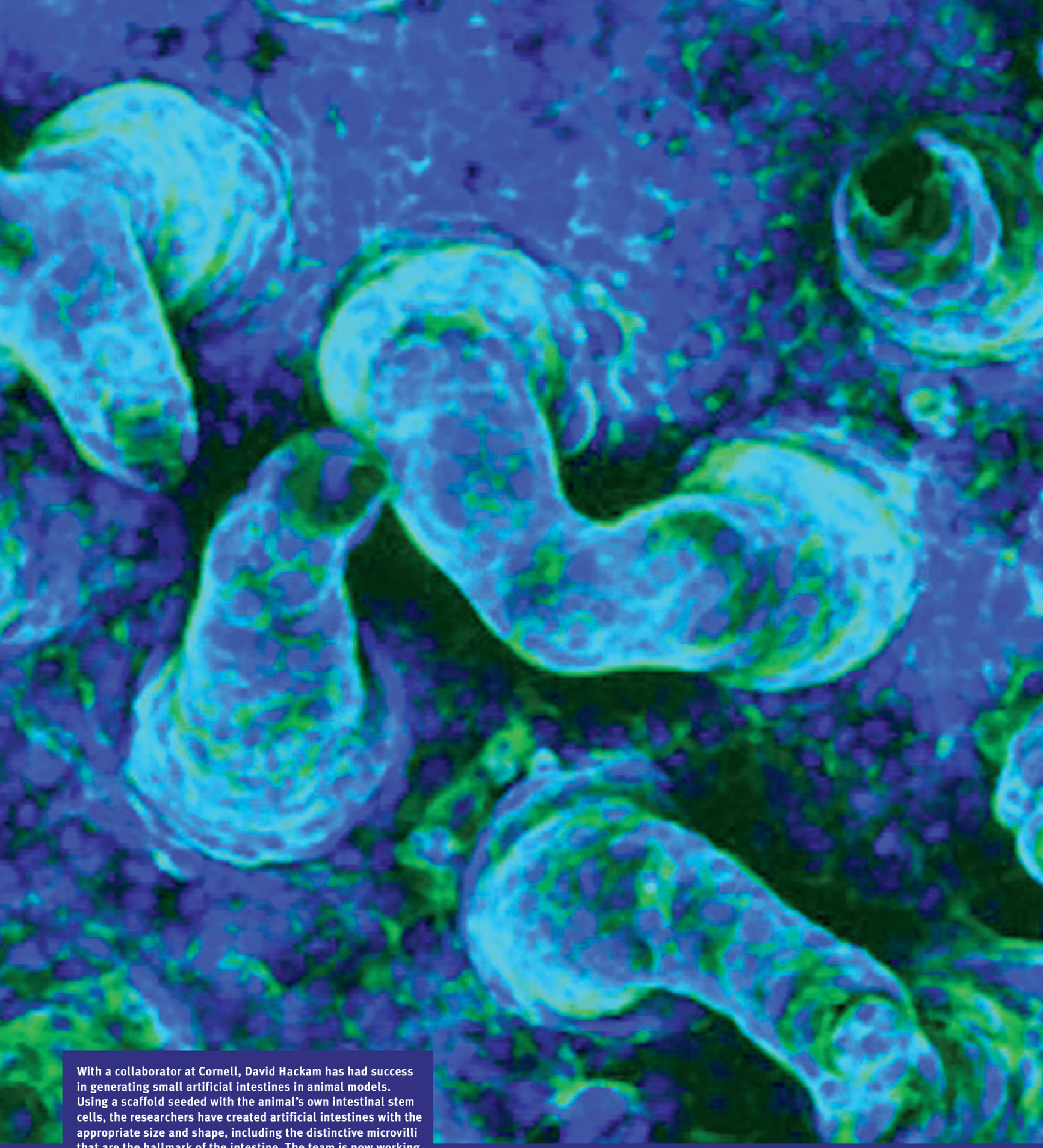


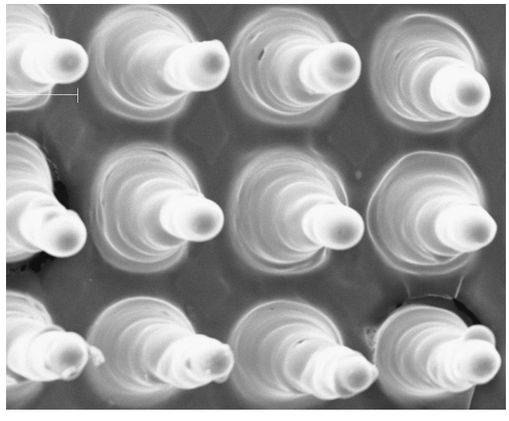
With a collaborator at Cornell, David Hackam has had success in generating small artificial intestines in animal models. Using a scaffold seeded with the animal's own intestinal stem cells, the researchers have created artificial intestines with the appropriate size and shape, including the distinctive microvilli that are the hallmark of the intestine. The team is now working to ensure that the artificial intestine can actually absorb food and have appropriate motility.

NEC MAY BE THE MOST IMPORTANT AND CONFOUNDING DISEASE YOU'VE NEVER HEARD OF

BY CHUCK STARESINIC

THE SCOURGE OF THE NICU

Expectant parents spend a great deal of time imagining the arrival of their baby, but they rarely expect major medical complications. While everyone knows that childbirth comes with risks for both mother and child, parents typically anticipate a pregnancy that lasts nine months (or close to it) and ends with a healthy mother holding and nursing a healthy newborn. Nevertheless, every year in this country, more than half a million babies arrive prematurely (before 37 weeks of gestation). That's around 12 percent of babies who are, to some extent, small, underdeveloped, and/or facing challenges; this is especially true of those born as early as 24 to 25 weeks, at the outer edge of viability. Capitalizing on scientific discoveries, new drugs, and greater understanding of the needs of these smallest of humans, specialists in neonatal intensive care units (NICUs) have become increasingly skilled at negotiating these challenges throughout the past few decades. The result is that many babies who would have died within hours or days of birth now regularly clear the hurdles of their first few weeks outside the womb.



Bioscaffolds used to grow cells into fingerlike projections that mimic those of the intestinal lining

SCIENCE IMAGES COURTESY CAIT COSTELLO

They may begin to gain weight and breathe without support. Parents are able to see their child's face free of oxygen tubes and adhesive tape for the first time; perhaps they allow themselves to think beyond just the next few hours.

In what seems to be a rather cruel bit of timing, this is often the moment when symptoms of NEC, a life-threatening intestinal disease that affects 12 percent of preemies, first appear. NEC rhymes with "heck," a term that's mild compared to the words it often elicits from parents and care providers. NEC is short for necrotizing enterocolitis. Simply put, it is an inflammation and dying off of the intestine.

"If you ask neonatologists or pediatric surgeons, 'What's one of the most challenging and frustrating diseases in the NICU?' they will immediately say, 'NEC,'" says David Hackam, a surgeon-scientist at Children's Hospital of Pittsburgh of UPMC and the Watson Family Professor of Surgery at the University of Pittsburgh. "It's challenging, because early on it's hard to diagnose. And there's no specific treatment. It's frustrating, because our success hasn't improved in the last 30 years."

The early signs of NEC can be subtle—feeding intolerance, lethargy, temperature instability—but they can rapidly progress to include vomiting bile and the appearance of blood in the stools. X-rays may show blockages and pockets of gas in the intestines, and this is when a pediatric surgeon gets involved.

Hackam operates on babies with NEC to remove dead and dying sections of intestine. It's impossible to know the extent of the problem until the intestine can be seen. At that point, Hackam says, it's obvious to even someone who isn't a doctor. Healthy tissue is pink. Diseased tissue is turning black. In the worst cases, the toxic stew in the abdomen sets off a cascade of inflammatory reactions from which the child cannot recover. Between 20 and 30 percent of infants with NEC do not survive, with sepsis and multisystem organ failure often contributing to death. In some survivors, not enough intestine remains to support normal digestion, and the child will require intravenous nutrition for the foreseeable future.

When asked to describe how he approaches the parents of a very sick child with NEC, Hackam sighs. "I'm very honest with the parents," he begins. "I'm also purposefully hopeful, but not falsely so. Because, if you have to choose between being optimistic and pessimistic, then you choose optimism—but be realistic. I tell them that it's not their fault. It's a consequence of early delivery, which they had no control over.

"At first it's hour-by-hour as to whether the [babies] will even survive. And if they make it through one hour, then they have hope for the next hour. And then, generally, if they make it through the night, they can say, 'We made it through 12 hours. We can make it through another 12 hours.' Then, after a few days, I tell them it's day-by-day. And that can be a huge relief, because a few days ago it was hour-by-hour. If I come and tell *you* it's day-by-day, that sounds like a death sentence. But not in the NICU, where one hour has huge consequences.

"So I'm as honest as I can be. But I hope for the best."

Hackam traces his commitment to treating NEC to one particular patient. We'll call him Kevin. He was born at about 26 weeks of gestation. He was small at birth and destined for a stay of several weeks in the NICU, but by the time he was 2 weeks old, he was gaining strength. When Kevin became ill, Hackam was the surgeon who met with the parents.

The family was at a hospital other than Children's Hospital of Pittsburgh of UPMC. It was a local hospital that Hackam and some of his colleagues covered at the time. The location did not affect treatment or prognosis, but there was a subtle difference in how Hackam went about his job. He wasn't with the large team of nurses, residents, and fellows that is ever-present in a large pediatric teaching hospital. At each step in the process, it frequently came down to him, the parents, and the baby.

While nobody can understand the emotional roller coaster parents go through in the NICU without having lived through it themselves, Hackam can relate to his patients on many levels. But for the surgical scrubs and white coat, he could be just another bespectacled new dad in the NICU. As a father of four children under 10, he is familiar with the joys and the fears of parenthood and the journey a family takes through pregnancy and childbirth. Hackam is friendly and talkative in a calming way, as Kevin's parents discovered in their many conversations with him.

"I got to know his parents," says Hackam. "They were a little bit older. They'd had kind of a tough journey to get pregnant. They were neat.

"But it was a typical story—their kid was born early and was a little sick. Then, within 24 hours, he was dying. There was no option but to operate, and most of his intestines were dead. And a few days before, he had been fine. It was that dramatic."

Kevin survived his bout with NEC, but he was left with a condition called SBS, or short bowel syndrome, which is common in kids who survive NEC. With insufficient intestine for normal digestion, he relied entirely on intravenous fluids for nutrition. For reasons that aren't completely understood, this can lead to liver disease, and that is what happened to Kevin. Before he could reach his first birthday, he was on the liver transplant waiting list. He died waiting for an organ to become available.

Ever since getting to know Kevin's family, Hackam has been driven to fix the problem of NEC. He is motivated, in part, by the frustration of having a surgical fix that is an imperfect fix. To really address Kevin's problem, a radically different solution was required—something like an artificial intestine. In addition, Hackam is drawn to the biological mystery of what causes NEC in the first place. What happens inside the gut of a preemie at 2 weeks of age to set off this terrible cascade of inflammation and cells bent on self-destruction? Why does it happen in preemies and not full-term babies? If doctors knew what was happening at the cellular level, could we turn it off?

NEC is exactly the sort of conundrum that Hackam had been trained to investigate. Years earlier, as a surgical resident at Toronto General Hospital, he had begun to feel that his medical education was missing something. Every disease, he realized, was essentially a failure of cells to work properly. But the art of surgery was practiced at the level of organs, organ systems, vessels, and layers of tissue. With his MD from the University of Western Ontario and a few

"If you ask neonatologists or pediatric surgeons, 'What's one of the most challenging and frustrating diseases in the NICU?' they will immediately say 'NEC.'"

PHOTOS COURTESY JENNIFER RATH

(Clockwise from top left) For Austin, life with short bowel syndrome involves large shipments of intravenous nutrition to his home. ◆ In 2012, Hackam hosted Austin at Children's Hospital so that the boy could meet the research team and see the work that Austin's Cupcake Fund was supporting. Shown from left to right are Thomas Prindle, Hongpeng Jia, Chhinder Sodhi, Austin, Hackam, Maria Branca, and Shahab Shaffiey. ◆ When news of Austin's Cupcake Fund made the local newspaper, Austin was thrilled to see his photo on the front page while at the grocery store. ◆ While visiting the Hackam lab and Children's Hospital, Austin brought his piggy bank to treat Hackam to popcorn, one of the few foods the boy can eat.

years of surgical training, he was on a career path that would allow him to perform surgical procedures to improve the health of his patients. Yet he found that he often lacked a complete understanding of what was wrong with them at the cellular level. He decided to pursue a PhD in cell biology.

Taking a leave of absence from his surgical residency, Hackam spent three years in the lab of a renowned cell biologist named Sergio Grinstein, a PhD senior scientist in the Hospital for Sick Children and a professor of biochemistry at the University of Toronto. Grinstein is internationally recognized for elucidating mechanisms underlying the immune response of white blood cells (in particular macrophages and neutrophils) against microbes. At first, Hackam was a fish out of water in Grinstein's lab—a surgeon among basic scientists. He felt like he and his colleagues were speaking different languages and didn't have much in common. But that didn't last long. Between 1996 and 2001, Hackam was a coauthor with Grinstein on a dozen scientific publications exploring the basic mechanisms of the immune system, including articles in *Proceedings of the National Academy of Sciences*, *Journal of Biological Chemistry*, and *Journal of Experimental Medicine*.

Following the completion of his doctorate and a subsequent year as chief surgical resident in Toronto, Hackam arrived in Pittsburgh in 2000 for fellowship training in pediatric surgery. He was drawn to Pittsburgh by what he describes as an unparalleled pediatric surgical training program led at the time by Eugene Wiener and Henri Ford (then chief of Pitt's Division of Pediatric General and Thoracic Surgery and now a vice dean of medical education at the Keck School of Medicine of the University of Southern California). Ford had trained at Pitt himself, completing a research fellowship in immunology in 1989 and pediatric surgery fellowship in 1993 and, after joining the faculty, conducting laboratory research into the cellular mechanisms behind NEC. Hackam was heavily influenced by Ford, as well as the surgeon who recruited him to Pittsburgh—Timothy Billiar, George Vance Foster Professor and chair of surgery. Billiar is widely known for his investigations into the cellular mechanisms of trauma and inflammation, especially the role of nitric oxide.

When Hackam arrived, the division included two pediatric surgeons running their own research laboratories in addition to pursuing clinical work. While it's not unusual for a major academic medical center to have surgeons who do basic science research, pediatric surgeons who do so are more of a rarity. Some centers have one, but most have none. Having multiple pediatric surgeons with NIH-funded labs, as Pittsburgh did then and still does, is practically unheard of. Far from being a fluke, the Division of Pediatric General and Thoracic Surgery includes four such labs today. (The division chief is George Gittes, who studies embryonic blood flow and organogenesis, particularly of the pancreas.)

The base of operations for these surgeon-scientists is the John G. Rangos Sr. Research Center, established in 1990 and moved to new quarters in 2008 alongside the new Children's Hospital of Pittsburgh of UPMC. Working closely with the University's Center for Biologic Imaging, the division has created a shared, top-notch imaging facility just steps away from the hospital. The proximity of the hospital to these labs is a great boon to the work of these surgeon scientists. When Hackam, who holds a secondary appointment in cell biology, leaves the operating theater at Children's, he simply walks down a few flights of stairs, along the covered walkway past families playing and eating in the courtyard

outside the cafeteria, and into the Rangos building. If he has tissue samples obtained during surgery, it takes just a few minutes to begin to examine them in the lab. One confocal microscope includes an incubator—a sealed chamber where researchers can control pressure, temperature, humidity, and other variables while viewing samples under magnification. Across the room is a microscope linked to a large video screen. When a sample of tissue is displayed on the monitor, researchers might draw on the screen with a stylus, encircling, for example, a single intestinal stem cell. A laser traces the same line in the tissue sample, making a precise cut so that the cell drops out of the tissue ready to be used in experiments on regenerating intestinal tissue. These facilities (supported, in part, with funding from Pitt's Clinical and Translational Science Institute) are critical to the work Hackam is undertaking to cure NEC and treat its survivors. In his office, with views of the rolling green hills of the Allegheny Cemetery and its raucous flocks of crows, Hackam shows pictures of NEC in action: tiny infants with swelling bellies, blackened and dying intestinal tissue.

"I'm going to show you in a minute that we have a treatment for [NEC], in mice, that works," he says. "That's the first step. It works in piglets, too—second step. And that is just one step away from humans." Though he is clearly optimistic, he also cautions that the FDA approval process of a new drug has many, many steps of its own.

When he and his colleagues started this work, they had no idea what even caused NEC. They knew that it was seen in association with bacteria—not in association with a particular pathogen, like salmonella. But bacteria were needed in the gut for NEC to occur. An important clue was the age at which an infant typically developed NEC—around two weeks. "You come out sterile," explains Hackam, of newborns. "After about two weeks all the bugs [bacteria] are there that you are going to have pretty much for life, though they change a little bit. That's when NEC develops."

At the time, it wasn't even known how the gut recognized bacteria, let alone how the process might go wrong in NEC. Just as Hackam was beginning to ask questions about NEC in the early 2000s, revolutionary discoveries about innate immune mechanisms that responded to the presence of bacteria were being published. A 2011 Nobel Prize would recognize the discoveries that elucidated aspects of the innate immune system, including the discovery of toll-like receptors (TLRs), molecules that span the membrane of the cell and react to the presence of bacterial products.

There are several TLRs in humans. One, TLR4, piqued the interest of Hackam and colleagues because it recognizes gram-negative bacteria, a large and diverse group of bacteria common in the gut and the environment. The investigators theorized that TLR4 was in the gut and learned that something went wrong with it in NEC. They successfully demonstrated that it was present in the gut and learned that in preemies the expression of the molecule sometimes seemed to get turned up really high.

In 2007, he and colleagues reported in the *Journal of Immunology* that expression of TLR4 was increased in patients with NEC, making it a likely target for treatment. Continuing this line of research, the Hackam lab published a potential breakthrough in *PNAS* in July 2012. Noting that healthy, full-term infants have relatively low levels of TLR4 in the gut, the researchers posited that something goes wrong with the TLR4 response when premature infants get colonized with normal gut flora.

"One big difference between a 34-week-old baby developing in its mother's uterus and one in the neonatal intensive care unit is that the first one is floating in and swallowing amniotic fluid," Hackam says. "Early delivery means that exposure to the fluid is over, so we speculated that components of the fluid could help prevent NEC by keeping TLR4 in check."

In the study, the researchers showed that injecting small amounts of amniotic fluid into the intestine of premature mice, or feeding the fluid to them, stopped NEC from developing. That's because the fluid is rich in epidermal growth factor (EGF), a wound-healing protein; when the researchers removed it from the fluid or blocked or removed the EGF receptor on intestinal cells, amniotic fluid no longer had a protective effect.

"It appears that EGF in amniotic fluid is able to shut off TLR4 activity and prevent NEC," Hackam says. "Perhaps if we one day banked amniotic fluid after premature delivery, we could give it to newborns at risk for the problem. We also could identify a drug that inhibits TLR4 activity to try to save these babies."

Hackam's group contacted Peter Wipf, Distinguished University Professor in Pitt's Department of Chemistry, Kenneth P. Dietrich School of Arts and Sciences. The team told him they were looking for a ligand for TLR4—a molecule that would fit the receptor like a key in a lock and shut it down. Thanks to computational tools, chemists like Wipf can search enormous databases of chemical structural information in order to identify candidates. The total number of chemicals in the databases Wipf searched is around 50 million.

After several rounds of narrowing the list based on cost and likely toxicity, Hackam's lab ordered 67 compounds and started the painstaking work of experimenting in mice. The mice had TLR4 highly expressed and were exposed to bacterial compounds known to trigger NEC. Working with Simon Watkins, PhD professor of cell biology and physiology, as well as of immunology, and director of Pitt's Center for Biologic Imaging, the team was able to make the intestinal tissue of the mice glow when TLR4 was active. If a drug silenced TLR4, it stopped glowing.

"Hi, Dr. Hackam. My name is Austin. I have short gut. My biggest wish ever is to get a new tummy. Work very hard! Bye."

"One of the compounds was almost perfect," says Hackam. Compound 34, as it was randomly called, is a type of saccharide—part of a family of molecules found in breast milk. Though no one has specifically looked for Compound 34 in breast milk, Hackam says it is likely present.

"I think the compound continues to have good properties," Wipf says. "The scientific data from David's lab are very encouraging, very interesting. But at this point we have about 5 percent of what it takes to put together a package that would allow us to move toward phase 1, which is the first kind of clinical trial—[it's] where you first ask the question about toxicity and dosing in humans. That's how strenuous that road really is. A lot of the data are easy to get, but nonetheless, they have to be collected."

In yet another groundbreaking scientific paper just going to press in *PNAS*, Hackam and several Pitt colleagues, including surgery chair Billiar, have elucidated new details on the mechanisms that cause infants to develop NEC. Based on these discoveries, the authors suggest additional therapeutic strategies. This line of research began with the theory that the death of intestinal tissue in NEC was related to inadequate circulation of blood. Collaborating with Mark Gladwin, professor of medicine, chief of the Division of Pulmonary, Allergy, and Critical Care Medicine, and director of Pitt's Vascular Medicine Institute, the Hackam lab demonstrated that TLR4 activation in blood vessels led to impaired blood flow to the gut, causing or contributing to NEC. Trying to determine why breast milk prevented NEC, the team discovered that breast milk contains sodium nitrate, which gets converted to the vasodilator nitric oxide and improves blood flow. To test whether sodium nitrate was breast milk's active ingredient preventing NEC, they added sodium nitrate to the infant formula fed to the mice. The supplemented formula was protective; the researchers were able to measure improved blood flow and show that NEC did not develop in these mice. Hackam and Gladwin are now planning a clinical trial to administer a similar therapy in infants at risk for NEC.

Austin, shown here at age 4, has been inspired by many doctors.

In December 2011, Hackam opened his e-mail to find a link to a video. When he clicked "play," he saw a chipper 5-year-old in front of a Christmas tree say, "Hi, Dr. Hackam. My name is Austin. I have short gut. My biggest wish ever is to get a new tummy. Work very hard! Bye."

Austin, who lives in Butler County, suffers from short bowel syndrome. Although it's not the result of NEC, he faces the same problems as many NEC survivors. He gets a great deal of his nutrition intravenously, though he can eat carbs and proteins. He spends about 17 hours each day connected to an IV line. No matter how thirsty he gets, he can't drink water, juice, or any liquids other than his medication. As if that isn't torture enough for a child, he also is not allowed sweets, including fruits, candies, and cakes.

One day at bedtime, Austin watched his little brother take sips of water after brushing his teeth. He broke down sobbing that he hated himself and hated having short gut; he just wanted to drink water like his brother and other kids. Austin's mother told him about Hackam's research, which she had just learned about. The next day, he insisted on making the video.

In 2010, Hackam was at a conference organized by the Hartwell Foundation. Hartwell has a goal of putting together innovators who are working on high-risk, high-reward studies to collaborate on applied biomedical research projects with potential to benefit children. And that is exactly what happened with Hackam and a bioengineer from Cornell University named John March.

The collaboration was born at a Hartwell conference. The way Hackam tells it, he was at one end of the bar talking about obstacles to building artificial intestine: He knew how to grow intestinal stem cells in a dish, but he had no way for them to grow into a structure that mimicked the complex shape of the human intestine—which, thanks to its folds and tiny fingerlike projections lining every inch of its interior, has roughly the same surface area as a tennis court. At the other end of the bar, the biophysicist and engineer March was talking about how he had developed techniques for making bioscaffolds with unique features, but seeding them with stem cells and coaxing complex structures to life were beyond his expertise. Somewhere between the two ends of the bar that night, a scientific collaboration was born. March and Hackam put their proposal for an artificial intestine together in 2011, news of which eventually prompted Austin's family to get in touch.

When Austin's family woke up on Christmas Day, they had an e-mail from Hackam, promising Austin that he would not rest until he could help him and other children who need "a new tummy." Austin has less than 10 percent of his intestines. The five-year mortality rate for children who are as sick as him is 20 percent. But in May 2012, he celebrated his sixth birthday. He told his parents that, instead of presents, he wanted to raise money to support Hackam's research. The family started a Facebook page, and when donations began pouring in, he decided to name his fund Austin's Cupcake Fund (www.facebook.com/AustinsCupcakeFund), hoping that one day he could eat lots and lots of cupcakes. The fund has since raised more than $70,000 to support the artificial intestine work.

It turns out that several groups around the world are working on an artificial intestine. Nanotech centers at many universities are busy creating scaffolds that mimic many anatomical structures for growing cells; they typically use a process called laser etching, which works well for detailing very small features. However, laser etching can't quite mimic the high aspect (width to height) ratio of the intestinal microvilli. (A skyscraper has a much higher aspect ratio than a pyramid; and in this respect, microvilli are more like skyscrapers.) March's secret to overcoming this challenge is to form the scaffold using a mold, which is then dissolved, leaving only the scaffold.

Viewing images of March's scaffold under a microscope, Hackam says, "It doesn't have cells on it, but that looks like a native intestine. This has been a major barrier in clinical medicine—to have a scaffold that looks like the intestine."

The team's scaffolds are made from an FDA-approved material—a compound similar to elastin and collagen naturally found in intestinal tissue. Their first experiments with it involved implanting it into the fatty abdominal tissue of mice. As the researchers hoped, the presence of the implant stimulated the growth of blood vessels, which are needed to feed and sustain the implant. Since then, the team has been able to implant and sustain artificial intestines in mice. Hackam and March have not yet published this work in a peer-reviewed journal.

Austin's Cupcake Fund allowed Hackam to hire dedicated staff and push the timetable for important experiments forward.

"If you had asked me six months ago how long it would take to do this—to take these cells and culture them on a scaffold and then have success in animals—I would have said maybe a year, and maybe more. But we've done it in the last six months really because of the funding we've had."

Austin recently visited Hackam at Children's; the boy arrived with his piggy bank. He wanted to treat his hero to some popcorn from the hospital snack bar. The two also stopped to admire the array of brightly colored candy treats in the gift shop. That day, they just looked, but both are determined to tear open some candy wrappers together someday and enjoy the sweet fruits of their labor.