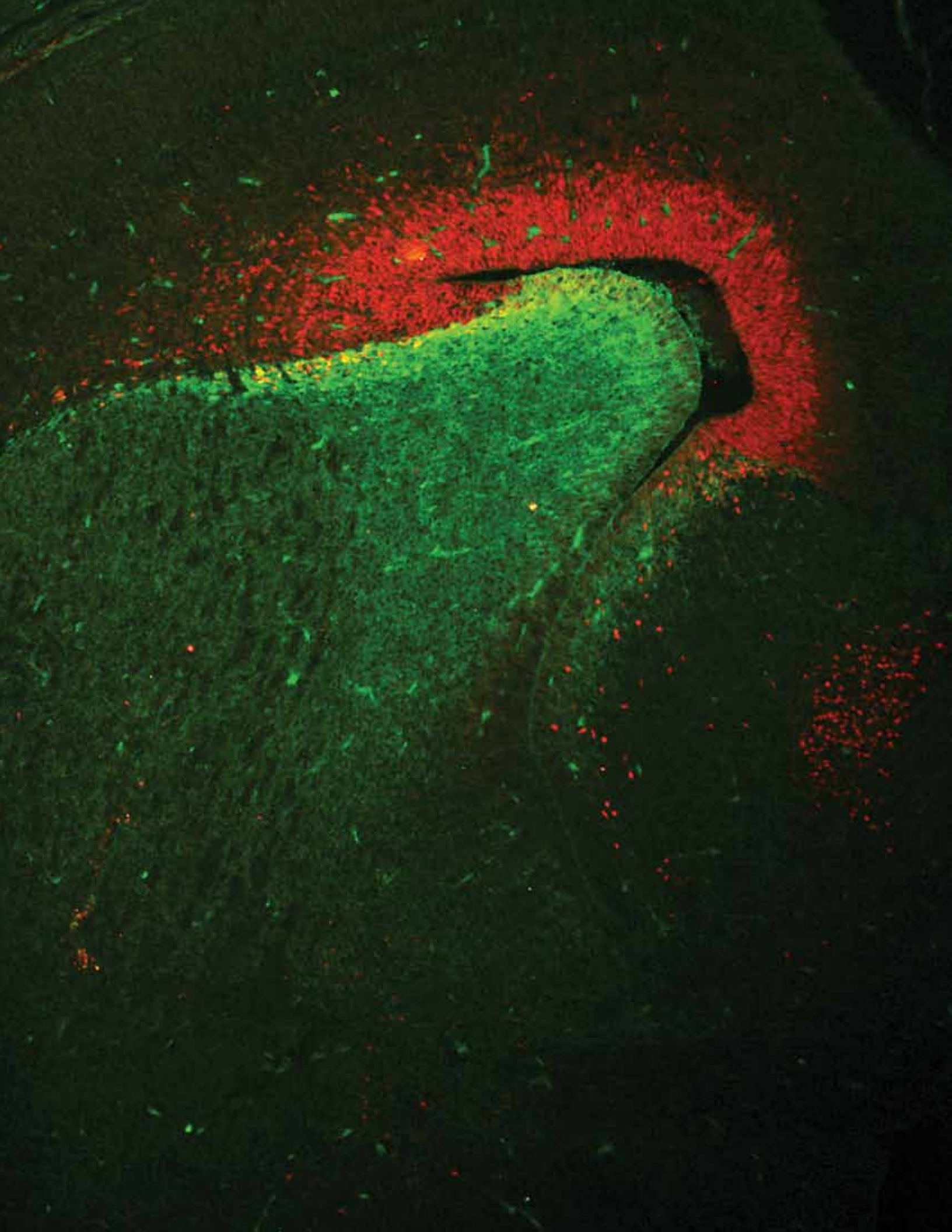
Research Horizons

A PUBLICATION OF THE CINCINNATI CHILDREN'S RESEARCH FOUNDATION
SUMMER 2014

How Life Takes Shape

Developmental biologists explore the frontiers within

Cincinnati Children's



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Front Cover: Scientists at Cincinnati Children's are using tadpoles of West African frogs to unlock the secrets of the earliest stages of organ development. Story, page 38

Samantha Brugmann, PhD,

Plastic Surgery, was awarded $1.9 million for five years by the National Institute of Dental and Craniofacial Research to study "The Role of Primary Cilia in Murine Craniofacial Development."

Kenneth Campbell, PhD,

Developmental Biology, will use a $3.3 million, five-year grant from the National Institute of Neurological Disorders and Stroke to study "Roles of Gsx Factors in Telencephalic Neurogenesis."

Carole Lannon, MD,

James Anderson Center for Health Systems Excellence, will use a $1.9 million grant from the Centers for Medicare/Medicaid over the next two years to work with Ohio University in reducing preterm births through the "Progesterone Quality Improvement Project."

Qing Richard Lu, PhD,

Brain Tumor Center, received a four-year, $1.2 million grant from the National Institute of Neurological Diseases and Stroke to study "Chromatin Remodeling Control of DNS Myelination and Remyelination."

Louis Muglia, MD,

Perinatal Institute, received a one-year, $2 million grant as part of the five-year, $10 million commitment from the March of Dimes for work on the Prematurity Research Center Collaboration.

Anjaparavanda Naren, PhD,

Pulmonary Medicine, received $1.5 million over five years from the National Institute of Diabetes and Digestive and Kidney Diseases, to study "Inhibition of an Apical cAMP/cGMP Transporter (MRP4) in the Gut Induces Diarrhea."

Joo-Seop Park, PhD,

Urology, received a four-year, $1.6 million award from the National Institute of Diabetes and Digestive and Kidney Diseases to study "Cell Fate Regulation of Nephron Progenitors."

Michael Seid, PhD,

Pulmonary Medicine, will participate with the University of North Carolina in a five-year, $2.4 million grant from the National Institute of Diabetes and Digestive and Kidney Diseases to study "FL3X: An Adaptive Intervention to Improve Outcomes for Youth with Type 1 Diabetes."

Research Horizons

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Project Closes Gap Between Evidence and Practice, Wins Quality Transformation Award

In March, the Society of Hospital Medicine presented its Excellence in Teamwork in Quality Improvement Award to a team of 60 healthcare professionals at Cincinnati Children's.

Their project was to close the gap between protocol-based evidence and applying that evidence to clinical care.

Dr. Samir Shah and a team of 60 undertook a successful project to put evidence-based goals into practice around use of antibiotics.

"We focused on infections, which are the most common reason for children being hospitalized," explains Samir Shah, MD, Director, Division of Hospital Medicine. "There can be large gaps between best practice, as demonstrated in published research or guidelines, and actual care. We decided to close that gap at our medical center, and we developed implementation strategies easily replicated at other institutions."

The team tackled issues with the greatest impact on patient safety and clinical outcomes. Specific projects included decreasing reliance on intravenous antibiotics and using oral antibiotics at hospital discharge instead; decreasing use of X-rays of the bladder for infants and children with their first urinary tract infection; increasing use of lactobacillus to treat acute gastroenteritis; improving antibiotic prescribing in community-acquired pneumonia; and shortening the duration of antibiotic use in treating skin and soft tissue infections.

The multidisciplinary team included staff from Hospital Medicine, General and Community Pediatrics; Emergency Medicine; Division of Pharmacy; Infection Diseases; Anderson Center for Health System Transformation; Information Services; Human Resources; Patient Services and Decision Support.

Hostetter Named Director of Research Foundation

Margaret "Peggy" Hostetter, MD, has been selected to serve as Director of the Cincinnati Children's Research Foundation, B.K. Rachford Professor and Chair of the Department of Pediatrics, University of Cincinnati College of Medicine, and Chief Medical Officer of Cincinnati Children's.

Hostetter assumes her new role on July 1. She succeeds Arnold Strauss, MD, who has served in these positions since 2007.

"Cincinnati Children's is a remarkable institution with leadership, faculty, and staff whose commitment to the improvement of children's health is extraordinary," Hostetter says. "I am honored to serve in this very special role."

Hostetter came to Cincinnati Children's in 2010 as the Albert B. Sabin Professor and Director of the Division of Infectious Diseases. She is funded by the National Institutes of Health for leading the Pediatric Scientist Development Program and by the Bill and Melinda Gates Foundation for her studies of *Candida albicans*, the yeast that causes vaginal colonization in pregnant women as well as potentially fatal bloodstream infections in premature infants and immunocompromised individuals. Hostetter and her laboratory hold six patents for discoveries in these areas.

Hostetter earned her MD from Baylor College of Medicine and completed her residency and fellowship in pediatric infectious diseases at Boston Children's Hospital. After leading the Division of Pediatric Infectious Diseases at the University of Minnesota, she served as Jean McLean Wallace Professor of Pediatrics and Chair of the Department of Pediatrics at Yale University until joining Cincinnati Children's.

Among her many honors are the American Academy of Pediatrics Award for Excellence in Research and the Maxwell Finland Lectureship of the Infectious Diseases Society of America. In 1993, Hostetter was the second woman elected to the presidency of the Society for Pediatric Research, 30 years after the election of her mentor, Mary Ellen Avery. She is a member of that organization as well as of the American Pediatric Society, the American Society for Clinical Investigation, the Association of American Physicians, and the Institute of Medicine of the National Academy of Sciences.

Adding to her list of accomplishments, Hostetter was recently voted President-Elect of the American Pediatric Society and will assume the presidency in May 2015.

"Peggy is an outstanding leader in pediatrics, dedicated to the development of physician-scientists and to discoveries that will change our care of children throughout the world," says Strauss.

Dr. Margaret “Peggy” Hostetter is the first woman to head the Cincinnati Children’s Research Foundation.

Twins Provide Clues for Battling Leukemia

Scientists have found a promising new target for treating a deadly malignancy, thanks to identical twins.

A recent study co-authored by a cancer researcher at Cincinnati Children's involved twin 3-year-old girls, one of whom was healthy while the other developed multi-lineage leukemia (MLL), an aggressive form of acute myeloid leukemia. By comparing the girls' blood cells, the scientists identified in the twin with MLL a cascade of cancer-fueling gene mutations and DNA misalignments that traced back to two mutations in the gene SETD2.

The research team from China and the United States reported their findings online Feb. 9 in *Nature Genetics*.

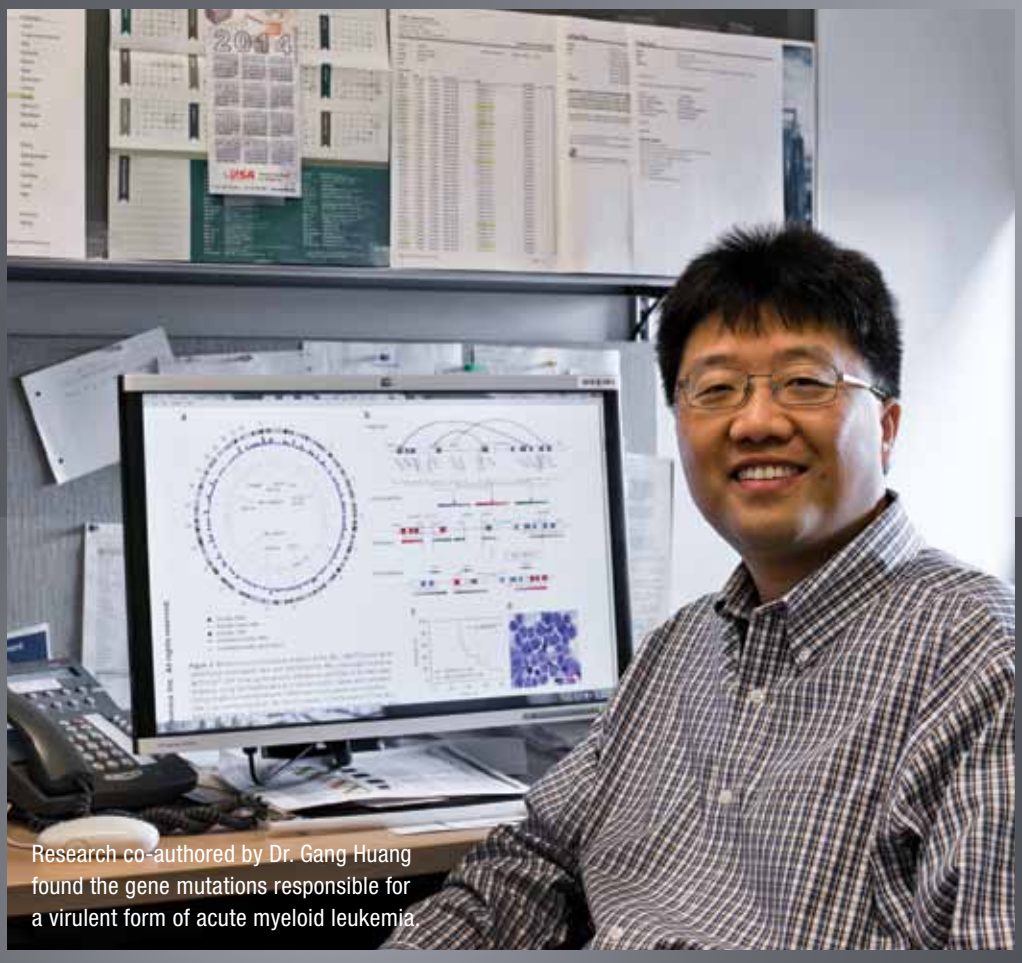
"These mutations contribute to the initiation and progression of leukemia by promoting the self-renewal potential of leukemia stem cells," says Gang Huang, PhD, the Cincinnati Children's researcher who was corresponding author of the study.

The SETD2 gene normally suppresses tumor growth, but mutations disrupt this process and fuel the development of MLL. The SETD2 mutations also activated two other genes (mTOR and JAK-STAT) known to contribute to cancer and leukemia.

Researchers found SETD2 mutations in 6.2 percent of the blood samples from 241 people with various forms of leukemia. The scientists tested mTOR-inhibiting medications on the pre-leukemic cells generated by the SETD2 gene mutations, which resulted in a marked decrease in cell growth.

Huang says these tests demonstrate that there are multiple opportunities to improve MLL treatment. The researchers plan to continue their work by looking for drugs that can target the MLL fusion-SETD2-H3K36me3 pathway. They also continue to look for additional pathways activated by SETD2 mutations.

The twin sisters' genomes were compared at the laboratory of co-corresponding author Qian-fei Wang, PhD, Beijing Institute of Genomics. Several other institutions in China and a research center in Chicago also collaborated on the study.



Research co-authored by Dr. Gang Huang found the gene mutations responsible for a virulent form of acute myeloid leukemia.

Receipts Could Pose Harm to Cashiers

A study led by Dr. Shelley Ehrlich was the first to show that the harmful chemical BPA can penetrate the skin.

Extensive handling of thermal receipts from cash registers significantly increases exposure to the chemical bisphenol A (BPA). BPA is used as a coating on the receipt paper.

The study, published in the *Journal of the American Medical Association*, is believed to be the first to show that BPA penetrates skin. The chemical has been implicated in health problems associated with ingesting food or drinks from cans or plastic bottles that contain it.

"We observed an increase in urinary BPA concentrations after continuously handling receipts for two hours without gloves," says Shelley Ehrlich, MD, an obstetrician/gynecologist and epidemiologist at Cincinnati Children's and the study's lead author. When the simulation was repeated using gloves, there was no significant increase in urinary levels of the chemical.

The study involved 24 volunteers at the Harvard School of Public Health who printed and handled receipts continuously for two hours without gloves. The researchers detected BPA in 83 percent of the students prior to handling the receipts and in 100 percent of the students after handling the receipts.

Urinary concentrations of BPA were more than three times higher after handling the receipts. Twelve participants who provided sequential urine samples over a 24-hour period had more than a five-fold increase in BPA urinary concentrations compared to baseline. There was no significant increase in urinary BPA when participants wore gloves.

This study shows that cashiers in supermarkets and gas stations, bank tellers, and librarians could be at higher risk by absorbing BPA through their skin when handling receipts.

"While our study findings showed that continuous thermal receipt handling without the use of gloves significantly increased urinary concentrations of BPA, a larger study is needed to confirm our findings and evaluate the clinical implications of this type of exposure," Ehrlich says.

Shock Value

Simple blood test works in adults and children to quickly predict severity of septic shock.

The biomarkers for risk of death in adults were nearly the same as those in children, says Dr. Hector Wong.

A doctor at Cincinnati Children's has developed a test that quickly and accurately predicts the risk of death for patients suffering from septic shock — a severe systemic infection that rapidly damages vital organs. It is one of the leading causes of death in hospitalized adults and children.

The simple blood test measures five biomarkers, or proteins, in the blood. Results are combined with other information about the patient to estimate the probability of surviving the illness.

The test should enable doctors to decide more quickly whether a severely sick person needs aggressive life-saving treatment, says Hector Wong, MD, Director of the Division of Critical Care Medicine. The study was led by Wong and Christopher Lindsell, PhD, of the University of Cincinnati College of Medicine.

Finding ways to treat septic shock has been tricky. Clinical trials of treatments to stop the infection are complicated by patients too sick to be saved by the therapies or not sick enough to warrant them.

"Substantial resources are invested in trying to find new treatments for septic shock, but the vast majority of them fail in clinical trials because mortality risk varies widely in septic shock patients," explains Wong.

Results of the test's effectiveness were reported in April in *Critical Care Medicine*. The researchers developed a similar test for children, the results of which were published in ***PLOS ONE*** earlier this year. The new study involved 882 adults in intensive care units in the United States, Finland and Canada. One important similarity in their findings was that three of the biomarkers identifying risk for adults were the same as those in children, providing potential targets for treatment.

Study funding came from the National Institutes of Health and the Center for Technology Commercialization at Cincinnati Children's.

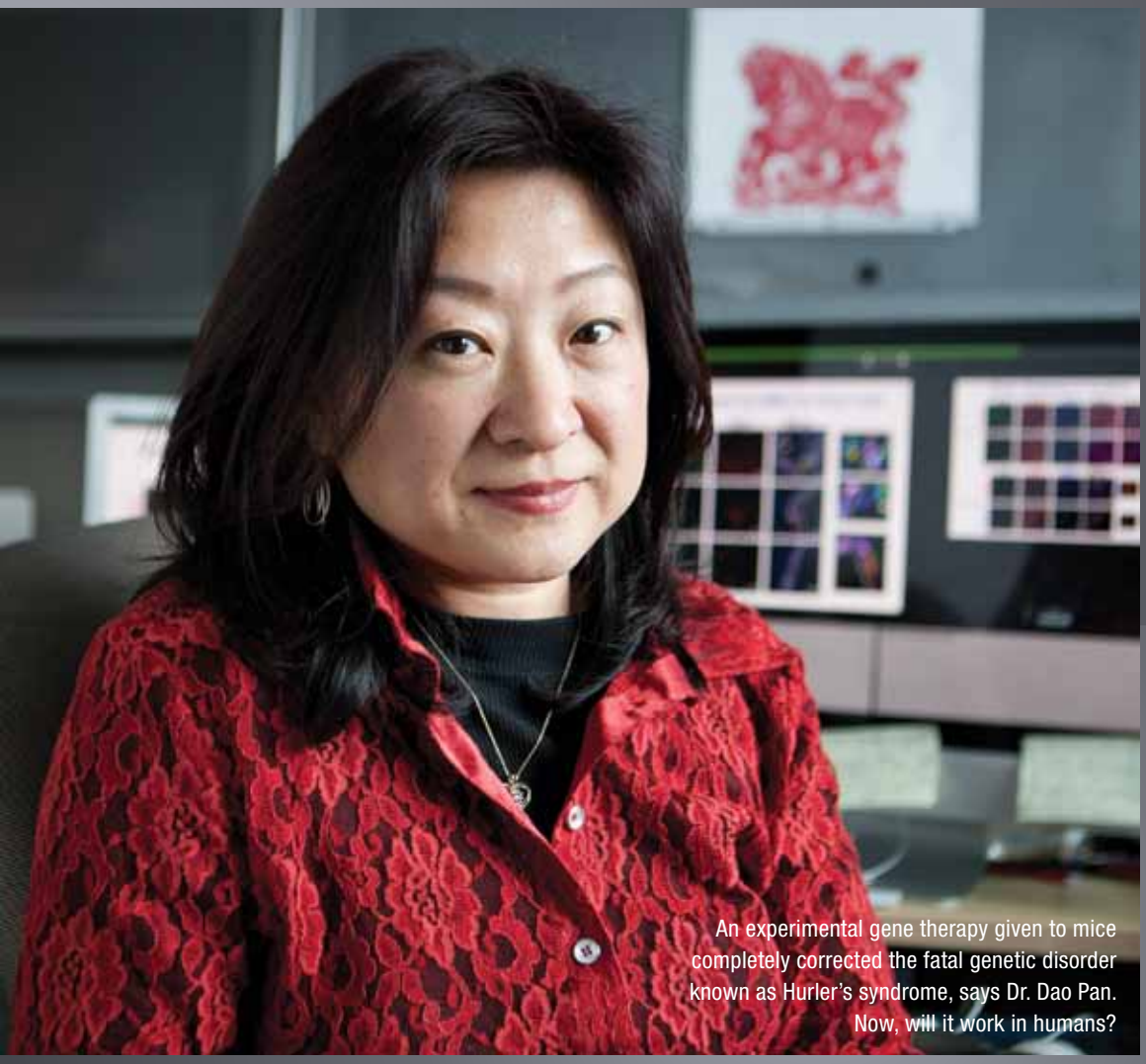
Gene Therapy for Hurler Syndrome Shows Promising Results

Scientists from Cincinnati Children's and the National Institute of Neurological Disorders and Stroke (NINDS) have developed an experimental gene therapy that could possibly cure Hurler syndrome, an unusual and often fatal genetic disorder.

Children with Hurler syndrome lack the critical lysosomal enzyme alpha-L-iduronidase (IDUA) and cannot break down sugar molecules called glycosaminoglycans. The molecules build up in the body and cause extensive damage. In the study, the researchers successfully used engineered blood platelets and bone marrow cells to deliver gene therapy to mice bred to exhibit the disease. The gene therapy enabled the cells to produce IDUA, resulting in a complete metabolic correction of the disease.

The results were published online in February, in the *Proceedings of the National Academy of Sciences*. "Our findings demonstrate a unique and somewhat surprising delivery pathway for lysosomal enzymes," says Dao Pan, PhD, corresponding author and researcher in the Division of Experimental Hematology and Cancer Biology. "We show proof of concept that platelets and megakaryocytes are capable of generating and storing fully functional lysosomal enzymes, which can lead to their targeted and efficient delivery to vital tissues where they are needed."

Pan and colleagues — including Roscoe Brady, MD, a researcher at NINDS — engineered human megakaryocytic cells capable of overexpressing IDUA. Once infused, the cells produced large amounts of functional IDUA and could cross-correct other cells. More study is needed to determine if the treatment would be safe and effective for human patients.



An experimental gene therapy given to mice completely corrected the fatal genetic disorder known as Hurler's syndrome, says Dr. Dao Pan. Now, will it work in humans?

Cincinnati Children's Named First U.S. INSPIRE Site

Cincinnati Children's is the first academic medical center in the United States to be named an INSPIRE (Investigator Networks, Site Partnerships and Infrastructure for Research Excellence) site by Pfizer.

Pfizer created the INSPIRE designation in 2013 to recognize medical facilities for their expertise and quality in conducting clinical trials. There are currently just over 100 INSPIRE sites in the world.

"Not only does this designation recognize our role as a leader in conducting treatment trials for children and adults," says John P. Clancy, MD, Medical Director of the Office for Clinical and Translational Research, "but it will also allow us to expand our program to deliver even more advanced and potentially lifesaving treatments to our patients."

INSPIRE sites receive early consideration for research opportunities with Pfizer and non-financial assistance with audits and other quality measures. Hospitals chosen as INSPIRE sites must meet strict criteria in how they conduct clinical trials, including having medical expertise in key disease areas, highly trained staff and dedicated resources for clinical research. They must also meet specific trial recruitment goals and timelines.

Annually, Cincinnati Children's has more than 2,250 investigator-initiated, industry- and federally-sponsored IRB-active protocols, including pediatric Phase I-IV and select adult (vaccine and cancer) Phase I-IV clinical studies.

Cincinnati Children's is accredited to conduct clinical research by the Association for the Accreditation of Human Research Protection Programs and has been conducting pediatric research since 1931.

As an INSPIRE site, we can further expand our trials of advanced treatments, says Dr. John Clancy, who oversees our Office for Clinical and Translational Research.

Drug Stops Cancer, But Not As Thought

Dr. Biplab Dasgupta's study of the diabetes drug metformin for cancer treatment showed the drug worked by inhibiting the mTOR molecule.

Researchers from Cincinnati Children's Hospital Medical Center who led a study of the drug metformin for cancer treatment have found that it works, but not the way it was believed.

In a report published Jan. 13 in ***Proceedings of the National Academy of Sciences***, the research team challenged the belief that the drug activates a molecular regulator of cell metabolism called AMPK to suppress tumor growth.

Extensive laboratory tests revealed that metformin does stop cancer, although not by activating AMPK. Instead, in tests involving glioma brain cancer cells, the authors found that metformin inhibits a different molecule called mammalian target of rapamycin (mTOR), which has been linked to many other cancers.

Originally developed to treat diabetes, metformin suppresses the actions of insulin and insulin-like growth factors — two molecules that support cancer growth, according to Biplab Dasgupta, PhD, principal investigator and a researcher in the Division of Hematology/Oncology at Cincinnati Children's. "Our findings do not suggest that clinical trials using metformin should be stopped. Metformin appears to be a very useful drug, but the drug's mechanism of cancer suppression is not clear," he says.

Dasgupta suggests that while one important cellular effect of metformin in cancer cells is inhibition of mTOR independent of AMPK, activation of AMPK itself may actually fuel growth of certain cancers. Recent unpublished work from his lab indicates this.

"Our findings unveil a potential role for AMPK as a tumor growth supporter, not a suppressor, in the type of cancer that we study. This is why clinicians using metformin in clinical trials should use caution during data interpretation."

Ultimately, improving targeted cancer therapies will require more accurate understanding of AMPK's role and a better understanding of how a drug like metformin stops cancer, says Dasgupta.

The study involved scientists from Cincinnati Children's; the University of Cincinnati College of Medicine; the Mayfield Clinic; the Mayo Clinic; Katholieke Universiteit and Antwerp University Hospital in Belgium; and the Inserm Institut Cochin and the Universite Paris Decartes in Paris.

Funding for the study came from CancerFreeKids, the Smith-Brinker Golf Foundation, a Cincinnati Children's Trustee Scholar Grant and the National Institutes of Health.

Dr. Raphael Kopan's discovery of how the Notch protein enters the cell nucleus changed the way scientists look at cell signaling. Now his team is exploring the protein's role in a variety of diseases.

Taking it Up a Notch

Simple protein wields tremendous influence in human development and disease

by Mary Silva

Raphael Kopan, PhD, started his research career with a rather modest goal, and failed at it. Luckily, he sees that as part and parcel of the scientific process.

"It's the inevitable reality of trying to discover how nature works. There's no manufacturer's manual," he says. "You never know where the path will lead."

Kopan was studying skin development — specifically, how to make a hair follicle. He admits it was not life-altering science, but thought it could be useful to certain hair-challenged individuals. Besides, he was having fun.

"It was an interesting project. I worked on it for about a year, tried lots of crazy procedures. Technically they all worked, but none of them resulted in anything remotely usable," Kopan says. "I was at the point of my career where it looked like there would be no career."

But Kopan's failed efforts with hair follicles had acquainted him with Notch protein, a molecule that appeared to influence the fate of early cell development. His advisor at the time — it was the late 1980s — was the late molecular biologist Harold Weintraub, PhD, who encouraged him to explore the protein further.

He did, and found that Notch had little to do with hair follicle induction but a lot to do with his future.

"My lucky break was, the piece of Notch I was studying was very small," Kopan says.

"Everybody else was studying the whole gene. I was the only guy whose wimpy little Notch needed help in order to work. And the help it needed was to travel into the nucleus."

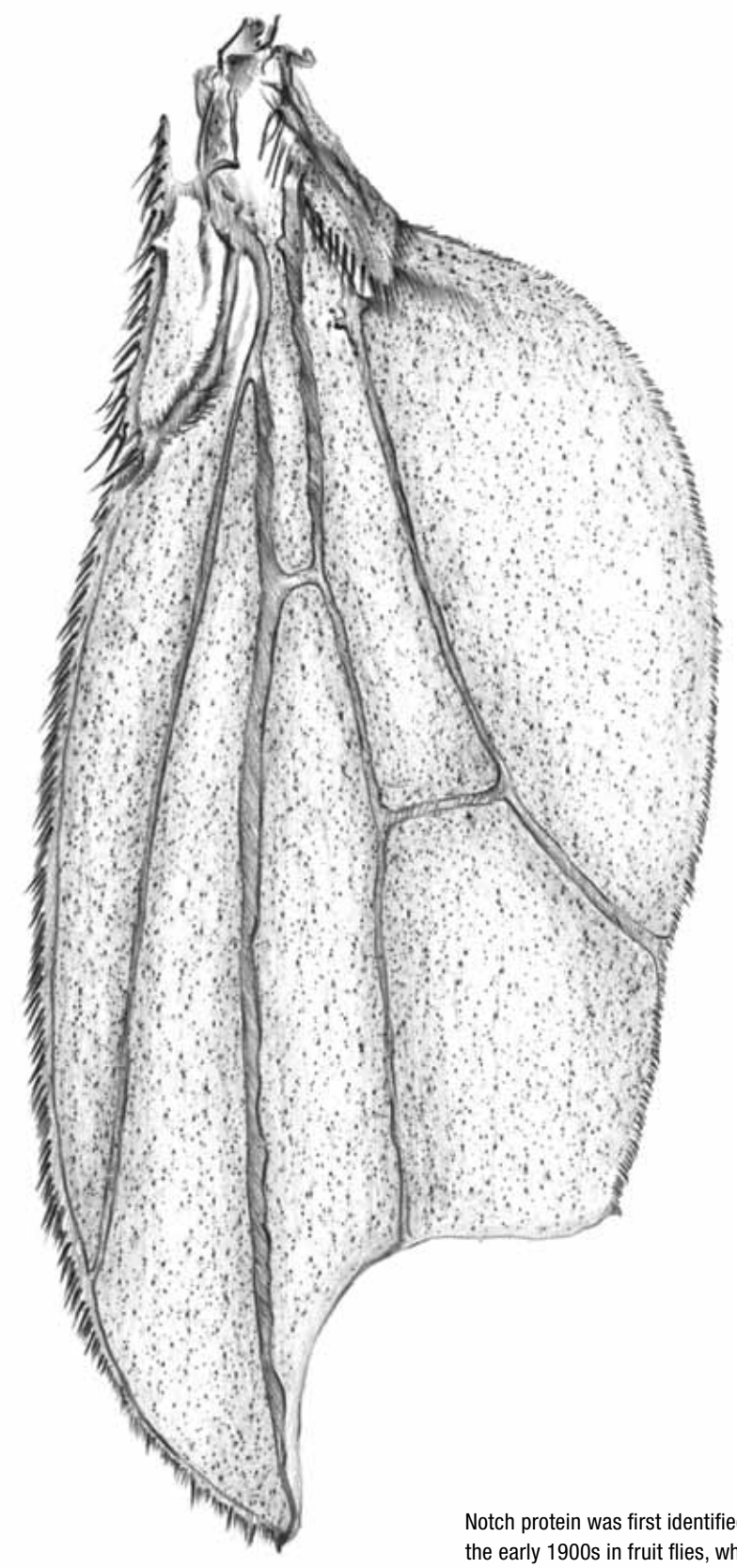
A BREAKTHROUGH BREAK

Notch protein was first identified in the early 1900s in a form of the *Drosophila* fruit fly, where mutations in the gene resulted in the insect's notched wings. Scientists now know that the protein is found in all multicellular animals and is responsible for the development of virtually every organ. Kopan was able to show how Notch influenced development by discovering how it got into the cell nucleus. His discovery changed the way scientists understood cell signaling.

"The idea people had about how cells translate a signal into the nucleus involved an army of secondary messengers," he says. "I found the sequence where the Notch molecule was broken. And I was able to show that this break only happened when the molecule encountered its ligand and not otherwise."

Kopan's discovery of Notch's breakage and subsequent journey into the nucleus was dubbed the "canonical pathway" by his fellow scientists. Notch protein straddles the cell, half in and half out. When the half that remains outside the cell's membrane encounters a specific binding protein - its ligand - it breaks in two. The half located inside the cell trav-

Kopan's discovery of Notch's breakage and subsequent journey into the nucleus was dubbed the "canonical pathway" by his fellow scientists.



Notch protein was first identified in the early 1900s in fruit flies, where mutations in the gene caused the insects to have notched wings

els to the nucleus, where it begins to issue genetic directives ranging from what the cell becomes to whether it lives or dies.

When Kopan and his team engineered Notch molecules that could not be broken, this cell signaling did not occur. "This was the strongest bit of evidence that the mechanisms for signals to be transduced required the molecules to fall apart," he says.

THE CELLULAR COIN TOSS

Understanding how Notch issues its orders was the first piece of the puzzle. Further study revealed the protein's masterful ability to regulate development.

Notch acts as a biological traffic cop, often helping one cell to adopt a particular fate — fate A — while stopping its neighbors from doing so. The cells signal to each other; the more Notch signal a cell receives, the less likely it is to activate. The cell receiving the least Notch signal moves on to differentiate into fate B. In this way, Notch organizes normal cellular structures and prevents equivalent cells from all adopting the same cellular fate. This can happen "stochastically," says Kopan - with a sort of controlled randomness - or in a very controlled manner.

"Notch is like the quarter you carry in your pocket," Kopan says. "If you have a binary decision to make, you flip a coin: Heads you go, tails you don't. That's Notch."

RANDOM EXPLORATIONS TIE TOGETHER

What led to Kopan's next discovery about Notch was less stochastic and more completely random.

Despite having discovered how it worked, Kopan was still puzzled by how Notch broke apart in the cellular membrane. Proteins are typically broken with water, a process called peptide bond hydrolysis or proteolysis, and the cell membrane is made up almost entirely of lipids, mostly fats. How does Notch come apart in that environment?

The answer came in the mid 1990s, when scientists researching Alzheimer's disease discovered the presenilin proteins that live in the cell membrane's fatty environs. Mutations in these proteins are a cause of inherited Alzheimer's disease. At the same time, scientists in another laboratory discovered that presenilin corrected an egg-laying defect caused by overactive Notch in the *C. elegans* worm. Kopan and his collaborators realized it is the presenilin protein that Notch depends on to break apart, enter the cell nucleus, and mediate its regulation of normal development.

This piecing together of what seem to be unrelated bits of exploration is what Kopan loves about science.

"In basic science, if you aim your arrow, you often

miss — it does not get you what you were after. If you don't aim where your arrow goes, but are mindful where it lands, you can extract deep meaning from most landing spots. Because everything is connected in biology, even this seemingly random process gets you back to the clinic."

IMPACT ON DISEASE

Now, Kopan is connecting what he has learned about Notch to determine how its presence or absence affects human disease. He and his team are exploring Notch's function in a variety of diseases and disorders, including kidney disease, immune disorders and cancer.

He knows what they learn from any species will be useful because Notch works the same way across all multicellular organisms.

"We are all made of the same building blocks; all metazoans use Notch signaling," he says. "We work with mutations that arise in an animal, but sooner or later a similar mutation will be in the human population. Or we can generate a mutation identified in humans in our model organisms, to better understand the mechanism of the disease and to see which levers we can push to get a better outcome."

CONNECTING THE DOTS

Kopan came to Cincinnati Children's just one year ago — his first stint in a pediatric setting — and likes working in an environment where both researchers and clinicians focus on the earliest stages of development. He considers all pediatricians developmental biologists.

"Here, I don't need to explain why developmental biology is important," he says. "There is a great flow of information from the human side to us and from us to them, to get to the bottom of various disorders and diseases, and to understand, treat and manage them. We try to accelerate the process by modeling the organism and coming up with intelligent ways to attack it." ■

"Notch is like the quarter you carry in your pocket," Kopan says. "If you have a binary decision to make, you flip a coin: Heads you go, tails you don't. That's Notch."

Tuning in to Facial Development

Research links malformations to cells' missed signals

by Tim Bonfield

Samantha Brugmann, PhD, studies how defects in primary cilia can lead to facial deformities, using an embryonic avian model (preceding page).

Long before an embryo develops the bones of its face, the progenitor cells of the facial skeleton must decide where to migrate, when to divide and when to differentiate. How do they know when to take these steps?

"Early on, you have neural crest cells — the progenitors of the facial bones — sandwiched between the developing brain, the neuroectoderm, and the developing skin, the surface ectoderm. Proper development depends on receiving molecular signals from both the neuroectoderm and surface ectoderm, but what happens if the neural crest cells cannot receive or transmit these signals?"

Asking this question is researcher Samantha Brugmann, PhD, an expert in craniofacial developmental at Cincinnati Children's who works in both the Divisions of Plastic Surgery and Developmental Biology.

CELLULAR ANTENNAE

Many cells, including neural crest cells, use the primary cilia to receive molecular signals. These tiny, finger-like protrusions play a large role in prenatal development by helping cells sense where they are and identify what surrounds them.

When primary cilia fail to do their job, the resulting disorder is called a ciliopathy. These disorders typically result in defects in the growing brain, kidney, skeleton and the developing face.

"It turns out there are multiple genes involved in the proper formation and function of the cilia," Brugmann says. "Different genetic mutations can result in different outcomes."

In facial development, ciliopathies are frequently characterized by cleft lip, cleft palate, hypertelorism (widely set eyes), micrognathia (a very small lower jaw) and other rare conditions.

Brugmann's team, which includes Ching Feng Chang, PhD, and graduate student Betsy Schock, studies ciliopathies in both chicken and mouse model systems. In the chicken model they have identified a novel avian gene, called C2CD3, that leads to cilia-related malformations. A paper identifying the gene and characterizing the affected molecular pathway is currently in revision for publication in the journal *Development*.

The findings are significant because Brugmann's team has classified a long utilized, but poorly understood avian mutant, talpid2, as a ciliopathy. This mutant exhibits features similar to human ciliopathies, such as cleft lip, cleft palate and micrognathia, which makes it especially useful for research.

MAKING A MODEL MOUSE

In January 2014, Brugmann was awarded a five-year, $1.25 million grant from the National Institute of Dental and Craniofacial Research to expand her study to a mouse model. She plans to explore the roles played by a specific ciliary protein, Kif3a, and how that protein functions in interpreting the Sonic Hedgehog (Shh) signaling pathway.

To conduct the study, the team is producing a line of "conditional knockout" mice, bred to lack the gene that makes the Kif3a protein — but only in the neural crest cells. Mice that lack the

gene entirely do not survive long enough to reach birth.

"These mice have a similar phenotype to what we see in the chicken — cleft palate, increased cartilage development, a wide midface and micrognathia with aglossia — the lack of a tongue," Brugmann says. "We believe the wide midface may be the result of too much Shh signaling, while the tongue and jaw defects may reflect too little Shh signaling."

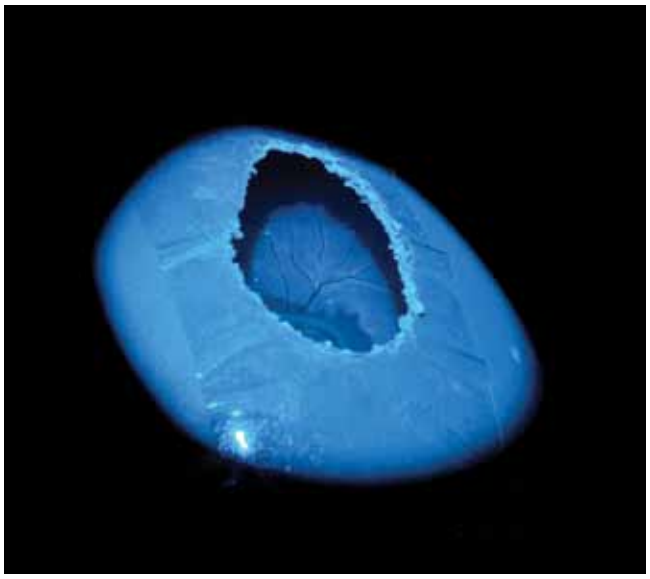
LONG ROAD AHEAD

When people are born with these sorts of defects, the only treatment has been surgery — often involving multiple invasive procedures. As Brugmann's work helps explain why these defects occur, it might lead to a preventive treatment. But that goal remains years away.

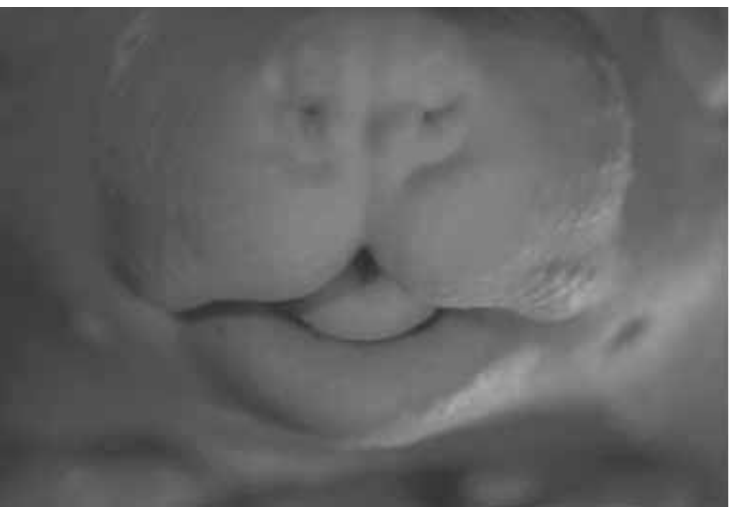
"A genetic therapy may be possible, but it would need to be performed before birth and that would be a potential ethical challenge," Brugmann says. "Still, understanding the flow of that molecular pathway could reveal other starting points for possible therapeutic intervention."

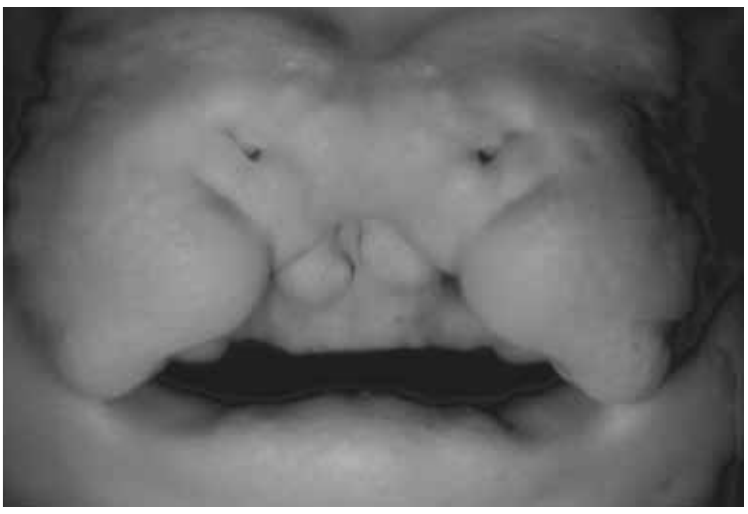
For example, scientists already know that cholesterol interacts with the Sonic Hedgehog pathway, which is one reason why pregnant women are advised to avoid certain cholesterol-lowering drugs. Could a cholesterol-based therapy prevent facial malformations? It's too early to tell. And it may turn out that other ways to manage the Hedgehog pathway would be more effective.

"First we have to learn the nuts and bolts of this process," Brugmann says. ■



Dr. Samantha Brugmann reviews some of the malformations produced in embryonic mice. She hopes to show that ciliogenesis is disrupted via the loss of ciliary protein Kif3a expression in neural crest cells.

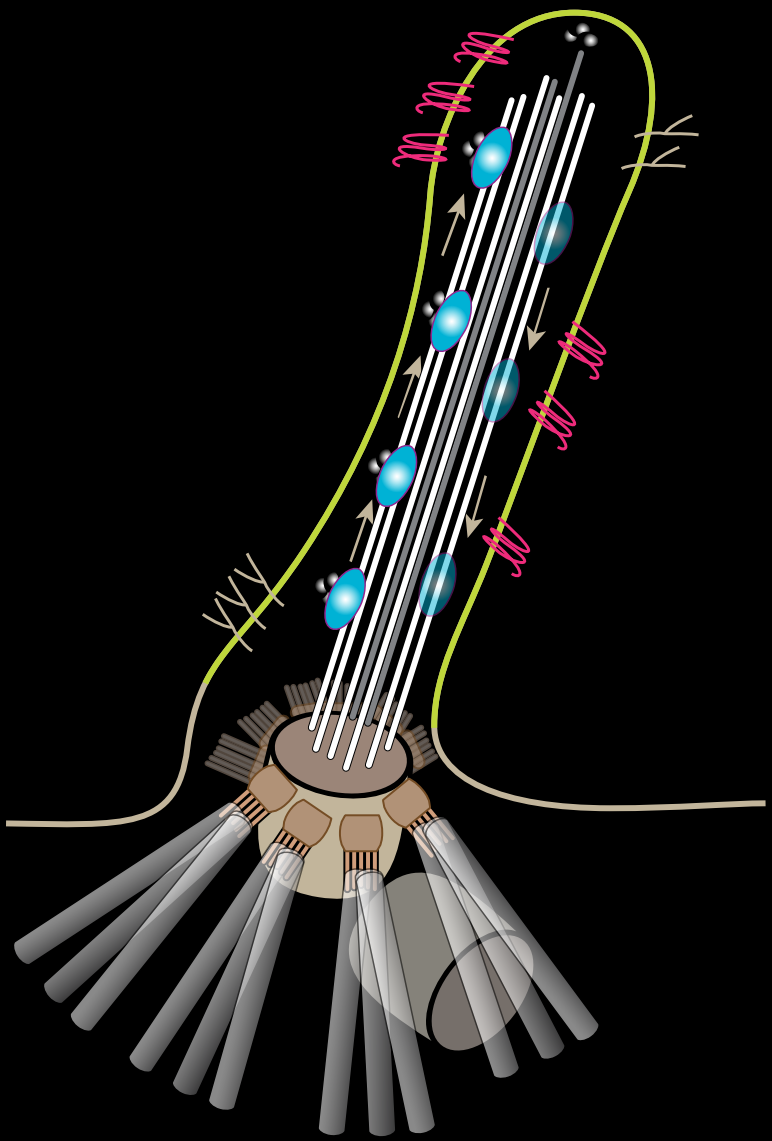




Anatomy of a Cilium

Cilia are microscopic, hair-like structures or organelles that extend from the surface of nearly all mammalian cells. There are two types of cilia - motile and non-motile, also known as primary cilia. For years, scientists believed primary cilia were useless vestiges of evolution. More recently, researchers have discovered that primary cilia play crucial roles during embryonic development. They act like tiny antennae that help cells sense their surroundings and pinpoint their location. This helps guide the developing cells to their ultimate fate. When primary cilia do not form properly or malfunction, it can disrupt the formation of bones, the brain, the liver and other organs. In recent years, a number of seemingly unrelated genetic birth defects have been re-classified as ciliopathies.

In this scanning electron microscope image, colored projections show avian cells extending primary cilia. Normally, 60 to 70 percent of cells are extending cilia at any given moment. However, when a gene mutation affects ciliogenesis, only 19 percent of cells extend cilia. (Images provided by a collaborator at Miami University.)



IFT proteins move along the microtubules to transfer signals to and from the cell.

Basal bodies anchor the antenna to the cell.

Membrane-bound receptors respond to chemicals, light and other signals from the cell's surroundings

Microtubules form the internal scaffolding.

A New Dimension in the Study of Human Disease

Researchers generate functioning, three-dimensional tissue to study what goes wrong - and right - in development

by Nick Miller

AS ORGANS GO, THE STOMACH DOES NOT GET MUCH RESPECT.

It takes in food and fluids, helps turn them into energy, and then begins converting it all into substances too often the subject of tasteless humor. Unlike the brain, heart or lungs, the stomach is not one of science's "glam" organs. Nor is it the subject of extensive developmental research literature - at least not yet.

Kyle McCracken, an MD/PhD student in the Developmental Biology laboratory of Jim Wells, PhD, hopes to change this. Wells is an expert in gastrointestinal development, diabetes research and stem cell technologies. When his team set out to see if they could use pluripotent stem cells (PSCs) to grow human stomachs in the laboratory to model and study human disease, McCracken started by looking for studies on basic stomach development.

"With organs like the pancreas, liver and the lungs, there are hundreds of publications about what controls their development," McCracken says. "But we went to the stomach and there is nothing. We think we are addressing some of these gaps in knowledge with the *in vitro* modeling system we've developed."

STOMACHS IN A DISH

Wells and McCracken successfully led a team of scientists to figure out how to generate in a petri dish a functioning, three-dimensional and critically important region of the human stomach called the antrum. They did this by using PSCs, some of them induced pluripotent stem cells (iPSCs). The iPSCs are made from human

Three-dimensional stomach tissue growing *in vitro* in the Wells laboratory

skin cells transformed with biochemical solutions to take on embryonic-like characteristics.

Like human embryonic stem cells (hESCs), iPSCs have the ability to become any cell or tissue type of the human body.

In early 2011, Wells' laboratory published a paper in *Nature* on using iPSCs to generate functioning, three-dimensional intestinal tissue in a petri dish. That study — the first time any research team had generated functioning, three-dimensional intestinal organoids from iPSCs — helped start a new chapter in life sciences research for studying diseases and therapeutic solutions.

Unlike generating intestine — where the literature gave researchers some clues on where to begin — the stomach project forced the scientists to start from scratch. It was a tedious and time-consuming process of testing different genes and biochemical combinations to get PSCs to form stomach tissue.

"Not only were we trying to generate gastric organoids for research and therapeutic purposes, we were actually using the new *in vitro* system as a primary research and discovery tool to determine what makes stomach to begin with because so little was known," Wells explains.

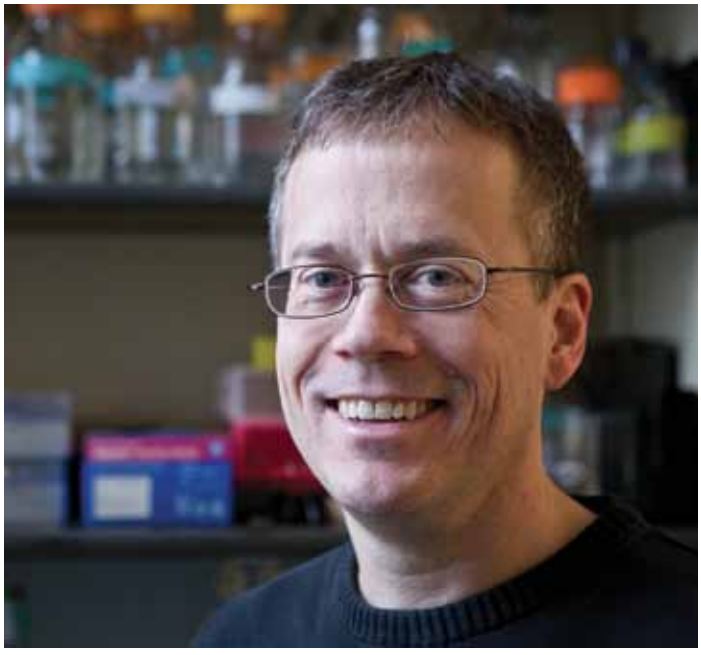
RECIPE FOR GROWTH

To grow distal stomach through what is called directed differentiation, the team used a precise combination of signaling by important developmental pathways — including FGF (fibroblast growth factor), Wnt (protein signaling pathway), and BMP (bone morphogenetic protein). This allowed the scientists to mimic the normal steps of development that occur in an embryo. Importantly, during this phase the researchers discovered that BMP needed to be repressed. Through the carefully timed manipulation of these and other molecular components, the researchers coaxed two-dimensional cultures of PSCs into becoming three-dimensional foregut tube structures — an embryonic starting point for stomach.

To get foregut tissues to become gastric tissue corresponding to the antrum in the distal region of the stomach, the scientists manipulated other cellular processes by stimulating the signaling of retinoic acid and epidermal growth factor. Over the course of a month, these steps resulted in the formation of 3D gastric tissues that grew into large organoids similar to the antrum in the distal stomach.

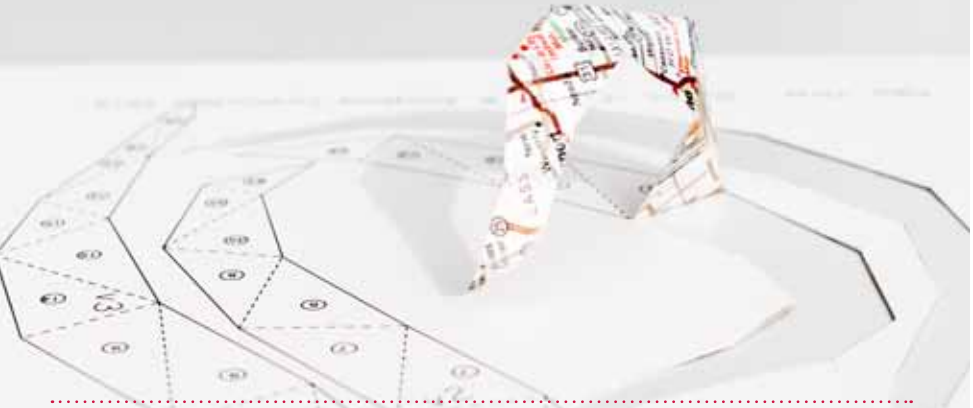
REPLICATING A VARIETY OF CONDITIONS

The new modeling system provides significant advantages for studying human disease, according to Wells. For example, the body's response to food intake starts in the stomach. Controlling this response could hold the keys to preventing obesity and diabetes — a growing health epidemic. The distal portion of the stomach generated by the Wells lab houses the body's satiety or "hunger" response. Wells says



Below, from left: Dr. James Wells and Kyle McCracken, an MD/PhD student in Wells' laboratory who helped lead the project to form three-dimensional stomach tissue.

ROAD MAP TO 3D HUMAN STOMACH



"Embryos aren't flat and we've figured out, at least partly, how the embryonic gastrointestinal system transitions from two-dimensional into three-dimensional, and then generated three-dimensional organ tissues with a fair level of complexity."

a breakdown in the hunger response is linked to obesity and resulting metabolic diseases like diabetes.

Stomach research may help explain why some gastric bypass surgery patients become diabetes-free even before they lose significant weight. And the new modeling system is already being used to study peptic ulcers and gastric cancers in unprecedented detail.

Wells, who has a dual appointment in the Division of Endocrinology, is part of a new research consortium at Cincinnati Children's formed to study the endocrine system. The effort is designed to bring a multi-laboratory and multi-disciplinary focus to studying the endocrine system — and the new iPSC modeling technology will be central to that effort.

Mouse studies — long a backbone of life sciences and disease research — are poorly suited for studying diseases of the stomach. For one, Wells says the stomach is one of the least evolutionarily conserved organs among mammals, so structural development differs between mice and humans. One possible reason for this may be the wide dietary differences between species. Pathogens that run amok in human stomachs will not, in many cases, infect the stomachs of mice.

Because the gastric organoids are derived from human cells, McCracken says they will allow scientists to study the biology of human stomach tissue.

THREE-DIMENSIONAL RESULTS

The new 3D organoids replicate in a laboratory what actually happens in a person far more precisely than do flat cell cultures.

"We haven't just made a bunch of flat cells in a dish," Wells says. "Embryos aren't flat and we've figured out, at least partly, how the embryonic gastrointestinal system transitions from two-dimensional into three-dimensional, and then generated three-dimensional organ tissues with a fair level of complexity." ■

Study of *H. pylori* is first to use 3D gastric model

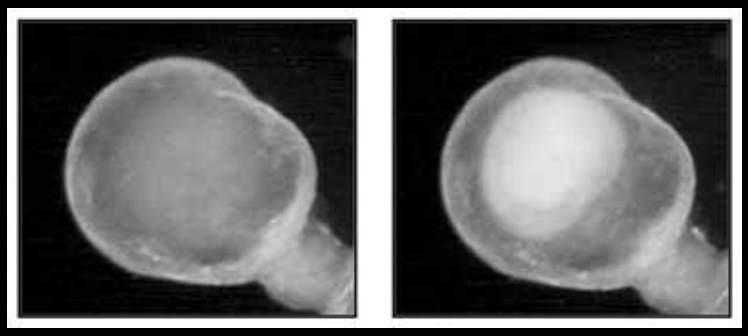


Image above shows gastric organoid before and after (white mass) infection with *H. Pylori*.

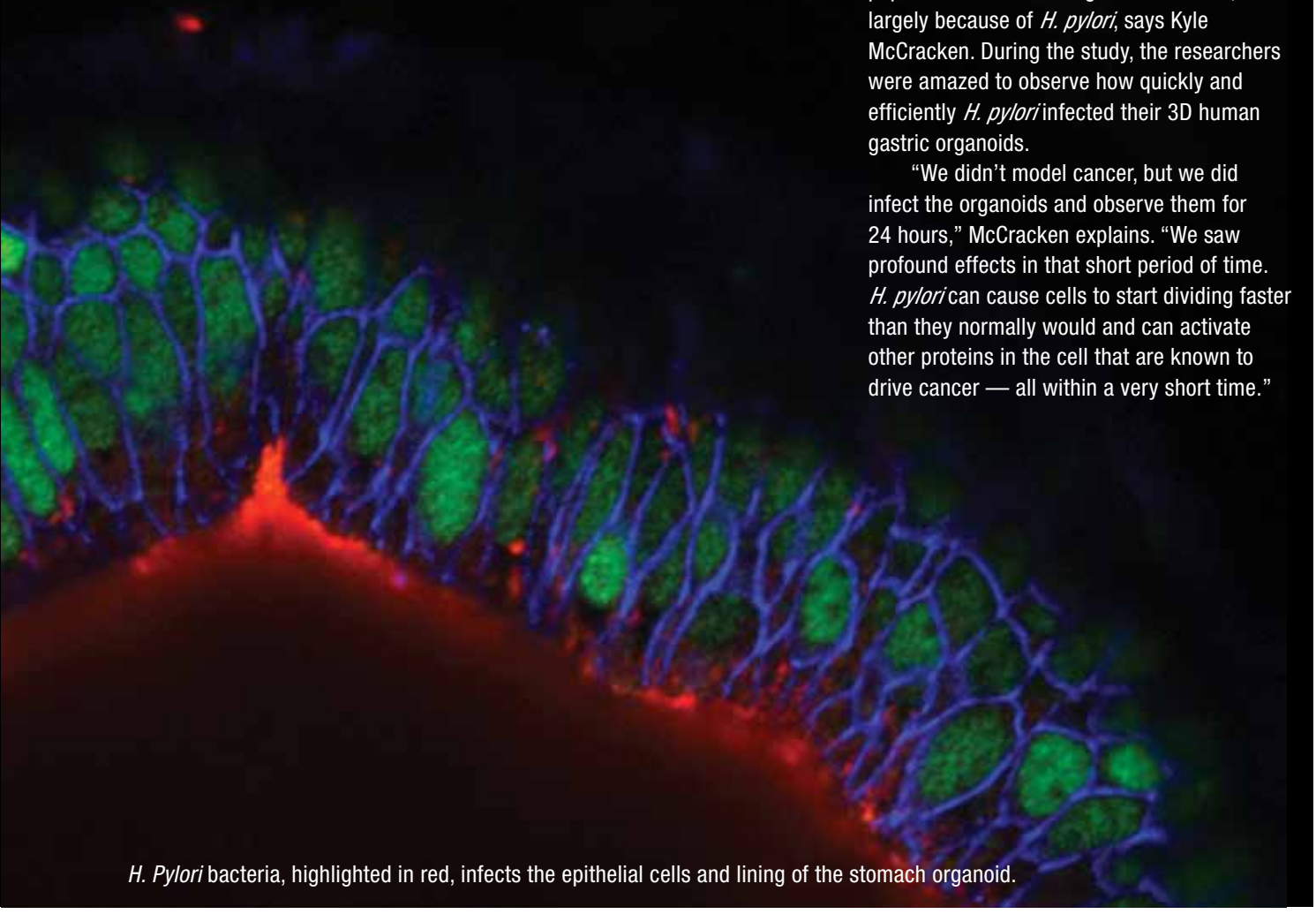
Although the digestive system is full of helpful bacteria, *Helicobacter pylori* are not among them.

H. pylori are perhaps the worst bacterial villains to afflict the stomach, and the chief culprit behind peptic ulcers and gastric cancers.

In collaboration with Yana Zavros, PhD, a researcher at the University of Cincinnati's Department of Molecular and Cellular Physiology who studies gastric cancer, one of the first studies using the new 3D gastric model is how *H. pylori* bacteria infect the human stomach.

An estimated 10 percent of the world's population suffers from gastric diseases, largely because of *H. pylori*, says Kyle McCracken. During the study, the researchers were amazed to observe how quickly and efficiently *H. pylori* infected their 3D human gastric organoids.

"We didn't model cancer, but we did infect the organoids and observe them for 24 hours," McCracken explains. "We saw profound effects in that short period of time. *H. pylori* can cause cells to start dividing faster than they normally would and can activate other proteins in the cell that are known to drive cancer — all within a very short time."



H. Pylori bacteria, highlighted in red, infects the epithelial cells and lining of the stomach organoid.

Stem Cell Lab Opens Doors *– and Minds –* to New Discoveries

By Nick Miller

Dr. Chris Mayhew with stem cell facility research assistants Amy Pitstick and Matt Kuhar.

Christopher Mayhew, PhD, appreciates the importance of converting skeptics into believers.

He finds this transformation happening more readily as time passes — certainly compared to four years ago. That is when Mayhew helped establish the Pluripotent Stem Cell Facility at Cincinnati Children's — one of the nation's first such core research laboratories at an academic medical center. The move was a significant investment and statement for Cincinnati Children's.

It signaled a strong commitment to an emerging and — at the time — somewhat uncertain technology called induced pluripotent stem cells (iPSCs). Only a few years later, the capabilities of iPSCs are resulting in dramatic advances in how scientists study disease.

For Mayhew, it has been rewarding to see fellow scientists learn they can use new, human-based modeling systems that do not require a reliance on animal models and their inherent functional or translational limitations.

"There has been a small cohort of pioneers and true believers from the very beginning, but what is exciting is to see people come into the fold with new ideas, and for the projects to actually reach a level of fulfillment," says Mayhew, who co-directs the facility with Jim Wells, PhD.

The technology uses gene-based biochemical solutions to transform human skin cells into cells capable of forming any tissue in the human body. Functionally based on the transformative powers of human embryonic stem cells (hESCs), which are derived from a fertilized egg, induced pluripotent stem cells were devised to accomplish the same goals, without the complications of hESCs.

For the purpose of regenerating tissues to be used for disease research modeling and eventual therapeutic purposes, iPSCs have the additional advantage of having the same genetics of the person who donated the cells. This means regenerated tissues should not risk rejection by that person's immune system.

"For years, people have been aware that this technology is out there and that you can do some interesting and novel studies with it," explains Mayhew. "But scientists are really starting to understand that iPSCs can be used for discovery research, and it's not just a novelty. This technology is being used to answer some very important questions, and providing new insights into the mechanisms of human disease is the next big explosion to come."

GROWTH IN SIZE AND SCOPE

As the technology of making iPSCs from skin cells becomes more refined, and as reputations and word-of-mouth grow, so does the amount of work at the facility and the types of projects it takes on. Mayhew estimates the lab's project load has quadrupled from its start four years ago.

With a staff that includes Mayhew, Wells, a full-time research assistant and a part-time assistant, the lab performs an array of very specialized and complex services. They can generate and maintain iPSC cultures for a research lab studying a specific organ or disease, or they can train the laboratory's staff to generate and maintain the cells themselves. The cells require constant and precise nurturing, Mayhew says.

It can take up to three months from the point of starting the reprogramming process to having iPSCs ready for a laboratory to start experiments. At that point, it is up the researchers receiving the iPSCs, with assistance from Mayhew and his team as needed, to turn the cells into an organ- or disease-specific model system.

In some cases, as in studies involving intestinal tissues, Mayhew's team can generate the organoids for researchers studying that organ. The lab also hopes to be able to generate other specific tissue types for researchers as those capabilities and needs develop.

Most of the lab's services are currently supplied to research labs at Cincinnati Chil-

In our stem cell laboratory, gene-based biochemical solutions transform human skin cells into induced pluripotent stem cells, capable of forming any tissue in the human body.

dren's or the University of Cincinnati. But the facility's growing reputation means its services are also being sought out by institutions from around the U.S. and as far away as Europe.

This means that, on any given day the lab might be generating iPSCs or training scientists who want to make heart cells. The day after, the focus might be on immune cells called macrophages, and the day after that the project may involve cells of the intestine or lung.

GROWING A BETTER STEM CELL

Some of the increasing acceptance of iPSCs comes with refinements in how they are generated from skin cells. When first described in 2006 by researchers in Japan, iPSCs were produced by introducing a set of genes that caused the cells to turn back their developmental clock to act like human embryonic stem cells.

Despite understandable excitement in the scientific community, the cells did come with a few challenges. Some genes in the original mixture (c-Myc in particular) were associated with causing cancer. This was not the only concern.

"The old method used viruses to deliver the genes that also jumped into the DNA of the recipient cells, and that can generate mutations and cancer," says Wells. "The method we use now is an approach called episomal reprogramming, meaning the genes don't jump into the host cell's chromosomes."

The new delivery method involves the use of plasmids containing the reprogramming genes, which enter the cells and float around. This process produces specific proteins that actually cause cellular reprogramming, avoiding insertion directly into the cells' chromosomes. "You don't have to worry about what is called insertional mutagenesis, and we have had great success with this method," Mayhew says.

Pluripotent Stem Cell Facility Supports Breakthrough Research

Gene Therapy for Pulmonary Disease

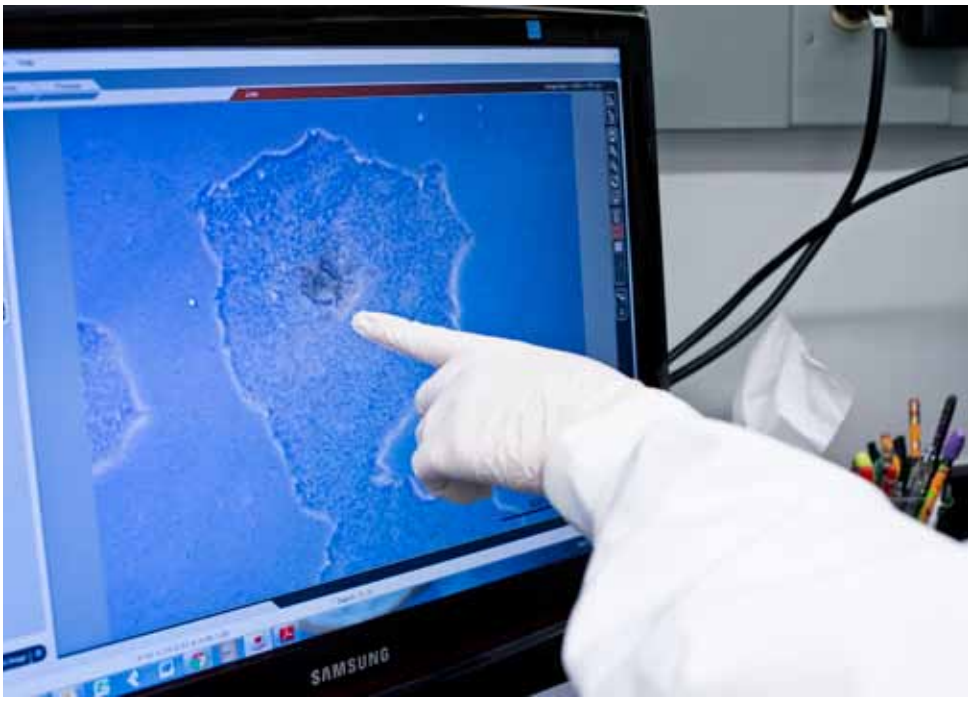
Bruce Trapnell, MD, and Takuji Suzuki, MD, PhD, Division of Pulmonary Biology, use induced pluripotent stem cells (iPSCs) to study a rare genetic disorder, pulmonary alveolar proteinosis (PAP). The lungs of children with PAP fill with surfactant, a thick fatty substance normally present in a small amount to help keep the lungs from collapsing. Current treatment is a whole-lung wash, an invasive procedure performed under general anesthesia to remove the excess surfactant.

The study used iPSCs derived directly from the cells of children with the disease. The iPSCs, which contained the mutated gene that causes PAP, were then converted into macrophages, immune system cells that become dysfunctional in PAP. Researchers analyzed the cells as they grew and differentiated into macrophages, acquiring the same PAP disease characteristics observed in patients.

The researchers then used an engineered virus to deliver a correct copy of the mutated gene to the diseased macrophages. This caused PAP disease manifestations in the cells to cease, providing a proof of principle for a potential gene therapy. The research was published earlier this year in the *American Journal of Respiratory and Critical Care Medicine*. Additional research is needed before the findings could be used clinically, but iPSC models of PAP will be central to the researchers' ongoing work.

Human embryonic stem cells are still used today, although much less than several years ago. They serve primarily as a gold standard and benchmark for measuring functional capabilities, and to make sure iPSCs are doing what they are supposed to do, according to Wells.

He expects an increasing shift toward using iPSCS to continue, especially as the technology continues to improve. ■



On-screen image shows a colony of induced pluripotent stem cells. A process called episomal reprogramming has eliminated the risk of genetic mutations associated with earlier stem cell methods.

Generating Human Intestine "Chips" to Test Therapies

Gastrointestinal disorders affect up to a fourth of the U.S. population. Many of these disorders involve a lack of spontaneous muscle contractions that help food and waste get through the body's digestive tract. There are few treatments — especially drugs — that target these ailments.

The laboratories of Jim Wells, PhD, and Samantha Brugmann, PhD, are using patient-specific iPSCs to generate human intestinal organoids with functioning epithelium. Wells' lab has already produced intestinal tissue, but this project aims to generate intestine with enteric nerves. The goal is to produce a system on a 3D chip with living cells and tissues to model functioning human intestine. The chip will be used to test prospective drugs for toxicity and safety.

Wells and Brugmann were selected in 2012 to be part of a 17-grant, multi-center consortium formed by the National Institutes of Health's National Center for Advancing Translational Sciences. The consortium's goal is to provide human tissue-based testing platforms to predict new drug safety in a faster, more cost-effective way.

New Clues to Understanding Genetic Diabetes

Researchers have long suspected that the gene neurogenin-3 might be a key to generating human pancreatic beta cells *in vitro*, and that its mutation may drive a genetic form of diabetes. Beta cells are vital to regulating blood glucose and preventing the disease. But some people born with mutations in the neurogenin-3 gene do not have diabetes, although they suffer from intractable diarrhea.

This sent researchers back to their labs. Doubt set in about whether mouse studies in this instance translated well to humans. But there was no way to study beta cell development in people.

Wells and Sean McGrath, a graduate student in Developmental Biology, decided to use iPSCs to model the human condition. They produced human PSCs in a petri dish and tested to see if neurogenin-3 is important for making beta cells.

So far, testing suggests that expression variance in mutated neurogenin-3 makes it possible for just enough gene function to produce beta cells, but not enough to manufacture intestinal cells critical for food absorption and preventing diarrhea. Although additional research on the findings is needed, Wells expects the study will demonstrate that complex human diseases can be effectively studied "in a dish." He believes the approach will increasingly replace the need to use animals in research.

Nature's Right-Sizing

Researchers explore what makes us the size we are

by Nick Miller

Dr. Jun Ma examines the role of Bicoid protein in controlling development.

That living beings come in all shapes and sizes is one of life's great mysteries to scientist Jun Ma, PhD. It is also the focus of his life's work.

The developmental biologist and his colleagues want to learn how — even in the environment of a frantic developmental process — embryos of different sizes manage to develop proportionally-sized body parts.

Ma's team focuses largely on probing the role of a protein called Bicoid, and analyzing its target genes in early embryos. This allows the researchers to identify the genes and molecular pathways involved with Bicoid in normal embryonic development. The team studies the various genes' expression levels, functionality and timing as they biochemically guide fruit fly embryos to form a normal developmental axis from head to tail.

Although fruit flies have miniscule brains and dine on rotten fruit, the insects are a valuable tool for studying clues about mammalian and human development. Through evolutionary conservation, many critical genes in fruit fly development are similar to genes in people.

Ma and his collaborators released two studies in the past year, published in *Nature Communications* and *Development,* that offer new insights on this developmental process. The *Development* study used female fruit flies selected to lay large or small eggs to show how the shape of the Bicoid protein gradient in the embryo adapts to its size. The *Nature Communications* study showed how Bicoid's potency is regulated to control development.

"Traditionally, most studies investigate the importance of where a gene is expressed in controlling the outcome of a developmental process, as does our study in *Development*," explains Ma, a member of the Divisions of Bioinformatics and Developmental Biology. "The *Nature Communications* paper reveals a new wrinkle. It shows that the final developmental outcome is also sensitive to how much a gene product is expressed, as a result of how long the gene is kept on by the Bicoid protein."

At their core, Ma says the studies help connect the concepts of time and space in explaining normal developmental patterns. The amount and distribution of the maternally-provided Bicoid protein give the embryo a first sense of its own size through activating a gene near mid-embryo. The length of time Bicoid activates this target gene determines how much gene product is made. Subsequently, this affects how body parts are further divided along the axis.

If the process goes well, then developmental patterning and the proportional sizes of body parts should be normal. If not, nature could take some interesting twists and turns.

Many questions remain about how embryos of different sizes have the precise gene expression, timing and spacing they need to grow proportionally-sized parts. But in the process of one discovery leading to the next, Ma's recent findings provide important clues.

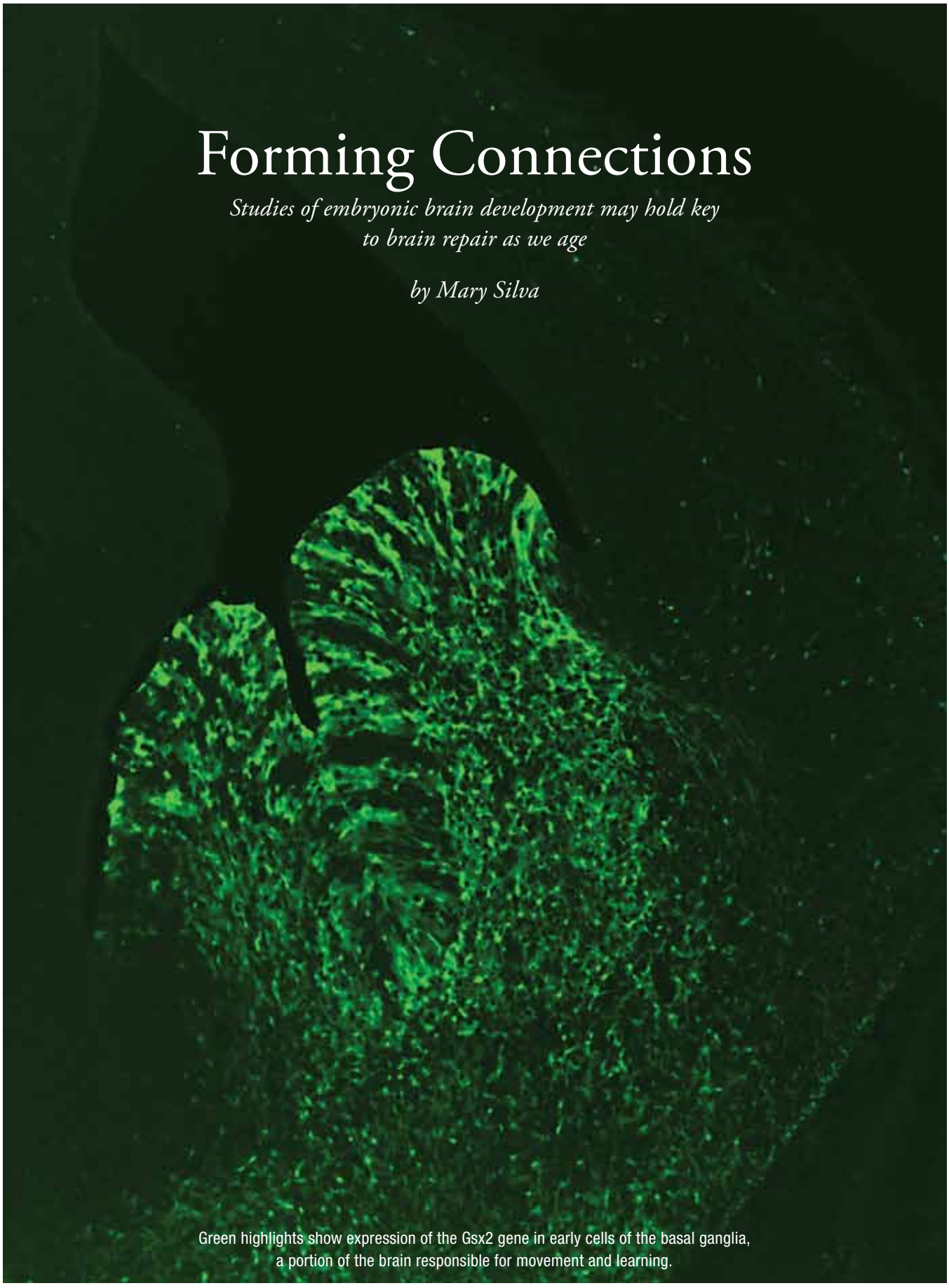
"Although this would still need to be validated, if a normal developmental process depends on biochemical reactions that need to take place correctly in space and time," he says, "this could help explain why different stages of a pregnancy may be sensitive to perturbations that can lead to defects." ■

Research Associate Dr. Junbo Liu was first author on a *Nature Communications* article that links developmental outcome to the amount of gene expressed.

Forming Connections

Studies of embryonic brain development may hold key to brain repair as we age

by Mary Silva



Green highlights show expression of the Gsx2 gene in early cells of the basal ganglia, a portion of the brain responsible for movement and learning.

Researchers at Cincinnati Children's have revealed that the developing brain of an embryo and the brain of an adult have much in common.

Developmental neurobiologists Kenneth Campbell, PhD, and Masato Nakafuku, MD, PhD, in the Division of Developmental Biology, discovered this while studying neurogenesis — the development of new neurons — at different stages of life. Campbell is focused on embryonic brain development; Nakafuku studies the adult brain as it ages.

The researchers have joined forces to find clues in early brain development that could help repair and restore function as the brain ages. Their findings could be helpful in understanding and even treating developmental diseases such as autism and ADHD, neurodegenerative diseases like Alzheimer's and Huntington's, and acute brain injury like stroke and trauma.

A number of these disorders are believed to stem from altered function of the basal ganglia, a group of nuclei located deep in the brain beneath the cerebral cortex. Basal ganglia control movement and play a role in how we learn and how we form habits, both good and bad.

Campbell and his team focus on how the fate of neurons is determined as well as how neural circuitry develops in the basal ganglia. "If you believe that alterations in the establishment of the circuitry could underlie neuropsychiatric disorders, then you have multiple points in the process where changes could occur," he says.

With a five-year, $3.3 million renewal of an NIH grant now entering its 12th year of

From left: Drs. Masato Nakafuku, Kenneth Campbell and Brian Gebelein

funding, Campbell and co-principal investigator Brian Gebelein, PhD, a molecular biologist in the Division of Developmental Biology, are studying the roles of Gsx1 and Gsx2, two genes expressed only in neural progenitor cells. Neural progenitors typically give rise to both types of brain cells, neurons and glia. But progenitors that express the Gsx factors appear to favor the creation of neurons rather than glial cells.

TWO GENES, TWO ROLES, MANY QUESTIONS

Campbell studies how Gsx1 and 2 control neurogenesis in basal ganglia progenitors. He believes that the Gsx proteins play different but complementary roles in this process.

"Gsx2 appears to keep neural progenitors in an undifferentiated state but 'poised' to become neuronal cells. On the other hand, Gsx1 seems to promote progenitor maturation toward neuronal differentiation," Campbell says. But questions remain about what prompts the genes to do what they do. "We've spent years using genetics and phenotyping to describe their roles, but that has left a hole in our understanding about how these factors work on the molecular level."

Gebelein's research helps fill that molecular gap. He has particular expertise in studying cis-regulatory elements, sites within the DNA that control gene expression. He and Campbell have already identified several of the cis-regulatory elements for Gsx1 and 2, and hope to learn more about the signaling pathways and transcription factors that control their expression.

Using mouse and fly models, they have explored an activity called phosphorylation in the Gsx proteins. Phosphorylation causes changes in a protein's shape and activity, altering its behavior — for example, whether it enters the cell nucleus or remains in the cytoplasm. Campbell and Gebelein are examining mitogen-activated protein (MAP) kinase phosphoryation as well as the interaction of other transcription factors to understand how they influence Gsx proteins in neurogenesis.

COMBINED EXPERTISE YIELDS BETTER RESULTS

Research grants that combine the expertise of multiple principal investigators on research studies is a new direction in funding for the NIH Research Project Grant and a boon to discovery in basic science, Gebelein says.

"Allowing researchers with different areas of expertise to tackle a developmental question is crucial to moving research forward," Gebelein says. "In this instance, we have been

able to demonstrate similar results in different genetic model systems, which lets us know more quickly that we are heading in the right direction."

CONNECTING TO THE ADULT BRAIN

The right direction is important, because the Gsx protein is also found in the adult brain, where it plays a key role in repairing neuron damage. Developmental biologist Masato Nakafuku, MD, PhD, is a co-investigator with Campbell on another multiple-PI study exploring the role of Gsx1 and Gsx2 in the adult brain.

"Stem cells in embryos and adult brains are similar in that they proliferate and differentiate in the same way," Nakafuku says. "But in embryos, every stem cell divides rapidly and creates new cells. In the adult, only a very small proportion of them do that. Most sit there and do nothing. Why is that?"

At about age 11 or 12, Nakafuku says, human brains slow their active regeneration of new brain cells to a trickle. Our brains retain a pool of stem cells that keep producing a small number of new neurons and glia for "plastic" activities in the brain — activities such as learning and remembering new things. But the vast majority of stem cells are kept as a reservoir presumably for repair purposes, much like other animals. When there is injury to the brain, the Gsx2 gene activates and attempts to recruit those stem cells for repair, Nakafuku says. But nothing much happens.

"In adults, you have the same set of genes at work, but at a time when it is critical to get more cells, the genes counteract each other. When the stem cell needs to decide whether to go back to quiescence or to make more cells, Gsx2 keeps it in check and the cells go back (to quiescence)."

NATURE'S RESTRICTION

Nakafuku proposes that this is likely the result of a built-in regulatory mechanism that controls how many new brain cells an adult brain can generate. Making too many new brain cells could cause problems with brain function, he says. But he would like to know how to call a reasonable supply of cells into action when there is significant injury to the brain.

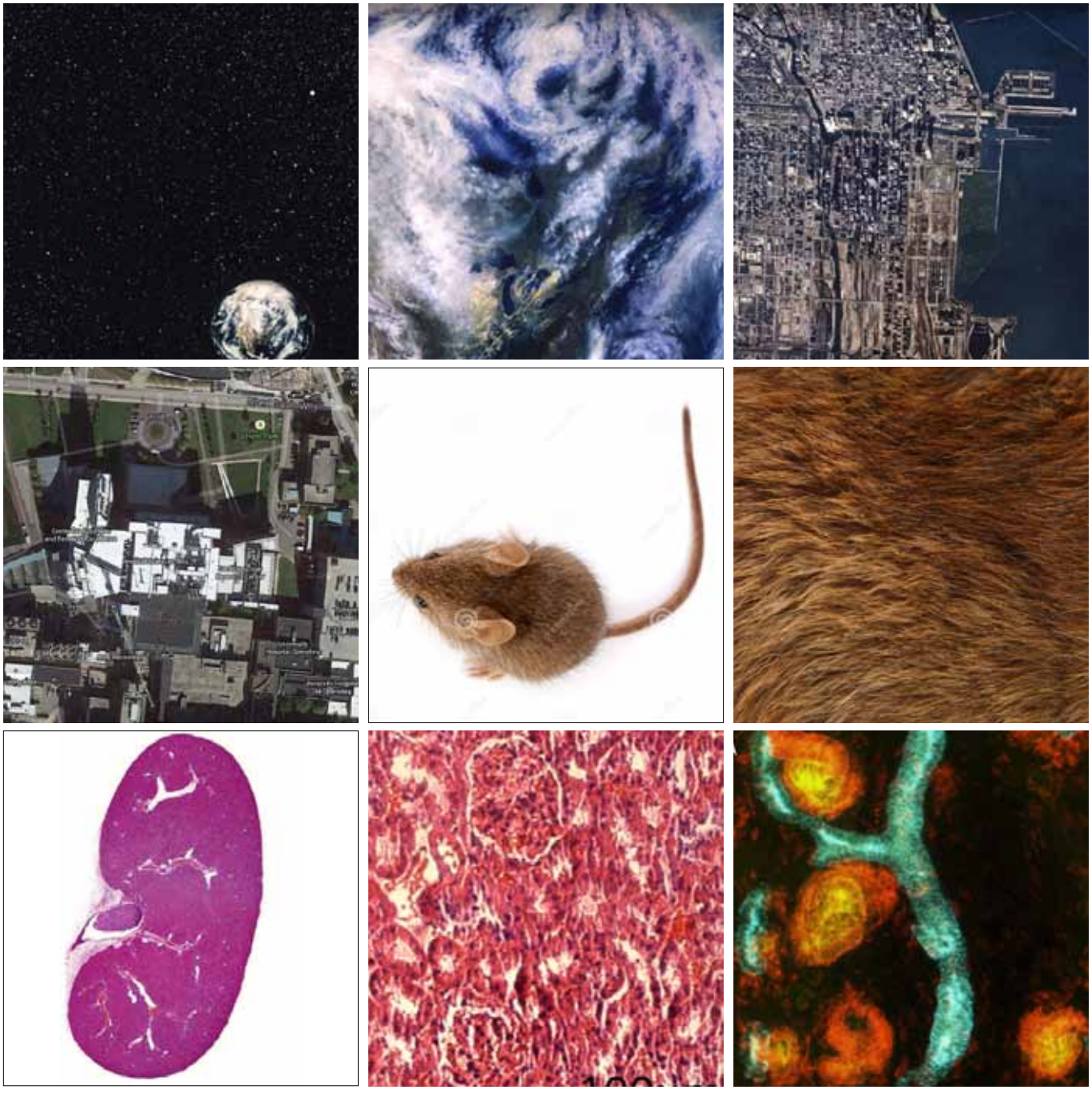
"We need to understand how to manipulate the process," he says. "There must be a reason for this stopping mechanism. But we need a broader view of other genes and how they interact with the Gsx factors so that we can draw on these cells when damage occurs." ■

Images below, second and third from left: Drs. Gebelein and Nakafuku; Drs. Campbell and Gebelein with postdoctoral fellow Kaushik Roychoudhury.

Zooming in on Kidney Development

Progress on a genetic atlas of kidney development shifts to single-cell studies and the tipping points that move cells along their earliest trajectories

by Sarah Stankorb



"There are many cases where if you study populations of cells, chunks of tissue, you don't really get the complete picture," says Steven Potter, PhD, a researcher in Cincinnati Children's Division of Developmental Biology.

Potter likes to get the complete picture. In late 2008, he published the world's first virtual map of murine kidney development, which he continues to improve upon to this day. Collaborating with Bruce Aronow, PhD, Division of Biomedical Informatics, Potter and a team of scientists defined the activity of nearly the entire kidney genome during normal development. It was a one-of-a-kind atlas, groundbreaking work. But it relied upon the law of averages, pooling data from groups of cells to provide an overview for that cell type. Potter's recent work is like moving from a standard Google Map to street view, then looking inside houses.

He is observing the smallest living unit of biology — the living cell — as it becomes differentiated.

For the initial atlas, Potter's lab used laser capture microdissection fluorescence-activated cell sorting and microarrays and RNA sequencing to define gene expression patterns. Put simply, Potter explains, "You have 1,000 cells in a pot, and you study their gene expression patterns. You're getting an average picture for those thousand cells that you're looking at." If you look at those ensemble averages or population pools, says Potter, "you miss the fine resolution differences."

SEEING THE FINE POINTS

Thanks to an investment of $150,000 in a Fluidigm C1 System, researchers can now isolate and reliably process individual cells for genomic analysis. Potter's lab uses enzymes to chew apart the connections that bind groups of cells and isolate them in single cell suspensions. From there, a series of biochemical reactions allow researchers to see how each cell is expressing each of its genes. What they have found represents a paradigm shift in the basic model of kidney development.

If you take a snapshot of embryonic kidney development, clusters of epithelial cells begin looking pretty much alike, but within a day's time, they follow different developmental pathways. "They're going different directions, and that's already happening, right here," says Potter, pointing at a slide of cells. A gene expression study of that collective population of epithelial cells misses the individual story happening within each cell — what directs one cell to become a distal tubule cell and another to become a podocyte of the glomerulus.

There are two competing models of development. One, the blank slate option, imagines a single cell expressing very little, "and then it starts to turn on a few genes that reflect its developmental destiny, where it's going to go," Potter describes. This is the model that many accept, but Potter's lab has found that some-

Thanks to single-cell studies, Dr. Steven Potter and his team have observed how early kidney cells decide their own fate based on gene expression.

thing else altogether is happening in the kidney.

It is not solely a matter of switching on the right genes.

When Potter's research team profiled 58 renal vesicle cells, they found that many cells were expressing genes associated with multiple cell types in the nephron. Potter explains that in a manner of speaking, "these cells haven't made up their minds yet. And not only have they not made up their minds—but they are seriously thinking about doing more than one thing." This is known as "multilineage priming."

As development progresses and a cell becomes a podocyte cell, for example, more genes associated with podocyte development are turned on, and genes associated with other cell types are repressed. It's a matter of switching on the right genes and dialing back others.

"We have very strong evidence from our single cell data that multilineage priming is the model that drives the progression of stem cells into adult differentiated cells," says Potter.

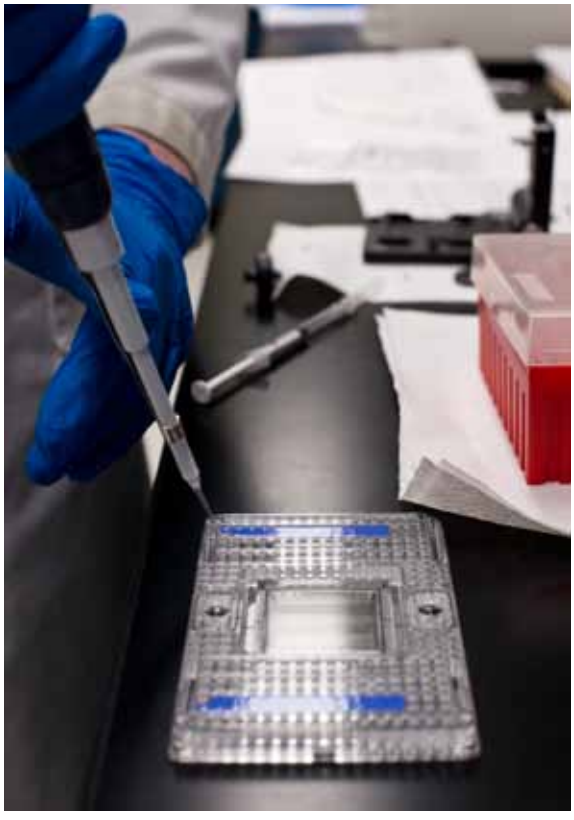
GETTING TO THE CAUSE

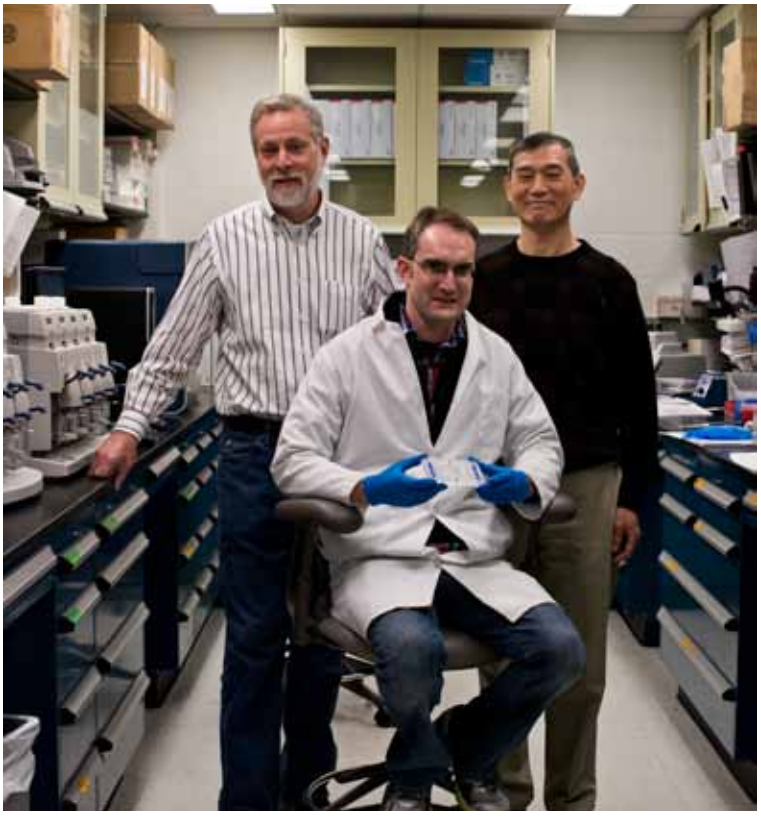
It remains unknown what triggers repression and activation of various gene expressions. Potter speculates that cells could be experiencing different growth factor environments, different cues from their environments, or signals that are directing them down different pathways. The challenge remains for working out the details of how that differentiation is accomplished for even one cell type, much less all the cell types within the kidney.

According to the National Kidney Foundation, some abnormality occurs in the development of the kidney or urinary tract in about one in 500 live births. But with so many development-related diseases of the kidney — ranging from renal aplasia to horseshoe kidneys — establishing how cells develop is crucial. "As we learn more about the genetic circuitry of how you make a kidney, we'll better understand the underpinnings of those diseases," says Potter. This knowledge, combined with the revolution in DNA sequencing technology, he adds, "will also help us better diagnose those diseases."

Potter's kidney research is part of a National Institute of Child Health and Human Development–funded consortium, the GenitoUrinary Development Molecular Anatomy Project (GUDMAP). His original kidney atlas was part of this broader investigation, which continues creating a genetic map of the kidneys, bladder and reproductive organs.

Potter is also collaborating on two earli-

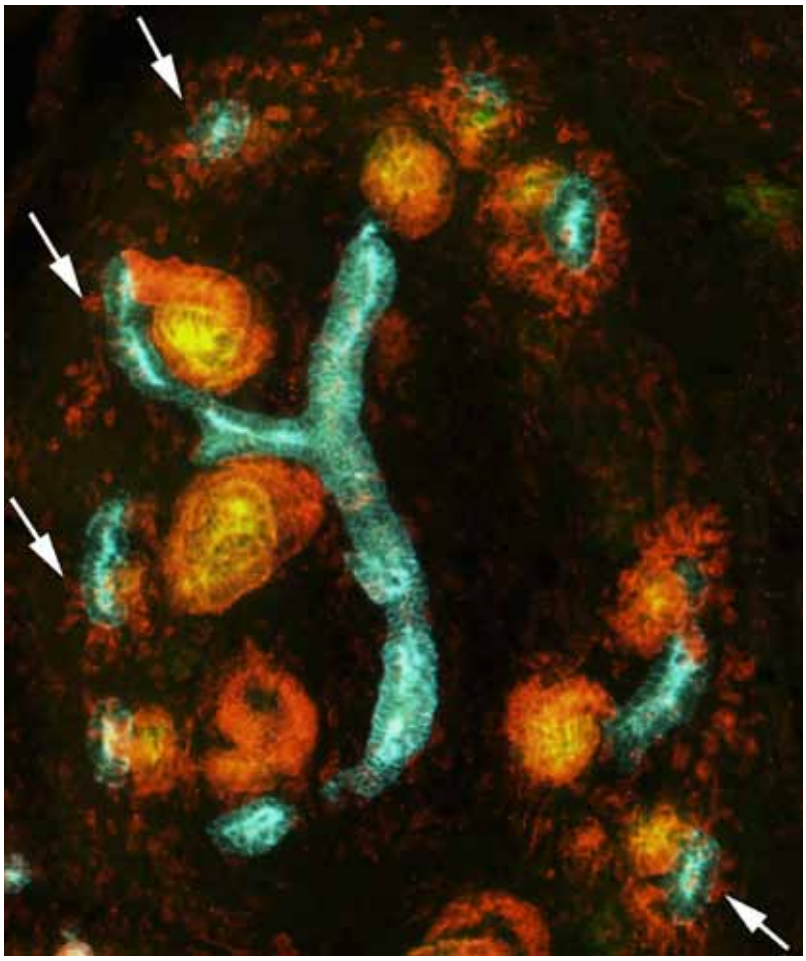




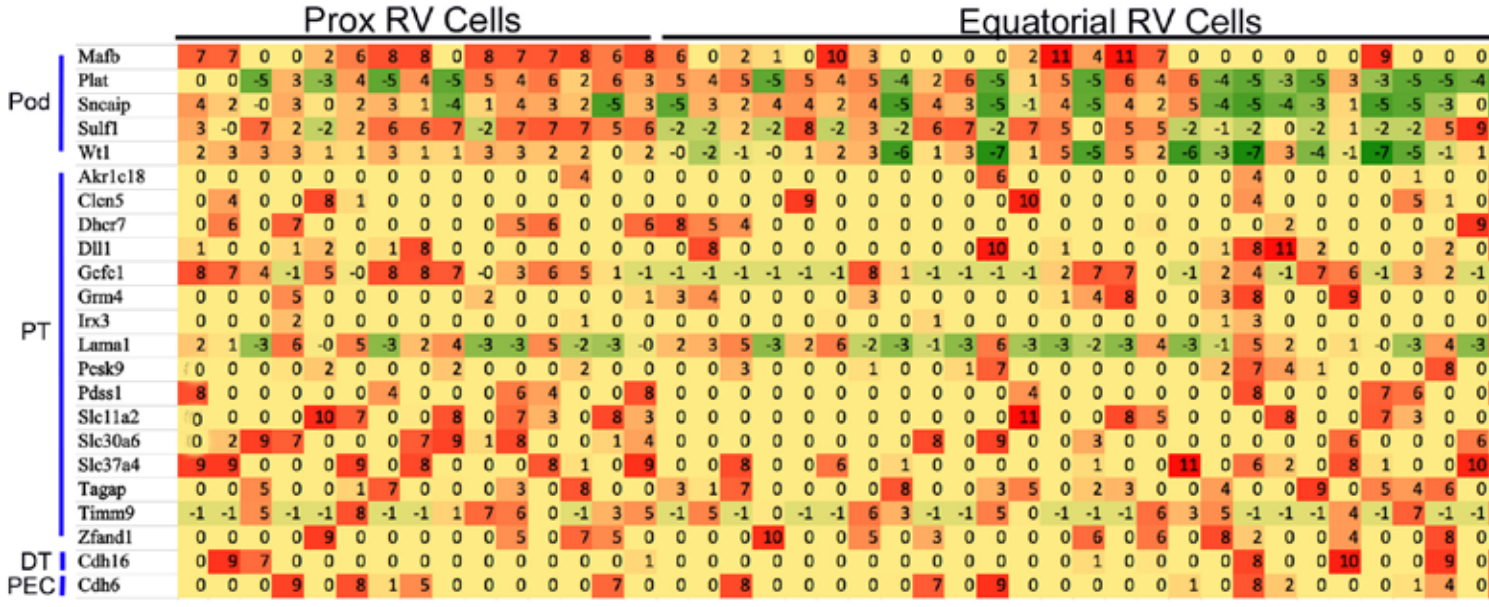
Dr. Steven Potter, research assistant Shawn Smith and Dr. Hung Chi Liang in the Gene Expression Core Laboratory, where they isolate individual cells to study kidney, lung and facial development.

er-stage studies, including the NIH-funded FaceBase, a scientific database that is compiling biological instructions to construct the middle region of the human face. He aims to use single-cell studies to define the genetics underlying normal and abnormal craniofacial development. For the similarly NIH-funded LungMap, Potter is working with Jeffrey Whitsett, MD, in Neonatology, Perinatal and Pulmonary Biology to begin developing a single-cell molecular anatomy for the developing lung.

The work has broad implications. Potter notes that in single-cell studies, "once you get it, you can just apply it to anything… heart, lung, kidney, spleen, pancreas." As his kidney investigation deepens and his team digs into studies of the gut, lungs and face, Potter's lab seeks to trace the lineage of cells from the moment they differentiate and observe these basic units of life as they transform. ■



In this image of a developing nephron, the blue cells are forming the collecting ducts, the orange are making nephrons. Arrows indicate cells that are progenitors of the nephrons and are expressing a Wnt4 gene not previously thought to be in those cells.



Technology now allows researchers to isolate and process individual cells for genomic analysis. This gridlike printout of single nephrons shows scientists how each cell expresses each of its genes. In this case of single nephrons, most cells express a marker of more than one lineage, suggesting that the cells are still "thinking" about becoming more than one thing.

Tracing Our Shifting Blueprint

Tadpoles help scientists unravel how progenitor cells do their jobs during organ development

by Tim Bonfield

DNA may be known as the blueprint of the human body, but during the many stages of prenatal development that blueprint is better understood as a full-blown book with pages turning every four or five hours.

At any given moment, only parts of the instruction manual are being used. And some parts get used over and over in combinations that scientists are just beginning to understand.

Aaron Zorn, PhD, a researcher with the Cincinnati Children's Division of Developmental Biology, works with this ever-shifting blueprint every day as he studies the progenitor cells that form the lung, liver, pancreas and gastrointestinal tract.

"It turns out that the liver, pancreas and lung originate from a common set of progenitors in the early embryo," Zorn says. "But how do these cells learn where they are supposed to go? It is clear that many, many decisions are being made right from the beginning of embryogenesis."

WHEN CELLS KNOW THEIR PLACE

Much like the real estate business, the ability of progenitor cells to form different types of tissue depends heavily upon location, location, location. For breathing to work, developing lungs must be connected to the surrounding vasculature. For digestion to work, the liver and pancreas have to interact properly with the intestines.

"The cell must know exactly where it is in order to coordinate its development with other tissues," Zorn says. "We know that cells talk to each other. We also know many of the genes, growth factors and transcription factors involved in that communication. What we still do not understand is, how this is all coordinated?"

Tiny differences in location can lead to huge differences in the fate of a progenitor cell. For example, at the equivalent of 28 days human gestation, a frog embryo has developed a simple gut tube made from nearly identical progenitor cells. Then some of these cells receive signals that tell them to become intestine — but not foregut, which later gives rise to the liver, pancreas and lung. Twelve hours later, those same signals instruct a subset of the foregut cells to become lung rather than pancreas or liver.

"That's an amazing thing. We are still trying to understand how is it that the same signals can produce dramatically different impacts in the space of just hours or days," Zorn says.

Finding the patterns within this constantly shifting blueprint requires skill, data and computing power. It also requires lots of experimentation, which is what makes frogs so important to developmental biology.

THE POWER OF XENOPUS

Zorn has helped Cincinnati Children's build a colony of about 1,000 West African frogs (*Xenopus tropicalis* and *Xenopus laevis*). These frogs produce hundreds of thousands of tadpoles a year for use in a wide variety of research projects. Zorn also is co-director of Xenbase, a huge multicenter online library of genomic data about African frogs and their use as models of human development and disease. This database was cited in nearly 900 peer-reviewed papers published in 2013.

Frogs are especially useful in developmental biology studies because females produce thousands of eggs the day after being stimulated with an injected hormone. Once fertilized in the lab, tadpoles develop quickly. "The first indications of lung development occur at about two days in frogs," Zorn says. "In mice, it takes nine to 10 days and 28 days in humans."

Not only do tadpoles grow faster and in far greater numbers than mouse embryos, they also can develop in a petri dish. "It can be diffi-

Frogs are valuable in research because fast-growing tadpoles can be observed at every stage, says Dr. Aaron Zorn.

cult to observe the dynamic processes of the first trimester *in utero*," Zorn says. "But with frogs, you can observe all of it in the dish. This allows you to ask more kinds of questions."

For example, the lining of the lung comes from embryonic tissue called endoderm while the connecting vasculature comes from nearby tissue called mesenchyme, but how the development of these two tissues are coordinated was poorly understood. By performing delicate microsurgery on tadpoles, Zorn and colleagues extracted both types of tissue and found that crucial signaling to begin lung development actually comes from the adjacent mesenchyme.

stem cells (iPSCs) to grow organs. In recent years, Zorn has worked closely with James Wells, PhD, Director of the Pluripotent Stem Cell Center at Cincinnati Children's. Wells' team has had significant success in growing functional intestinal and stomach tissue from stem cells. (See related story, page 20) More recently, the two have teamed up with John Shannon, PhD, and Jeffrey Whitsett, MD, in the Division of Pulmonary Biology at Cincinnati Children's, to work on lung development.

"Compared to other organs, we have not had good models for studying very early stages of lung formation," Zorn says. "Our understanding of the early stages of lung development has been murky. Now, we have about half our lab working on this."

"We still have a great deal of work to do understand these gene regulatory networks," Zorn says. "But as we do, there will be many applications."

Such findings will help clinicians better understand why human development can sometimes go awry.

"Most principles of organ development are the same in fish, frogs, mice, and ultimately humans," Zorn says. "In frog embryos we've been able to work out that this communication between cells during development actually uses a rather small handful of growth factors. They are used over and over in different combinations with dramatically different roles at different times."

TO GROW A LUNG

This information also helps inform efforts to use induced pluripotent

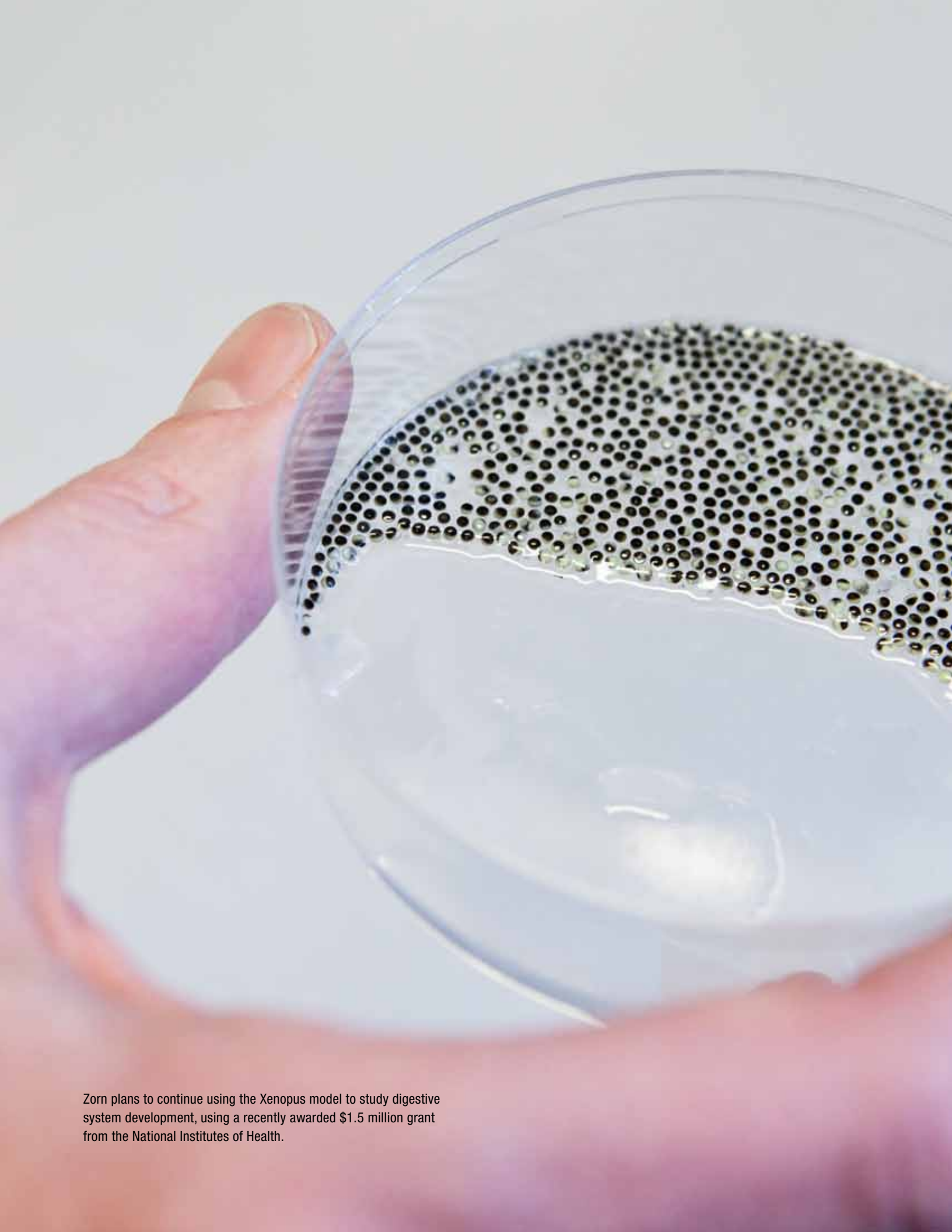
The research team already has applied some of the signaling information learned from studying frogs to human stem cell cultures. This has helped them grow spheroids of lung progenitor cells that include both mesenchyme and endoderm.

"This is very exciting because it more closely mimics the natural process of lung development than previous attempts," Zorn says. ■

INSIGHTS WITH POSSIBILITIES

These fundamental research questions about early development are not as distant as they seem from day-to-day medical practice. Doctors at Cincinnati Children's often care for the 3 to 4 percent of babies born each year in the U.S. with major organ defects. Understanding how organs form helps reveal how and why the process can go wrong, and how it can be corrected, says Aaron Zorn, PhD.

- Even if it takes years to figure out how to grow full-sized replacement organs, the ability to use stem cells to grow small amounts of organ tissue opens the door to vastly improved drug screening.
- As research reveals gene mutations that disrupt development, gene testing could inform would-be parents if they are carriers.
- Research already shows that healthy prenatal development can be disrupted by environmental conditions, such as alcohol exposure, vitamin deficiencies or high cholesterol. Identifying the timing of key organ development signals may reveal particularly sensitive times during pregnancy where a lifestyle adjustment or a preventive treatment could save a life.
- Improved testing could lead to earlier detection of major-but-survivable defects. This in turn could help guide expectant mothers to better-equipped hospitals and the best available surgeons.
- Identifying the pathways used during embryonic development also could reveal how organ repair and regeneration works later in life — and suggest ways to speed up or amplify the process.



Zorn plans to continue using the Xenopus model to study digestive system development, using a recently awarded $1.5 million grant from the National Institutes of Health.

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