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CCR connections

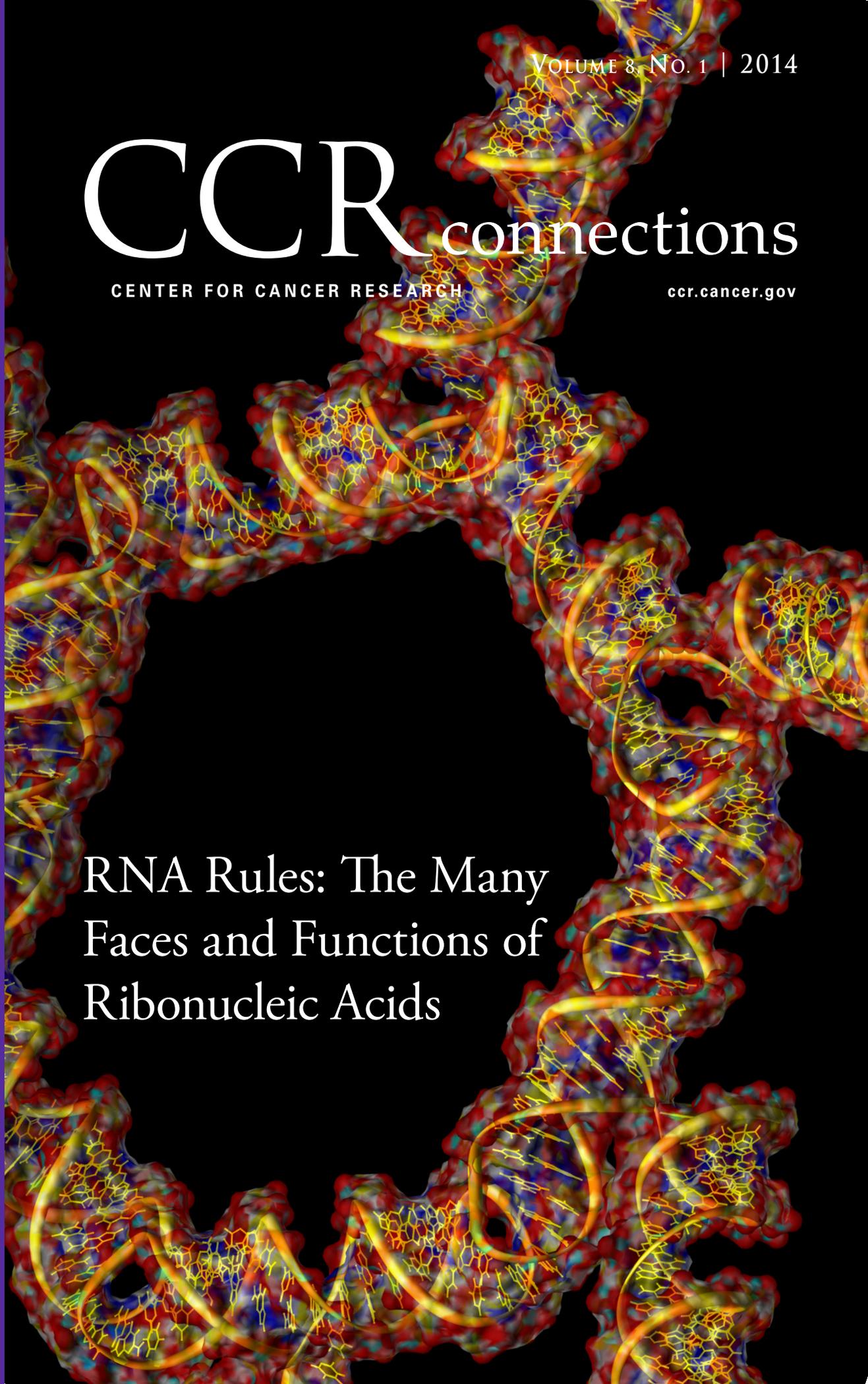
CENTER FOR CANCER RESEARCH

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RNA Rules: The Many Faces and Functions of Ribonucleic Acids

U.S. DEPARTMENT
OF HEALTH AND
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Center for Cancer Research

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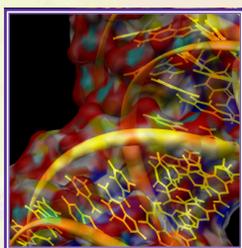
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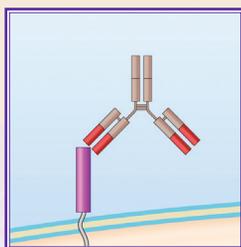
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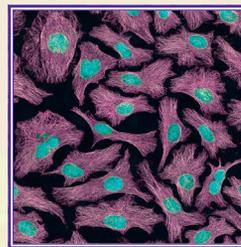
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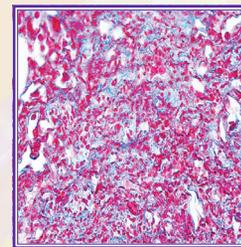
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The mission of CCR is:

To inform and empower the entire cancer research community by making breakthrough discoveries in basic and clinical cancer research and by developing them into novel therapeutic interventions for adults and children afflicted with cancer or infected with HIV.

<http://home.ccr.cancer.gov/connections>

The Long View

Many CCR Investigators see patients regularly as part of their clinical research programs. As Steven Rosenberg, M.D., Ph.D., points out in “Adopting Bodily Defenses to Cure Cancer,” in this issue of *CCR connections*, our patients are often here because they have no other options and their prognosis is far from good. So the urgency to translate research into improved patient care is always a priority.

As scientists, however, we also understand that strong research foundations are required to truly break new therapeutic ground. As a part of the NCI Intramural Research Program, our mission includes the goal of mapping uncharted scientific territories and developing important research questions that may require extended time for exploration.

Many of the investigators featured in this magazine, including Rosenberg, have been doggedly pursuing lines of research that have garnered increasing attention from the scientific community. As we learn in “Precision in Targeting with Anti-Mesothelin Therapies,” Raffit Hassan, M.D., has built a series of clinical trials upon the work that he and Ira Pastan, M.D., have done to create a new class of immunotoxins.

Meanwhile, Sanford Markowitz, M.D., Ph.D., took what he learned about solid tumors during his NCI Fellowship many years ago to Case Western University; there, he built a nationally recognized program to study the fundamental mechanisms of colon cancer, which he describes in “Gut Check: A Career in Colon Cancer Research.”

Shioko Kimura, Ph.D., began studying the molecules that regulate thyroid hormone production in the 1990s and discovered a transcriptional master regulator in the lungs, thyroid, and ventral forebrain. As described in “Influence of a Master,” the mouse models she has created to study this developmental system have generated many fruitful collaborations and new directions for her own cancer research.

Kimura is not alone in sharing the fruits of her labors; CCR investigators have invested in resources from cells to tissue microarrays and databases, some of which are highlighted in “Tools of the Trade.” For instance, Michael Gottesman’s multidrug-resistant cell lines are used throughout the world for cancer research and drug development.

Bruce Shapiro, Ph.D., remembers a time when RNA was considered rather a boring molecule. Now, as recounted in “RNA Rules: The Many Faces and Functions of Ribonucleic Acids,” he and his CCR colleagues who are studying RNA biology are part of an NCI-wide RNA initiative to share resources and knowledge in this fast moving field. Shapiro is using his carefully cultivated understanding of the basic structural features of RNA to create entirely new classes of nanoparticles as therapeutics.

Javed Khan, M.D., and his Clinical Fellow, Jack Shern, M.D., have taken on the challenge of providing a complete genomic survey of rare pediatric cancers, results from which were recently published in *Cancer*



(Photo: B. Branson)

Robert Wilttrout, Ph.D.



(Photo: R. Baer)

Lee Helman, M.D.

Discovery and highlighted here in “Seeing the Forest and the Trees.” They hope that this work will lead to new precision cancer therapies. As Shern concludes in this issue’s “In Conversation” article, the only way to make real progress in science is to be persistent.

Robert Wilttrout, Ph.D.

Director
Center for Cancer Research

Lee Helman, M.D.

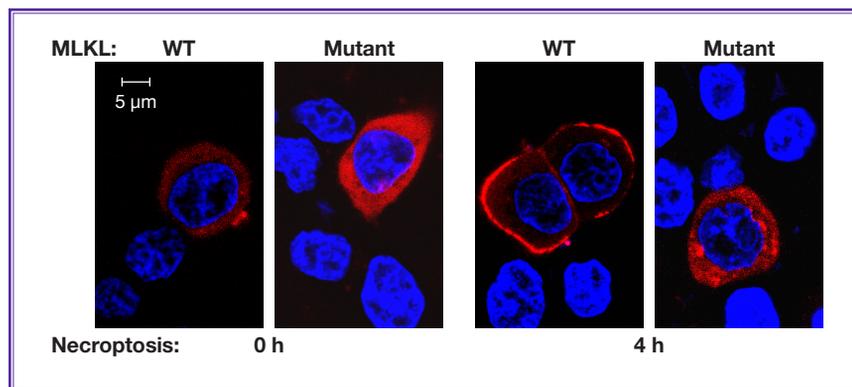
Scientific Director for Clinical Research
Center for Cancer Research

Spying on Cellular Suicide

Short-circuiting a recently discovered form of programmed cell death could lead to new cancer treatment strategies.

The underworld of cell death was once divided into the molecularly programmed suicide of apoptosis and the messier, undirected disruption of necrosis. Necrosis leads to rupture of the plasma membrane, releasing cellular contents into the tissue milieu. As with many morphological descriptions placed under modern molecular scrutiny, however, necrosis turns out to have more nuanced underpinnings than previously suspected. In particular, scientists have discovered a form of programmed necrosis—now called “necroptosis”—that relies on a macromolecular complex containing receptor-interacting protein 3 (RIP3) and is characterized by a rise in intracellular Ca^{2+} , generation of reactive oxygen species, intracellular acidity, and depletion of ATP. Because necroptosis exposes intracellular antigens to immunological attack, it may have evolved as a mechanism to fight viral infections. However, this newly identified form of cell death has also been found to play a role in a wide variety of diseases from neurodegeneration to cancer, and, as such, represents a novel target pathway for therapeutic intervention.

In 2012, Zheng-Gang Liu, Ph.D., Senior Investigator in CCR’s Laboratory of Genitourinary Cancer Pathogenesis, and his colleagues discovered a key protein involved in necroptosis called mixed lineage kinase domain-like protein (MLKL). More recently, in an issue of *Nature Cell Biology*, Liu’s team tracked the movements of MLKL throughout the cell and discovered that the molecule initiates



Cells in which the gene for MLKL is silenced were imaged with confocal microscopy immediately after and 4 hours after the induction of necroptosis. Cells transfected with wild-type MLKL bound to a fluorescent tag (red) showed more localization of the protein to the cell’s plasma membrane, away from the cytosol (blue clumps), than cells transfected with a mutant form of MLKL.

necroptosis by triggering the influx of Ca^{2+} .

Liu’s team found that MLKL is recruited into the necrosome upon phosphorylation by RIP3. Using imaging to visualize fluorescently labeled MLKL and RIP3 in human embryonic kidney cells further revealed that MLKL escorts RIP3 and the rest of the necrosome to the plasma membrane, a journey found to be critical for necroptosis.

Knowing that Ca^{2+} influx is a characteristic of necroptosis, the researchers explored whether the MLKL that reaches the plasma membrane plays a role in facilitating the ion’s movement into the cell. Short hairpin RNA-silencing of the gene that encodes MLKL resulted in complete blockage of Ca^{2+} influx, which was restored when MLKL expression was induced. Next, the team discovered that blockers of non-voltage-sensitive ion channels spared cells from necroptosis. In a knockdown experiment in which a genetically encoded fluorescent

Ca^{2+} indicator tracked the ion’s movement, the team found that TRPM7, a non-voltage-sensitive ion channel previously implicated in necroptosis, was the channel activated by MLKL.

Taken together, this work identifies MLKL as a key component in the initiation of necroptosis, making the molecule a potential novel drug target for inflammation-related cancers. Kick-starting necroptosis can also be an effective weapon against cancer. “It has been reported that many anticancer drugs trigger necroptosis in cancer cells,” said Liu. “Therefore, the induction of necroptosis in cancer is also a promising strategy for potential cancer therapy, particularly in apoptosis-resistant cancer cells.”

To learn more about Dr. Liu’s research, please visit his CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=zgliu>.

Interspecies Cooperation to Fight Cancer

Microbes in the gut may be helping a variety of cancer therapies.

Bacteria were here first, colonizing everything in sight with a two billion-year head start over eukaryotes. In hindsight, it is not surprising, therefore, that bacteria would colonize us, too, as we evolved on the planet. But we have only recently become aware that we share our bodies with a microbial population that outnumbers our own cells by a factor of 10.

We are beginning to understand that our immune system is profoundly shaped by these unseen fellow travelers. Microbes in the gut, which include bacteria, archaea, fungi, viruses, protozoans, and even multicellular helminthes, affect inflammation and immunity systemically as well as locally, leading Giorgio Trinchieri, M.D., Chief of CCR's Laboratory of Experimental Immunology, to wonder whether they might affect inflammatory processes associated with cancer and its therapy.

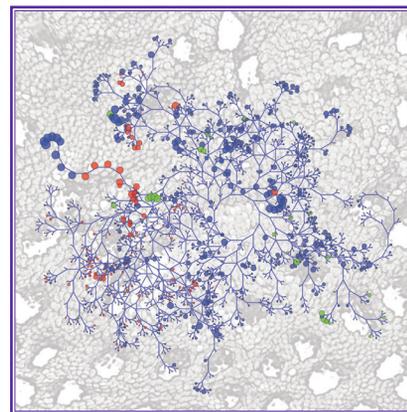
To model this question, Trinchieri and Staff Scientist Romina Goldszmid, Ph.D., led their colleagues in a study of three different cancer types—lymphoma, colon, and melanoma—that could be injected under the skin of mice. The cell lines were chosen for their sensitivity to specific therapies. Preceding the tumor initiation, mice were either treated with a cocktail of antibiotics (vancomycin, imipenem, and neomycin in drinking water) to deplete their gut microbes or they were raised from birth in a germ-free environment. Then, once the tumors reached a certain size, the mice were challenged with an immunotherapy regime (intratumoral injection of

CpG oligonucleotide, a TLR9 ligand, and systemic anti-interleukin-10 receptor) or a conventional platinum-based chemotherapy.

Regardless of the tumor type or treatment examined, mice depleted of their gut microbes responded less well to therapy than controls, as measured by reduction in tumor volume and long-term survival. Moreover, the response of myeloid-derived cells that normally infiltrate the tumors after therapy was reduced. Mice treated with immunotherapy had reduced tumor necrosis factor (TNF) expression and those treated with chemotherapy showed reduced reactive oxygen species (ROS) production. The findings were reported in the November 22, 2013, issue of *Science*.

Tumor-bearing mice that lacked the *tnf* gene did not respond to immunotherapy, regardless of the presence or absence of gut microbes. The researchers found that administration of bacterial products could restore TNF production by tumor myeloid cells in animals depleted of their gut microbiota. Allowing microbial colonies to reestablish after antibiotic treatment, they further determined that bacterial composition—not just abundance—was important for restoring the TNF response.

“The use of antibiotics should be considered as an important element affecting microbiota composition. After antibiotic treatment the bacterial composition in the gut never returns to its initial composition,” said Trinchieri. “Our findings raise the possibility that the frequent



(Image: A. Dzarisser, CCR)

Mouse gut bacteria phylogenetic tree superimposed over a microphotograph of colonic tissue, showing the abundance of bacteria by the size of the circles. Red circles indicate bacteria that primes the mice for a response to immunotherapy, while the green circles show bacteria that suppress antitumor response to the drug.

use of antibiotics during a patient's lifetime or to treat infections related to cancer may affect the success of anticancer therapy.”

The researchers plan to continue their work in mice to fully understand the signaling between gut microbes and tumors in distant organ sites. However, the commensal microbial ecosystem is enormously complex and the extent to which results can be directly translated into humans is still unclear. Thus, important future directions for the team include studying the role of gut bacteria (and antibiotic interventions) in the human inflammatory response and tumor response to therapy.

To learn more about Dr. Trinchieri's research, please visit his CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=gtrinchieri>.

Bringing Breast Cancer Technologies to Market

CCR research is recognized in novel competition to encourage the commercialization of breast cancer inventions.

Start-up companies are instrumental in bringing the fruits of scientific research to market. Recognizing an opportunity to bring entrepreneurial minds to bear on the diagnosis and treatment of breast cancer, the Avon Foundation for Women partnered with NCI and the Center for Advancing Innovation to launch the Breast Cancer Startup Challenge. The Challenge has brought together teams of university students and entrepreneurs to create strategic business plans to develop and commercialize patented technologies. Nine of the 10 inventions chosen

in the competition were developed by scientists at NCI: five by CCR scientists and two by scientists formerly with CCR.

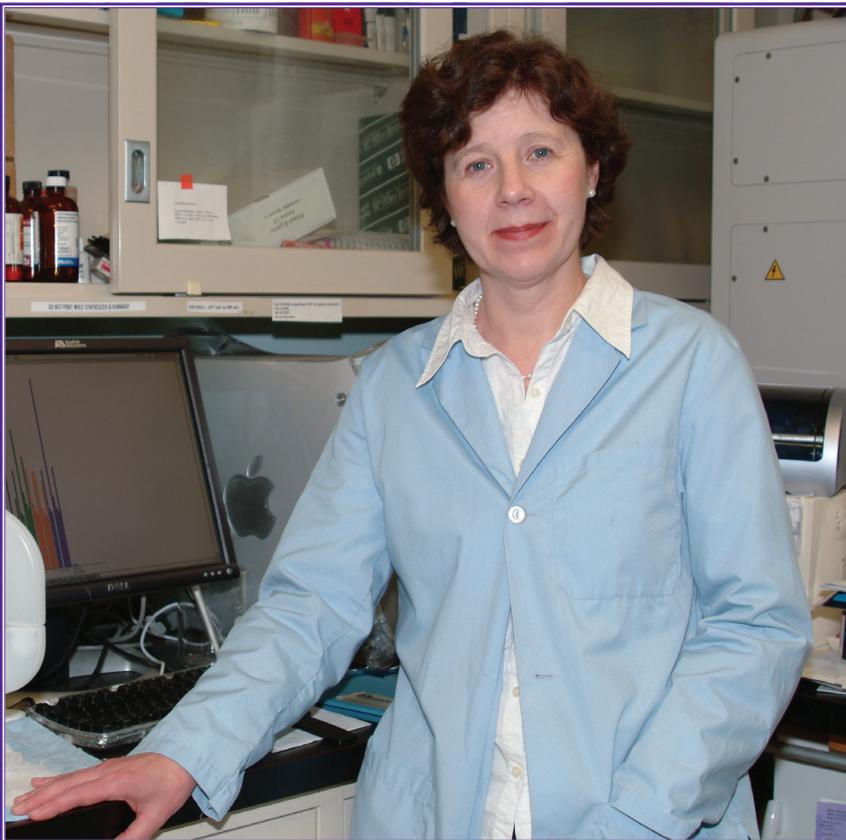
“NCI has always had a strong interest in fostering young investigators and the fact that this challenge pairs each student team with entrepreneur-mentors to assist in the development of the business plans is another example of how we can bring new ideas and energy to cancer research,” said Douglas Lowy, M.D., Deputy Director of NCI and Chief of CCR’s Laboratory of Cellular Oncology.

The chosen technologies include cancer diagnostics, therapeutics, prognostics, one medical device, one vaccine, one delivery system, and one health IT system. The 10 winning teams will each be encouraged to launch start-up companies to develop the technologies.

One of the winning teams, based at Rutgers University in N.J., has formed a company called OncoLinx to push forward research into a class of cytotoxins that boost the effectiveness of antitumor agents. Developed by Nadya Tarasova, Ph.D., Head of the Synthetic Biologics and Drug Discovery Facility in CCR’s Cancer and Inflammation Program, the cytotoxins are easy to synthesize, stable in circulation, and are cell permeable. In an unpublished study, Tarasova and her colleagues discovered that when one of the cytotoxins was conjugated to the antibody Herceptin, the combined therapy killed 98 percent of cancer cells *in vitro* compared to 46 percent of cells killed by the antibody alone. An important step for the Rutgers team will be demonstrating the cytotoxins’ safety and effectiveness in preclinical studies. Key to this will be developing a method for transporting the cytotoxin directly to diseased cells within patients’ bodies, while avoiding surrounding healthy cells.

For any given cancer type, a number of treatment options exist. A major challenge for clinicians is determining which option is best for their patient without having to test them one by one. To address

Image: Courtesy of Scientific Publications, Graphics & Media



CCR researcher and Breast Cancer Startup Challenge inventor Nadya Tarasova, Ph.D.

this issue, Stephen Hewitt, M.D., Ph.D., Staff Clinician in CCR's Laboratory of Pathology, devised a tissue assay for up to four protein biomarkers. The presence or absence of the various biomarkers can indicate the optimal therapy for a patient and predict that patient's survival rate for a given treatment. "The invention came out of a single study where we measured multiple biomarkers looking for a correlation between biomarker and drug response," said Hewitt. "None of the biomarkers alone predicted response, but in ratios, based on the pathway interactions, they did." A team of students at McGill University in Montreal has formed a company called ProVivoX that plans to develop a test based on Hewitt's biomarker research that can predict breast cancer relapse. The team hopes to begin clinical trials by 2015.

Another particularly promising cancer therapy invented at CCR helps patients' immune systems target tumors more effectively. Dennis Klinman, M.D., Ph.D., Senior Investigator in CCR's Laboratory of Experimental Immunology, discovered a method for customizing synthetic immune system stimulators called CpG oligonucleotides that interact with immune cells that express Toll-like receptor 9 and thus drive an innate immune response. When linked to an apoptotic tumor cell vaccine derived from the patients' own tumor biopsies, the oligonucleotide-vaccine conjugates reduce tumor size, as well as prevent cancer recurrence and metastasis in mice. A team from Washington University in St. Louis, Mo., plans to apply CpG oligonucleotides to triple-negative breast cancer. They also hope to expand their treatment, which is dubbed TheraProVax, to other cancer types.

As for Klinman, he appreciates the chance to see his research, as well as that of his colleagues, brought out of the lab and into the hands of doctors. "The Breast Cancer Startup Challenge provides a wonderful opportunity to focus on inventions for the treatment of cancer and

improves the likelihood that they will find clinical application," he said.

To learn more about the Challenge, please visit <http://www.breastcancerstartupchallenge.com>.

CCR Inventions and Business Plan Winners*

Diagnostic from Biopsies with Software Analysis

Category: Diagnostics/Health IT

Lead Inventor: Tom Misteli, Ph.D., CCR

Winner: University of Cambridge

Immunotherapy Using Modified Self Tumor Cells

Category: Therapeutic

Lead Inventor: Dennis Klinman, M.D., Ph.D., CCR

Winner: Washington University in Saint Louis

Human Monoclonal Antibody-Based Cancer Therapies

Category: Therapeutic, Diagnostic

Lead Inventor: Mitchell Ho, Ph.D., CCR

Winner: Stanford University

Immunotherapy Using Granulysin Activated Monocytes

Category: Therapeutic

Lead Inventor: Alan Krensky, M.D., Northwestern University
(formerly with CCR)

Winner: Northwestern University

Anticancer Toxin

Category: Therapeutic

Lead Inventor: Nadya Tarasova, Ph.D., CCR

Winner: Rutgers, The State University of New Jersey

Genomic-Based Diagnostic Assay

Category: Diagnostics and Prognostic

Lead Inventor: Steven Libutti, M.D., Albert Einstein College of
Medicine (formerly with CCR)

Winner: University of California, Berkeley

Tissue-Based Diagnostic Assay

Category: Diagnostic

Lead Inventor: Stephen Hewitt, M.D., Ph.D., CCR

Winner: McGill University

* This listing does not constitute CCR/NCI's endorsement of the companies or potential products and does not guarantee a grant of license for any federally-owned technology.

Recent CCR Awards

2014 Fellows of the American Academy of Microbiology

Alan Rein, Ph.D.

Senior Investigator, HIV DRP Retroviral Replication Laboratory

Zhi-Ming Zheng, M.D., Ph.D.

Senior Investigator, Gene Regulation and Chromosome Biology Laboratory

Bert and Natalie Vallee Award in Biomedical Sciences

American Society of Biochemistry and Molecular Biology

For outstanding accomplishments in basic biomedical research

Michael Gottesman, M.D.

Chief, Laboratory of Cell Biology

F.K. Mostofi Distinguished Service Award

United States and Canadian Academy of Pathology

For outstanding service to the International Academy of Pathology and its U.S.-Canadian Division

Frederic Barr, M.D., Ph.D.

Deputy Chief, Laboratory of Pathology

2014 Abbott Award in Clinical and Diagnostic Immunology

For outstanding contributions to clinical or diagnostic immunology

Robert Yarchoan, M.D.

Chief, HIV and AIDS Malignancy Branch

2014 Award for Research Excellence in Cancer Epidemiology and Prevention

American Association for Cancer Research and American Cancer Society

For outstanding research accomplishments in the fields of cancer epidemiology, biomarkers, and prevention

Curtis Harris, M.D.

Chief, Laboratory of Human Carcinogenesis

Elected to the National Academy of Sciences

Shiv Grewal, Ph.D.

Chief, Laboratory of Biochemistry and Molecular Biology

2014 AACR Team Science Award

American Association for Cancer Research

For participation on the Duke University, Johns Hopkins University, and NCI Malignant Brain Tumor Team and the immense impact of their research on the understanding of the biology of glioblastoma multiforme

Ira Pastan, M.D.

Co-Chief, Laboratory of Molecular Biology

Blue Faery Award for Excellence in Liver Cancer Research

For contributions to the advancement of scientific knowledge in the diagnosis, treatment, prevention, or understanding of liver cancer

Xin Wang, Ph.D.

Deputy Chief, Laboratory of Human Carcinogenesis

Intel Science Talent Search Semifinalists

For high school students who have performed independent scientific research

Ben Freed

Cancer and Inflammation Program

Tina Ju

Laboratory of Experimental Immunology

Madelyne Xiao

Laboratory of Experimental Immunology

Jessica Yau

Laboratory of Protein Dynamics and Signaling

New Tenure-Track Scientists

(Photo: B. Brantson)



Shuo Gu, Ph.D.

Shuo Gu joins CCR's Gene Regulation and Chromosome Biology Laboratory as an Earl Stadtman Tenure-Track Investigator. Gu studies the mechanisms of miRNA biogenesis, post-transcriptional modifications, and biological functions in mammalian systems with the goal of developing new therapeutic approaches for cancer treatment.

(Photo: B. Brantson)



Brid Ryan, Ph.D., M.P.H.

Brid Ryan joins CCR's Laboratory of Human Carcinogenesis as an Earl Stadtman Tenure-Track Investigator. Ryan focuses on genetic and biological factors that contribute to health disparities in lung cancer and applies an integrative molecular epidemiological framework to understanding the biological underpinnings of lung carcinogenesis.

Partners Across the Beltway: University of Maryland and CCR

Graduate students bring energy and focus to collaborations between UMD physicists and CCR biologists.

Now in its fourth year, the Partnership for Cancer Technology was established between NCI and the University of Maryland (UMD) to bring UMD's expertise in the physical sciences to bear on the problems of cancer biology (See "Teaming Up to Fight Cancer," *CCR connections* Vol. 4, No. 2). The program has thus far enrolled 15 UMD graduate students to conduct research under the joint supervision of a CCR investigator and a university faculty member. Sixteen NCI investigators and 21 university faculty members have participated in the program.

"Formalization made a huge difference," said Carole Parent, Ph.D., Deputy Chief of CCR's Laboratory of Cellular and Molecular Biology, who co-directs the partnership with Dan Larson, Ph.D., Investigator in CCR's Laboratory of Receptor Biology and Gene Expression, and UMD's Wolfgang Losert, Ph.D. "Collaborations work best when you involve students and postdocs to go in between labs and be part of the research. The program has made it a lot easier to recruit students and allow them to move freely between the laboratories." In addition to logistical support, the Partnership has also allocated competitive funding for joint projects.

Setting the stage for an official agreement between their institutions, Parent and Losert first

began collaborating in 2003. "My lab is interested in understanding, at the molecular and signal transduction level, how cells communicate with each other and migrate through the body," said Parent. "We like to see how things move." Her laboratory works with three models: the single-celled social amoebae, *Dictyostelium discoideum*, neutrophils, and human breast cancer cell lines. Using time-lapse imaging, Parent could observe patterns of movement that changed depending on the substrate or the cell-cell interactions involved, but she lacked the means to quantify those changes. Quantification became particularly challenging in the study of aggregate cellular behavior, which occurs, for example, when epithelial cells form sheets. Losert was able to help her analyze the changes she was observing.

"Over the years, we've shared many graduate students and postdocs who have moved on," said Parent. "Currently, we have two graduate students who split their time roughly 50-50, trying to apply their physics backgrounds to biological questions."

Chenglu Wang is a fifth-year Ph.D. student at UMD, working with Parent and Losert. She is interested in both the chemical signals and mechanical forces that guide cell migration. "At UMD, our lab has both physicists and biophysicists. When I work in Carole's lab at NCI,

I can talk to students and postdocs that have a biology background and they have different ways of thinking; even for the same scientific question, they have different approaches to solve problems. I get to know what they really care about in cancer biology," said Wang. She also enjoys the opportunity to attend cancer research seminars and lab meetings.

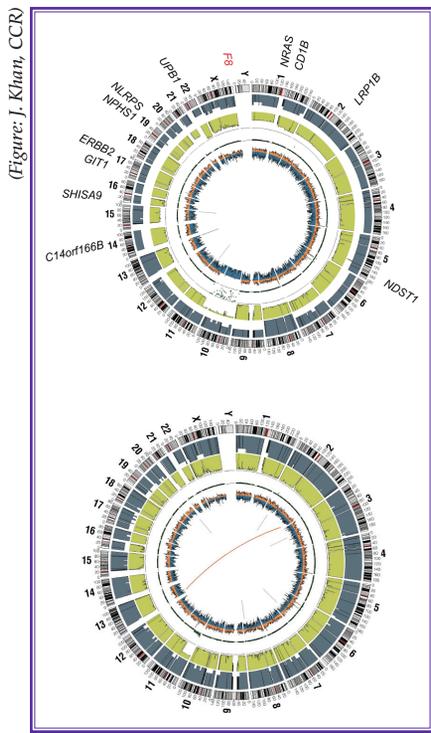
The Partnership hosts symposia twice a year, during which students and young investigators present their work. It is also an opportunity to introduce new researchers and students to the possibilities for collaboration.

"Now other institutes are becoming interested in this endeavor. They are working with UMD to add grad students from different departments within UMD. We now have six different NIH institutes participating in this program," said Parent. "I think it is very important to employ a multi-disciplinary approach to help solve complex biological questions. The partnership with UMD is a great mechanism to help NIH investigators attract motivated students that bring unique complementary skills to the study of specific biological problems."

To learn more about the UMD-NCI Partnership for Cancer Technology, please visit <http://cancertechnology.umd.edu>.

Seeing the Forest and the Trees

Comprehensive genomic analysis gives new insight into a highly malignant childhood tumor.



(Figure: J. Khan, CCR)

Circos plots of genomic alterations in two of the 147 rhabdomyosarcomas examined for this study.

As little as 15 years ago, sequencing a human genome required the cooperation of multiple research centers and millions of dollars. Today the time and costs associated with genomic analysis have plummeted by orders of magnitude, giving cancer researchers an unprecedented ability to examine entire tumor genomes for changes that may drive disease.

Javed Khan, M.D., Deputy Chief of CCR's Genetics Branch and an Investigator in CCR's Pediatric Oncology Branch, and his Clinical Fellow, Jack Shern, M.D., have taken this survey approach to the childhood cancer, rhabdomyosarcoma. Rhabdomyosarcoma, the most common soft-tissue sarcoma in children, is still relatively rare, affecting 350 patients per year. While many patients are successfully treated with existing therapeutic regimens, some go on

to develop metastatic disease and have a five-year survival rate of only 30 percent.

Khan, Shern, and their colleagues studied 147 tumors and compared them with matched control tissue. They used a combination of whole-genome sequencing (WGS), whole-exome sequencing (WES), and whole-transcriptome sequencing (WTS), along with high-resolution single-nucleotide polymorphism (SNP) arrays, to provide a comprehensive genomic landscape for this disease. The results were published in the February 1, 2014, issue of *Cancer Discovery*.

The researchers found two broad classes of tumors separable by the presence or absence of a novel gene resulting from the fusion of *PAX3* or *PAX7* with the *FOXO1* gene on a different chromosome. This fusion leads to altered expression of many genes because it acts as an aberrant transcription factor. The presence of a fusion gene was associated with very few additional genetic alterations (usually an amplification or deletion), and with poor clinical outcomes. Absence of the fusion gene was associated with a broader collection of genomic abnormalities including chromosomal rearrangements, aneuploidies, and mutations. Many previously identified genes were implicated, as well as some new suspects like *FBXW7* and *BCOR*. Tumors lacking the PAX fusion gene expressed mutations in genes that were induced or suppressed by the PAX fusion gene.

Recurrence of alterations to the same gene in multiple tumors suggests it may be central to the disease process. Because this cancer has a relatively low mutation rate, the team was faced with assigning

significance to genetic alterations in potentially interesting signaling molecules, which could be found in a few, or even only one, tumor samples. However, when they analyzed the context of these rare and singleton mutations, they discovered that many were part of the same signaling pathways. In particular, they found alterations in the receptor tyrosine kinase/RAS/PIK3CA signaling pathways in 93 percent of the tumors.

Khan and Shern are continuing their work with the Children's Oncology Group, which has identified an additional 650 clinically annotated samples to enable follow up work on the genes identified in this study. "I think we've described the tip of the iceberg. In the next couple of years, we'll hopefully be able to associate prognosis and therapies based on the particular mutations we find," said Shern.

Building on this research, the team also plans to design and test interventions that target the genetic drivers they have identified.

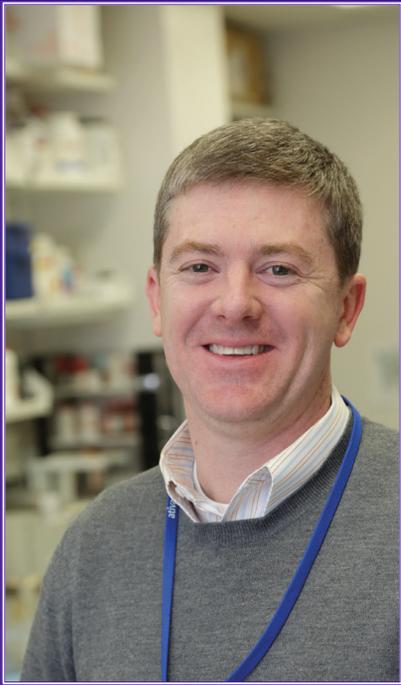
"These studies are very difficult to do because tissue acquisition and validation is so complex," said Khan. "This work would not have been possible without our brave pediatric patients and their families. In the face of their life-threatening disease, they offered their tumors for study knowing that they may not personally benefit from this work but in the hope that investigators might learn lessons that would help children diagnosed with rhabdomyosarcoma in the future."

To learn more about Dr. Khan's research, please visit his CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=khan>.

In Conversation:

Clinical Fellow Jack Shern, M.D.

(Photo: B. Branson)



Jack Shern, M.D.

CCR: Pediatric oncology is a challenging field. How did you choose to work with kids?

Jack: I knew very early that I was interested in pediatrics. We have a pretty good cure rate, which means we can make a huge difference. If you save an infant, they have their whole life ahead of them. Plus, pediatrics is a fun specialty. It's amazing to see, even when they are sick, kids can keep smiling, laughing, and having fun.

CCR: You have a joint fellowship between NCI and Johns Hopkins. Tell us about it.

Jack: First, there was a very intense clinical service year of seeing patients all the time, half at Hopkins, half at NCI. For research, I knew pretty early that I wanted to be at

NCI. I met my wife when I came here as a summer research fellow, and we both knew we wanted to come back. So I started looking around the Pediatric Oncology Branch for a research opportunity. And Javed was the best salesman.

CCR: That's Javed Khan, Deputy Chief of our Genetics Branch and an Investigator in our Pediatric Oncology Branch. Humor aside, what interested you in his work?

Jack: I saw high-throughput genomics as the best way to characterize these tumors. We can survey the entire picture of what these tumors do with a couple of experiments. When I joined in 2011, his laboratory was already actively sequencing about 140 rhabdomyosarcomas and Javed suggested I lead that project. I had no experience in genomics, but it was a good place to learn. There is so much expertise in the Branch; for a novice, it is reassuring to have the world's experts mentoring you.

CCR: What was the biggest challenge to the project?

Jack: The biggest challenge was collecting enough tumors, then pooling all the data and figuring what story all this information was trying to tell us. Tumors are heterogeneous and the sequencing data can be very hard to interpret. You have to come up with statistical tools to carefully balance the false positives and negatives. It is even more challenging in a rare tumor where you have low sample numbers and recurrent mutations are infrequent.

CCR: What surprised you most about the results?

Jack: How simple, yet deadly, some of the tumors are. There was a one-year old with only two genomic alterations, one of which generated the fusion oncogene, *PAX3-FOXO1*. Of course, that change affects hundreds of genes downstream. In another big project, we are building some cell lines that can be screened by groups at the National Center for Advancing Translational Sciences (NCATS) and the RNAi core facility to help us figure out what the fusion gene is doing, who it is interacting with and ultimately, to develop novel therapies.

CCR: What advice would you give to your colleagues?

Jack: This project had so many moving parts and collaborators that it took a lot of persistence to get it to where it is today. There are lots of times in science when you get disappointed. To succeed, you need persistence.

CCR: Where do you see your future career?

Jack: I still love to see patients, but I would one day like to head my own lab. The NIH is a remarkable community where this type of "big science" can be done. It's hard not to aspire to be a principal investigator in this exhilarating environment. For me, that means I'll try to stay here and use genomics to translate our findings into new treatments for children who currently have incurable cancer.

RNA Rules: The Many Faces and Functions of Ribonucleic Acids

Long overshadowed by its more famous DNA cousin, RNA is enjoying a molecular renaissance. RNA serves as a middleman between DNA templates and protein machinery. However, RNA molecules have catalytic activity of their own (a discovery for which Thomas Cech, Ph.D., and Sidney Altman, Ph.D., won the Nobel Prize in Chemistry in 1989). And small segments of RNA can silence the gene expression of their brethren (a discovery for which Craig Mello and Andrew Fire won the Nobel Prize in Physiology or Medicine in 2006). Researchers are even learning how to create entirely new types of RNA molecules. Today, CCR investigators are exploring and reinventing the roles of RNA in health and disease.

“For years, people thought of RNA as a not very interesting molecule,” said Bruce Shapiro, Ph.D., Senior Investigator in CCR’s Basic Research Laboratory. “Essentially, there was the central dogma of going from DNA to RNA to protein; and, RNA was just an intermediary. Then a revolution started. Much of what people used to call junk DNA is in fact transcribed, conferring whole new levels of cellular control.”

A new RNA Biology Initiative is bringing together CCR investigators to exchange ideas and expertise on the structure, function, and biological roles of RNA. Through workshops, shared technology development, collaborations, and mentorship, the Initiative is building on the strengths of CCR’s ongoing research in this influential and rapidly evolving area of biomedical research.

Errors in Transcription

Even the processes supporting basic RNA transcription from DNA are more complicated than foretold. Mikhail Kashlev, Ph.D.,

Senior Investigator in CCR’s Gene Regulation and Chromosome Biology Laboratory, has uncovered novel disease-promoting mechanisms by focusing his research on the molecular interactions that support efficient RNA synthesis. “We really want to know the role of transcriptional fidelity in cell physiology,” said Kashlev. “This is a very under-investigated question.”

When RNA polymerase II (Pol II) is recruited to the promoter of a gene to be transcribed, it does not rush to get the job done. Instead, it pauses first, in so-called promoter proximal elongation arrest. This pause in transcription may last for just a second or it may last for hours. “This is very important because the enzyme is thereby poised for a quick transcriptional response to

additional signals,” said Kashlev. “It is also important to coordinate the timing of transcriptional elongation for alternative splicing.”

Kashlev’s laboratory uses biochemical approaches to discover elements in DNA (*cis* acting signals) that modulate transcriptional arrest and, in collaboration with others, has analyzed factors that bind DNA (*trans* acting signals) to similarly modulate the process. They have developed techniques to reconstitute the elongation process *in vitro*, with the purified polymerase, synthetic DNA, and RNA oligonucleotides. “We can reconstitute the elongation of any sequence you want with up to 90 percent efficiency,” said Kashlev.

Recently, his laboratory discovered that in addition to *cis* and *trans* factors, transcriptional

“Much of what people used to call junk DNA is in fact transcribed, conferring whole new levels of cellular control.”

(Photo: R. Baer)



Maria Kireeva, Ph.D., and Mikhail Kashlev, Ph.D.

errors can strongly arrest Pol II. The erroneous incorporation of a single ribonucleotide creates a mismatch between the DNA template and the growing RNA molecule, resulting in a physical disturbance to the active site of Pol II. “Elongation can’t continue until the error is removed,” said Kashlev. “But Pol II has a very limited ability to excise errors.” Left uncorrected, paused elongation complexes could litter the genome.

This finding gave Kashlev and his Postdoctoral Fellow, Masahiko Imashimizu, Ph.D., a way to study transcriptional fidelity *in vivo*, where the transcriptional error rate is normally very low—on the order of 1 in 10,000 to 1 in 100,000—making it difficult to isolate these instances. “What if instead of sequencing all the mRNA isolated from a cell, we isolated all the elongation complexes that are paused?” asked Kashlev. “We should be able to enrich our population of mRNAs with errors.” Isolating all the complexes from *E. coli* and performing high-resolution sequencing of the RNA to look for errors at the 3’ nascent end of the mRNA, Kashlev discovered that about one percent of all pausing events were due to errors.

“When errors arrest transcription very strongly, the genes are not expressed,” said Kashlev. “Even more importantly, errors can also generate problems for DNA replication. When DNA polymerase collides with the transcriptional fork, replication is aborted. If that happens again, a double-stranded DNA break occurs.”

Somewhat paradoxically, error-prone transcription can be beneficial for the cell. Celine Walmacq, Ph.D., a Postdoctoral Fellow, and Maria Kireeva, a Staff Scientist who initiated investigation of transcriptional fidelity mechanisms in Kashlev’s laboratory, established that mutations in the Pol II catalytic subunit, rendering transcription fast and error-prone, reduce transcription arrest at UV-induced DNA lesions *in vitro* and increase cell resistance to UV irradiation.

The discovery of several classes of mutations in Pol II that alter transcriptional fidelity and mismatch extension by Kashlev’s lab in collaboration with Zachary Burton, Ph.D., Professor, Michigan State University, and Jeffrey Strathern, Ph.D., Chief of CCR’s Gene Regulation and Chromosome Biology Laboratory,

opens new research directions. Indeed, errors in RNA transcription have been proposed as a route to cell pathophysiology. But the assumed mechanism has been transcriptional errors propagating to a particular protein, for example, transcriptional errors leading to mutated DNA polymerase, which in turn would produce faulty DNA replication.

“People have suggested that low fidelity transcription may be a priming event in cancer,” said Kashlev. Such a mechanism is very hard to prove because it would occur at the individual cell level before any cancerous transformation. Kashlev’s work suggests that errors in nascent mRNA may have an important direct effect on DNA through transcriptional arrest, an effect that may become even more important in the deteriorating cellular conditions of cancer or aging.

Viral RNA Dynamics

Viruses co-opt their hosts’ transcriptional machinery to replicate. Retroviral RNA is reverse transcribed into DNA, which integrates into the host genome. The DNA is then transcribed to create more viral RNA, which is exported from the nucleus to join its protein cohort in the formation of a new virus. During DNA synthesis, genetic information from both packaged RNAs can be combined and genetic recombination becomes clinically significant as the viral progeny can acquire new properties and vulnerabilities. Wei-Shau Hu, Ph.D., Senior Investigator in CCR’s HIV Drug Resistance Program Retroviral Replication Laboratory (RRL), wants to know exactly what happens to HIV RNA within the environment of a cell.

While studying recombination, Hu realized that a number of basic facts about viral RNA packaging were unknown or based on untested assumptions. For example, how many viral RNA molecules are

(Photo: R. Baer)



Olga Nikolaitchik, Ph.D., Wei-Shau Hu, Ph.D., and Jianbo Chen, Ph.D.

packaged in an individual HIV virus? If you take 100 virus particles, how many contain viral RNA at all?

The prevailing answers were based on biochemical data. “When you do a biochemical study, you know the amount of protein and the amount of RNA,” explained Hu. “You correlate these numbers and assume an even distribution. But there are error rates in both measurements, which lead to big differences in predicting how many of those particles have RNA genomes and can be infectious.”

Staff Scientist Jianbo Chen, Ph.D., working with Research Biologist Olga Nikolaitchik, Ph.D., took the lead in designing a reporter system based on RNA-binding proteins to detect viral RNA in a single viral particle. This involved directly engineering a stem-loop structure into the HIV genome that could be bound by a fluorescent reporter.

“We discovered that HIV is really quite good at packaging two, and only two, copies of the RNA genome per virus,” said Hu. “It’s a highly regulated process. But, how?”

Nikolaitchik led a study to first distinguish between two hypotheses: regulation based on RNA mass versus number of molecules. She generated viral genomes of dramatically different sizes and showed that regardless of size, the virus always packaged two copies of the RNA genome. The RNA molecules of the viral genome do not anneal to one another through base-pair complementarity along the entire molecule the way DNA does; instead, they have been shown to pair off through a short dimerization sequence affixed to one end of each molecule. When Nikolaitchik engineered RNA that could self-dimerize, she found that only a single copy was packaged.

“We are very interested in finding out exactly when and where these RNAs find each other and dimerize,” said Hu. Her laboratory has shown that this must happen in the cytoplasm, not the nucleus. Furthermore, full-length viral RNA can exploit host nuclear export pathways to exit the nucleus, but Hu

and her colleagues have shown that viral RNAs that use different export pathways do not recombine nearly as well.

To continue studying RNA sorting and packaging, Chen is developing a system to visualize its transport through the cytoplasm in living cells to its assembly site at the plasma membrane. “The live-cell imaging is technically very challenging,” said Hu. “RNA moves extremely fast; you need fast cameras (25 pictures/sec) to capture its movement. But, thanks to Jianbo’s efforts now we can track a single molecule of viral RNA moving inside a cell. Because HIV uses the host machinery to transport RNA, these studies also help us understand how human cells transport cellular RNAs.”

Viral Havoc

Joseph Ziegelbauer, Ph.D., Tenure-Track Investigator in CCR’s HIV and AIDS Malignancy Branch, focuses on the role of RNA in the lifecycle of a DNA virus, the Kaposi’s sarcoma-associated Herpesvirus (KSHV). After infecting cells, these herpes viruses can exist quietly in a latent phase, remaining under the immunological radar by expressing only a handful of protein encoding genes while expressing an abundance of miRNAs. Like endogenous miRNAs, these viral miRNAs modulate cellular gene expression, presumably in the service of increased viral survival and propagation.

“We are trying to use these miRNAs to tell us humans what the virus already knows about genes that are important for infection,” said Ziegelbauer.

Identifying the cellular targets of viral miRNAs is more complicated than it might seem. Bioinformatic approaches to look for sequence complementarity between miRNAs and potential targets turn up many false positives, relying as they do on very small segments of nucleotide

“...these studies also help us understand how human cells transport cellular RNAs.”

pairing. False negatives also arise when the miRNA operates despite imperfect complementarity.

As a postdoctoral fellow in the laboratory of Don Ganem, M.D., at the University of California, San Francisco, Ziegelbauer began studying the recently discovered viral miRNAs by examining gene expression patterns in cells infected with the virus. When he joined CCR in 2008, he brought these data with him and has since expanded his search for significant viral miRNA targets.

“Our approach has been to use multiple ways of either introducing or inhibiting the miRNA and then integrating those data sets to find the genes that are changing the most across different experiments,” said Ziegelbauer. “When we started, we were looking at a short list of 50 predicted targets.”

Many of these targets are responsive to more than one of the 18 individual miRNAs encoded by the herpes virus. “Redundancy is a central theme, and that gives us another way to filter these targets. We take redundancy to mean that the target is potentially important and worthy of follow-up.”

More recently, Ziegelbauer has been using network analysis to examine how the target genes are interacting with one another as an additional tool for identifying the important players and forming hypotheses about their cellular roles.

“I’m really excited about the next steps, one of which is a collaboration with the trans-NIH RNAi facility,” said Ziegelbauer. “They are testing knock downs of some of our targets to figure out how they affect particular phenotypes of infection. They do the hard work of optimizing your assay for an RNAi screen and then they conduct the screen. That would be really difficult for us to do on our own.”

Ziegelbauer is also collaborating with his Branch colleagues to use proteomic approaches on fresh clinical samples. “In our Branch, there are clinicians treating patients with KSHV infections,” said Ziegelbauer. “It gives us a way to learn about the biology of the disease directly.”

Using two-color quantitative immunoblotting, Ziegelbauer can look at vastly different levels of protein abundance in individual

samples. Proteins give yet another view into the function of miRNAs and they may reflect a more faithful view of their function. Ziegelbauer recently published a study in *PLOS Pathogens* highlighting viral miRNA targets implicated in angiogenesis and immune evasion.

“I wouldn’t ever rely on just the clinical or just the cell culture data,” said Ziegelbauer. “But if we can show a miRNA target is repressed in a cell culture and we then look at uninfected versus infected lymph nodes and see the same target gene is repressed, they are pieces of the puzzle that can be put together to say that these genes are really repressed in patients.”

RNA on the Offensive

Shapiro has learned a lot about the fundamentals of RNA structure and function, since he began his RNA research in the 1980s. Now, he is using that knowledge to build nanostructures out of RNA that Mother Nature has not yet dreamed of. He wants to then put those structures to use in curing disease.

“RNA is interesting because it can naturally fold into fairly complex



(Photo: R. Bucr)

Left to right: Anna Serquiña, M.D., Ph.D., Joseph Ziegelbauer, Ph.D., Xiaoyan Liu, Ph.D., Christine Happel, Ph.D., and Dhivya Ramalingam, Ph.D.

(Photo: R. Baer)



Bruce Shapiro, Ph.D., and Kirill Afonin, Ph.D.

structures. It can serve as a catalyst or as an information storage medium. It has lots of natural functionality. And you can control the self-assembly process by understanding base-pairing interactions and known structural geometries," said Shapiro.

To define the elements of RNA structures, Shapiro and his colleagues, Eckart Bindewald, Ph.D., and Wojciech Kasprzak, M.S., went to the Protein Data Bank (PDB), a database of molecular structures that is more expansive than its name implies, and extracted three-dimensional "motifs" that are found among the known RNA structures. "We ultimately identified about 13,000 motifs and we view them like LEGO blocks that we can piece together to generate the types of 3-D patterns we want," said Shapiro. These motifs are freely available online at the RNA junction database that they produced (<http://rnajunction.abcc.ncifcrf.gov>). Shapiro's group also developed software to aid in the nanoscale structure design and in the design of sequences that can self-assemble into nanostructures.

With his Research Fellow Kirill Afonin, Ph.D., Shapiro has experimentally created some of these computationally designed shapes. One is a nanometer-scale hexagon formed from individual elements shaped like dumbbells with

so-called "kissing loops" of RNA on each end. In collaboration with Luc Jaeger, Ph.D., from the University of California, Santa Barbara, they experimentally verified the formation of these hexagonal structures and by programming each kissing loop differently, they gained greater control over the assembly and purity of these hexagonal shapes. They have also experimentally formed cubes from either 6 or 10 computationally designed motifs, so that the individual strands do not self-fold. They can verify the shapes through atomic force microscopy, or cryo-electron microscopy but they have also incorporated specialized RNA molecules—aptamers—that they engineered to fluoresce when pieces come together properly.

As much fun as building nanostructures from RNA can be, this research is not merely academic. Shapiro is driving this work towards novel therapeutic applications.

RNAi is both a naturally occurring phenomenon and a research tool for silencing the translation of specific genes. It may also be a very powerful tool for therapeutically altering gene expression in disease. One difficulty has been delivery of the siRNA agents, which are easily degraded by nucleases. Shapiro has shown that he can attach multiple siRNA molecules to the RNA nanostructures his group has created. These nanodelivery

vehicles enter the cell intact, whereupon the siRNAs are cleaved by an enzyme (Dicer) to become functionally active. Experimentally, he has shown that siRNA delivered via the nanostructures can silence the expression of the reporter gene—EGFP—in cell culture and in xenograft mouse models.

In addition to a variety of pure RNA structures, Afonin and Shapiro have also experimented with DNA/RNA hybrid structures that confer added stability and functionality. They have shown, in collaboration with Eric Freed, Ph.D., Senior Investigator, RRL, that the activated forms have the ability to silence targeted HIV. Also, they have silenced multiple cancer targets in cells and in xenograft mouse models. "We have a database of structures that are computationally designed, but have not yet been tested experimentally," said Shapiro. A lot of the work the group has done so far validated the fundamental concepts of design, self-assembly, and delivery of functional RNA nanoconstructs, but Shapiro says, "We are working towards targeting specific cancer pathways and viral diseases with these designer molecules."

To learn more about Dr. Hu's research, please visit her CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=hu>.

To learn more about Dr. Kashlev's research, please visit his CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=kashlev>.

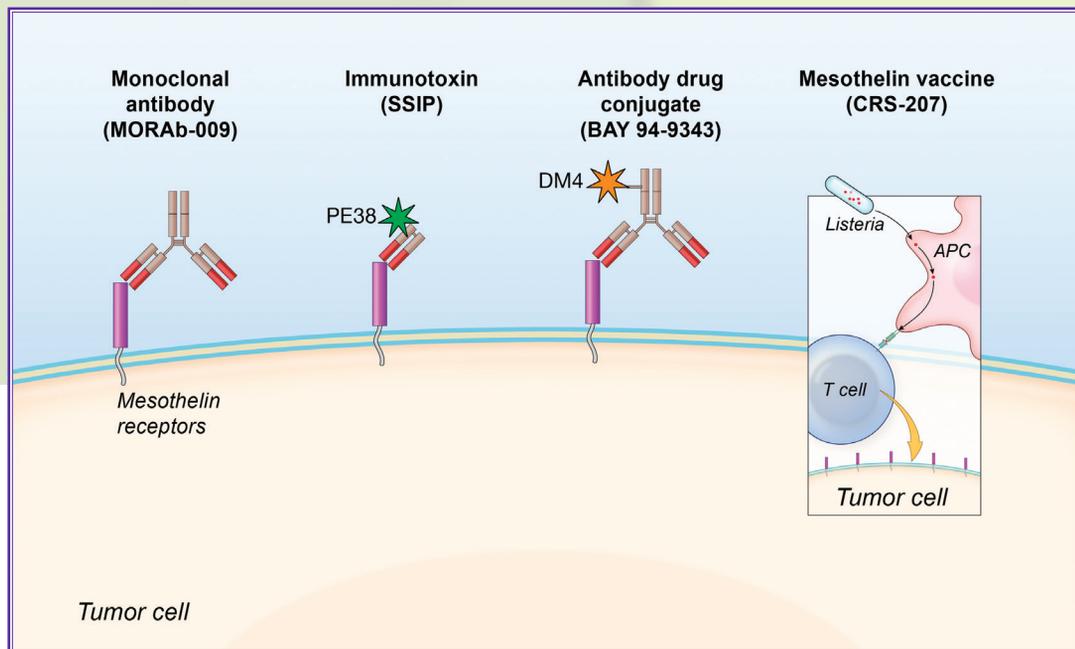
To learn more about Dr. Shapiro's research, please visit his CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=shapiro>.

To learn more about Dr. Ziegelbauer's research, please visit his CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=jzielbauer>.

Precision in Targeting with Anti-Mesothelin Therapies

For two decades, Raffit Hassan, M.D., Co-Chief of CCR's Thoracic and Gastrointestinal Oncology Branch, and Ira Pastan, M.D., Co-Chief of CCR's Laboratory of Molecular Biology (LMB) have been systematically plotting a new era of targeted cancer therapeutics. Building on Pastan's discovery of mesothelin, a protein that is expressed almost exclusively by cancer cells in the adult, Hassan has led efforts to capitalize on this rare selectivity by exploiting mesothelin-directed "smart bombs" developed in the Pastan lab to attack solid tumors, while sparing healthy tissue.

Early clinical trials are validating mesothelin's potential as a selective cancer target. These trials are increasingly showing that mesothelin-directed agents can be used effectively in mesothelioma and have the potential to treat other malignancies, including ovarian, lung, and pancreatic cancers. Hassan and Pastan hope that these new agents will do for solid tumors what precision therapies are already achieving in various blood cancers.



(Image: A. Hoofring, NIH Medical Arts)

Different strategies to target mesothelin for treatment of mesothelioma

A New Target for Solid Tumors

Pastan discovered mesothelin in 1994 during a search for new drug targets in ovarian cancer. At the time, he was collaborating with Mark Willingham, M.D., previously a

Senior Investigator in CCR and now at the Wake Forest Baptist Medical Center's Comprehensive Cancer Center, in N.C. While studying ovarian cancer cells in immunized mice, the team encountered a unique antibody. Though it bound readily

to cancer cells, this antibody—now called mAB K1—ignored normal cells from almost all healthy tissues. In fact, the only normal cells expressing the antibody's target antigen—a 40-kilodalton cell surface glycoprotein—were mesothelial

(Photo: R. Baer)



Raffit Hassan, M.D., and Ira Pastan, M.D.

cells from the pleura (which lines the lungs), the pericardium (which lines the heart), and the peritoneum (which lines the abdominal cavity). That finding got Pastan's attention, given that patients can lose mesothelial cells during cancer treatment without experiencing life-threatening consequences. (See "A Better Immunotoxin," *CCR connections* Vol. 2, No. 1).

Pastan and colleagues sequenced the target gene, now called mesothelin, but even today, much remains to be discovered about its biology. Deleting mesothelin has no apparent effect in mice or their offspring, so the protein does not appear to be necessary for normal growth and development. Yet, according to Pastan, many tumors express mesothelin, and mounting evidence implicates it in cancer progression and metastatic spread.

Shortly after mesothelin's discovery, Hassan—then a Clinical Fellow in CCR's Medical Oncology Branch—joined Pastan's laboratory and began to investigate if mesothelin expression in tumor cells could be exploited for cancer therapy. Hassan knew that mAB K1 homed in on mesothelin-expressing tumors in mice. So he chemically attached the antibody to a potent bacterial poison,

called PE38, to build an immunotoxin that kills malignant cells by inhibiting their ability to synthesize proteins. The new therapy eliminated cancer cells in mice, but using mAB K1 also posed clinical drawbacks: it bound poorly to mesothelin, and with its large size, it did not penetrate easily into the murky interiors of solid tumors.

To solve the issue of size, Pastan's Postdoctoral Fellow, Partha Chowdhury, Ph.D., isolated a smaller protein fragment with much higher mesothelin binding affinity. Dubbed SS1, the fragment was linked to PE38 to create a new immunotoxin that proved to be highly effective against cancer cells in preclinical studies. This complex, which they called SS1(dsFv)PE38, or SS1P, has since been the focus of extensive clinical research.

Better Results with Combination Treatments

In 2002, Hassan launched a mesothelioma clinic at the NIH Clinical Center. Now one of the largest mesothelioma clinics in the U.S., it offers care to approximately 100 new patients each year, most of them are enrolled in clinical trials investigating mesothelin-directed treatments.

Hassan chose mesothelioma as a "proof-of-concept" disease for evaluating mesothelin-directed therapy, in part because new treatments are badly needed for this rare and aggressive malignancy. "Findings from mesothelioma studies can be easily translated into other cancers that also express mesothelin," he explained. "Our ultimate goal is to treat a range of solid tumors with mesothelin-targeted approaches."

Hassan's first published phase 1 study showed that SS1P monotherapy is safe, but only minimally effective in human patients. Robert Kreitman, M.D., Senior Investigator in LMB, conducted a phase 1 clinical trial of SS1P using a slightly different schedule of administration that produced similar results. According to Pastan, SS1P monotherapy is less effective than desired because after just a few doses, the patient's immune system recognizes and then inactivates SS1P's foreign bacterial toxin. "So we started asking 'What are we going to do with this agent that by itself has low efficacy in solid tumors?'" Pastan recalled. The answer, Pastan and Hassan concluded, was to give SS1P in combination with other drugs.

In ongoing research, the two scientists have shown that SS1P in combination with chemotherapy completely eliminates mesothelin-positive tumors in mice. Based on these findings, they designed a new clinical trial that enrolls newly diagnosed mesothelioma patients who are also given standard chemotherapy for their disease: pemetrexed and cisplatin. Results for the trial, which is now closed, will soon be published. "The data look promising," Hassan reported. "Patients treated with the combination do better than what you would expect from chemotherapy alone." Chemotherapy breaks up the tumor cell's exterior layer to facilitate SS1P's access to the tumor's interior.

(Photo: R. Baer)



Raffit Hassan, M.D., and his team meet with Kristi Lescalleet and her husband, Roy Lescalleet, to discuss her case. From left to right: Jeannette Nashed, N.P., Andrew Miller, M.D., Raffit Hassan, M.D., Kristi Lescalleet, Roy Lescalleet, Yvonne Mallory, R.N., and Lisa Bengtson, R.N.

While more effective than SS1P alone, this combination regime leaves untouched the immune response generated against the foreign toxin at the heart of SS1P. But Daniel Fowler, M.D., Senior Investigator in CCR's Experimental Transplantation and Immunology Branch, has found a novel way to suppress this immune response, which Hassan and Pastan have incorporated into their therapeutic strategy.

Delaying Immune Responses

Fowler developed his immunosuppressive method while working with allogenic stem cell transplants, which are procedures that deliver bone marrow-derived stem cells into patients whose own marrow has been damaged by disease. Immune reactions against foreign stem cells (obtained from donors), also complicate these procedures. But in preclinical and clinical studies, Fowler has found that pretreating with pentostatin and cyclophosphamide suppresses and delays the body's immune response to perceived threats. Pioneered at NCI and used in treating different leukemias, pentostatin primes T cells to commit suicide by

apoptosis in response to fairly minor challenges, including low doses of cyclophosphamide. Fowler's method is to give pentostatin first and then to follow with cyclophosphamide over several days. "That combination gives us a safe and effective way to bring the immune system down over time," he explained. Moreover, the two drugs preserve the neutrophil and monocyte arms of the immune system, so that patients can avoid near-term risks of infection arising from T cell depletion.

Pastan reasoned that pentostatin and cyclophosphamide would also delay immunity against the SS1P toxin.

The results of their pilot study to test Fowler's immune suppression technique on the development of SS1P antibodies surpassed the team's expectations and were recently published in the journal *Science Translational Medicine*. "The results were very exciting and unexpected in this group of heavily pretreated patients who had failed all standard therapy," said Hassan. "Three of the 10 patients had major tumor regressions and are alive more than two years later, which is unprecedented for this tumor type."

In 2009, Kristi Lescalleet, a 55-year-old wife and mother of two grown children, was diagnosed with pleural mesothelioma, which is a cancer of the lining of the lungs. For Lescalleet and her family, the diagnosis was unexpected. "I had never even heard of mesothelioma," said Lescalleet, who attributed her initial symptoms—shortness of breath, a cough, and back pain—to bronchitis or pneumonia. Lescalleet underwent treatment and did not develop anti-SS1P antibodies. "This was a very good result—something we have never seen before," Hassan said. Kristi continues to be in partial remission more than two years later. She has received no treatment since she went on the protocol and continues to maintain the partial response without any additional therapy. "I have been feeling just great," said Lescalleet, who has once again resumed an active lifestyle.

Mary Hesdorffer, Executive Director of the Mesothelioma Applied Research Foundation, referred Lescalleet to CCR. She agrees that Lescalleet and other patients have enjoyed "unexpectedly positive responses" to mesothelin-directed treatments. "You would not expect to see this type of response in patients

with advanced disease,” she said. “That is what makes these treatments so exciting. But we have to be cautious—we still do not know if the findings will translate to the general mesothelioma population.”

Based on the results of their pilot study, Hassan and his colleagues have moved directly into a phase 2 clinical trial to evaluate the efficacy of this regimen in patients with pleural and peritoneal mesothelioma.

Other Approaches to Target Mesothelin

In addition to SS1P, Hassan is evaluating other mesothelin-directed agents that also look promising. A chimeric antibody called MORAb-009, or Amatuximab, for instance, which was developed by the biotech firm Morphotek, in collaboration with LMB, is now in phase 2 clinical trials for mesothelioma in combination with pemetrexed and cisplatin, with Hassan as Principal Investigator. Hassan also collaborates on a mesothelin-directed cancer vaccine, CRS-207, developed by scientists at Aduro Biotech, Inc. This vaccine is being evaluated in a phase 1 clinical trial in combination with chemotherapy for newly diagnosed patients with mesothelioma. Finally, Hassan is also working on a new antibody-drug conjugate called BAY 94-9394. Developed by Bayer Healthcare, this compound is currently in a phase 1 clinical trial at



(Photo: R. Bauer)

The Hassan laboratory staff: Neetu Kalra, Ph.D., and Jingli Zhang, Research Biologist

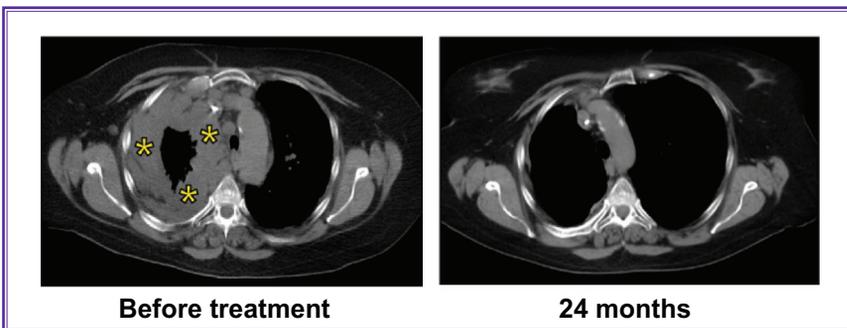
NCI, Sarah Cannon Research Institute in Nashville, Tenn., and the University of Texas M.D. Anderson Cancer Center in Houston. BAY94-9394 links a mesothelin-directed antibody with a potent chemotherapy agent called DM4. Preclinical research showed the drug was highly active against mesothelin-expressing cell lines and tumors. Now the phase 1 study will assess safety, pharmacokinetics, and maximum tolerated doses in patients with advanced solid tumors. In addition, Steven Rosenberg, M.D., Ph.D., Chief of CCR’s Surgery Branch, in collaboration with Pastan and Hassan, has initiated a clinical trial of adoptive T cell therapy in patients with mesothelin-expressing cancer, providing yet another

treatment option for patients whose tumors express this protein antigen.

“We’re looking at mesothelin in a very broad, holistic manner,” Hassan said. “There are very few antigens that can be targeted for cancer therapy, and the attraction in this case is that mesothelin is not expressed by important human tissues. Even if treatment damages healthy mesothelial cells, this is not a life-threatening problem. I am heartened that the work done by Dr. Pastan and me, in addition to our many collaborators, is now leading to new treatment options for patients with cancer.”

Meanwhile, Lescalleet’s tumor continues to show good responses. “Every three months I go back for another CT scan (computed tomography scan), and so far, SS1P is keeping the tumor under control,” she said.

(Image: R. Hassan, CCR)



A computed tomography (CT) scan of a patient with pleural mesothelioma before and after treatment with SS1P.

To learn more about Dr. Hassan’s research, please visit his CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=hassan>.

Tools of the Trade

Among the best ways to accelerate scientific progress is through the direct sharing of data, tools, and biological materials. Within CCR, most laboratories are contributing directly to this greater scientific good by developing resources for their fellow scientists. Here we profile a few of the many examples of CCR investigators providing cells, techniques, and data sets that are having a worldwide impact on the study of cancer.

Multidrug Resistance

In the 1980s, Michael Gottesman, M.D., now Chief of CCR's Laboratory of Cell Biology (LCB), and Ira Pastan, M.D., now Co-Chief of CCR's Laboratory of Molecular Biology, began a very successful collaboration to understand the increasingly apparent problem of multidrug resistance (MDR) to chemotherapies (see "All in Good Fun," *CCR connections* Vol. 4, No. 2). Their work led to the discovery of the *MDR1* gene, which codes for P-glycoprotein, an ATP-dependent transporter (ABC transporter). This widely distributed membrane protein uses the energy of ATP to actively pump out a broad range of foreign substances from cells, including chemotherapeutic agents. During the course of their research, the team created human cancer cell lines in which drug resistance was enhanced through expression of *MDR1*.

Gottesman's laboratory not only continues to study ABC transporters, it has also developed and become a key source of cell lines, plasmids, and antibodies for research and drug development.

Along with researchers seeking to understand whether ABC transporters act on their drugs of interest, pharmaceutical companies also have a strong interest in these biological resources. "Pharma companies use our cell lines, which are highly characterized, to screen drugs that they are planning to use for cancer treatment," said Gottesman.

(Photo: R. Baer)



Michael Gottesman, M.D., and Carol Cardarelli, B.A.

"In that sense, our cell lines have probably influenced many anticancer drugs in development. The U.S. Food and Drug Administration, in fact, now requires companies to report on whether their drugs are substrates for specific transporters."

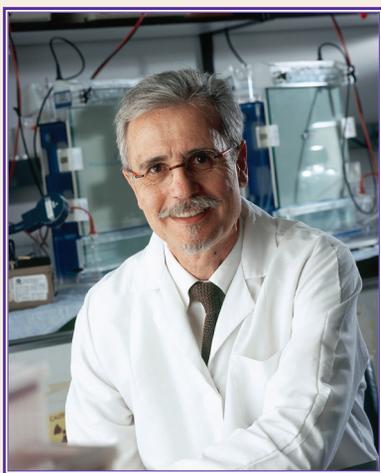
The cell lines are each derived from a single cell and genetically characterized. The most commonly requested cell lines are known as KB-3-1 (drug sensitive parent) and KB-C1, a human cell line derived from the KB-3-1 HeLa line that was initially selected for resistance to high levels of the chemotherapeutic agent colchicine, and then screened for cross-resistance to other unrelated anticancer drugs to characterize their cross-resistance to a broad range of cytotoxic agents. "They are used worldwide as a drug-sensitive control

and MDR-derivative line to study the mechanism of MDR," said Carol Cardarelli, B.A., who has worked with Gottesman since 1983, when the MDR project was just beginning.

Similarly, the KB-V1 cell line, which was the source of the original mRNA used to clone the *MDR1* gene, is routinely used as a drug-resistant positive control for high expression of the P-glycoprotein.

In addition to MDR studies, the research tools are also valuable in the study of drug transport across epithelial barriers. MDCK-pHaMDR is a dog kidney cell line virally infected with the *MDR1* gene. The P-glycoprotein is expressed in a polarized manner on the apical surface of the cells, just as it is in a normal kidney. "One of the major ways drugs are excreted is through

(Photo: R. Bauer)



Yves Pommier, M.D., Ph.D.

the kidney,” said Gottesman. “By studying monolayer cells that form epithelial barriers, like the kidney, pharma companies can study how the drug is handled in the body: its uptake, distribution, and efflux.”

Cardarelli personally handles the MDR-related biological material transfers that are approved by Gottesman and covered by either a NCI Material Transfer Agreement (MTA) or a NIH Office of Technology Transfer (OTT) Licensing Agreement. From 2000–2010, a total of 335 MTAs, were arranged to fill requests, both domestic and foreign, for either cell lines (484), plasmids (173), or antibodies (37) developed in LCB. (One MTA can cover requests for multiple cell lines, plasmids, and/or antibodies.) Over a longer period of time (1997–2014), the OTT issued 55 Licensing Agreements to the private sector. Currently, there are 10 active OTT Licensing Agreements between the NIH and pharmaceutical companies using LCB cell lines for research and development. “We make these resources available because we want to help cancer patients. The more researchers who have access to these materials, the more help there is to develop better anticancer drugs and treatments,” said Cardarelli. “LCB’s cell lines have gone to every continent except Antarctica.”

NCI-60 and CellMiner

Around the same time that Gottesman began creating MDR cell lines, NCI was also developing a new screening tool for anticancer drugs, a panel of 60 human cancer cell lines derived from nine tissues of origin. Now administered by NCI’s Developmental Therapeutics Program (DTP), the NCI-60 represents some of the most thoroughly studied and carefully curated cancer cells. Over the years, laboratories around the world from academia and industry alike have taken advantage of this resource to test their compounds.

“It emerged very quickly that drugs with similar mechanisms of action had similar activity profiles across the 60 cell lines,” explained Yves Pommier, M.D., Ph.D., Chief of CCR’s Developmental Therapeutics Branch. “One cell line would be consistently sensitive to taxol and another would be completely resistant. The responses of the 60 cell lines to a drug define a profile; compounds that have similar targets have similar profiles.”

The DTP created a database to capture the results from these compound screens and Pommier estimates that around one million compounds have been added to it.

Meanwhile, Pommier became interested in the genomic profiles of these cells and how they might relate to drug sensitivities. His laboratory and others began to extensively characterize these cells across several parameters, including gene expression, copy number variation, whole-exome sequencing, miRNA profiles, and karyotype analysis.

“It became obvious to me that if we could bring all this genomic information together with the drug studies, and provide the tools to enable their use, we could produce a wonderful resource for the community,” said Pommier.

His group decided to expand the publicly available CellMiner (<http://discover.nci.nih.gov/cellminer/home.do>), a web-based open access tool, to include both the DTP drug database and their own genomic database. The goal was to create a user-friendly tool to allow anyone to analyze drug responses with respect to a variety of genomic properties.

Paul Liu, M.D., Ph.D., Senior Investigator, National Human Genome Research Institute (NHGRI), for example, has been studying the interaction between transcription factors RUNX1 and CBF- β in certain forms of leukemia. From a high-throughput assay of close to 250,000 compounds, one hit emerged that directly inhibited interaction of these transcription factors. Using the newly expanded CellMiner, the team could show that sensitivity to this compound correlated with the expression of RUNX1 and CBF- β in NCI-60 cell lines.

The power of the database continues to grow. A recent paper in *PLOS ONE* describes newly added comparative genomic hybridization (array CGH) data; and, through a collaboration with Paul Meltzer, M.D., Ph.D., Chief of CCR’s Genetics Branch, methylation and RNA sequencing (RNAseq) data are soon to be added.

Pommier notes that both the Broad Institute in Mass. and the Sanger Institute in the U.K. have more recently adopted similar, larger-scale approaches through the Cancer Cell Line Encyclopedia (CCLE) and the Cancer Genome Project (CGP), respectively. Each encompasses 1,000 cell lines. The majority of the NCI-60 cell lines are included in these databases. “It’s a rich environment for us to do cross comparisons and mine the uniquely vast NCI drug database,” said Pommier.

(Photo: R. Baer)



Stephen Hewitt, M.D., Ph.D.

Tissue Microarrays

The Tissue Array Research Program (TARP) was created in 2000 to distribute tissue microarrays (TMAs)—slides containing up to 500 different formalin-fixed, paraffin embedded tissues—representing the most common human malignancies, which could be used for immunohistochemistry staining to identify the presence of particular proteins of interest. The technology for construction of TMAs had just been developed by NHGRI and its value to cancer research was quickly recognized.

In addition to applying these standard TMAs, TARP now works with NIH investigators with more specialized needs. “We provide complete lifecycle support,” explained Stephen Hewitt, M.D., Ph.D.,

Staff Clinician in CCR’s Laboratory of Pathology. “We assist in identifying a source of the material, construct the tissue microarray, perform the immunohistochemistry, and provide interpretations.” They have also adapted the NCI-60 cell line as an array for immunohistochemistry, which is available through NCI’s Cancer Therapy Evaluation Program (CTEP).

Among his many collaborations, Hewitt has been working with Udayan Guha, M.D., Ph.D., Investigator in CCR’s Thoracic and Gastrointestinal Oncology Branch. Guha uses mass spectrometry to identify potential therapeutic targets for lung cancer in cell lines, focusing on the epidermal growth factor receptor (EGFR) pathway. “With a postdoc in my lab, we obtained a cohort of 300 lung cancers from Japan and were able to confirm with immunohistochemistry that targets he had identified are clinically relevant,” said Hewitt. This effort has now moved forward to support Guha’s rapid autopsy protocol.

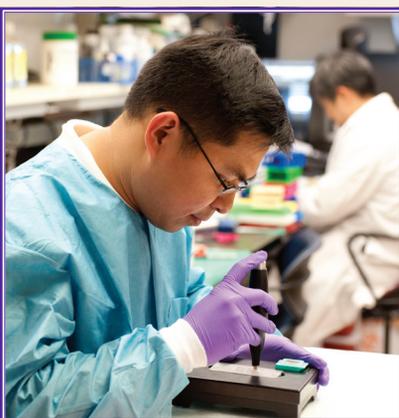
“It’s really not uncommon for studies to take many years to come full circle,” said Hewitt. He has worked with Philip Taylor, M.D., Sc.D., Senior Investigator in NCI’s Division of Cancer Epidemiology and Genetics since 2002, when they

constructed a tissue microarray of squamous cell carcinomas from patients in China. They followed up initial biomarker analysis with a study of patients that were undergoing screening, to identify distinct roles in tumor metastasis. “Recently, we built an array to compare normal tissue with tumors, looking for markers of the earliest stages of disease.”

In addition to tissue microarray work, Hewitt’s group performs antibody validation, troubleshoots biospecimen handling, and develops instrumentation. They filed a patent for a low-cost tissue arrayer in 2003 that was commercialized via the Small Business Innovation Research (SBIR) program. They are in the process of adapting that instrument to construct tissue microarrays out of frozen tissue.

“Target proteins come from all over—mRNA microarrays, mass spectrometry, proteomics, and now genome-wide association studies (GWAS). But at the end of the day, when someone wants to translate them into clinically relevant pathophysiology in large populations and look at outcomes, they basically come down to tissue microarrays and immunohistochemistry,” said Hewitt. “We provide the expertise for that validation.”

(Photo: R. Baer)



Kris Ylaja demonstrates the template arrayer, the latest generation of tissue array instruments.

To learn more about Dr. Gottesman’s research, please visit his CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=mgottesman>.

To learn more about Dr. Hewitt’s research, please visit his CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=shewitt>.

To learn more about Dr. Pommier’s research, please visit his CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=pommier>.

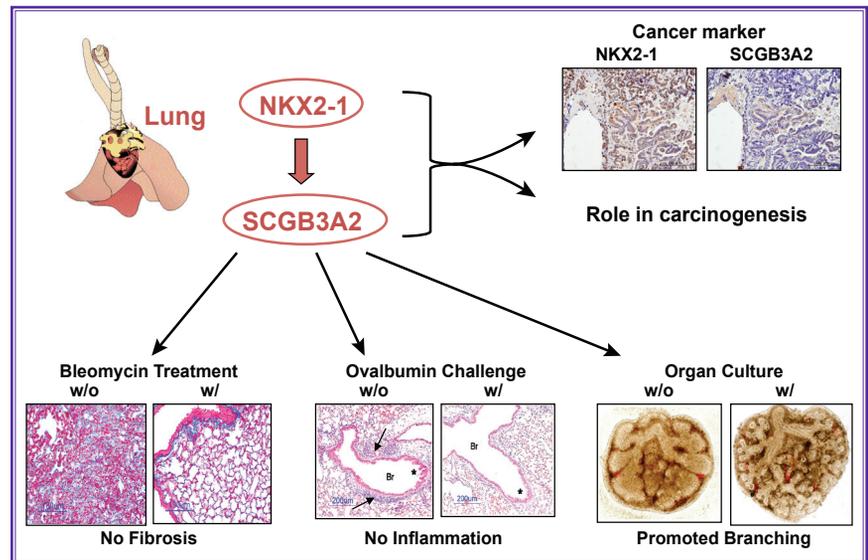
Influence of a Master

In 1991, Shioko Kimura, Ph.D., Senior Investigator in CCR's Laboratory of Metabolism, cloned the transcription factor *Thyroid-specific enhancer binding protein (T/EBP)* based on its ability to bind to an enhancer region in the promoter of the thyroid peroxidase gene. *NKX2-1*, as the 38-kDa protein is now known, plays a central role in early development of the lung, thyroid, and ventral forebrain; its expression is also associated with cancers of the lung and thyroid. Kimura's laboratory has made several genetically engineered mouse models that she has shared with the research community, while continuing to focus her interests on development and cancer of the thyroid and lung. Her laboratory has discovered a molecule downstream of *NKX2-1*, secretoglobin (*SCGB*) 3A2, on which they have recently filed patents for its therapeutic potential.

Master Regulator

"Years ago, I was working on the regulation of thyroid peroxidase gene expression," began Kimura. Thyroid peroxidase is an important enzyme in the production of thyroid hormones from thyroglobulin. "I cloned the thyroid peroxidase gene and was analyzing the promoter, which led me to a protein binding 5.5 kB upstream of the transcription start site." This protein, which she called T/EBP, was also found by a group in Italy and named thyroid transcription factor-1 (TTF-1), a transcriptional activator of the thyroglobulin gene which binds a homeodomain with strong homology to the *Drosophila* NK2 homeodomain. *NKX2-1*, as the transcription factor is now known, is a master regulator of many more thyroid and lung-specific genes, and the proteins encoded by these genes are essential for the function and homeostasis of the respective organs.

Kimura's work on *NKX2-1* as a developmental transcription factor led her to its role in cancer. Her laboratory has created several mouse models to study *NKX2-1* function, including mice that express *Nkx2-1* in the thyroid at half the level of the normal mouse thyroid. However, the expression varies at the individual cell level (as distinct from heterozygous mice that have *Nkx2-1* expression reduced by half in all cells within the



(Figure: S. Kimura, CCR)

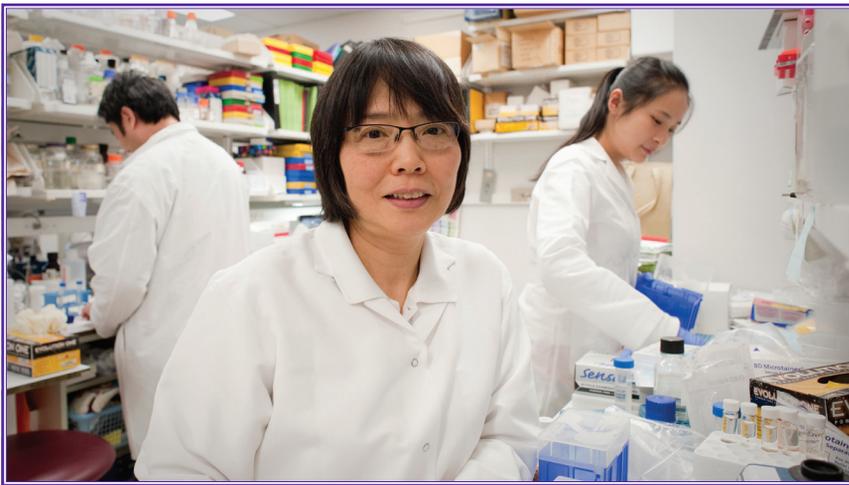
SCGB3A2 was identified as a downstream target for *NKX2-1*, one of the master transcription factors in lung. *SCGB3A2* demonstrated 1) anti-fibrotic activity in the bleomycin-induced pulmonary fibrosis model mouse, 2) anti-inflammatory activity in the ovalbumin allergic inflammation model mouse, and 3) growth factor activity promoting lung development as shown by *ex vivo* embryonic lung organ culture. *SCGB3A2* can be used as a tumor marker just like *NKX2-1*, which has a role in lung carcinogenesis and is an established diagnostic marker for lung adenocarcinomas. The role of *SCGB3A2* in lung carcinogenesis is unfolding.

tissue). Approximately 20 percent of these mice exhibited atrophic/degenerative thyroids and frequent adenomas, while the rest developed thyroids with extraordinarily dilated follicles, suggesting a role for *NKX2-1* in disease. Based on these results, her team decided to test the susceptibility of these mice to cancer induced by a genotoxic carcinogen. They found an increased incidence of thyroid adenomas associated with a doubling of the normal cell proliferation rate.

Human thyroid cancer association studies have also found a link with the human homologue *NKX2-1*.

Kimura has followed the trail of *NKX2-1* as it relates to thyroid development and carcinogenesis, while collaborating with other laboratories to explore its roles in the lung. "We are also interested in the forebrain, and have shared our mice with collaborators who specialize in brain development and function, but so far this line of investigation has

(Photo: R. Baer)



Shioko Kimura, Ph.D., (center) with Shigetoshi Yokoyama, Ph.D., and Yan Cai, M.D., Ph.D., working in the lab

been slow due to the complexity of working with this organ.”

Most recently, she has shared her expertise and transgenic mice with the laboratory of Tyler Jacks, Ph.D., at the Massachusetts Institute of Technology; and, they recently co-authored an analysis of conditional *Nkx2-1* disruption in normal and cancerous lungs. *NKX2-1* expression is seen in 75 to 85 percent of human lung adenocarcinomas, where it is associated with a better prognosis. Their work suggests *NKX2-1* is important for maintaining a pulmonary differentiation state that results in a less aggressive cancer.

Downstream Hope

In 2001, Kimura and her colleagues identified a novel target gene of *NKX2-1*, Uteroglobin/Clara Cell Secretory Protein-Related Protein (*UGRP1*), which is primarily expressed in lung airway epithelial cells, but also found in the thyroid. It was later recognized as part of the secretoglobin (*Scgb*) gene superfamily, which is involved in lung inflammation; it was thus renamed *SCGB3A2*.

SCGB3A2 is a secretory protein, whose receptor is unknown. Like the most studied founding member of the *Scgb* gene superfamily, *SCGB1A1*, it has significant anti-inflammatory activity. Kimura’s laboratory has identified roles for the protein as a growth factor

required for fetal lung development. It also has antifibrotic activity.

In 2007, based on her work on this molecule, Kimura and NCI filed a patent to use *SCGB3A2* or a molecular mimic to treat the development of neonatal respiratory distress, promote lung development, and reduce lung damage due to fibrosis that results from certain anticancer agents. She is working with a local biotech company to advance this agent to the clinic.

“When we found that this protein has anti-inflammatory activity combined with growth-promoting activities, I felt there should be a connection to cancer,” said Kimura. She and her colleagues went on to demonstrate that *SCGB3A2* is overexpressed in lung carcinomas, particularly in human adenocarcinomas. More recently, they discovered that *SCGB3A2* may also have anticancer activity and have broadened their intellectual property accordingly. They have shown in more than one mouse model, that administration of *SCGB3A2* reduces tumor burden and metastatic migration to the lung.

“We are working hard to clarify the secretoglobin signaling pathway and how its antitumor activity works; we want to understand how widely this can be applied to adult cancers and whether, if we can target this protein, we can decrease lung cancer incidence and metastasis,”

said Kimura. And of course, if they can identify *SCGB3A2*’s receptor, they will address many more questions about its function.

“We have been working for 7 or 8 years to find the receptor for this molecule. We’ve done everything: cDNAs, overexpressions, pull downs... Receptor isolation and identification is just not that easy to do,” said Kimura. “But recently, we’ve found a candidate through protein-protein interaction arrays which is a potential receptor in tumors and normal tissues. Finally, we see a light at the end of the tunnel.”

Under Construction

Kimura’s laboratory is also working on establishing cell lines from adult thyroid stem cells, a challenge all the more daunting because there are no known thyroid stem/progenitor cell-specific surface markers. “The thyroid is so small in mice that for one study, you need 30 to 40 mouse thyroids and then you can only do one or two experiments,” said Kimura. “We are establishing a cell line to see if we can produce a more stable source of thyroid stem cells.”

Once established, the cell line could be manipulated to address several research questions. It could be transformed as a model of cancer initiation. Kimura also suspects that *NKX2-1* may be involved in stem cell niche maintenance, a hypothesis she plans to test once she can establish the cells.

As with her work on the thyroid peroxidase gene, which started her on the trail of *NKX2-1*, Kimura knows that the true value of an experimental pursuit is as much in the new questions it enables as in the answers it provides.

To learn more about Dr. Kimura’s research, please visit her CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=kimura>.

Gut Check: A Career in Colon Cancer Research

Sanford Markowitz, M.D., Ph.D., is the Markowitz-Ingalls Professor of Cancer Genetics at the Case Western Reserve University School of Medicine. He is also Principal Investigator of the Case GI SPORE, an NCI-Designated Specialized Program of Research Excellence in Gastrointestinal Cancers. After studying at Yale and completing his medical residency at the University of Chicago, Markowitz became a Fellow at the NCI-Navy Medical Oncology Branch, headed by John Minna, M.D. From there, he accepted a position at Case Western, where he built the foundations of a colon cancer clinical research program with his colleague and fellow NCI alum, James Willson, M.D. Over the years, his research has led to the identification of key genetic drivers of colon cancer, including the first oncogenic mutations associated with the transforming growth factor-beta (TGF- β) pathway, and molecular tests for early detection.

(Photo: Courtesy of S. Markowitz)



Sanford Markowitz, M.D.

Working in the NCI-Navy Branch was a wonderful opportunity; the program attracted an extraordinarily talented group of people. John Minna's interest was in lung cancer, but the work he led became a roadmap for studying solid tumors. Back then, there were no disease models; one really had to start at the very beginning to create the cell lines

and the tissue banks. The seminal cancer discoveries were being made in blood cancers, in part, because cells were readily available from a blood draw and many were easy to culture. I had a long-standing interest in colon cancer for personal reasons. My father was diagnosed with the disease when I was an intern. So when I developed my own research

agenda, it was natural to combine that personal motivation with ideas stimulated by my exposure to solid tumor research. The Case Western leadership, under Nathan Berger, M.D., was committed to building a solid tumor research program, making my appointment a natural fit.

One of the early successes of our program was the discovery that TGF- β receptors were tumor suppressor genes. We found a unique sequence in these receptors that required DNA repair mechanisms for normal processing, thereby simultaneously providing the first genetic proof that TGF- β signaling was a tumor suppressor pathway and a mechanistic explanation for the appearance of colon cancer in Lynch Syndrome, which is associated with an inherited defect in DNA repair. We now know that one-third of all colon cancers are associated with alterations in TGF- β receptors, with or without concomitant defects in DNA repair mechanisms, and that different defects in TGF- β signaling are present in many other colon cancers.

Causation

In the last four years, we've finally been able to answer a key question that led from our initial discovery: What does TGF- β do in the gut that makes it so important as a tumor suppressor? Through a series of studies, we found that TGF- β metabolically suppresses an important oncogenic pathway, the cyclooxygenase 2 (COX2) pathway. COX2 is involved in inflammation; it is the target of drugs like aspirin and Celebrex. Interestingly, it has been known for many years that taking aspirin can lower the risk of colon tumors.

The gut also produces a naturally occurring inhibitor of the COX2 pathway, 15-hydroxyprostaglandin dehydrogenase (15-PDGH), which in turn is activated by TGF- β . Where COX2 promotes the synthesis of prostaglandins, 15-PDGH causes their degradation. Normally, 15-PDGH is strongly expressed in the gut; in colon cancers, this activity is downregulated. In disease models, we have shown that 15-PDGH can suppress tumor activity and that removal of 15-PDGH promotes vulnerability to tumors.

Moreover, people have individualized levels of 15-PDGH in the gut. Our collaborator, Monica Bertagnoli, M.D., at Brigham and Women's Hospital in Boston, led a clinical trial which demonstrated that Celebrex could reduce the recurrence of colon polyps by almost 50 percent. When we looked at the nonresponders, we found that they had low levels of 15-PDGH. We have now confirmed in similar studies with Andy Chan, M.D., Charlie Fuchs, M.D., and Shuji Ogino, M.D., Ph.D., that low levels of 15-PGDH are also associated with resistance to colon tumor prevention with aspirin. Thus, we have found a marker that is important in determining who would benefit from aspirin-type drugs as part of a tumor prevention strategy.

We are also beginning efforts to develop drugs that target the 15-PDGH pathway. Gratifyingly, that means we are once again working with Jim Willson, who moved from Case Western to the University of Texas (UT) Southwestern. UT Southwestern has made a significant investment in drug development infrastructure, recruiting Bruce Posner, Ph.D., to lead high-throughput screening, Joe Ready, Ph.D., to lead medicinal chemistry, and Noelle Williams, Ph.D., to lead pharmacology and formulation.

Early Detection

The importance of early detection has been strongly established for colon cancer. Through colonoscopies, we know that if you can catch it early, you can prevent death from the disease. But colonoscopies have been around since I was a medical student and colon cancer is still the second leading cause of cancer death in the United States. A few colon cancers are probably so aggressive they develop after screening, but many people simply do not get screened.

We've been developing non-invasive methods to detect colon cancer. A decade ago, a fellow in my laboratory, Bill Grady, M.D., (who now has his own lab at the Fred Hutchinson Cancer Center), found abnormally methylated DNA in the blood in about 15 percent of colon cancers. Five years ago, we did a genomic screen and found that the vimentin gene was methylated in about 80 percent of colon cancers. We've since shown that we may be able to detect up to 80 percent of early stage cancers (those that can be cured surgically) and even polyps by screening stool samples for this marker.

Methylated vimentin is part of a national multicenter trial to validate biomarkers for early detection against colonoscopies in 6,000 people

through NCI's Early Detection Research Network. Through our GI SPORE, we are asking an associated question, namely what does it mean if a biomarker test is positive but the colonoscopy is negative? We've launched a clinical trial here at Case Western to follow people with positive stool tests and normal colonoscopies to see if the DNA test was right even though the lesion was not detected.

Translation

There is a gap that everybody recognizes and nobody yet knows how to fill between finding something in a lab that could actually have application as a therapy or diagnostic test and bringing it to clinical fruition. Capital, expertise, and commercial dedication are required to bring innovations through all the appropriate regulatory requirements. There are a few places in the country that have excelled at attracting venture capital for spinning companies out, but opportunities are often geographically confined.

This is particularly important now because research and development within Big Pharma is downsizing and, meanwhile, academia is generating fantastic opportunities. When I started out, I would have never guessed that we would be where we are now. The revolution in genetics and genomics and our ability to screen hundreds of thousands of compounds to find new drug leads is all beyond our wildest imaginings when I was in training. The possibilities still have a freshness and excitement to them that really make coming into the lab everyday a joy.

Adopting Bodily Defenses to Cure Cancer

Steven Rosenberg, M.D., Ph.D., Chief of CCR's Surgery Branch since 1974, is a genuine pioneer in the development of immunotherapies for cancer. In 1985, he was the first to demonstrate that an immunotherapy—specifically, the administration of interleukin-2 (IL-2)—could cure certain patients with metastatic disease. A few years later, he opened the doors to cell-based immunotherapies by showing that tumor-infiltrating T lymphocytes (TILs) could be isolated from melanomas, stimulated to proliferate, and reintroduced into patients to promote cancer regression. Since that time, Rosenberg and his colleagues have discovered and developed innovative ways to improve upon cell transfer therapies. He was the first to insert foreign genes into humans in 1990 and the first to demonstrate that genetic modification of T cells could mediate cancer regression in patients with melanoma, sarcomas, and lymphomas. Rosenberg has written more than 1,100 scientific articles, as well as eight books, and was the most cited clinician in the world in the field of oncology between 1981 and 1998.

(Photo: R. Baer)



Steven Rosenberg, M.D., Ph.D.

My colleagues and I are trying to develop curative treatments for patients with metastatic cancer. While I was still a surgical resident in Boston, I came across a patient who had spontaneously recovered from an untreatable, aggressive stomach cancer. The patient's own body had cured his disease. Since that time, I've seen the immune system as our best source of untapped therapeutic potential.

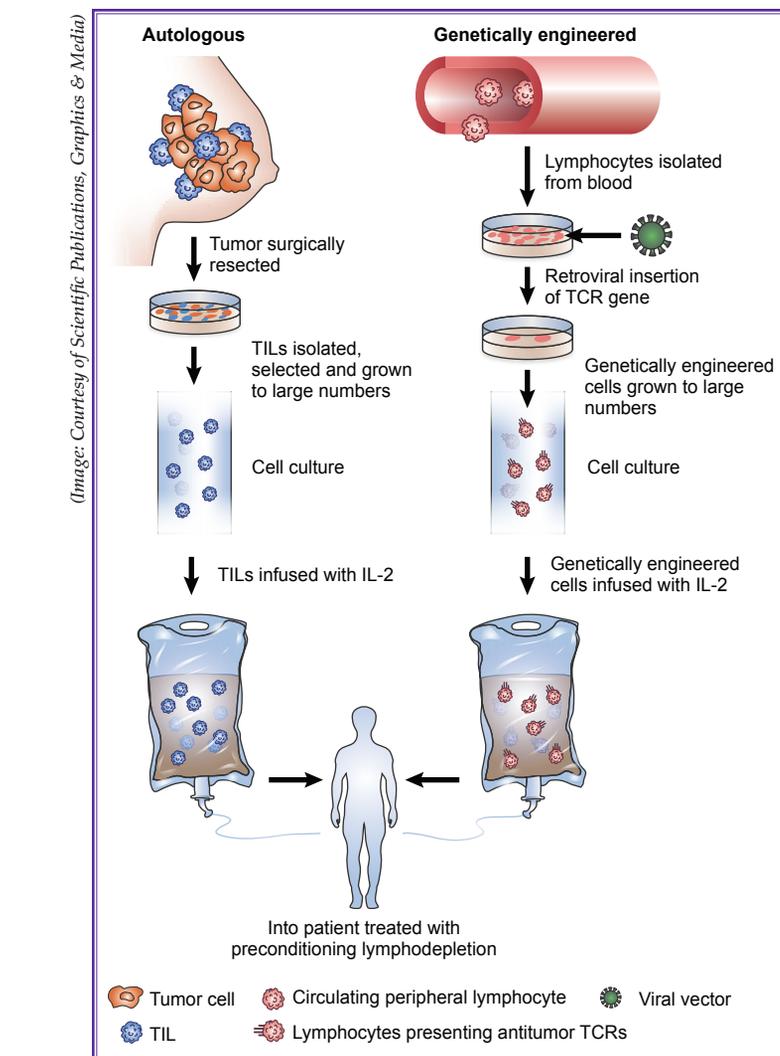
After several years of research, we developed IL-2 as a systemic treatment for metastatic disease in the 1980s, and found that it could cure a small fraction (seven to eight percent) of patients with metastatic melanoma and renal cancer. IL-2 is a cytokine that stimulates the growth

and proliferation of lymphocytes. We have also worked with other systemic immunomodulators and were the first to demonstrate the cancer regression properties of an anti-CTLA-4 antibody in humans with metastatic melanoma.

However, most of our current research is based on the development of anticancer T cells that can recognize and destroy melanomas and other cancers. TILs are naturally occurring T lymphocytes that are capable of attacking tumors but have apparently been unsuccessful in fending them off unaided in our patients. In 1988, we described a procedure for extracting TILs from the surgically resected tumors of cancer patients, allowing the cells to proliferate outside the body, and then reinfusing them in sufficient numbers to successfully eliminate tumors. Since then, we have worked on a number of strategies for optimizing this adoptive cell transfer immunotherapy (ACT) approach.

Destroying the Competition

In 2002, we demonstrated that we could increase the therapeutic efficacy of ACT dramatically, by first extracting TILs, then depleting the patient's remaining lymphocytes with a combination of drugs (cyclophosphamide and fludarabine) before reinfusing the expanded population of TILs into the patient. We recently reported that among the first 93 patients with metastatic melanoma who were treated in this way, 20 had complete regressions. Of those 20, 19 maintained their tumor-free status for more than six years and some have been followed for more than 10 years. We reported these data from three successive pilot trials; in the last trial, 40 percent of patients experienced complete cancer regression.



Adoptive cell transfer immunotherapy using autologous tumor-infiltrating T lymphocytes (TILs) extracted from patients' tumors or using lymphocytes isolated from blood and genetically engineered to express antitumor T-cell receptors (TCR). After cells are stimulated to proliferate *in vitro*, they are reintroduced into patients whose remaining lymphocytes have been depleted.

We don't know why some patients respond completely to the therapy and others only respond partially or not at all. There is no relationship between the size of the tumors, where they are located, or any prior treatments and the likelihood of regression.

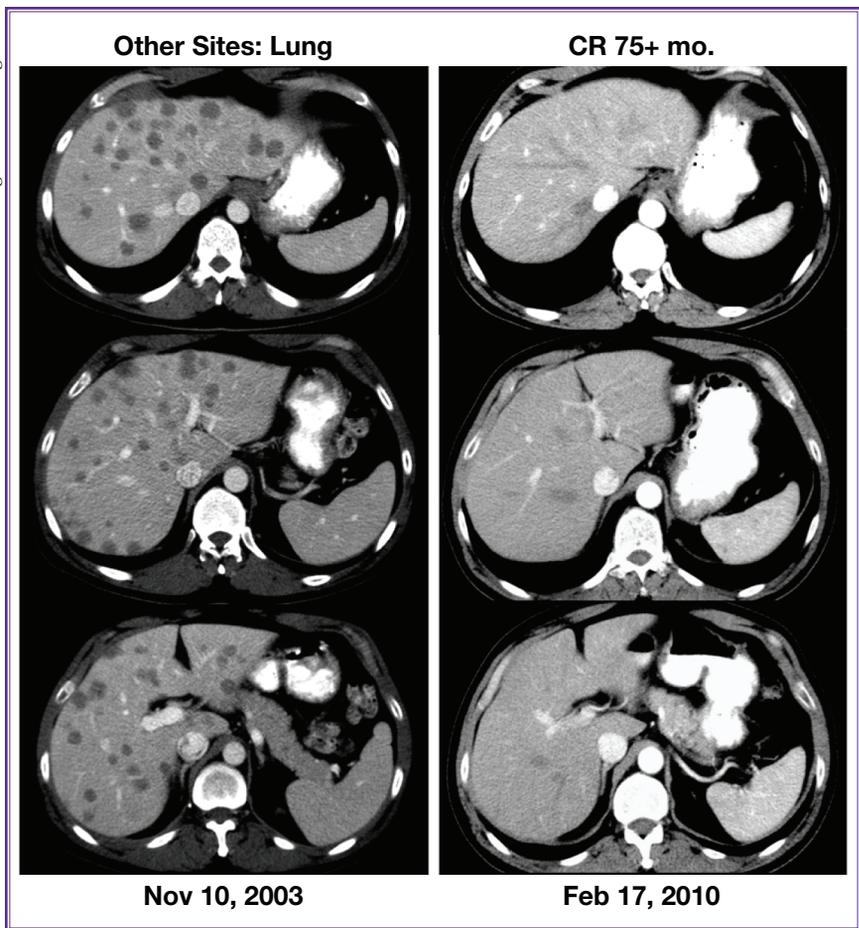
We have studied the molecular and cellular mechanisms underlying the enhancement of ACT therapy by depletion of lymphocytes, both in our clinical work and in animal models. There are many types of lymphocytes in the body and some, such as regulatory T cells

and myeloid-derived suppressor cells, actually dampen the immune response. Moreover, without other lymphocytes competing for and depleting circulating cytokines like IL-7 and IL-15, TILs can benefit from exclusive access to their growth-promoting abilities. In fact, IL-15 is not normally found in the blood of patients, but is found in high levels after the chemical depletion of lymphocytes.

Better, Stronger T Cells

We're working hard now to extend the development of our cell transfer

(Image: S. Rosenberg, CCR)



Computed tomography (CT) images of a melanoma patient before and after treatment with adoptive cell transfer immunotherapy. The patient has an ongoing complete regression for more than 10 years.

therapies from melanoma to other cancer types. Melanoma is one of the few tumors that give rise to TILs naturally. In order to treat other kinds of solid tumor metastases, we have developed methods for genetically engineering a patient's own circulating lymphocytes to recognize the cancer.

In 2006, we showed for the first time that genetically modified lymphocytes could mediate tumor regression. In patients with melanoma, we used a retrovirus to introduce the gene for a tumor-specific T-cell receptor into patients' T cells. The receptor bound a molecule found on the surface of melanoma cells known as "melanoma antigen recognized by T cells 1" or MART-1. There are

a series of known cancer-related antigens that can be used. Some mutations are shared among cancers; others like CD19 are present on normal B cells as well as lymphomas. We've gone on to successfully treat patients with synovial cell sarcomas with genetically modified T cells targeting the cancer-testis antigen NY-ESO-1, which is present on many common epithelial cancers. The NY-ESO-1 testis antigen is expressed during fetal development but has very little expression in adults. It is, however, re-expressed in many adult cancers.

T cells can be engineered with receptors that are not found in the naturally occurring repertoire of immune responses. Chimeric antigen receptors (CARs) can be designed that not only bind to tumor

antigens, but also contain additional components that stimulate the T cell to respond and proliferate. In 2010, we demonstrated that we could successfully treat patients with B-cell lymphomas and leukemias by engineering their own T cells to produce a CAR that recognized the B-cell antigen CD19. Some patients from that trial have remained progression free of lymphoma for over four years.

We're also looking at ways to genetically enhance T cells, beyond their ability to identify tumor antigens. For example, T cells could be engineered to produce their own source of IL-2 or IL-12, subverting the requirement for supplemental cytokines.

We have over a dozen trials underway to use ACT for a variety of cancers and T-cell strategies. A full list is available here: https://bethesdaclinicaltrials.cancer.gov/clinical-trials-search-physician?field_investigator_name_value=Rosenberg.

Identifying New Targets

For the immune system to recognize cancer as different from "self," it must recognize and attack unique protein features. We know some of those targets, but the majority still eludes us. The future of ACT lies in finding ways to engineer cells to attack the unique mutations on an individual's cancer. Melanomas are distinct among cancers by virtue of having a much larger number of mutations, i.e. several hundreds, than most cancers, which have on the order of 20 to 70 mutations. Tumors with larger numbers of mutations are likely to give rise to immune reactions that are capable of affecting a growing cancer, which may explain the ready availability of TILs in melanomas.

However, just because a protein contains a mutation, that does not mean it is going to be recognized

and attacked by the immune system. One of the major efforts in our laboratory is to identify mutations in individual cancers that are capable of eliciting a T-cell response. Last year, we published a paper in *Nature Medicine* in which we described a new technique—combining whole-exome sequencing and immunology—to identify mutations in melanoma samples, which generate a T-cell response. We used a bioinformatics approach to predict which mutations would generate protein fragments that would be presented to T cells as a threat. Then we tested those predictions using TILs from patients whose tumors had completely regressed after ACT. More recently, we have developed a new technique to identify unique immunogenic mutations in patients with common epithelial cancers. We published in *Science*, the successful use of this approach in a patient with a metastatic bile duct cancer. We believe this approach may be a generally applicable method for identifying mutated antigens expressed in a variety of tumor types.

Commercial Development

The immune reaction against cancer is very complex, but the fact that TILs can be used to cure patients with metastatic melanoma shows that this kind of therapy is capable of eliminating every last tumor cell. The challenge is enormous. Last year 580,000 Americans died of cancer; but, I believe these immunotherapies have unlimited potential if we can develop clever ways to create T cells that identify cancer mutations.

There are many companies that are involved in developing adoptive cell transfer strategies to treat cancer; NCI has a Cooperative Research and Development Agreement (CRADA) with Kite Pharma to develop our gene modification



(Photo: R. Baer)

Patient Jay Lake and Steven Rosenberg, M.D., Ph.D., discuss Jay's case the day before his surgery.

approaches for ACT. Kite was founded in 2009, specifically to commercialize cancer immunotherapy approaches. NCI also has a CRADA with Lion Biotechnologies to commercialize TIL therapy. Many other larger pharmaceutical companies are also becoming very interested in these cell transfer therapies. The Seattle-based biotechnology company Juno Therapeutics just raised one of the largest early private investments to develop CAR-based immunotherapies.

Our Patients

My own laboratory has about eight to ten scientists at any given time. But our Tumor Immunology Section in CCR has 30 to 40 people that are all working towards better cancer immunotherapies. I make rounds every day, visiting all the patients we are treating. We often have about a dozen patients with advanced cancer at any one time, each receiving these new immunotherapy approaches. I spend probably a third of my time on clinical work and most of the rest of the time on laboratory research.

When information is published

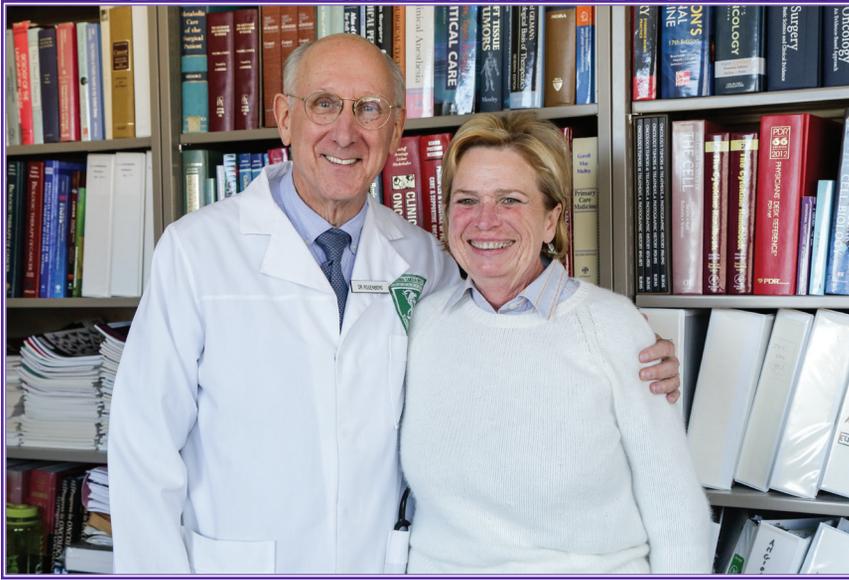
about new treatments for patients who have otherwise untreatable diseases, we are besieged by patients asking to be included in trials. Whereas 20 years ago, all our referrals came from oncologists, now half of our inquiries come from the patients themselves. Many who call us have been through other treatments and are desperately seeking something that might be of use. Some we can treat, but some are not eligible given the criteria we have.

Every patient we treat at CCR has a disease that cannot be successfully treated by today's standard-of-care medicine. All have advanced cancer that is refractory to existing treatments. The patients we see have a very limited life expectancy. Our goal is not to administer today's treatments; we are here to create the treatments of tomorrow. But tomorrow no longer seems so far away.

To learn more about Dr. Rosenberg's research, please visit his CCR website at <http://ccr.cancer.gov/staff/staff.asp?name=rosenberg>.

Immunotherapy's First Cure

(Photo: B. Brattson)



Steven Rosenberg, M.D., Ph.D., and former patient Linda Taylor, who has been in complete cancer remission for 30 years.

Linda Taylor was first diagnosed with melanoma in 1982. Taylor was a self-described hard-charging, ambitious, “workaholic” officer in the Navy at the time. After surgery to remove her tumors and being told she had a 50-50 chance of survival, Taylor took an assignment to Guam as a Flag Secretary. When several small nodules appeared on her arm some weeks later, the Admiral sent Linda to Bethesda Naval Hospital, which is now the Walter Reed National Military Medical Center. “They told me that there wasn’t much they could do for melanoma once it hits the system,” said Taylor. “They thought maybe I had 17 months, but they didn’t give me any real hope.”

The Hospital is just across the street from the NIH Campus in Bethesda and during her stay, a doctor visiting from NCI reviewed her case and recommended an experimental treatment. She was treated under two protocols, one of which involved a month-long hospital stay. When neither treatment was successful, one of the doctors suggested a new protocol, using interleukin-2 (IL-2) to treat

advanced metastatic cancer, run by Steven Rosenberg, M.D., Ph.D.

“I have to be honest, I was pretty reluctant to go through with another experimental treatment,” said Taylor. “The last two had been very draining and I felt like my quality of life had become nonexistent. But my family encouraged me to talk to Dr. Rosenberg. He was a very unassuming, low-key guy. He told me that he had not had a tremendous amount of success so far with the new therapy, but had great hope. He was very convincing.”

During her month-long treatment, Taylor was followed closely by Rosenberg’s team, including Stephen Ettinghausen, M.D., and Research Nurse Claudia Seipp. Taylor experienced severe chills, gained a lot of weight, and often had trouble breathing because of fluid build-up in her lungs. “I did well until the middle of December, but then I stopped breathing,” said Taylor. “Steven intubated me and eventually, I pulled right back out of it. I had one chipped tooth from the intubation and then the treatment was over.” Everyone was surprised when follow-up biopsies

revealed the complete death and disappearance of all the tumors.

“When I went into remission, nobody really thought it was going to last. They just didn’t know what it meant. So we just waited and watched,” said Taylor. The Navy had put her on the Temporary Disability Retirement List (TDRL) and stamped “Death Imminent” on her personnel file. After five years on TDRL, she returned as a Commander and was eventually promoted to Captain. She became Dean of Administration at the Naval War College in Newport, R.I. Taylor retired in 2001.

Taylor’s story is described in much more detail in Dr. Rosenberg’s book *The Transformed Cell*, where she is identified as Linda Granger. “When he first published the results of the IL-2 trial in the *New England Journal of Medicine*, all of sudden he was everywhere. His picture was on magazines. It took him by surprise,” said Taylor. “I was still on disability retirement from the Navy, and I wasn’t too keen on having a lot of visibility. So he changed my name in the book to protect my identity.”

“They say if you stay in remission for about five years, that’s a good rule of thumb that it won’t recur,” said Taylor. “But when you do an experimental protocol, they make it very clear you are breaking new ground and there are no rules. When I went to visit Dr. Rosenberg last December, he told me that he has never had a patient have a recurrence after 5 years’ complete remission. So he said, ‘I think you’re good to go.’ After 30 years, it’s still very emotional.”

During Taylor’s recent visit with Rosenberg, they were filmed for an upcoming PBS documentary on the history of cancer. Overseen by Ken Burns, the three-part series titled, *The Story of Cancer: The Emperor of All Maladies*, is scheduled to premiere in spring 2015.

CCR connections is available online at <http://home.ccr.cancer.gov/connections>.

Websites with More Information about CCR

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Additional Links

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