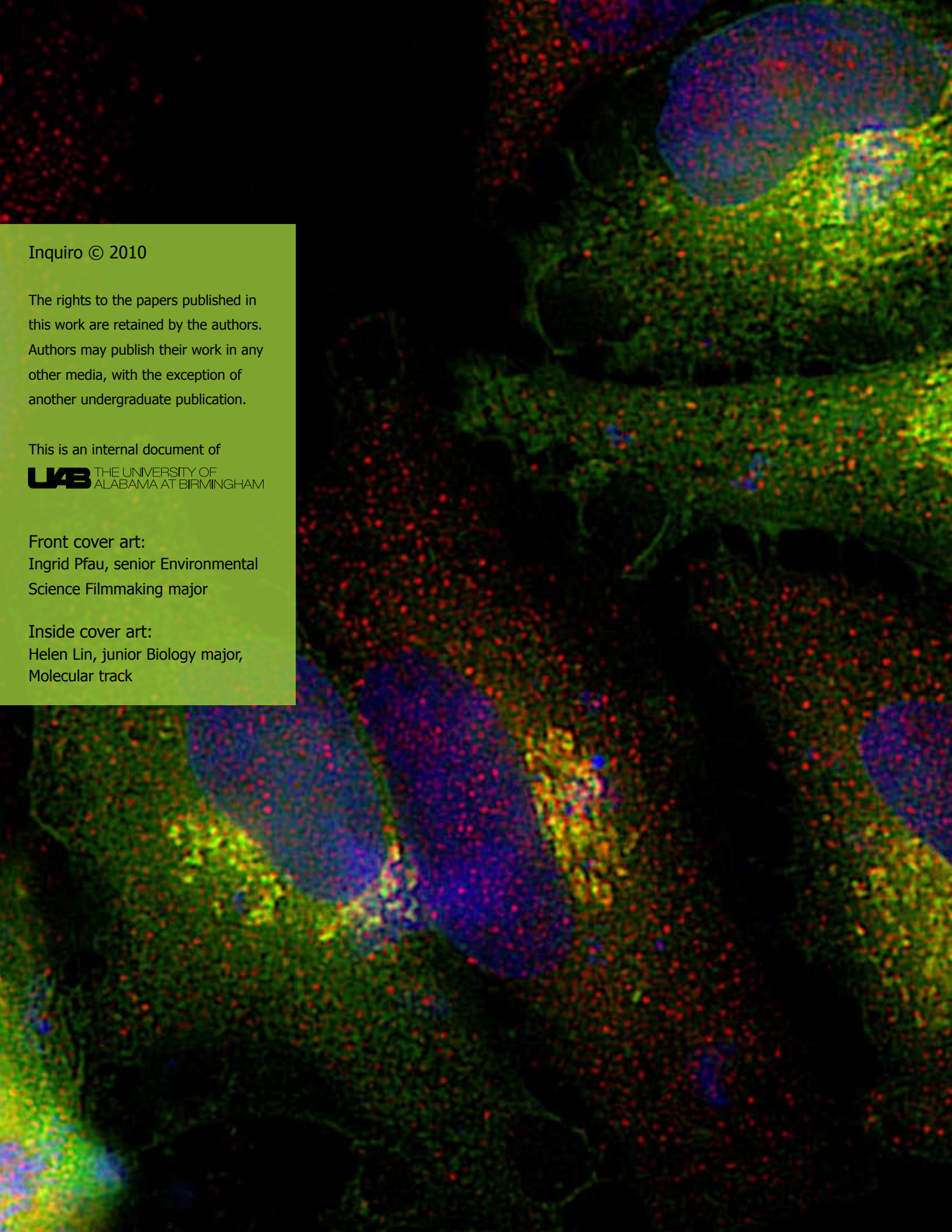
inquiro

Volume 4 • 2010

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM



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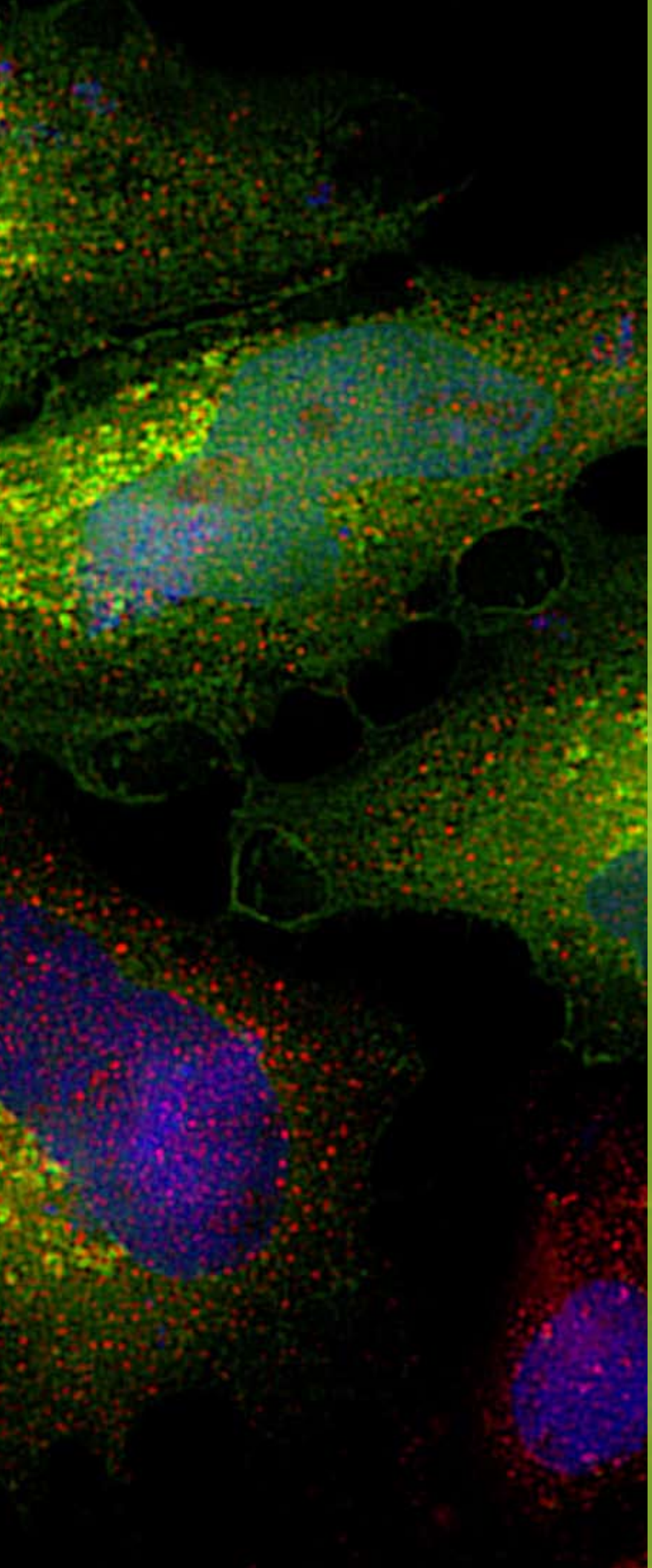

inquiro

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Founded and staffed by undergraduate students at the University of Alabama at Birmingham, *Inquiro* is an annual research journal produced as an outlet for the publication of undergraduate scientific research. UAB is an excellent undergraduate research university, and with the addition of a journal such as *Inquiro* in which to publish their findings, the package is complete. Any undergraduate student at UAB, as well as any student participating in a summer program at the university, is eligible to submit research. The rights to every paper published in *Inquiro* are retained by the author, leaving each individual free to submit to and publish in a larger national journal or magazine. Students are invited to submit research papers, short reports derived from posters or research narratives throughout the year.

inquiro staff

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from the editors

Inquiro: to search; to know.

Curiosity about the natural world has been a defining trait of our species since the beginning of time. Ancient civilizations all over the world developed methods to harness the power of nature and to explain the mysteries of the universe. The human spirit of discovery has survived millennia and flourishes now more than ever before. As we uncover the secrets of the human genome, the laws of modern physics, and the delicate balance of our environment, we embark on unprecedented journeys into the unknown. Furthermore, it is a journey that allows us to escape national borders, age differences, and even language barriers. This journey is for all humanity.

The journey of inquiry flourishes on the campus of the University of Alabama at Birmingham (UAB) through the cultivation of inquiring minds with dedication to scientific research. This environment entices students and faculty members alike to find solutions to countless issues throughout the world. Once discoveries have been made, an important part of research is the sharing of these findings. Shweta and I have experienced this desire to explore the unknown and have since decided to continually provide UAB an outlet with which to share their passion for research. This outlet assumes the form of a yearly publication known as *Inquiro*.

Within the first year of our undergraduate education, Shweta and I experienced the publication of the first volume of *Inquiro* in 2007. The launch of the journal initiated the beginning of our involvement with it. Both of us realized the impact such a journal has on the surrounding community, and we quickly pursued positions on the editorial board in 2008. Organized, edited, and published solely by undergraduates, we would be partially responsible for the success of the editorial. Already prepared from previous experiences with high school publications, the entire *Inquiro* process of editing and reviewing submissions, writing articles, and conducting interviews was very appealing to us both. We were specifically enthused about its relatedness to the scientific world, as we were both set on obtaining degrees in this field, Shweta in Biology and I in Chemistry.

After a successful second issue of *Inquiro*, we wanted to remain a part of the editorial board. Our growing passion for the journal and constant need to seek the next tier of challenges motivated us to apply for the Chief Editor positions. Receiving this position, we were overwhelmed with excitement to play an integral part in the publication of the journal. We had ambitions of making the journal bigger and more diverse, a feat we like to think we accomplished. The third volume of *Inquiro* exceeded all of our expectations, providing us with more submissions than previous years. These submissions came in a variety of disciplines, allowing us to publish the first ever computer science and neuroscience paper in that year's journal. Ultimately, the admiration and support of the journal received from students, faculty, and administration convinced us that we were successfully continuing the legacy started two years prior. The success of *Inquiro* stands as a testament to the unique atmosphere at UAB that encourages undergraduate students to participate in research.

Convinced that *Inquiro* would only continue to grow as a publication, we chose in 2010 to remain as Chief Editors for the fourth issue. Working as Editors in Chief the previous year had left Shweta and I with a sense of com-

mitment, and we desired to maintain the success and wellbeing of the journal for as long as our undergraduate careers would allow, especially through the transition from the School of Natural Sciences and Mathematics to the College of Arts and Sciences. In this past year, we have witnessed yet another increase in submission number, a greater diversity in the types of submissions, and even more submitted research narratives from outside of the editorial board. This year's journal boasts a psychology paper, a public health highlight, and the journal's first physics paper, allowing this edition the honor of being the largest and most diverse issue published thus far.

Because UAB is research-oriented, *Inquiro* was established from the need of an outlet through which UAB undergraduates can display their groundbreaking research. Every year, students have the opportunity to work in research labs, whether it is through summer programs, departmental honors, or independent studies. Although many university departments hold research symposiums throughout the year, such as the annual UAB Expo held in April, it is rare for students to have the opportunity to display their work before peers and faculty from other disciplines, as well as to the university community as a whole. These symposiums provide undergraduates ample opportunity to perfect their presenting skills; however, *Inquiro* allows undergraduates to polish their scientific-writing skills. While undergraduates may work in labs for a few semesters or a summer, it is unusual for students to publish their work in internationally peer-reviewed journals, simply due to time constraints.

This fourth issue of *Inquiro* helps to embody the unyielding legacy of the University of Alabama at Birmingham. This journal gives students the opportunity to partake in the scientific process and prepare their research for publication. Each paper is reviewed by at least one or two faculty members, so that students get a feel for submission and revision processes, preparing them for the graduate and professional world. Other universities such as Harvard, Columbia, and Yale have previously embraced the concept of the undergraduate journal. With the continued success of *Inquiro*, UAB students now have the opportunity of ascending to the undergraduate publishing ranks with the best and brightest students in the nation. Please join us as we blaze the trail for the future of undergraduate research at the University of Alabama at Birmingham!

—Andrew Buie and Shweta Patel
Chief Editors

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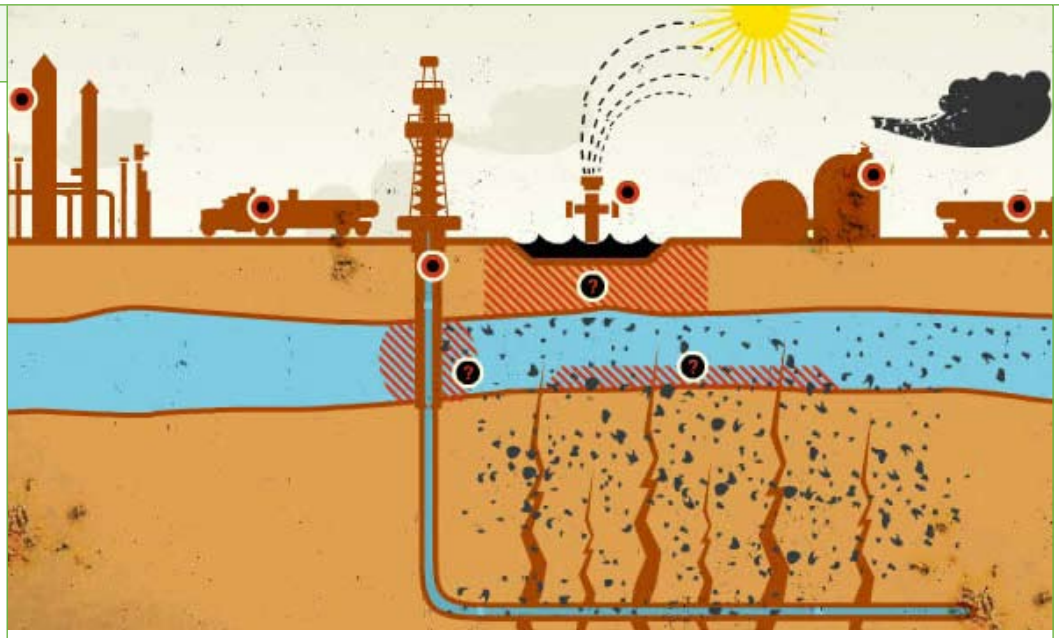
The Perils of Natural Gas

Helen Lin



With the recent trend towards energy independence, natural gas is becoming a popular alternative. There are an estimated 100 years worth of natural gas reserves within the United States, and it is cheap – cheaper than gasoline. In addition to its economic incentives, natural gas also burns with less polluting by-products than petroleum and coal.

A popular means of drilling for natural gas is to use hydraulic fracturing or fracking, which is commonly used in deep wells. Water and chemicals are pumped underground through a wellbore at a rate sufficient to increase the pressure enough to cause fraction formation in the rock. To keep fractures open, solid proppants, commonly sieved round sand, are added to the fracture fluid. Other proppants include ceramics, resin-coated sand, and radioactive sand to measure the fracture trace along the wellbore. Fracture fluids include water, gels, foams, nitrogen, carbon dioxide, and air. Volatile chemicals such as benzene, toluene, and xylene are commonly used. Presently, drilling companies are not required to disclose the chemicals used in their fluids. Drilling companies maintain that any chemicals used in their fractioning fluids are in dilute amounts. However, one to eight million gallons of fractioning fluid may be used while drilling, which could translate to several gallons of chemicals. Some of the chemicals used are carcinogens, and others can damage kidneys, the immune systems, and affect reproductive development even in low dosages.

The drilling of natural gas is a NIMBY (Not In My Backyard) activity. People living around natural gas wells protest the hazard to their lives. Fracturing fluid in particular has the potential to be especially hazardous. Mishandling of the fluid could lead to air pollution and contamination of ground water. Evaporated volatile fluids could mix with diesel exhaust from trucks and generators, producing ozone, and fumes can travel as far as 250 miles. Additionally, dissolved natural gas reaches the surface of water, creating highly toxic waste water, which contains about 596 chemicals. Protocol dictates that waste water should be trucked to water treatment plants before reentering the water system, but thirty to seventy percent of the water used in fracturing is left in the ground. Of the waste water that is removed, some evaporates before treatment. Clean ups from damages caused by mishandling of waste water and fracturing fluid will be monetarily expensive. Externalities such as loss of land value and both human and animal health concerns are difficult to measure.

Livestock and animals near drilling wells have been affected. Cows have stopped delivering healthy calves. Bulls have gone sterile, and some herds have stopped going into heat. A herd of sheep suddenly produced an unusually high number of still births. Farms blame the proximity of natural gas wells and contaminated water for the effects on livestock.

Livestock have not been the only ones affected. Larry and Laura Amos lived close to a natural gas well. One day, the water well exploded, spewing mud and water. The water was contaminated

with methane. Sometime later both Laura Amos and her infant daughter were diagnosed with rare adrenal tumors, likely caused by chemicals found in their drinking water.

Because the composition of the fractioning fluid is deemed proprietary, it is not disclosed to the public or professionals, making any treatments of injured personnel extremely difficult. A few days after treating a wildcatter who had been sprayed by fractioning fluid, a nurse was hospitalized for multiple organ failure. The drilling company refused to disclose the composition of their fractioning fluid so that the nurse could be treated. The physician eventually determined what caused the organ failure but was contractually silenced from ever disclosing it, even to the patient.

The main cause for concern for many nearby citizens is the contamination of drinking water with natural gas. Natural gas wells average 8000 feet deep, while most drinking aquifers are 1000 feet deep. The process of fracking could potentially allow natural gas to enter the water table. Fracturing fluid could also enter the drinking water because of its unpredictable migration path through different rock layers. The first federally documented contamination of the water table occurred in 2008, and, since then, more than 1000 cases have been reported.

The town of Dimock, Pennsylvania is a prime example of the dangers associated with natural gas drilling. New Year's 2009, a water well exploded, leading to an investigation that found the ground water to be contaminated with natural gas. The water in Dimock is undrinkable; the water is actually flammable, as demonstrated in the Josh Fox *Gasland* documentary.

While natural gas may seem like a good solution to energy dependence and greenhouse gas emissions, the industry needs to be held accountable for safety. Better safety measures and extraction techniques need to be developed to prevent further contamination of the water table and the air.

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Photo Source

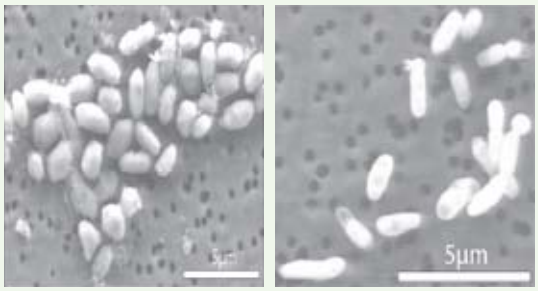
Fox J. Gasland. http://gaslandthemovie.com/whats-fracking.

Does the Poisonous Arsenic Support Life?

Atbin Doroodchi

A group led by Felisa Wolfe-Simon through a NASA funded project discovered a strain of bacteria called GFAJ-1 that uses arsenic as a metabolite. This strain of bacteria lives in the harsh conditions of Mono Lake in California. Conventionally, it was thought that the basic building blocks of life are carbon, hydrogen, oxygen, nitrogen, phosphorus and sulfur. However, the addition of arsenic to this category is revolutionary. "The definition of life has just expanded," said Ed Weiler, NASA's associate administrator for the Science Mission Directorate. Weiler also mentioned that in order to find life in outer space, we have to "broaden our definition of life." GFAJ-1 bacteria are bacillus bacteria, which belong to a common group of bacteria called Gammaproteobacteria. In this strain, phosphorus, which is commonly found in many other organisms, has been replaced with arsenic. Phosphorus is an essential element present not only in the DNA and RNA backbone but also in the metabolic pathways in the form of ATP. Arsenic—belonging to the same periodic group as phosphorus—is toxic because of its ability to replace phosphorus in the metabolic pathways in other organisms which will result in death.

Interestingly, GFAJ-1 showed a significantly higher growth rate in the presence of arsenic than they did in the presence of phosphorus. Wolfe-Simon and her group insist that GFAJ-1 has replaced phosphorus in their DNA and RNA backbone with arsenic. However, biochemist Steven Brenner expressed doubt on whether this strain uses arsenic in its DNA and RNA backbone. He thinks that a trace contamination can be a source of phosphorus for the bacteria. Brenner suspects that arsenic is used somewhere else in the cell. Nonetheless, this discovery is an important breakthrough in the fields of biochemistry, cellular and molecular biology, and astrobiology. This discovery changes our understanding about the definition of life and the chemical structure of DNA and RNA.



GFAJ-1 in the presence of arsenic (right) and phosphorus (left)
Image Credit: Jodi Switzer Blum, obtained from nasa.gov

science news

Discovery of Two New Horned Dinosaurs

Natalie Mitchell

Lukas Panzarin's reconstruction of the Kosmoceratops richardsoni

Scientists recently discovered two new species of horned dinosaurs in the Grand Staircase-Escalante National Monument (GSENM), a 1.9 million acre land tract in Utah. *Utahceratops gettyi* and *Kosmoceratops richardsoni* are predicted to have lived over 76 million years ago in Laramidia, a land mass formed when water flooded the central region of North America and isolated the eastern (Appalachia) and western (Laramidia) portions of the continent.

The bigger of the two dinosaurs, *Utahceratops gettyi*, stood 18 to 22 feet tall and weighed between 6600 to 8800 pounds. It had a skull 7 feet long, a large horn over the nose, and short, blunt eye horns that projected to the side. The second new species, *Kosmoceratops richardsoni*, stood 15 feet tall, weighed 5500 pounds, and had 15 horns, making it the most ornate-headed dinosaur known. While there is speculation about the purpose of the horns, it is likely that they were used to enhance reproductive success by intimidating members of the same sex while attracting the opposite sex.

This finding shrinks a large scientific gap that existed regarding dinosaurs of the area. Little crossover occurred between species in the northern and southern parts of Laramidia; however, scientists were unable to theorize reasons for this due to a lack of fossils from the GSENM. The discovery of these two new species will allow paleontologists to finally piece together an explanation for this isolation.

Source: Time Magazine

Embryonic Stem Cell Research: The Next Step

Khushboo Jhala

On the cornerstone of one of the most contentious areas of science and American politics, the first U.S.-government-approved clinical trial involving human embryonic stem cells injected a patient with millions of the potentially lifesaving cells on October 11, 2010. The patient, who remains anonymous to the media, is being cared for at The Shepherd Center in Atlanta for a paralyzing spinal cord injury. In accordance with the trial's protocol, the patient received injections within 14 days of the injury. The study focuses only on spinal cord injuries, one of medicine's most debilitating conditions, for which there are few, if any, effective treatments. If the trial proves to be a success, however, it could make way for treatments to cure or prevent Alzheimer's disease, Parkinson's, diabetes, and more. Because of this, the trial is considered a landmark case in this line of research. Evan Y. Snyder, director of the stem cell program at the Sanford-Burnham Medical Research Institute in San Diego, explains, "There's a lot of angst around these trials. There's going to be this perception that if the cells do not perform well, the entire field will be illegitimate."

The debate on embryonic stem cell therapy has been a major source of controversy on the political platform for years. The Bush Administration banned federal funding for research using newly created embryonic stem cells, citing ethical concerns that the cells represented viable human life. The Obama administration overturned the ban. However, in late August of this year, U.S. District Court Judge Royce Lamberth ruled that federal funding of embryonic stem cell research violated the 1996 Dickey-Wicker Amendment, which stated that Congress prohibited funding any research in which a human embryo was destroyed. Proponents of embryonic stem cell research argue that in March 2009, President Barack Obama set a precedent in addressing arguments in relation to the amendment when he reversed the executive order of former President George W. Bush, allowing research on cells derived from embryos that would otherwise be disposed of after in-vitro fertilization procedures. Sure enough, the Obama administration appealed the decision, and, soon after, an appeals court issued a temporary suspension of the reinstituted ban until it could hear full arguments over the next few weeks. During this waiting period, U.S. government officials announced that researchers at the National Institutes of Health would resume working with embryonic stem cells.

For many institutions, including UAB, the wait for a solid decision is not one of anguish. At least, not with a recent ability to produce induced pluripotent stem (iPS) cells, cells artificially derived from non-pluripotent cells, typically an adult somatic cell. The benefit of these cells is that, although they may be taken from a form of differentiated cell, the skin cell, they can be cultured and converted into cells which then can derive any of the 200 cell types in the body. Ability to induce iPS cells, therefore, steals a bit from the unique traits of embryonic stem cells. Already, research labs such as that of Dr. Tim Townes, chair of the UAB Department of Biochemistry and Molecular Genetics, are harnessing the power of iPS cells to help in research for treatment of diseases such as sickle-cell anemia. Unfortunately, many studies have found that the ginks and glitches of these newly discovered iPS cells lead to their differentiation less efficiently and effectively than their embryonic counterparts. Because of this, embryonic stem cell research is still in high demand for many researchers. The federal government is scheduled to file a reply to the court appeal for use of embryonic stem cells the week of November 1, 2010.

Gulf Oil Spill: UAB Department of Biology's Approach

Toral Patel

The 2010 Gulf of Mexico Oil Spill, also known as the Deepwater Horizon Oil Spill, lasted 3 months and resulted in over 185 million gallons of crude oil escaping into the ocean. The spill has caused damage to the Gulf's fishing and tourism industries, as well as to marine and wildlife habitats. The three fundamental strategies to address the spilled oil were to contain it on the surface, to dilute and disperse it, and to remove it from the water. By July 2010, oil on the surface of the Gulf had largely dissipated, but the concern still remains for underwater oil and ecological damage.

Factors such as petroleum toxicity, oxygen depletion, dispersant compounds, and crude oil components threaten the environment and wildlife. Researchers have begun to monitor turtles, coral reef, microbes, crustaceans, fish, mollusks, and birds. The UAB Department of Biology has proposed to investigate the effect of the crude oil and remnants on organisms ranging from sea grass and microbes to fish and turtles.

Oil Degrading Bacteria

Dr. Asim Bej, from the Department of Biology, and Dr. Elizabeth Gardner, from the Department of Justice Sciences, are studying the effect of the oil spill on the response of oil degrading bacteria. While marine biology will consume the remaining oil over the next years, Nutrient Enhanced Bioremediation (NEB) can accelerate this process without negatively impacting the environment. Bioremediation is the process of using microorganism, in this case oxidative hydrocarbon degraders (OHD), and their biological systems to destroy contaminants in soil and water. In order to obtain data on the effectiveness of NEB, Dr. Bej and Dr. Gardner will be establishing baseline measurements from the affected areas, determining the optimal nutrient concentration to culture beneficial OHD, and applying the OHD to the affected coastal areas. Finally, they will monitor the OHD populations and degradation of crude oil.

Concurrently, Dr. Bej and Dr. Gardner will investigate the role of oil contamination and dispersant on pathogenic *Vibrios* (PV). Some of these PVs are oil consuming bacteria that cause seafood born infections and are found in oysters, shrimp, and crabs. The use of the dispersants has increased the surface area of oil micelles, allowing oil degrading bacteria to enhance the degradation process. The contamination increases pathogens that affect the Gulf of Mexico ecosystem as well as human health. PVs are studied using the *Bacterial Analytical Manual* (BAM) with molecular methodologies from oil affected sediments and oysters that will be compared to samples collected from unaffected locations. This data will allow scientists to assess the ecological impact of the oil and dispersants, provide information to guide the recovery process to natural conditions in the Gulf, and prevent human health consequences.

Turtlegrass

Thalassia testudinum (turtlegrass) is the largest of the seagrasses and a vital component of the Gulf of Mexico's unique ecosystems. Turtlegrass is very effective in stabilizing sediments, inhibiting erosion, producing oxygen and organic matter, regenerating nutrients, serving as a food source, and providing a nursery for many fish and shellfish. The hydrophobic oil molecules can limit the growth and survival of the seagrass by multiple mechanisms. Dr. Karolina Mukhtar, from the Department of Biology, and collaborators Drs. Shahid Mukhtar and Stephen Watts (Department of Biology), and Dr. Stephen Barnes (Department of Pharmacology and Toxicology), are evaluating the molecular response of the turtlegrass to sublethal concentrations of crude oil and dispersants. They will evaluate bioaccumulation patterns in plant tissues and the immunological stress response to the contaminants, dispersants, and byproducts by comparing samples from unaffected areas to samples from heavily impacted areas by the spill.

In order to determine the extent of the impact of petroleum hydrocarbons and dispersant components on turtlegrass systems, Dr. Mukhtar et al, will evaluate the biomass production of the turtlegrass as well as the photosynthetic rates correlated to the bioaccumulation of toxicants. Petrochemicals are able to penetrate through the lipid bilayer and accumulate on the thylakoid membranes of the chloroplasts, reducing photosynthetic efficiency contents of anthocyanins, ascorbic acid, and riboflavin; expression levels of genes coding for enzymes involved in their biosynthesis will be analyzed to reflect the degree of environmental pollution. These experiments will provide necessary information on how the seagrasses respond to oil and dispersants, insights into the long-term effects of the toxicant exposure, and possible remediation strategies.

Zebrafish

To screen for low level toxicity of dispersants and residue oil, Dr. Mickie Powell from the Department of Biology and collaborators Dr. Stephen Watts and Dr. Vithal Ghanta (Department of Biology), Dr. Alex Szalai (Department of Medicine), and Dr. Bob Peters (Department of Engineering) have proposed the use of molecular biomarkers in zebrafish. Non-lethal levels of crude oil and bioremediation by-products can negatively impact organisms through physiological, biochemical, and pathological changes. Animals respond to environmental stresses by changes in the expression of different stress proteins, such as the C-reactive protein (CRP). Increased serum levels of CRP can identify a response to bacterial toxins and other inflammatory agents. Dr. Powell et al, have previously identified several stress-inducible mRNAs in zebrafish, including CRP, vitellogenin, and serum amyloid A (SAA). They have also confirmed the increased expression of their mRNAs in response to injection of bacterial endotoxin.

The levels of CRP, SAA, and vitellogenin gene expression will be measured in zebrafish exposed to crude oil contaminants and dispersants. Dose-dependent response curves will be generated to quantify the response to environmentally relevant exposure levels. These same proteins will be analyzed in fish exposed to remediated water to test the effectiveness of various bioremediation techniques. The data collected will form the basis for the development of techniques to monitor wild populations of fish for possible toxin exposure (acute, chronic, and recovery post) in the future.

Blue Crab

In order to understand the biological consequences of the 2010 Deepwater Horizon oil spill, it is important to determine the effects of endocrine disrupting compounds (EDCs) present in crude oil and dispersant on aquatic animals. EDCs are environmental pollutants that mimic or block the effects of naturally occurring hormones. This has adverse effects on the organisms, their offspring, or both. Constituents of oil spilled into the Gulf of Mexico and chemical dispersants used in remediation are known and are potential EDCs. Dr. Douglas Watson from the Department of Biology, along with collaborators Dr. Helen Kim (Department of Pharmacology and Toxicology) and Dr. Christine Duarte (Department of Biostatistics), are testing the effects of constituents of crude oil (polycyclic aromatic hydrocarbons, PAHs) and chemical dispersant (Corexit 9500A) singly and in combination on markers of endocrine disruption in blue crabs *(Callinectes sapidus)*.

They hypothesize that these constituents of oil and dispersant will act individually as endocrine disruptors and that dispersant will enhance the uptake and bioavailability of oil and oil byproducts, thus making animals more susceptible to the effects of the compounds. The overall approach by Dr. Watson and colleagues will be to assess established markers of endocrine disruption and proteomic changes that may represent previously unrecognized responses to the oil and dispersants. The blue crab is both economically and ecologically significant to the Gulf of Mexico. The research is designed to determine the effects of oil constituents and dispersant on this key sentinel species in the Gulf of Mexico and, in future experiments, to permit assessment of the impacts of the oil spill in wild populations during remediation and recovery.

Turtles

To lessen the impact of the oil spill on ecosystems, the National Oceanic and Atmospheric Association (NOAA) and the U.S. Fish and Wildlife Service (USFWS) decided to relocate all loggerhead sea turtle eggs from the beaches in Alabama and Florida panhandle. All eggs were moved to the mid-Atlantic coast of Florida so that the hatchlings could enter the Atlantic Ocean. Dr. Thane Wibbels and Jenny Estes (Ph.D. student) from the Department of Biology plan to evaluate the genetic implications of translocating the loggerhead eggs and relate it to future ecological and conservational translocations. DNA sequence analysis on the tissues will be conducted and compared

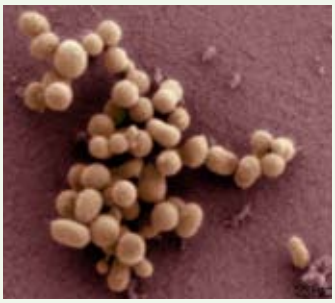
to previously published data on loggerheads nesting along the mid-Atlantic coast of Florida. By performing genetic analysis of tissue samples from hatchlings, Dr. Wibbels and Estes will be able to evaluate if the translocation strategy is altering the genetic composition of loggerhead subpopulations inhabiting the coastal waters of southeastern U.S.

A key species in salt marsh habitats of the Gulf of Mexico are diamond back terrapins. These turtles are pivotal in the diversity of animals and plants found in the salt marsh. Terrapin populations are sensitive to contamination in the salt marsh. They also consume prey that accumulate PAHs (i.e. clams and mussels) and store the PAHs in their fatty tissues for prolonged periods, increasing their toxicological exposure. Dr. Wibbels and Dr. Ken Marion (Department of Biology) propose to evaluate physiological markers that document the impact of oil contamination on the terrapins. PAHs will be analyzed in both tissue and blood samples and hormone systems that respond to chronic stress. Terrapins will also be marked in order to be repeatedly recaptured and resampled over a 1-year period to assess chronic oil exposure effects. Data from these experiments will generate a baseline on the impact of oil on the terrapins, as well as facilitate long-term studies on the effects of the oil spill on the ecology and survival status of the terrapins.

science news

Scientists Solve the Mystery of Life?

Kavita Nadendla



Scanning electron micrograph image of M. mycoides *JCVI-syn1.0*

In his 15-year-long and 40 million dollar quest to create synthetic life, Dr. J. Craig Venter, founder of the company Synthetic Genomics, successfully synthesized an entire bacterial genome, generating the first "synthetic cell." His first success occurred in 2007 when he showed that natural DNA from one bacterium could be inserted into another to take over the host cell's operation. His second success followed in 2009: synthesizing a strand of DNA containing 1,080,000 bases.

The team of scientists from labs in Rockville, Maryland and San Diego, California wrote out the organism's entire genetic code as a digital computer file and ordered pieces of DNA 1,000 units in length from Blue Heron. They developed a technique using the natural capabilities of yeast and other bacteria to meld genes together into a complete genome. By replacing the genome in one bacterium with the one they synthesized, Synthetic Genomics turned *Mycoplasm capricolum* into a novel variant of an existing species called *Mycoplasm mycoides*. Dr. Venter reported in *Science* that the synthetic DNA took over the bacterial cell, making the cell generate the proteins specified by the new genetic information. He described it as "the first self-replicating species we've had on the planet whose parent is a computer."

Applications for the synthetic cell include production of biofuels and biochemicals, as well as new tools for vaccine and pharmaceutical development. Venter's company has a contract with Exxon Mobil Corp. to build "an entire algae genome so we can vary the 50 to 60 different parameters for algae growth to make super productive organisms."

Along with necessary genes for survival, the researchers added "watermark" DNA sequences to differentiate it from true DNA found in natural organisms, including the names of the scientists who worked on the project, a website address for the new species, and quotations from author James Joyce and physicist Richard Feynman. Instead of giving the organism an original species name, the researchers labeled it as a version of its parent bacterium, *Mycoplasm mycoides* JVCI-syn1.0.

Although the new bacterium's ancestor is biological, the assertion that a "synthetic cell" has been created has alarmed people who think Dr. Venter generated an artificial organism. "My worry is that some people are going to draw the conclusion that they have created a new life form," said Jim Collins, a bioengineer at Boston University. "What they have created is an organism with a synthesized natural genome. But it doesn't represent the creation of life from scratch or the creation of a new life form." If Venter has really cracked the mystery of life, his work suggests that an artificial genome is not limited to the laws of nature.

Source:

J. Craig Venter Institute, Popular Science.

"We came, we saw, but I am not sure we conquered" The Effects of New Vaccines and Emerging Virus Strains

Ashruta Patel

The implementation of newly researched vaccines will help control a potential pandemic, especially if the numbers of illnesses associated with different strains of viruses continue to spread, such as the recent cases of H1N1 (swine flu). There are numerous diseases capable of causing causalities worldwide. Some have been discovered, while others have the possibility of initiating a pandemic. The incidence of illnesses that once caused significant morbidity and mortality has drastically decreased because of vaccination programs (Ravanfar 110). The first vaccine created was in 1796, allowing for the eradication of smallpox in 1980. Smallpox was a highly virulent, contagious, and easily transmittable disease with a mortality rate of at least thirty percent (Stewart 329). Edward Jenner's immunization discovery significantly reduced the mortality rates and controlled the virus at a considerable rate. In spite of the eradication of smallpox over thirty years ago, "smallpox remains a serious public health threat both through the possibility of bioterrorism and the intentional release of smallpox and through natural outbreaks of emerging infectious diseases" (Kennedy 314). Many viruses have the ability to adapt or mutate into a different form. After the outbreak of smallpox, humans have faced many other diseases that have been eliminated through immunology research and the implementation of certain vaccines. Subsequent antiviral vaccines have been developed, and these existing antiviral vaccines permit further potential eradication of viral diseases worldwide (Ravanfar 110). Immunizations have been promising in the past for numerous illnesses, such as yellow fever, malaria, measles, mumps, influenza, and rabies. New technology has provided better results from the past, and implementing vaccines recently discovered can significantly impact how populations around the world react to probable illnesses.

Vaccination represents an important medical breakthrough, and to this day, vaccination remains the most effective means available for combating infectious disease. Although vaccines must demonstrate clinical efficacy in order to receive U.S. Food and Drug Administration (FDA) approval, the correlates of immunity vary remarkably between different vaccines and may be based primarily on animal studies, clinical evidence, or a combination of these sources of information (Kennedy 314).

The recent H1N1 pandemic primarily targeted populations that consisted of youth around the world. World Health Organization (WHO) declared reduced numbers of H1N1 cases after the implementation of the correctly discovered vaccine. The vaccine was successful in controlling the prevalence of cases; however, the lacked knowledge about the virus strain had consequences (Weintraub 24). For example, some doctors did not know how many vaccines they would receive, which made it difficult for many of the high risk populations (i.e. pregnant women and health workers). Newly discovered vaccines encounter many misconceptions that have the potential to hinder the success of its usage. Many erroneous beliefs are eliminated by educating the public through various media sources. Even with infection rates dropping, many flu experts are concerned about certain factors associated with swine flu. H1N1 could have another virulent strain emerge and continue to target specific populations, such as children (Weintraub 24). The cases of swine flu decreased once a vaccine was discovered and put to use, but educating vulnerable populations and continuing to develop immunizations is a fundamental aspect in managing its spread.

Transmission of various viruses occurs through different mediums. "Previous studies suggest that the majority of the contacts for school-aged children are with their peers, which may explain quick spread of viruses through certain youth groups" (Calatayud 185). A school in London showed cases of staff members who became ill after taking care of sick students, suggesting the transmission of H1N1 requires close contact. Many of these cases can be avoided through appropriate preparation, for example, "prompt notification of cases is important to allow early detection of school outbreaks and implementation of public health measures" (Calatayud 183). Several schools have pandemic plans which incorporate public awareness. Communication of certain precautions is essential to control prevalence cases. Through continuing research, newly emerging viruses can be defined under certain conditions, such as where and who the strain can possibly target. Research can be conducted to determine what strains might arise; however, it is necessary for countries to be prepared and ready to take any preventative measures.

Recognition of certain viruses has strengthened pandemic preparedness over the past years. Many individuals are aware of the general procedures that need to be conducted if need be. Many strains target different populations. "Older individuals may have a degree of cross protection conferred by neutralizing antibodies directed against them" (Maritz 11). Pandemic H1N1 affect all age groups, with a preponderance of younger cases. According to the WHO, risk factors for severe illness due to pandemic H1N1 seem to be similar to those of seasonal influenza. This information can serve to help understand what vaccines can be used and what changes need to be made in the future. Not all viruses have vaccines, primarily because they can

be cured with medications already discovered or because there simply has not been enough information found to produce a potential vaccine.

Many vaccines assist in preventing the occurrence of diseases that have the ability to cause death such as the recent cervical cancer vaccine, Gardasil. Another future trend will consist of reducing the pain when administering a vaccine, especially in infants and toddlers. Possible strategies under study include, oral and nasal vaccines, bioengineered plants (can be used as a prospective ingredient), microencapsulation (protection from side effects), DNA vaccines, and applying liquid to skin (National Foundation for Infectious Diseases 2006). Many of these discoveries occurred from information obtained in the past from the effects of vaccines, diseases, pandemics, or epidemics. As new research is conducted and preventative measures are taken, deadly diseases prevalent today can successfully become eradicated. One promising discovery in the near future might be the implementation of a universal vaccine capable of eliminating many strains or diseases with one dosage.

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2010 Nobel Prizes Have Been Awarded

Atbin Doroodchi

2010 Nobel prizes were awarded in October in Stockholm, Sweden by the Royal Academy. In the field of Medicine and Physiology, Robert G. Edwards received the award "for the development of *in vitro* fertilization." *In vitro* fertilization, or IVF, is a process by which the sperm fertilizes the egg inside a test tube, and the fertilized zygote is implanted into another female's uterus. This procedure has been used for treating infertility, cloning animals, and creating mouse models of genetic disease. In Physics, Andre Geim and Konstantin Novoselov shared an award for "groundbreaking experiments regarding the two-dimensional material graphene." Graphene is a one-atom thick planar carbon molecule, in which all of the carbons have sp^2 hybridization. The carbon atoms are arranged in a honeycomb fashion. Graphene is a useful compound in nanometerials. Geim has a paper published with his hamster in 2001. In Chemistry, the award was shared between Richard F. Heck, Ei-ichi Negishi, and Akira Suzuki for "palladium-catalyzed cross couplings in organic synthesis." Palladium catalyzed coupling reaction is a synthesis reaction by which two hydrocarbon reactions are joined with the aid of palladium. This method is widely used in the industry, which uses less expensive metals such as nickel or copper instead of palladium.

Robert Edwards *Andre Geim* *Richard Heck*

Lessons in Failure

Helen Lin

As I leaned over the light, UV rays bathed my protective face mask, *"Please! Let it show products!"* For many months, I had been working on creating a chimera of my proteins. The lack of bands on the gel meant another failure, and another week of work. This project would be my first and it was not progressing well.

I am working with two proteins: Alpha and Beta. Each protein targets to separate areas of the cell. My goal is to discover what domains in Alpha target it to the membrane. The data I collected over the summer indicates that the N-terminal of Alpha is necessary for membrane targeting. To confirm this hypothesis, we proposed a "swapping" project: replace the N-terminal of another protein, Beta, with that of Alpha to build a chimera. If HeLa cells transfected with this chimera show colocalization of Beta at the membrane then the N-terminal of each is responsible for targeting. A simple polymerase chain reaction (PCR) protocol would do all the work.

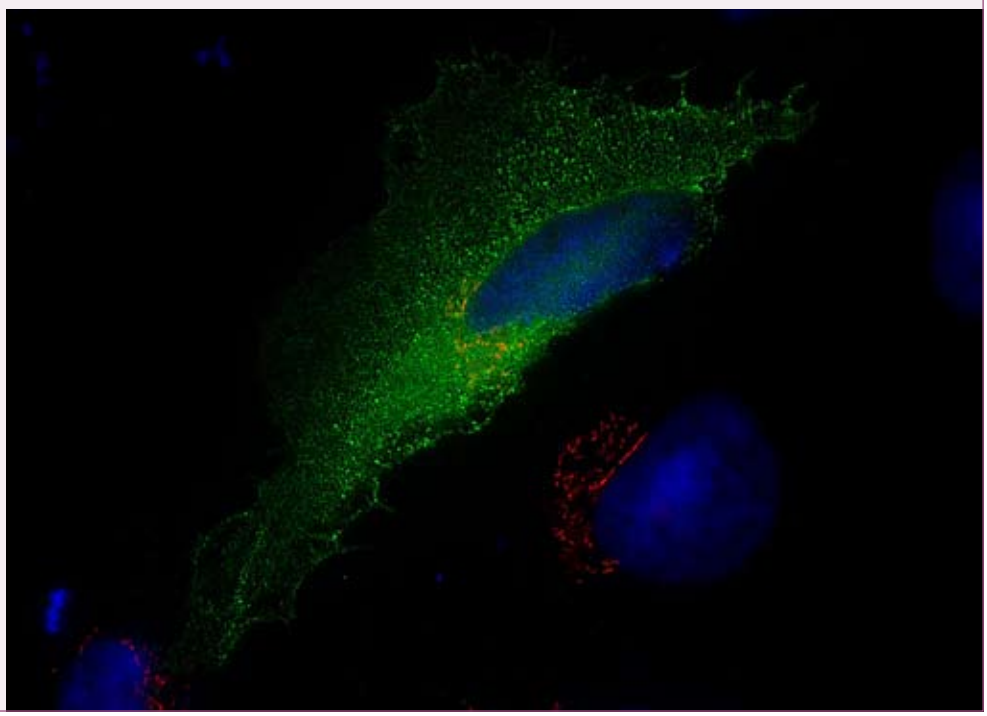
I underestimated the whimsical nature of PCR. This simple undertaking proved more difficult than said. At first I tried to make blunt-end pieces of each protein to ligate together. While I received PCR products for each individual piece, the pieces did not ligate properly. "If at first you don't succeed, try, try again." I tried altering the PCR annealing temperature to make it less specific but still no product. Next I tried overlapping PCR to eliminate the need for ligation. In this method, each individual piece contains a portion of the other and so serves as a template for each the other. The same problem persisted. Again I changed the annealing temperature to no avail. I could make the individual Alpha and Beta pieces but the chimera continued to be elusive. After consulting with the post-doctoral fellow, who I work with, we decided to redesign the primers to make them longer. The new primers did not solve the problem. In fact, now I could not even produce the protein fragments.

After months of arduous work, I still possessed no results. The post-doc continued to insist I was doing something wrong. In my mind, this possibility did not exist. I added every necessary ingredient to the mix and in the correct amounts. I double, triple, and quadruple checked my primers and the mix before running the PCR. No reason existed for the experiment to fail and yet it did.

The post-doc wanted to observe me performing the experiment insisting that I must have left out a step or done something wrong. I prepared my lab bench setting out the necessary reagents. I pulled gloves onto my hands and began combining reagents. Because of the small volumes used in the experiment I made sure to observe the reagent in the pipette tips. Having performed the protocol as specified, I turned to my post-doc with a victorious look. He shattered that victory with a single question, "Did you mix it? Do you mix it every time?"

"What mixing?!" Having performed the protocol many times over, I could recite it from memory at any moment. Mixing was not one of the steps. He informed me, much to chagrin, that the mixture must be homogenized because the polymerase is in a glycerol solution, which causes it to sink to the bottom of the reaction tube. As such, the polymerase does not mix with the other elements of the reaction resulting in low concentrations or none of the desired products. Turning back to the PCR tubes, I reached for the p20 and homogenized the mixture. This time I got products.

The saying "if at first you don't succeed, try again" is lacking. If the first attempt does not succeed, find out why. Change something in the next attempt. Only then will knowledge be gained and success one step closer.



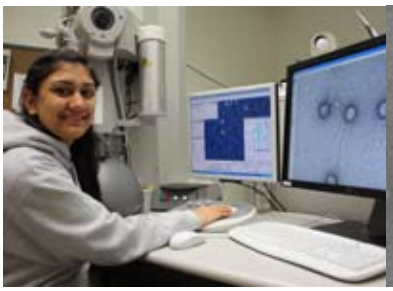
My Phage, My Miracle

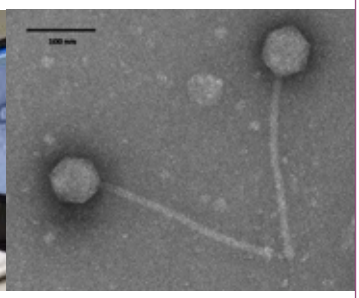
Mira Patel

"Research? What do you mean by that?"

When my older sister decided to join a research lab as an undergraduate at the University of Alabama at Birmingham, I was caught off guard. I always knew that she wanted to join the medical field, and I thought that her ultimate goal was to be a doctor. It was only when she joined the Science and Technology Honors Program at UAB that she seriously considered another career choice. I was skeptical of her new decision because I knew how focused she had become on being a doctor and was surprised at her new choice of career. However, I never knew how fun research could be until my freshman year at UAB.

A new research initiative was implanted my freshman year for Biology majors who are freshmen and sophomores. This new class is known as Phage Genomic Explorations programmed by the Howard Hughes Medical Institute. In this class, I and 15 other students are learning the various techniques and disciplines of working in a research lab. Our goal for this semester is to isolate and purify our own unique phage. At the end of the semester one of us has the chance to send our phage to the Howard Hughes Medical Institute in order to be DNA sequenced and result in a genome.





I believe that there are two aspects of this program that are very appealing to applicants. The first is that this opportunity is available to freshmen who are usually not able to gain such experience until their late sophomore or junior years in college. The second is that this class provides an escape into the other side of the medical field. For many, like me, being a doctor meant treating patients and working in a hospital; however, this class shows the differences and commonalities between the clinical and practical science settings. I have learnt so much about myself and science through this program that I truly consider myself privileged to be part of a community that emphasizes its education so much.

Phage not only teaches one the techniques in a lab and how to apply biology lectures with lab experience, but it also sets an example of how research is set up as a class. Our professor, Dr. Denise Monti, has structured this class with Journal Club and days when we present our experiment thus far. These special editions to our class prepare us for reading research articles if we decide to enter a lab or one day write a research paper ourselves; it also helps our career as a medical student to interpret research findings in a scientific journal atmosphere. With these presentations and research articles, we are more prepared for the lab environment when it comes to reading such papers and being comfortable with presenting scientific information to professors, doctors, and our peers.

Phage has prepared me for many life situations. Phage Genomics has instilled many virtues in me, such as patience. With the procedures required to isolate and purify mycobacterial DNA, it seems that patience is as you have to wait for 24 or 48 when the results are visible. Along with these findings the results are not always what one has anticipated. This has happened to every one of us in the class and the reactions were disappointing. We did not want to accept that something had gone awry or that we did not have phage particles the first time we enriched our soil samples. That was our first disheartening experience in phage, and that so early on in the class; we did not think it was a good sign but we were surely wrong. As it always goes in any situation, we just wanted everything to work out without any problems, but research was teaching us that it does not always happen that way and that is not necessarily bad thing. This can instead help us with accepting setbacks in other daily life situations. As a result we moved on with the encouragement of Dr. Monti, Angelina our TA, and Kyle our undergraduate student.

Research is a "behind-the-scenes" job that helps run the big picture. If it were not for scientists and researchers working in their labs, doctors would not have the medicine to treat patients with or the medical equipment to do so. Phage has brought to light just how researchers are the people working behind the curtains who are trying to help patients as much as the doctors upfront. As research is applied to the other end of the medical field, everyone benefits and people come to learn something new; discovering something you never knew before is the basis of life and medicine, I believe, because it helps one to always keep their mind open and look forward to new and refreshing knowledge.

In short, opportunities like Phage offered at UAB and other institutions, open new windows through which you see yourself and your career. With this experience, you can decide whether research is the way for you or not. My personal experience has been enlightening and I hope that it will continue to be for the rest of my first year at UAB. I encourage students who are looking into the medical field to explore every horizon and try something that you might not have thought was for you, because you never know how one experience can change your life forever.

Lab Bench to Bedside

Toral Patel

Since childhood, being interested in pursuing a medical degree and becoming a physician just was not enough. I wanted to work behind the scenes and develop novel conclusions that could further the growth and development of medicine in the future.

Many students like me join labs and take opportunities that lead them closer to their careers by conducting laboratory science and observing the effect of findings on drug or therapy development, for diseases and other medical conditions. However, working only in a laboratory can give insufficient opportunities for clinical observations and can persuade students to discontinue pursuing research and apply only to medical school. Clinical research allows one to conduct research in a clinical setting with patients. But what are the limitations? How would I be able to use models to assist in the progression of experiments? Where could I incorporate community outreach and education into this experience?

One day I came across an interesting article by the National Institutes of Health (NIH) discussing a new program consisting of Clinical and Translational Science. This program would allow students to execute basic science and translate it to the bedside for patients. In May 2008, the University of Alabama at Birmingham was awarded a Clinical and Translational Science Award and the new UAB Center for Clinical and Translational Science (CCTS) was formed. The center's mission is "to develop an infrastructure that encompasses UAB research from preclinical research to the translation of bench research to a clinical application, and on to community implementation of research findings."

But how would an undergraduate student be able to pursue research and translate it to a clinical setting? Through this center, a novel society emerged to further educate students about the CCTS and create interactions and relationships between faculty and students. The Pittman Society for Clinical and Translational Science, named in honor of James A. Pittman, MD, former Dean of the School of Medicine at the University of Alabama, and founder of the UAB Center for Advanced Medical Studies, was formed in Fall 2009. Its purpose is to serve as a campus wide forum for discussions between students, faculty, and speakers on all aspects of clinical and translational science.

I was intrigued by the Pittman Society as I discovered more depth in this program. The Society hosts quarterly evening meetings that feature an invited lecture by a noted clinical and/or translational researcher. The Society held its first meeting and reception on Tuesday, September 29, 2009 with guest speaker *Dr. Edward W. Hook, III MD*, Professor, Departments of Medicine, Microbiology, and Epidemiology, and Co-Director of the CCTS Training Component. He presented a lecture on "*Translating Research into Practice: Personal and Population Based STD Research using Recent Technological Advances.*" The Society also holds "Leadership in Clinical and Translational Science" dinner series which provide an opportunity for leaders in Clinical and Translational Science at UAB to informally discuss inspirations for their careers, challenges they have faced, and opportunities they have received. This intimate setting encourages relationships to be built between students and faculty.

After becoming an active member in the Pittman Society and participating in the UAB CCTS seminars, I have found a path that will help me reach my career goal. I enjoy being able to observe and discuss research that is translated into practice and create informal and formal relationships with faculty and nationally as well as internationally acclaimed clinical and/or translational researchers.

...membership provides students with the unique opportunity to learn about ongoing clinical and translational research at UAB and meet potential research and career mentors.

I encourage all undergraduate, graduate, and professional students interested in clinical and translational science to join the Pittman Society and explore fields of sciences that they find exciting or interesting. Joining this society from freshman year will be highly beneficial as membership provides students with the unique opportunity to learn about ongoing clinical and translational research at UAB and meet potential research and career mentors. This is a great way to begin your undergraduate research in the field that intrigues you the most.

Anthony and Rob's

Excellent IACUC Adventure

Rob Wians and Anthony Todd

What has blue feet, and is yellow all over?

A: Anthony and Rob – at least on the day we visited the Institutional Animal Care and Use Committee (IACUC). Finding the IACUC facilities would prove to be harder than we thought, however. A series of misfortunate events would eventually lead us to finding the facilities we would be touring: disrupting multiple classes held in Volker, almost being run over by a semi-trailer, and being chased down by a woman who worked at IACUC.

"Go ahead and have a seat. Dr. Hines will be with you shortly."

While we waited for others to join us (we would be the only two), we took every brochure available to us to learn more about IACUC before the tour began. Our purpose for touring was to learn how animals are used for and treated during research here at UAB. In particular, we wanted to see how the committee itself contributed to research on breast cancer.

Dr. Hines eventually walked in and greeted us amiably. We introduced ourselves, and asked him if we could take pictures to use as a visual in our presentation.

"Absolutely….not!" he replied enthusiastically. IACUC turns out to be pretty confidential when it comes to revealing what goes on in the facilities. This is to protect the rights of everybody and make sure there are no liability issues.

Our tour began after we left our backpacks and cameras in Dr. Hines's office, and putting on our surgical gear, with difficulty. We followed Dr. Hines into a room full of animal taxis and a loading dock. The loading dock was like something out of the Die Hard movies. A truck would back in, and then henchman would lower the garage door. The animals would then be transported out of the truck onto the loading platform.

Adjacent to the animal taxi rooms was the processing and labeling room, where animals were labeled according to which labs they were assigned to. Then, we were brought to a gigantic room full of all of the food the animals were given to eat. Everything from rat pellets to scientific dog food formula was included, and the expiration dates on the food were closely monitored. Next, we saw a slew of mice, one set of which were particularly interesting to us because they were being used to study the effects of estradiol on breast cancer.

We discovered that cancer metastasis research can only be done using animal models. The animal of choice in most cases is mice, but rats are also used. The mice we saw were being studied to observe the effects of breast cancer metastasis in response to increased levels of estradiol, and to test the effects of a certain diet on cancer progression. These mice are placed in a controlled environment and given doses of estradiol. Some mice are given a special diet rich in soy, which is thought to contain a special "anti-estradiol" agent that blocks the production of excess estradiol while other mice continued with their normal, balanced diet. This diet fluctuation is based on the fact that people in the eastern part of the world do not suffer from breast cancer as much because the soy diet prevents excess estradiol, which acts as a carcinogen. Through this method, researchers can study how the soy-diet effects the production of cancerous breast cells in mice, and hopefully relate their findings to humans.

After passing more and more rooms filled with copious amounts mice, rats, and all other varmints, we came to a big glass window through which we saw three massive pigs undergoing a cholecystectomy. Their gall bladders were being removed by medical students ranging from first year to third year students. We were allowed to walk into this room and stand side-by-side with the medical students while they learned about lacroscopic techniques. After seeing the pigs, we walked into the adjacent room to see an animal surgery room. These rooms are equipped just like a surgical room for humans. We also saw a surgical room for mice and rats. These rooms were designed with two metal tables in which the mice were prepped, operated on, and cared for in the same room, unlike humans who have separate rooms for each part.

Next, we walked through a hallway which led us to our buddy Peter, the petrified hemophilic dog. Peter was alone shaking violently in the first cage visible to us. He was surrounded by other dogs of the same breed, but they were slightly more excited to see us. Dr. Hines pointed out to us that the dogs always come to the cage when familiar people come in because they are treated very well at the facilities.

"However," he said, *"They are very apprehensive when new people come in, and that is why the dogs go to the far end of the cage away from us."*

The next room we were taken into was full of wickedly big albino rabbits. These rabbits were the meanest looking animals in the facility as their eyes were blood red and their fur was pure white.

Lastly, we saw some Macaque monkeys being transported to another room. Dr. Hines told us that these monkeys carried an extreme form of herpes that could kill a human within three days if any of the monkeys' waste got into the mucus membranes. In addition to seeing the monkeys being transported (complete with feces on the floor), Dr. Hines showed us another room full of monkeys and told us not to stare at them because the monkeys would become upset and agitated, causing them to rattle the cages.

This completed our tour of the IACUC facilities. We ripped off our gear, got hand sanitizer, and walked back into the office where it all began.

Our purpose for going on this tour was fulfilled. We had investigated how the animals were treated within the facilities and concluded that IACUC is a fantastic place to support animal research. The animals are treated very well and given the proper attention and nutrients needed to be optimal test subjects. We would recommend anyone to take a tour of the IACUC facilities and see for themselves what a great committee IACUC is to have around.

research narrative

It's just not Science, It's Art!

Uma Srivastava

The first day in the lab, I had a million questions running through my head. Am I capable enough to handle the fancy equipment? What if I don't know a term or a technique? What if I cannot make the connection? What if I don't see the big picture? What if I don't know how to analyze the data properly? I had always seen fancy laboratories in movies and on TV, but I never imagined I would be working in one. With no experience in high school, I was anxious and concerned about my research experience. With the help of Dr. Datta and Dr. Tucker, I cruised through my project.

My mentor's colleague, Dr. Chaddha, told me on my first day in the lab that doing research involves both science and art. Whether running electrophoresis gels or Western Blots, the actual application of science is needed along with the art. Science, to me, is the thinking process that is involved including all the scientific names and methods. Art, on the other hand, is the experience of seeing the bigger picture. Art enters the picture when making sure all the bands show up and they are the right colors. Art is where I modify a basic technique to make it my own. The science comes back into the picture afterwards when analyzing the data. As time went on, I slowly learned the tricks of the game. Dr. Datta and Dr. Chaddha slowly taught me the secrets.

The only real science based experience I had previous to working in the lab was job shadowing doctors. When I came to UAB, I learned about the various research opportunities. With the Science and Technology Honors Program, Dr. Tucker motivated me to enter the world of research. She wanted me to go out of my comfort zone of being in a hospital to being in a laboratory. She assisted me in finding my niche and my interests. In the beginning, it felt weird working in a laboratory because I did not always know the outcome; there was no right or wrong answer. I had always seen the applications in a doctor's clinic. In that environment, I knew what the outcome of every procedure was. The only other lab experience I had was my high school

chemistry lab. Even there, my teacher did all the experiments and we just watched her. Once I was in Dr. Datta's lab and I began to do my own work, I had a sense of pride that I'm helping out the field of science-even if it is a little drop in the ocean.

Working with Dr. Datta made me realize that all the tiny pieces are required to make the puzzle perfect. With one piece missing, it is not possible to finish an experiment. At first, I thought that doing just one piece of the puzzle was enough. But once I started looking at the data, tying everything to the big picture was difficult yet necessary. With Dr. Datta's help,I began to think of my research project as a big tree, and my goal was to find the smallest branch. I began with the stump which was atherosclerosis. Then the big branches such as HDL and LDL appeared. After that, came the smaller branches; the major protein, apo A-1. The smallest branches were the peptide chains and how they reacted to changes. Once I began visualizing this image in my head, the entire puzzle came together.

Now that I have completed my research with Dr. Datta, my big tree makes sense. Although I was intimidated by the entire research process, Dr. Datta and Dr. Tucker assisted me throughout. With research in my pocket, I can now work out problems where I don't necessarily know the answer to the question. For emerging scientists, research sounds intimidating but thinking of it as an art simplifies it to an extent. The science aspect cannot be neglected but looking at it from a different perspective makes it easier to handle. Research is not just science—it is also an art!

The Fruit of Locke's Experience

Yvetta D. Riley

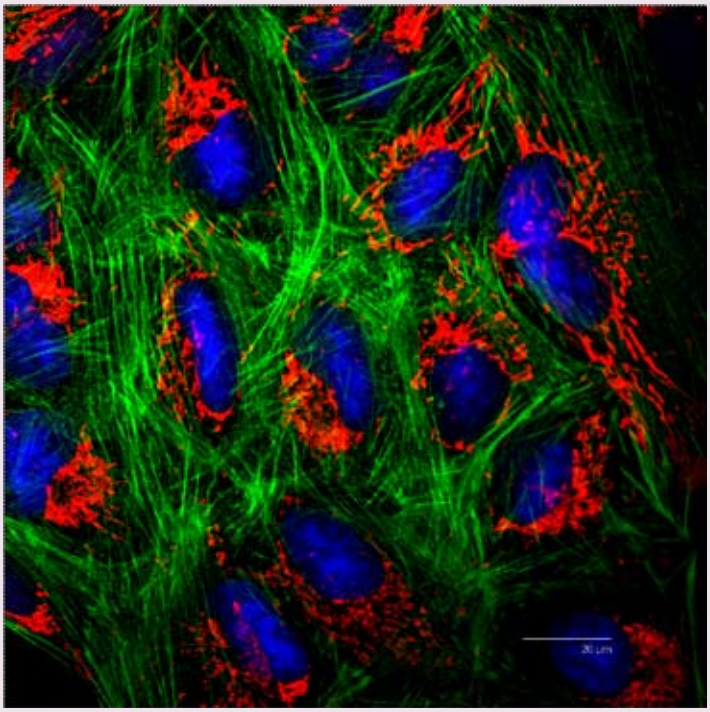
John Locke believed that every mind is a blank slate and that learning through experience and not through books was the only way to truly gain knowledge. Independent Research in Histology provides the setting for John Locke's belief that everyone is called upon to face the question for themselves. The class is led by Dr. Dana Peterson and is open to both graduate and undergraduate students. This quote from Dr. Peterson demonstrates how her outlook on science, research, and learning closely mirrors John Locke's.

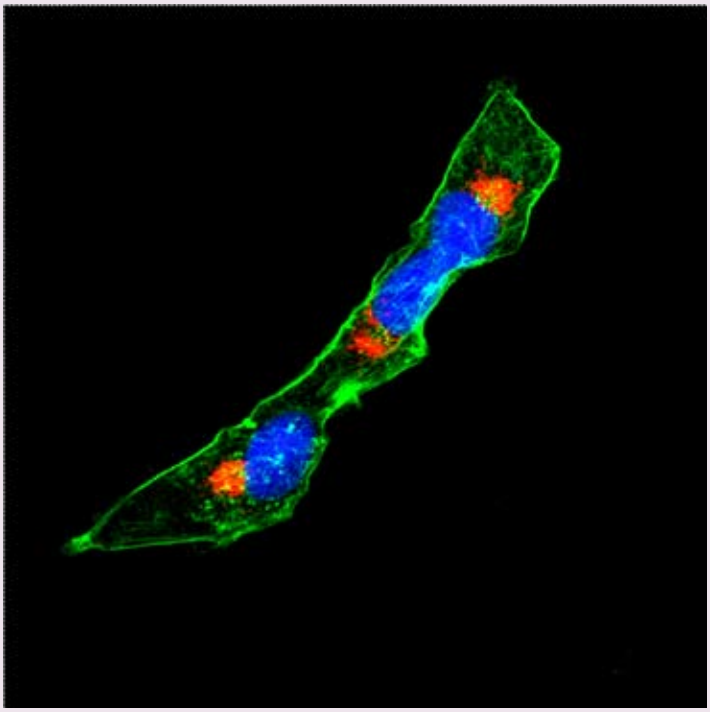
"I have been a scientist off and on for the last twenty years. I have done science for government. I have done science for industry. And I have done science for academia. The science described in textbooks and in peer-reviewed journals is not the science of reality. Textbook science is depicted as clean, neat, methodical, and clinical. It is science that asks discernable questions and produces publishable conclusions. Textbook science describes success stories. Science as practiced is messy. It can be mind-numbing in its repetitiveness and it's routine. In a single moment, it can provide an epiphany that will last a lifetime. Science as practiced rests not on a foundation of success but of resilience. The conclusions it generates are rarely wholly satisfactory and the questions it asks even less so. Science as practiced is about seeking rather than knowing. Science as practiced is about possibilities."

Textbook science is depicted as clean, neat, methodical, and clinical. It is science that asks discernable questions and produces publishable conclusions. Textbook science describes success stories. Science as practiced is messy. It can be mind-numbing in its repetitiveness and it's routine. In a single moment, it can provide an epiphany that will last a lifetime.

This informal class encompasses so much more than a focus on histology techniques. Imagine an environment where individuals are actually encouraged to generate and explore new ideas. No preplanned syllabus exists and the initiative to work independently is essential. Previous research experience is not needed, although there are some academic prerequisites. No two classes will be the same.

This semester two graduate students and four undergraduate students make up this small class with diverse interests, experiences, and skill sets. The graduate students hope to learn new histological laboratory techniques that will help them, not only in their current research, but also in their future career. The undergraduate students have a variety of reasons for taking the class, mostly the opportunity to expand their knowledge and appreciation of research. Thuy Kim, one of the undergrad students in Dr. Peterson's class, said "Training means that we get a chance to apply what we learned in





the classroom. In the microbiology class that I'm currently taking, we learned about different types of microscopes and if I weren't taking this independent research course, it would just be another thing to memorize now and forget right after the test. To be able to actually get on one (of the microscopes) and use it, really makes a difference on if and how we, as students, actually learn the differences of the microscopes and their importance."

The class meets once a week for thirty minutes to discuss accomplishments, goals, expectations, and areas of interest and to create a timeline that is related to the decisions of the group. Everything the students do is a collaborative effort. The flexible, cooperative nature of the class encourages the students to pool their various talents and resources to achieve common goals. The students' plan this semester includes; working on cell culture techniques, practicing advanced histological staining methods, training on advanced microscope use, assisting in the purchase of an epifluorescence microscope, and getting other lab equipment back into operation. Additionally, each undergraduate student will design and implement an independent research project of their choice. The graduate students serve as mentors to the undergraduate students. The group will also be attending an abstract and poster conference to sharpen their documentation and presentation skills. The grand finale will be to make a portfolio of the semester accomplishments which will include digital images from confocal microscopy training. Dr. Peterson will guide the class through each endeavor.

Although Dr. Dana Peterson is the research supervisor, she is more of a resource engine for the Independent Research in Histology group. She supports all histological processes and is instrumental in narrowing the target focus to optimize the outcomes. Professor Peterson provides the background information and materials that are relevant to the students' current chosen task. She is always up to date on current research and fosters cooperative, active learning. The group will use her expertise in biology for many things including following lab protocols correctly and using appropriate safety equipment. Dr. Peterson's valuable skill set will open unique doors to academic growth.

Overall the class will gain an intimate understanding and transformative experience that will contribute to their knowledge and appreciation of research. This independent enterprise motivates leadership, investigation, and enthusiasm for the field of research. Increased research productivity of each student will result in the following: implementation of previous course learning, improvement in current laboratory techniques, and groundwork to future applications. The course is a step building process for personal ownership in one's capacity to contribute to science. The collective experience of the class unifies the group as a part of the research community by contributing new knowledge that helps raise issues and new questions that are pertinent to our ever-changing world. The class will be an insurmountable foundation for success. Come enjoy the journey.

Acknowledgements:

Teneasha Washington, Thuy Kim, and Mallory Schultz

A Rewarding Chase

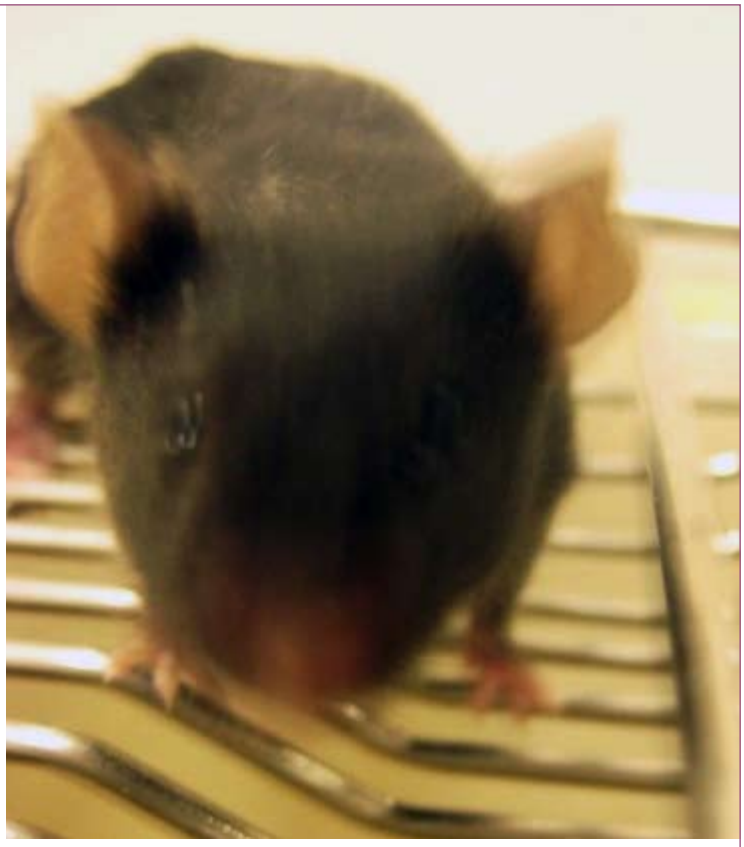
Alisa Trinh

I gasped and the mouse dropped out of my hands. The bite had not so much hurt as scared me. I immediately scanned the room for the mouse's whereabouts, but the initial sweep proved fruitless. Out of the corner of my eye, I caught a tiny brown blur dash to the back corner. I lunged toward it only to see the mouse scurry past my fingertips. Now on my hands and knees, I spotted the mouse's new hiding place, behind the table. I repositioned my body tangent to the table's back leg and waited for the opportune moment to capture the runaway.

Until that moment, I had always pictured research as staring deeply into glass bottles filled with chemical solutions. Little did I realize that as a research assistant, I would find myself crawling around a room to hunt down a mouse. But then again, there were other misconceptions I had about research.

I had always been interested in science and eager to utilize the knowledge that I had learned in classes in real life applications. For months, I searched for a lab to work in where I would be able to acquire a more hands-on experience. It wasn't until I received an email from my Neuroscience advisor with a list of open research labs that I was able to find a position.

From the list, I found Dr. Rita Cowell's lab which investigates transcriptional regulation in early postnatal development and neurological disorders, an area that seemed fitting to my interests as a Neuroscience major. After an interview, I was ecstatic to find out that I would be assisting doctoral student Beth Lucas in analyzing the effects of the transcriptional coactivator PGC-1α, a protein that is believed to play a major role in Huntington's Disease, on the behavior of mice.



I began to feel less like an assistant and more like a detriment.

I was overwhelmed with learning from the lengthy immunohistochemistry protocol to the meticulous sectioning brains procedure. Beth would demonstrate the tasks effortlessly and as soon as she turned it over to me, disaster would ensue. Whether it was miscalculating a solution ratio or trapping bubbles underneath the brain slices, everything I did seemed to go wrong. To add to that, I was assigned to read journal articles that might as well have been written in Farsi. I seemed neither to understand nor perform anything correctly that I was supposed to do in the lab.

Many of the procedures were tedious, and if they weren't tedious, they were definitely not fun. Most of my work with the mice consisted of cleaning up their excrements while running their behavior tests. Of course there were the occasional perks of being bitten or having to chase down a few escapees here and there.

Little did I realize that as a research assistant, I would find myself crawling around a room to hunt down a mouse. But, then again, there were other misconceptions I had about research.

I dived into research a week after classes ended in May, working full-time throughout the summer. On the first day, I walked in nervous, yet excited to get started on what all my science and lab classes had prepared me to do. After the first few weeks, however, As the weeks rolled on however, I began to notice a change. I was slicing and transferring the brain sections onto the slides with fewer bubbles and folds. The staining from the immunohistochemistry became darker and clearer. I developed better

techniques for handling the mice. I still couldn't understand the majority of the journal articles, but I at least understood the basic mechanisms that the papers were describing. Beth even allowed me to assist her in the fascinating mice dissections that would become one of my favorite parts of lab.

I collected the data from all the behavioral tests that I had been running in the past few weeks and analyzed the information. From the graphs I produced, I was able to visualize the correlations. For the first time, I began to understand why I was doing all the different procedures and how they pieced together. I actually felt a sense of pride from the work that I had done.

When I started research, I had naively assumed that I had already learned all the principles from science classes and that working in a research lab was a merely a job in which to apply those principles. But research is not a job. It is a skill. It is something that needs to be learned and demands patience. Working in a lab requires techniques that cannot be picked up from a reading a textbook. Instead, they are learned through actual execution of the techniques that takes practice and time. Research is not necessarily about the individual experiments and procedures, but rather taking information from those several different components and piecing them together to create a new way of thinking. Whether it's chasing down mice or slicing brains, with the right attitude, working in research can become an incredible learning experience with rich rewards.

faculty interview: mathematics

Don't Be Afraid of Math

Rachael Rosales

Dr. Nandor Simanyi graduated from the University of Budapest with his doctoral degree in 1987. He worked with the Mathematical Institute of the Hungarian Academy of Sciences starting in 1982, and he was a professor at the University of Szeged from 1996 until 1999. He came to UAB eleven years ago from Hungary in order to work more closely with Dr. Nikolai Chernov.

Dr. Nandor Simanyi is going to start his first research project with an undergraduate student soon. Although he has served as an advisor to young students in the past, Dr. Simanyi regrets that students rarely approach him for any research opportunities. He is excited to work with a talented young student and encourages others to seek research opportunities early. Dr. Simanyi's eagerness to help undergraduate students mirrors his development of his love for math at a very young age.

When Dr. Simanyi started going to math competitions in 5th grade, he was fascinated by math problems. As a high school student, he spent every afternoon poring over different problems. "Some people say that mathematicians are a little eccentric. There is certainly some truth in it," he joked. When he started college at the University of Budapest, he was introduced to the theoretical basis of mathematics, and he found it very challenging at first. "I remember it was a shock, a cultural shock, to become an undergraduate student," he confessed. However, Dr. Simanyi did not let this challenge intimidate him, and, after a lot of hard work, he adjusted to a different facet of the diverse field of mathematics.

Dr. Simanyi's current research interest is in dynamical systems. This field focuses on the time evolution of complex chemical and physical systems with large amounts of data. Dr. Simanyi has recently become especially interested in the biological applications of his field of study due to the wide range of applications to living systems. His ongoing projects focus on topics like highly unstable, nonlinear systems; much of his current research relates to problems in the foundations of statistical physics.

When asked about specific advice he would give to young students interested in research, Dr. Simanyi hesitated at first: "Mathematics is so rich and broad that I cannot mention anything specific right away. Mathematics is beautiful because there are so many different areas." After thinking for a few seconds, he explained that math research projects don't always require extensive preparation. With a good advisor, a student can get to a point where he or she has good results after a few semesters. Although there are a multitude of areas that can be explored by undergraduates, there are a few particular fields that have experienced a large increase in demand in previous years. Any type of research that uses computer animations or models is very much sought after in many different scientific disciplines; models make information easier to confirm or refute. Additionally, any type of research that deals with biological models is becoming increasingly important in interdisciplinary studies.

Dr. Simanyi's most emphasized piece of advice was this: "Don't be afraid of math. Please love math – be enthusiastic about it like I am. It's not as hard as people conceive it to be."

short report

Bizarre Parosteal Osteochondromatous Proliferation (Nora's Lesion): A report of the first case originating in soft tissue

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University of Alabama at Birmingham (UAB) Undergraduate Honors Program[1], UAB Departments of Diagnostic Radiology[2] and Pathology[3]

Abstract
Many tumors are unique to the organs from which they arise. Over the last 20 years, however, most tumors that were thought to be primary in soft tissues (derived from the primitive mesenchyme) and thought not to have counterparts in bone, were found to, in fact, rarely arise as unique lesions from bone. Some examples include synovial sarcoma, rhabdomyosarcoma and leiomyosarcoma, to name but three. We now have begun to see the reverse with lesions that were initially thought to be unique to bone arising in soft tissue. While this has been well reported with osteosarcoma and Ewing's sarcoma, it has never been reported with Bizarre Parosteal Osteochondromatous Proliferation (BPOP), also known as Nora's lesion. This study explores the first reported case of Nora's lesion originating in soft tissue. While BPOP was originally thought to be a reactive (non neoplastic) process, molecular genetics suggest it has a non-random unique molecular signature. Moreover, it is clonal, which many consider the ultimate indicator of a true neoplasm. In this study, clinical, histopathological, and radiological studies help in further clarification of this entity.

Introduction
Bizarre parosteal osteochondromatous proliferation (BPOP) mostly commonly presents as an exophytic outgrowth from the cortical surface of bones. It was described in 1983 by Nora and colleagues, resulting in the eponym, Nora's lesion. Since then, approximately 160 cases have been presented in the peer-reviewed literature with a wide age range peaking in the 4th decade (1). BPOP usually affects the metacarpal or metatarsal bones, or the proximal and middle phalanges; however, long bones, skull, maxilla and metatarsophalangeal sesamoid have been reported to be affected as well (2-4). The first line of treatment is generally surgical excision (5). The frequency of recurrence of this benign lesion as well as its clinical presentation may cause it to be mistaken for malignant processes (5-6). While BPOP was originally thought to be a reactive (non-neoplastic) process, molecular genetics suggest it has a non-random unique molecular signature (7). Moreover, it is clonal, which many consider the ultimate indicator of a true neoplasm.

There has been a trend building over the last 2 decades, to recognize tumors that were thought to arise primarily in soft tissues (derived from the primitive mesenchyme) to, in fact, rarely arise as unique lesions of bone. Examples include synovial sarcoma, rhabdomyosarcoma and leiomyosarcoma. While still exceedingly rare, the recognition of such primary bone lesions is now well accepted. Synovial sarcoma arising in bone for example, has been reported in three cases since its first report in 1997 (8). Even rarer is the recognition of the reverse, with lesions that were initially thought to be unique to bone now identified as having arisen in soft tissue. While this has been well documented with osteosarcoma and Ewing's sarcoma, it has never been reported for Bizarre Parosteal Osteochondromatous Proliferation. This study explores the first reported case of Nora's lesion originating in soft tissue.

Case Report
The patient, a 48 year old hypertensive, dyslipidemic African American woman, status post hysterectomy, was first referred to our institution because of coronary artery disease with unstable angina. After admission, acute myocardial infarction was ruled out and she underwent cardiac catheterization and right coronary artery percutaneous transluminal coronary angioplasty (PTCA) with stent placement in her right ventricular marginal branch coronary artery which initially showed 90% stenosis and 70% stenosis of the right ventricular marginal branch. The 90% RCA lesion was reduced with a stent to 0%. The right ventricular marginal stenosis was reduced to 10% utilizing a balloon. There were no complications. She did well but only for several months when she started having recurrent episodes of retrosternal chest pain with shortness of breath on minimal exertion. After a sudden increase in shortness of breath and pain radiating to the left arm, she went to her local emergency room. Again an acute infarction was excluded and she was transferred to our institution for left heart catheterization with coronary angiogram and left ventriculogram showing end stent restenosis of her RCA. This was dilated to 0% successfully. Several months later a third episode occurred with similar symptomatology and the addition of intermittent claudication in her right groin area and calf with walking but with no prior history of peripheral vascular disease. As before, the patient underwent left heart catheterization with coronary angiogram the same day. The left main stem artery was patent while the LAD and left circumflex demonstrated minor irregularities. Again, the RCA showed 90% restenosis and this stenosis was reduced from 90% to 20%.

Three years later she presented because of near complete incapacitation by pain in her right wrist progressing over a three month period and a mass on the lateral volar aspect of the ulna at the wrist joint identified on physical examination. It was very tender to touch resulting in lack of full supination, keeping her awake at night. Conventional radiography revealed calcification at the rim of a cystic appearing lesion adjacent to the distal ulna. MRI had both fluid and solid characteristics and was suspicious for contact with, and invasion of, the cortex of the ulna. Microbiologic cultures at the same time of excision were negative.

She had an uneventful recovery without sequela Seven years have now past following removal of her wrist lesion. She has had no problems related to the lesion or its excision.

Results

Radiographic Imaging

Best seen on CT was a 1.5 x 0.9 cm soft tissue mass in the volar aspect of the wrist immediately anterior and separate from the distal ulna at the level of the distal radial ulnar joint. The lesion was slightly cephalad and anterior to the pronator quadratus muscle. It demonstrated curvilinear ossification peripherally and proximally that was well defined while a more irregular calcified component was observed on its radial aspect (Figure 1).

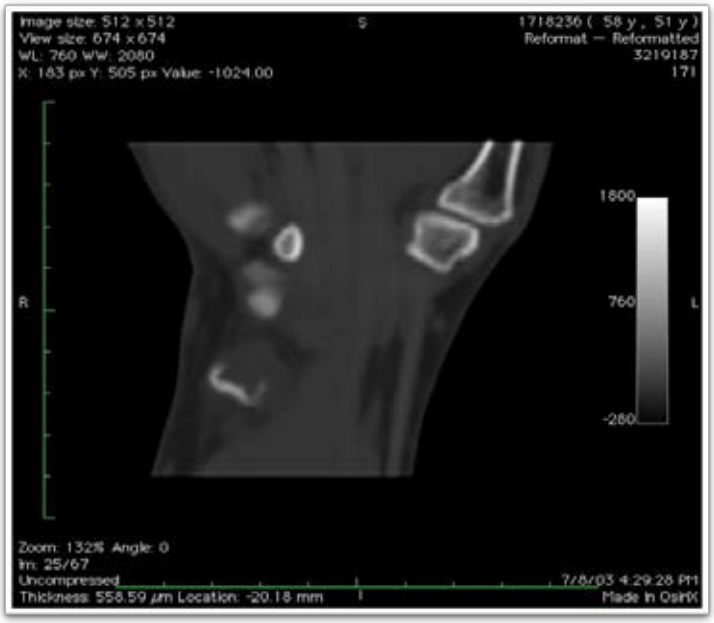

Figure 1. Coronal view

Pathology

On gross examination, the specimen was a glistening, tan-white, 2.0 x 1.7 x 1.5 cm portion of bony tissue that appeared to be partially covered by a glistening blue-gray cartilaginous cap. This cartilaginous cap measured up to 1.3 cm in maximal thickness. Upon sectioning, only minute portions of bone admixed with mottled tan-yellow cartilage was noted throughout the specimen.

The specimen was entirely submitted following fixation and decalcification. The lesion demonstrated interdigitation of fibroblastic, cartilaginous and osseous elements with focal atypia. At higher power, a transition from one tissue type to another was evident (chondroosseous metaplasia). Still higher power demonstrated large number of stromal cells with nuclear hyperplasia, hyperchromasia and contour membrane irregularities abutting bizarre fibroblastic cells, osteoclastic giant cells, osteoblasts and vascular elements (Figure 2). Other fields showed less worrisome zones of hyaline cartilage and fibrocartilage.

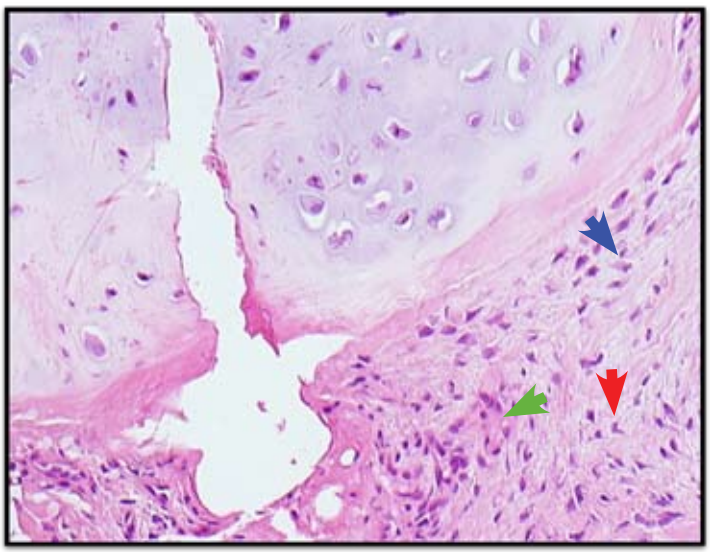

Figure 2. High power pictomicrograph (H & E 400x). Red arrow indicates stromal cells with hyperchromasia and nuclear contour abnormality. Blue arrow indicates bizarre fibroblastic cells. Green arrow indicates osteoclastic giant cell.

Discussion

Histologically, BPOB demonstrates well formed trabecular bone centrally surfaced by hyaline cartilage. The bone has blue tentorial properties by conventional H & E staing (blue bone). The cartilage is often lobulated and the connective tissue stroma hypervascular and admixed with atypical (bizarre) fibroblasts & mineralized bone mimicking sarcoma. This case fulfilled all these criteria and was considered a benign myxocartilagenous proliferation with non-zonal ossification separated by small amounts of bizarre spindled cells of fibroblastic lineage surrounding rare osteoclastic giant cells. No abnormal mitoses were appreciated and necrosis was not seen to be present. The differential diagnosis was broad and included malignant tumors such as osteosarcoma, reactive lesions such as periosteal chondroma and benign conditions including soft tissue chondroma. However, all were rejected because of clinical, radiologic or histologic features incompatible with those options leaving Nora's lesion the most reasonable possibility.

Conclusion

In conclusion, we, for the first time, report here that Nora's lesion can originate in soft tissue. While we explored here the histological and radiological demonstration of this uncommon entity, molecular genetics may help to further characterize the pathogenesis of this entity.

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short report

The Effects of Temperature on Growth of Endophytic Antarctic Algae in Culture

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Abstract

The current study investigated the effects of temperature on the growth of endophytic Antarctic brown algae. Four species of Antarctic macroalgae, *Ectocarpus siliculosis*, *Geminocarpus 89-07*, *Geminocarpus austrogeorgia*, and *Geminocarpus 89-2*, were selected for this present study. Each endophytic species was exposed to -1°C, +1°C, +4°C, and +10°C temperatures in culture. The goal of this study was to evaluate specific growth rates and morphological developments in culture of *E. siliculosis*, *G. 89-07*, *G. austrogeorgia*, and *G. 89-2* in varying temperatures to help determine the biogeographic affinities and basic ecophysiological information. *E. siliculosis* had optimum growth in the warmer temperatures of +4°C and +10°C. *G. austrogeorgia* had optimum growth in the -1°C condition. *G. 89-2* and *G. 89-07* had optimum growth at +4°C. This suggests that *E. siliculosis* is better adapted to warmer temperatures. Because of the variable growth in different temperatures, *G. 89-2* and *G. 89-07* are better adapted to the Subantarctic and *G. austrogeorgia* is better adapted to the Antarctic. Temperature plays an important role in the growth rate and the biogeographic affinities of these endophytic strains.

Introduction

The marine environment of Antarctica has been exposed to low temperatures and isolation for twenty-four million years (Ehrmann and Mackenson 1992). Polar marine environments are characterized by extreme variations in irradiance and seasonal changes of day length and low water temperatures, ranging from -1.8°C to +2°C and up to +10°C in shallow intertidal waters (Kirst and Wiencke 1995). Even in these harsh conditions, Antarctic species have strongly adapted to their surroundings and function efficiently within their environment. Temperature is a crucial factor within Antarctic ecosystems as it influences some aspects of an alga's life-history (Rautenberger and Bischof 2006). Four species of Antarctic macroalgae, *E. siliculosis*, *G. 89-07*, *G. austrogeorgia*, and *G. 89-2*, were selected for this present study. All four subtidal algal were isolated as endophytes growing within larger macroalgae at Anvers Island off the western Antarctic Peninsula (Amsler *et al.* 2009). Antarctica is unusual in that filamentous, endophytic algae are very common, but it is rare to see macroscopic growths growing out of the macroalgae as epiphytes (Peters 2003, Amsler *et al.* 2009). It is believed that this is because of high levels of amphipod mesoherbivores that consume any filaments that do grow out from within the chemically-defended macroalgae (Peters 2003, Amsler *et al.* 2009). Because the epiphytic forms are rarely or never seen in nature, their biogeographic and other characteristics are largely unknown. Observations of each species' development in different temperatures can help determine the biogeographic affinities and provide other basic ecophysiological information. The goal of this study was to evaluate the growth response of *E. siliculosis*, *G. 89-07*, *G. austrogeorgia*, and *G. 89-2* to varying temperatures and to determine specific growth rates and morphological developments in culture.

Materials/Methods

E. siliculosis was collected in *Delesseria lancifolia* growing at the Palmer Station pier on Anvers Island. *G. 89-07* was collected in *Desmarestia antarctica* from Bonaparte Point

on Anvers Island. *G. 89-2* was collected in *D. antarctica* from the from Bahia Paraiso shipwreck off DeLaca Island. *G. austrogeorgia* was collected in *Ascoseira mirabilis* from Shortcut Island. All were collected as endophytes.

Growth rates of *E. siliculosis*, *G. 89-07, G. austrogeorgia*, and *G. 89-2* were determined across a range of temperatures. Using clonal cultures, each alga was microdissected into fragments a few cells long with no reproductive structures. Three fragments of each species were placed into three separate wells of 6-well culture plates. The temperatures of -1°C, +1°C, +4°C, and +10°C were used for all the experiments. All temperatures were ±1.0°C and maintained by lighted incubators. In all the algal cultures, the irradiance was kept constant of 28 µm photons m^{-2} s^{-1}. The irradiances were measured with a LI-COR Biosciences model 250A light meter and quantum sensor. To prevent depletion of nutrients, Provasoli's enriched seawater medium was changed twice a week. Each culture was observed every three days to note the occurrence of branching, the formation of reproductive structures, or the presence of germlings. The length of each event's duration was recorded in days. When reproductive structures were present, the type of reproductive structure was noted. When germlings were present, they were removed from the parent to avoid competition for nutrients and were noted. The duration of the experiments for the *E. siliculosis* and *G. 89-07* was 75 days, and 40 days for *G. austrogeorgia*, and *G. 89-2*.

Final dry weights were determined by drying the algal filaments onto pre-dried, preweighed herbarium paper and weighing them on a Mettler MT5 analytical micro-balance. The final weights varied for each temperature and a typical final weight for a large individual was approximately 14 mg for *E. siliculosis*, 8 mg for *G. 89-07*, 1.25 mg for *G. 89-07*, and 0.9 mg for *G. austrogeorgia* .

Results

Temperature had a significant effect on the specific growth rates of all the Antarctic endophytes. *G. 89-07* had the greatest growth rate at +4°C. *G. 89-2* had the greatest growth rate at +4°C. *E. siliculosis* had the greatest growth rates at +4°C and +10°C. *G. austrogeorgia* had the greatest growth rates at +1°C (Fig. 1). No growth was observed at +10°C except in the culture of *E. siliculosis*.

Discussion and Conclusion

In Antarctica, *E. siliculosis* grows as an epiphyte; however, *E. siliculosis* is found all around the world in various temperatures. The average growth data reveals that *E. siliculosis* can tolerate and grow in Antarctic temperatures. However, because *E. siliculosis* had the greatest growth in the +10°C and +4°C, the data suggest that *E. siliculosis* is better adapted to warmer temperatures.

Unlike *E. siliculosis*, the *Geminocarpus* species is unique to the Antarctic and Subantarctic. The average growth data proves this assumption as all the *Geminocarpus* strain algae died in the highest temperature of +10°C. Moreover, the data suggest that there is a relative growth difference between temperatures. This variance would imply that *G. austrogeorgia* is better adapted to the Antarctic, while *G. 89-07* and *G. 89-2* are better adapted to the Subantarctic.

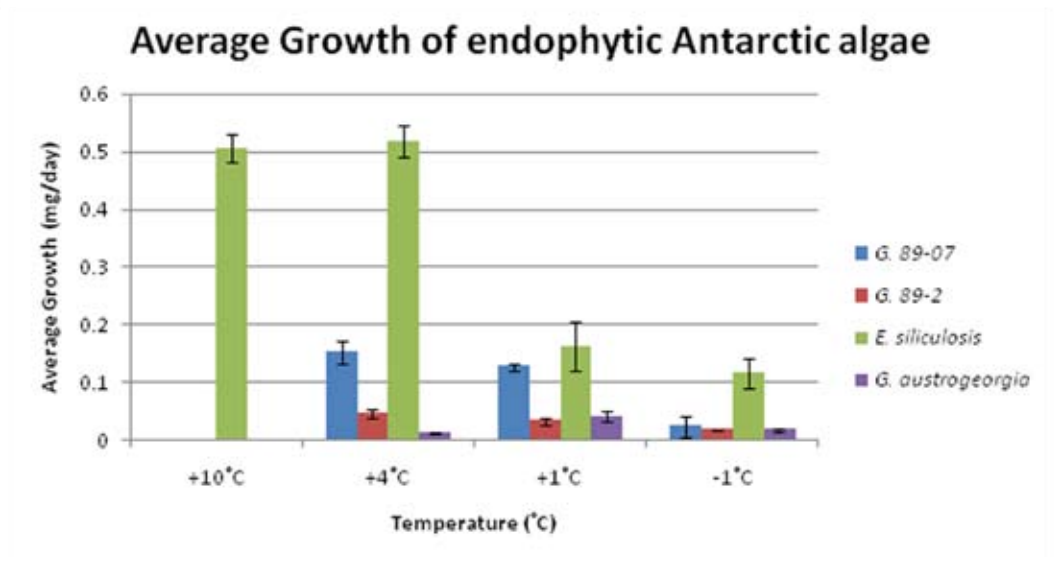

Fig 1. Growth Rates of endophytic Antarctic algae at varying temperatures

Global warming presents itself as a threat to the Antarctic ecosystem. As global climate change alters the composition of the marine community in Antarctica, one can speculate that more Antarctic-adapted algae will be forced to adjust to warmer temperatures and that the endophytes that adapt to a warmer climate will extend the range in which they are

found. Moreover, a warmer climate change would select for those endophytes that can adapt and grow well in such temperatures and many Antarctic-adapted algae will be eliminated.

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short report

Orthodenticle Homeobox 2 (OTX2) and its Influence on Mammalian Development: A Scientific Review

Ashruta Patel

Introduction

Gene expression takes information from a gene to perform regulated functions, such as synthesizing a protein. The functionality of a gene can be affected by the timing, location and degree of its expression. The overexpression and underexpression of proteins produce numerous effects. Homeobox genes are developmental control genes regulating aspects of morphogenesis and cell differentiation. OTX2 is a protein involved in creating a structure around certain neurons that mature within the brain. The result of OTX2 overexpression or underexpression influences visual experience, postnatal embryonic formation, development of neural and sensory structures and the critical period onset for plasticity (Westenskow 2505). The *OTX2* gene encodes a member of the bicoid subfamily of homeodomain-containing transcription factors that is essential in brain and sensory organ development (Bunt 2010). This review will focus on the role of OTX2 in mammalian development and its involvement in neuronal functions.

Required critical period onset of OTX2 for the proper development and function of the retinal pigment epithelium

The presence of OTX2 plays an important role in the development of the retinal pigment epithelium. Its emergence leads to structural maturations which positively promote OTX2 uptake to strengthen the critical period onset. The critical period is a limited time for OTX2 to play its role in an organism. If the stage displays high sensitivity to certain stimuli but the stimulus is not received, it may prove difficult for certain function development later in life. Simultaneoulsy, the mutation of *OTX2* can cause malformations and/or brain anomalies, seizures and developmental delay of the retinal pigment epithelium (Sugiyama 370). The expression of OTX2 in developmental human fetal eyes from 6-10 weeks post-conception was studied to determine how the localization of the protein depends on its timing. The x-ray film signals show protein variation over time (Figure 1). The peak in Figure 2 reveals the highest level and strongest signal of OTX2 (Larsen 488).

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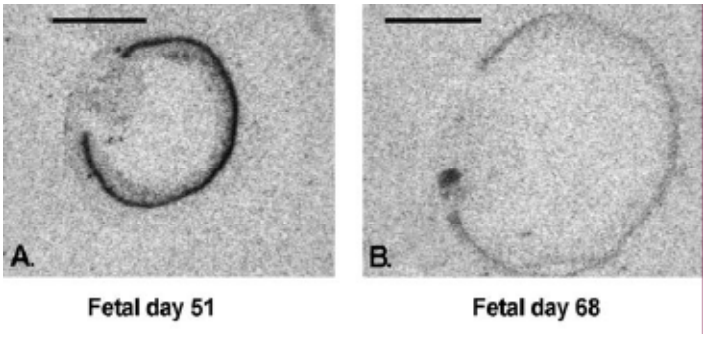

Figure 1. X-ray image of retinal OTX2 signal in fetus day 51 (critical period) and 68 (Larsen, 488)

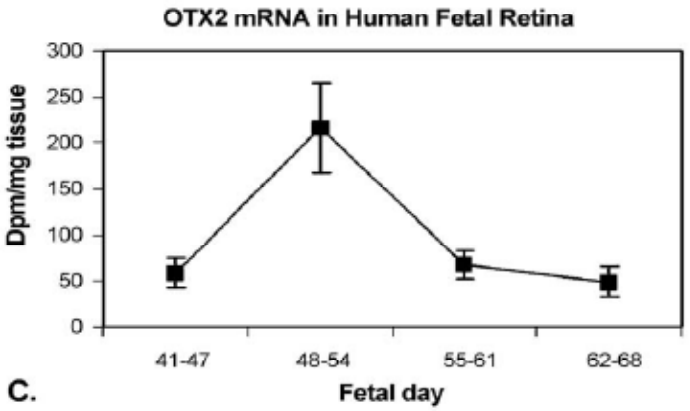

Figure 2. OTX2 expression peaks at different fetal days -protein in expressed early in the human retina from the 6th and 10th weeks of fetal development (Larsen 489)

Prevalent OTX2 levels allow the human fetal retina to develop during the onset of the critical period. The involvement in retinal development allows for differentiation of the retinal pigment epithelium (Larsen 489).

During development, OTX2 expression persists in the pigment epithelium, whereas OTX2 expression of the neural retina becomes progressively restricted to the outer nuclear layer and the outer part of the inner nuclear layer. This pattern of OTX2 expression through developmental stages plays a crucial role in the general maturity of the vertebrate eye. (Rath 65). Stages of progressive developmental growth are shown below.

Retinoblastoma tumors, on the other hand, originate from cells that have the ability to express OTX2, suggesting a correlation between the cell-of-origin of retinoblastoma tumors and cells expressing OTX2 (Glubrecht 250). Figure 4 shows the expression of OTX2 on retinoblastoma tumors.

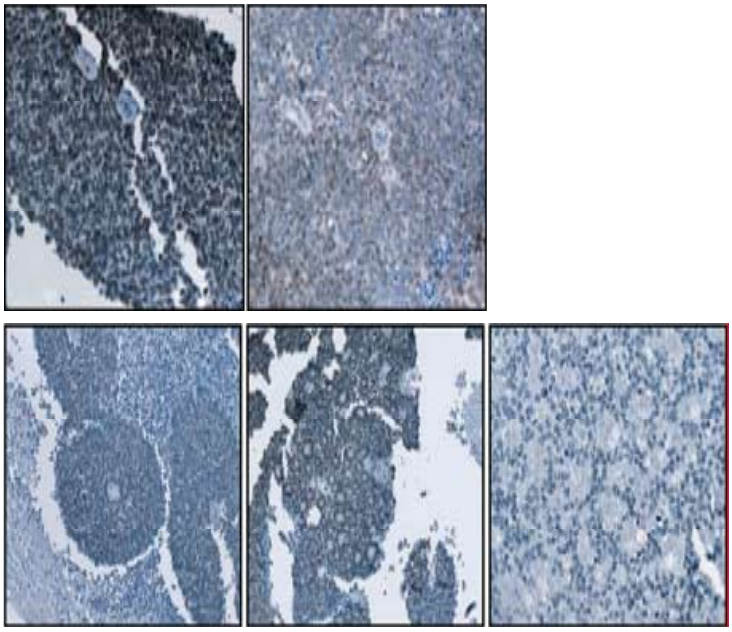

Figure 4. OTX2 expression in tumors - An analysis of retinoblastoma tumors indicates that OTX2 is widely expressed (Glubrecht 258)

Complications related to OTX2

OTX2 mutations can lead to many other diseases, such as anophthalmia, a disease caused by a mutation in fore and midbrain development and retinal differentiation leading to learning difficulties in humans (Mihelec 412). Humans with heterozygous mutations can have a variety of structural eye malformations. For example, ophthalmological abnormalities can arise in patients with Combined Pituitary Hormone Deficiency (CPHD) (Diaczok 4351). CPHD is caused by an insertion mutation that occurs from premature stop codon and the lack of a C-terminal region on the protein which prevents the activation of the target gene (OTX2) (Tajima 314).

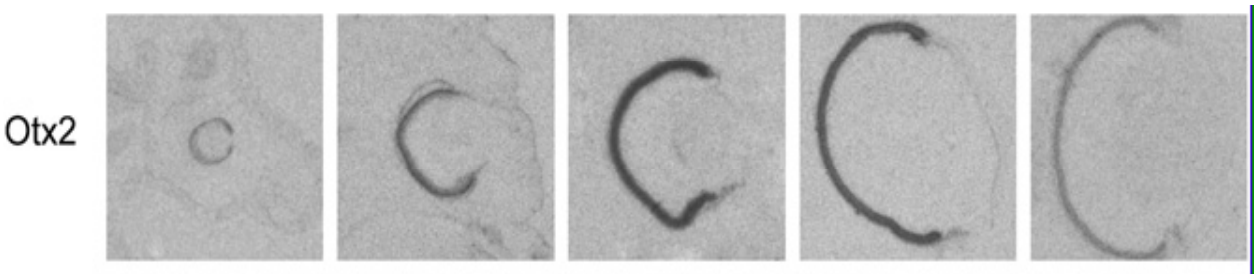

Figure 3. Retina at developmental stages (Rath 71).

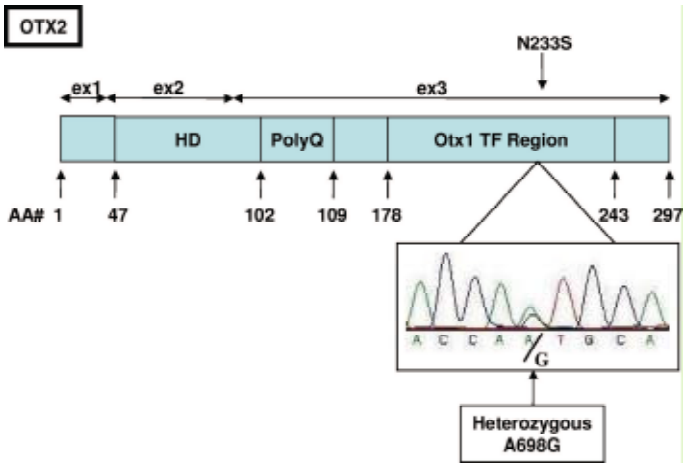

Figure 5 . Insertion herterozygous OTX2 mutation that is capable of causing a variety of structural eye malformations and ophthalmological abnormality (Diaczok 4354)

OTX2 is one of the genes associated in medulloblastoma tumorigenesis and its elevated expression suggests its role as an oncogene in medulloblastoma (Bunt 2010). The emergence of OTX2 can further affect other neural and sensory structures such as the brain, ear, nose and eye.

OTX2 involved in visual experience

Visual experience using the eye leads to the emergence of the OTX2 protein, which in turn triggers the further development of the visual and sensory system. The removal of OTX2 from the visual pathways (i.e. raised in complete darkness) can cause a delay in plasticity (Sugiyama 509). Enucleation by the removal of both eyes before the onset of the critical period also shows a decrease in OTX2 protein levels (Sugiyama 509).

There is a delay in ocular dominance plasticity when animals (mice, rats etc.) are nurtured in complete darkness from birth (dark-rearing). The influence of visual experience can be determined by counting OTX2 cells at the critical period peak after 2 sensory deficiencies. One deficiency is the removal of both eyes (enucleation) from eye-opening and another is dark-rearing from birth. OTX2 protein levels reduce in the superficial layer of the brain (cortical) with enucleation (Sugiyama, 510). Experiments have shown that OTX2 is reduced in the visual cortex after dark rearing indicating that its emergence is experience-dependent.

OTX2 localization in Parvalbumin (PV) cells

Dark rearing also delays maturation of PV (parvalbumin) cells; however, the cells have the ability to mature when OTX2 is delivered directly into the visual cortex. The maturation of PV cells triggers the onset of plasticity. OTX2 is localized to PV cells and early OTX2 infusion increases its expression, whereas a conditional deletion of the protein decreases PV cell expression (Sugiyama 511). Thus, visual experience promotes the accumulation of OTX2, which leads to accelerated parvalbumin (PV) cell network development and critical timing period. It has been noted that the conditional removal of OTX2 from the visual pathway abolishes plasticity (Sugiyama 511).

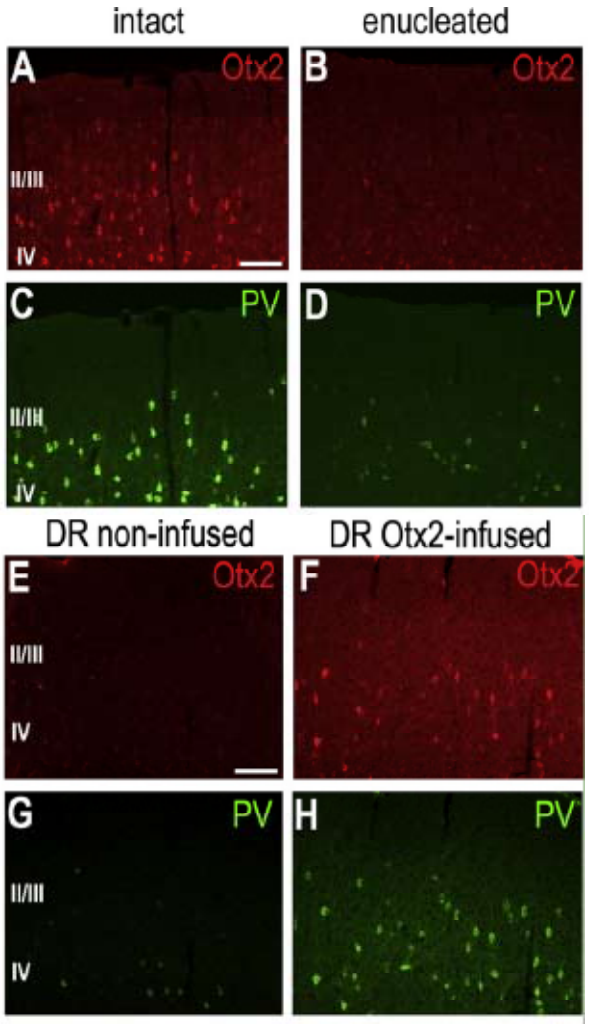

Figure 6. Decreased expression of OTX2 and PV when enucleated at eye-opening and dark-rearing (Sugiyama 511)

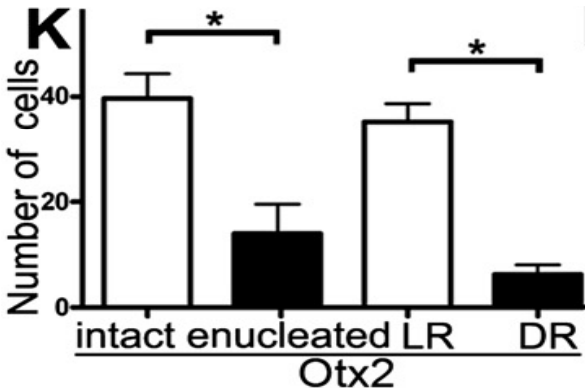

Figure 7. Decreased number of OTX2 cells at critical period after enucleation and dark rearing (DR). Higher number of cells when eyes are intact and light reared (LR)] (Sugiyama 511) This suggests that OTX2 may influence late PV cell maturation by re-emerging in response to visual experience after eye-opening.

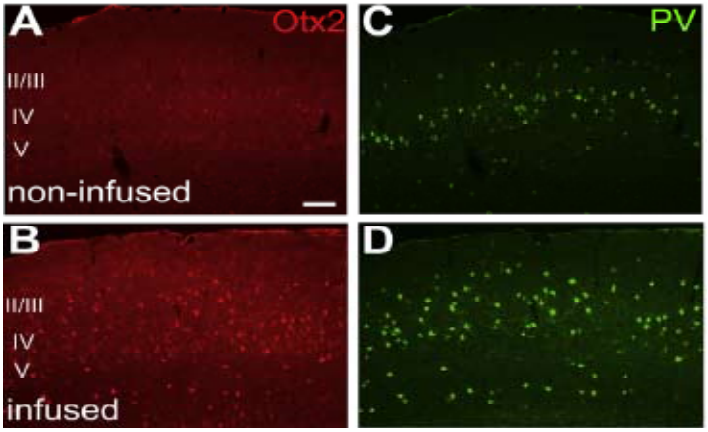

Figure 8. PV (parvalbumin) acceleration once infused with OTX2 for 5 days (Sugiyama 512)

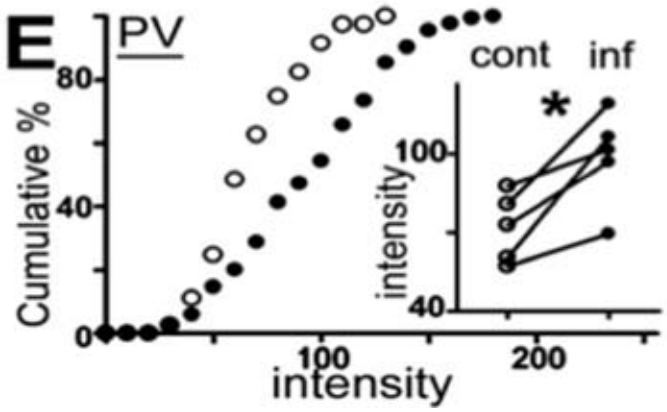

Figure 9. PV (parvalbumin) acceleration once infused with OTX2, filled symbol = OTX2, open symbol = control (Sugiyama 511)

OTX2 is weakly expressed before the critical period and strongly expressed in PV cells during the critical period and into adulthood. OTX2 expression in the mouse visual cortex is also dependent on activity, because dark-rearing or surgical removal of the eye(s) reduces both parvalbumin (PV) and OTX2 signals, implicating a link between OTX2 and PV-cell maturation (Rebsam 386).

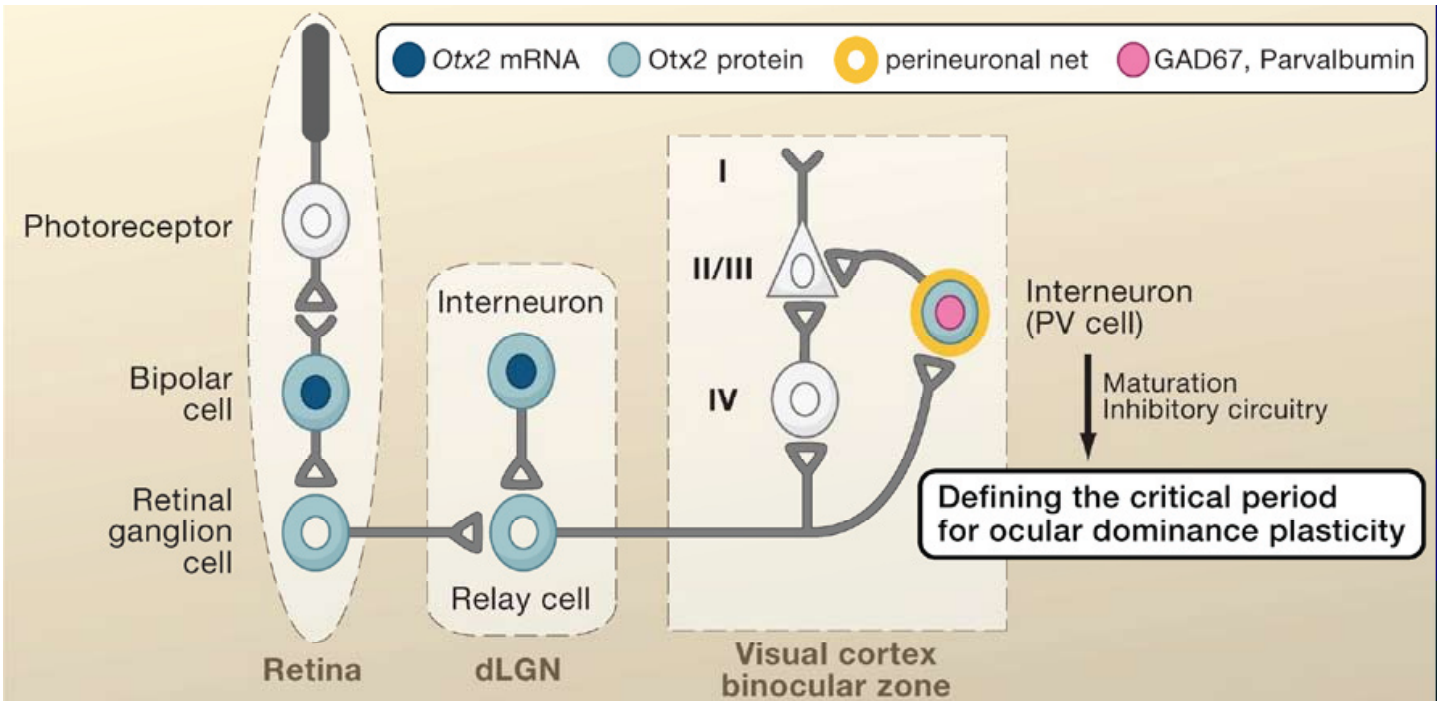

Figure 10. OTX2 Expression in the Visual Pathway (Rebsam 386)

Conclusions and Future Directions

Therefore, it can be concluded OTX2 genes translate proteins that have many causes related to its presence or deficiency. If an individual were to contract certain visual or sensory malformations, a potential cause might be linked to OTX2. There are many benefits associated with gene/protein studies, and OTX2 research could provide a prospective outlook on how certain postnatal deficiencies are detected to prevent birth abnormalities. The Psychiatry and Behavioral Neurobiology department affiliated with the School of Medicine at the University of Alabama at Birmingham is just one example of a subdivision dedicated to such insights. Several case studies have analyzed the possible causes of certain effects of OTX2 which may potentially assist individuals diagnosed with retinoblastoma tumors, OTX2 gene mutations, combined pituitary hormone

deficiency (CPHD) or anophthalmia (Tajima 314). Findings deciphering the effects of specific proteins can lead to innovative discoveries for treatment and diagnosis, essential tools for scientific researchers and clinical physicians when addressing patients. One such example may be detection of an OTX2 mutation before birth, an asset which may be used to prevent congenital abnormalities. Therefore, experiments conducted on OTX2 can provide hope for individuals who require certain regulations. Additional experiments could help discover regulatory controls and functional domains to define properties required to control developmental pathways. Identification of molecular partners and downstream targets can contribute to additional findings related to the complexity of the mammalian brain (Simeone 1998). Future studies may seek to explore when and where protein transfer would be required in evolution and how development of essential functions can be altered over a period of time.

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short report

Upregulation of the 1,25-Dihydroxyvitamin D_3 Receptor in Neonatal Brain Injury

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Abstract

The Vitamin D_3 receptor (VDR) has been found to play a role in brain development through its identification in brain and spinal cord neurons of developing fetal rats. Also, studies have shown that Vitamin D_3 increases the amount of transcripts coding for its own receptor, VDR, and also for neurotrophins, further indicating a role in neurogenesis and protection.

Objectives:

To investigate the neuroprotective role of 1, 25-Dihydroxyvitamin D_3 and its receptor (VDR) in neonatal brain injury.

Methods:

Neural Stem Cells (NSC'S) were cultured and treated with various concentrations of glutamate. Samples were collected and analyzed through a protein assay and Western Blots. The unilateral carotid ligation model and the use of a hypoxia chamber were used to induce brain injury. In immunohistochemistry, brains previously extracted from 5-and 12-day-old mice kept in room air, hypoxic, and hypoxic-ligated conditions were stained for VDR and Cleaved Caspase-3 proteins. The stained slices were observed using a fluorescent light microscope.

Results and Conclusions:

In neural stem cells, the amount of VDR protein increased by 3.6-fold under 0.50 mM glutamate conditions, 4.2-fold under 0.75 mM glutamate, and up to 6.9-fold under 3.0 mM glutamate conditions. In hypoxia-ligation mouse brain tissue, the VDR receptor was found in areas of apoptosis, indicating that damaged or stressed cells produced more VDR than cells in normal conditions. These results suggest a role of Vitamin D_3 Receptor in brain injury; however, further studies must be completed to understand the role of Vitamin D_3 in such injury.

Introduction

Premature neonates are vulnerable to many conditions including neonatal brain injury. Neonatal brain injury affects thousands of children yearly leading to conditions such as cerebral palsy. In 2006, it was estimated that the preterm birth rate was 12.8% of the roughly 4 million live births in that year[1]. MRI data of these infants showed that about 50% exhibited some form of cerebral white matter injury[2]. Many researchers are investigating the potential role of many candidate mechanisms to confer neuroprotection. One of the difficulties in studying this condition is the multifactorial nature of this condition. As we learn more about the disease process we may one day be able to decrease the amount of destruction to the premature brain.

The main causes that play a role in the preterm brain's susceptibility to injury include hypoxia-ischaemia and infection/inflammation. Due to their inability to breathe effectively and maintain cerebral blood flow, preterm infants commonly have reduced oxygen levels in vital organs such as the brain[3]. This leads to cell death, particularly in the premyelinated oligodendrocytes (pre-OLs), an important type of glial cell which functions in neuronal insulation and conduction of action potentials throughout the central nervous system. Additionally, maternal or fetal infection/inflammation also contribute to perinatal brain injury through downstream mechanisms such as increased glutamate levels. These can lead to excitotoxicity, or neuronal death, due to the over activation of receptors for the excitatory neurotransmitter glutamate. It has been found that elevated levels of glutamate correspond directly with the amount of white matter injury present in the brain[3].

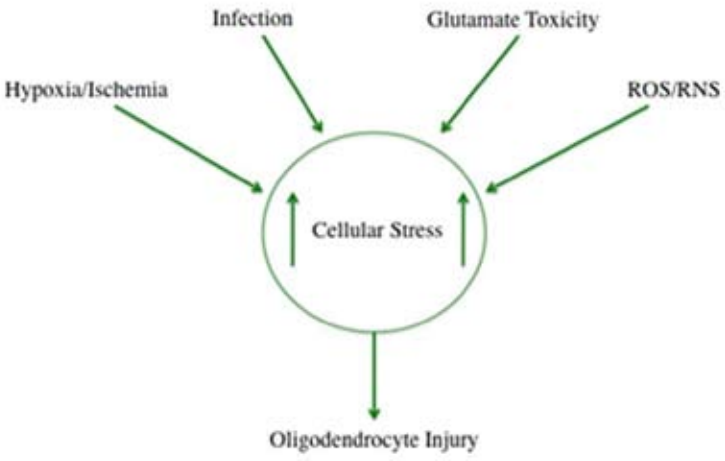


Figure 1.Known mechanisms of oligodendrocyte injury

In the developing brain, neurogenesis occurs through the differentiation of neural stem cells (NSCs). NSCs possess the capacity to differentiate into many cell types including pre-OLs, the targets of white matter injury in preterm infants. Due to their multipotent nature, NSCs are an ideal candidate for research in perinatal brain injury, and specifically in its prevention at an early stage.

Although the relationships between Vitamin D_3 and calcium absorption are understood, recent research suggests that 1,25-dihydroxyvitamin D_3 plays a role in brain development. The Vitamin D_3 receptor (VDR) has been identified in the brain and spinal cord neurons of developing fetal rats[4], indicating that vitamin D_3 must have some role in neurogenesis. Furthermore, Vitamin D_3 has been found to have a neuroprotective role, as it regulates the production of nerve growth factor (NGF) and the expression of other neurotrophins, or neuroprotective compounds. Studies have also shown that VDR transcripts existed in rat oligodendrocytes and VDR-positive cells were present in brain white matter. Vitamin D_3 increased the amount of transcripts coding for its own receptor, VDR and also for NGF, further con-

firming a role in neurogenesis and protection[4].

Methods and Materials

Cell Culture and Sample Collection

Experimental cell types (Neural Stem Cells) were cultured in sterile conditions at 37°C. NSC cells were treated with increasing millimolar concentrations (0, 0.25, 0.50, 0.75, 1.0, and 3.0 mM) of glutamate. Samples were collected, Ripa Buffer was added, and stored at -20°C.

Protein Analysis

A Bradford Protein Assay was completed for all samples to determine their respective protein concentrations. Samples were then prepared and loaded into a 10% Tris-HCl gel for Gel Electrophoresis. The gels were allowed to run and equilibrate in a 1X Transfer Buffer. They were then transferred onto a nitrocellulose membrane using a semi-dry transfer apparatus. After transfer was complete, blots were probed for various proteins (VDR, Cleaved Caspase-3, and Actin) using the SNAP i.d.™ Protein Detection System. Probed blots were then developed in a dark room using western blotting detection reagents.

Unilateral Carotid Ligation

Mice were anesthetized using 2% isofluorane per IACUC protocol. Temperature regulation occurred using a warming blanket. Using sterile technique, a midline tracheal incision was made and the left common carotid was exposed and then cauterized. The incision was closed using Dermabond. Animals were placed back with mother after 15 minutes. The animals were then placed in a plexiglass hypoxia chamber at 12% overnight. The brains were collected after 24 hours.

Immunohistochemistry

Five- and twelve- day- old mice kept in various conditions (Room Air, Hypoxia, and Hypoxia-Ligation) were euthanized. Their brains were extracted, fixed with 4% Paraformaldehyde (PFA), and cryosectioned at 16 microns. Brain slices were fluorescently stained for the VDR protein and Cleaved Caspase-3. Analysis was completed using a Zeiss fluorescent light microscope.

Results

The role of the Vitamin D_3 Receptor, VDR, in injury or protection is not fully understood. To examine the role of VDR in injury we used undifferentiated neural stem cells in the presence of increasing concentrations of glutamate, to model glutamate toxicity, a known mechanism of oligodendrocyte injury. Glutamate toxicity concentrations used were from 0-3mM. It was found that as glutamate increases, there is a concomitant increase in VDR protein level (Figure 2A). To assess the amount of cell death, we probed the same blot for Cleaved Caspase-3 and probed for Actin to normalize the results (Figure 2A). Cleaved Caspase-3 levels were normalized to Actin and represented in Figure 2B. There is a statistical increase in cell death at 0.25 and 0.50mM, $p < 0.05$, compared to control. VDR levels were also normalized using Actin as the"housekeeping" protein and Figure 1C demonstrates a general increase in VDR levels with a statistical increase of protein concentration seen at 0.75, 1 and 3 mM glutamate concentrations, $p < 0.01$. The increase seen in VDR almost reaches 7 fold as seen at 3mM.

If VDR levels change during stress, they should increase during certain pathological states. One such pathological state is in hypoxia-ischemia, which we were able to mimic by using the unilateral carotid ligation method. Full details of the procedure are described in methods section, but animals were anesthetized, common carotid ligated, and then placed in hypoxia. The VDR levels in control hemispheres (right) were compared to VDR levels in ligated hemispheres (left). In Figure 3, A (nuclear), C (Cleaved-Caspase 3), E (VDR), G (merged) of control hippocampus are compared to the ligated hemisphere in Figure 3, B (nuclear), D (Cleaved-Caspase 3), F (VDR) and H (merged), respectively. There is a dramatic increase seen in VDR shown in

Figure 2.(A)

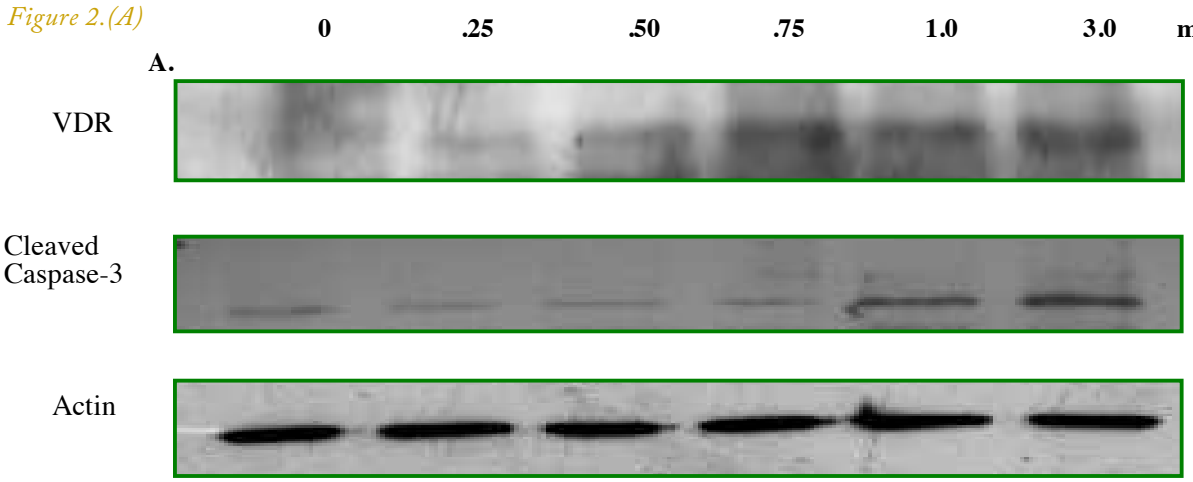
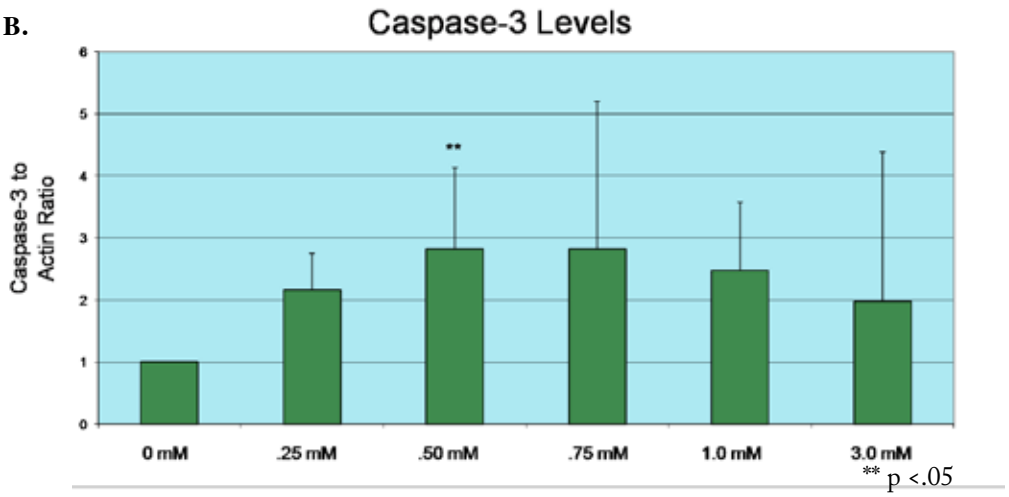


Figure 2 (A-C). Protein analysis of neural stem cells under increasing glutamate concentrations

(A) Representative immunoblot probed for VDR, Cleaved Caspase-3, and Actin proteins

(B-C on page 32)

B.



C.

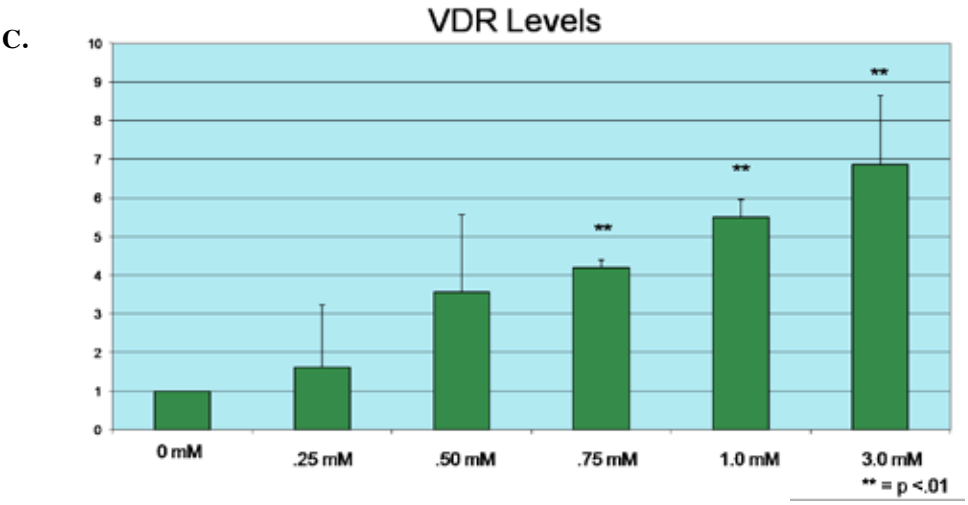


Figure 2. (B-C)

*(B) Caspase-3 levels in neural stem cells increase, p<0.05 in ** experiments, in increasing concentrations of glutamate: 0, 0.25, 0.50, 0.75, 1.0, and 3.0 mM*

*(C) VDR levels in neural stem cells increase, p<0.01 in ** experiments, in increasing concentrations of glutamate: 0, 0.25, 0.50, 0.75, 1.0, and 3.0 mM*

Figure 3F compared to Figure 3E, which additionally seems to correlate with cells that are Cleaved-Caspase 3 positive. Controls are not shown in this figure but no primary antibody controls were used to normalize the fluorescence.

In the experiment in Figure 3 these brains were not assessed for the specific cell type that responds to stress. The distribution of VDR in the brain is not well understood. Therefore, this experiment was designed to demonstrate specific cell types that express VDR. In Figure 4 (B and D), we demonstrate that VDR is expressed highly in NeuN positive cells.

Discussion

Currently, there is little understanding of brain injury and even less understanding of neonatal brain injury. There is a growing need in the vulnerable population of premature neonates to gain more insight into the pathology of this condition. It is well known that certain conditions such as infection and inflammation are linked to neonatal brain injury[3]. The current animal models to study neonatal brain injury use animals such as mice at different stages of development. The current model we use at 5 days will assess potential white matter injury but older animals are better models for grey matter injury as assessed in our current experiments. There is no evidence that links brain injury to the vitamin D receptor.

In our current study, we have linked VDR to excitotoxicity in neural stem cells. Neural stem cells are the most vulnerable cell population in neonatal brain injury. We have made a first step in understanding the role of VDR in neonatal brain injury by demonstrating that this receptor responds to stress. It can be argued that increases in VDR may be harmful to the cell but we have not done experiments to address these issues. The cells could be exhausting all potentially protective pathways before cell death. We can address both of these scenarios through genetic manipulation of the VDR in vitro and in vivo by experiments that involve RNA silencing.

The response of VDR in neuropathology is under investigated. Therefore, our study suggests that there may be a potential role in VDR in brain injury. Our preliminary findings suggest there is an increase in VDR in our model of hypoxia-ischemia. Using the unilateral carotid ligation we can tease out the role of hypoxia alone on the VDR and also do time-course experiments in these animals to further elucidate the role of VDR to see if it is an early or late responder.

Neural stem cells can also be labeled in brain slices similar to how we determined the presence of VDR on NeuN positive cells. Our study has opened up many doors of investigation that will

Immunohistochemistry

Non-Ligated (Right Hemisphere) Ligated (Left Hemisphere)

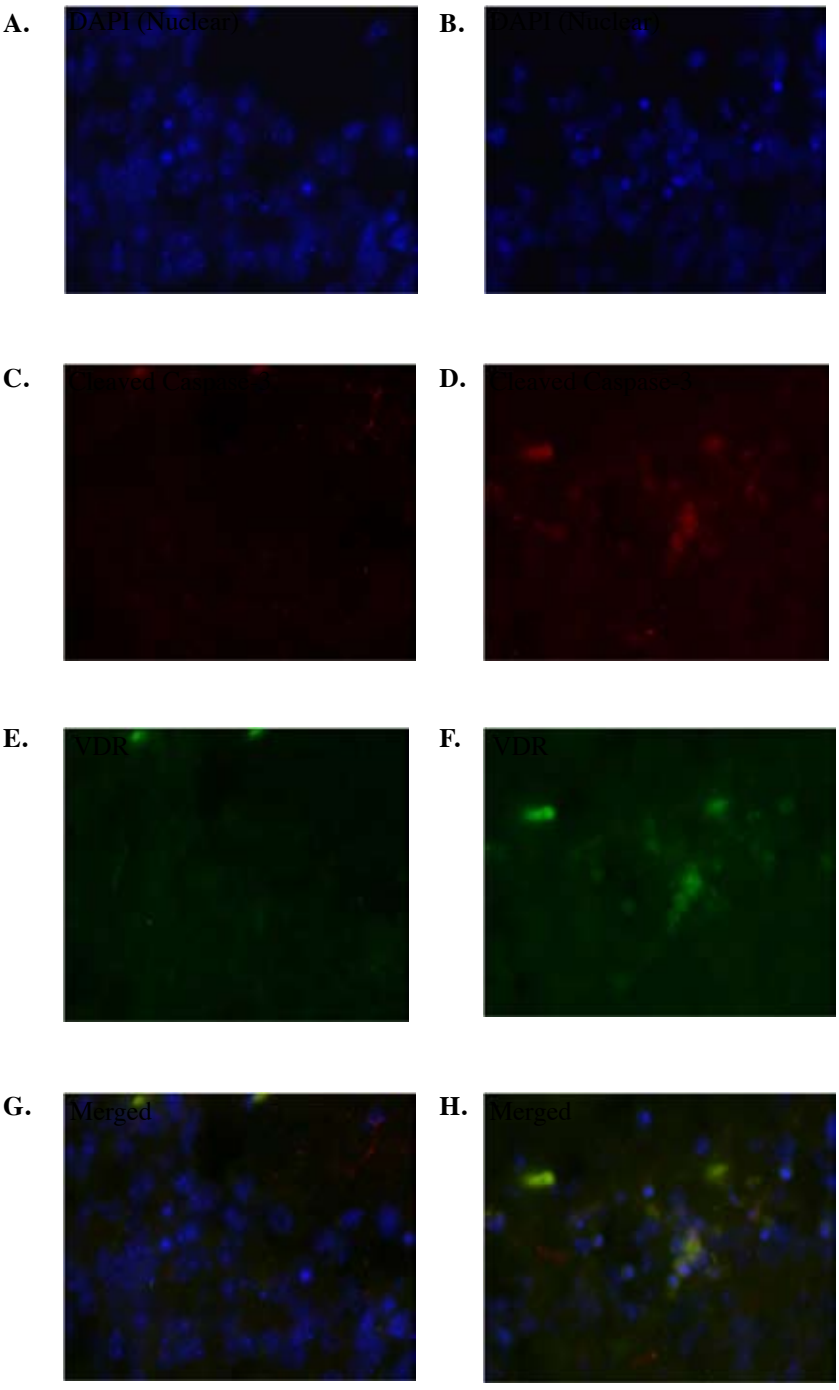


Figure 3. Immunohistochemistry analysis of neonatal mouse brain tissue in hypoxia-ischemia with effects from a unilateral carotid ligation (right column images) and no ligation effects (left column images)
(A) and (B) Nuclear-Dapi. (C) and (D) Cleaved Caspase-3-Cy3. (F) VDR-FTIC increases in ligated (left) hemisphere as compared to non-ligated (right) hemisphere of brain (E). Also, VDR co-localizes with Cleaved Caspase-3 in areas of brain injury (H).

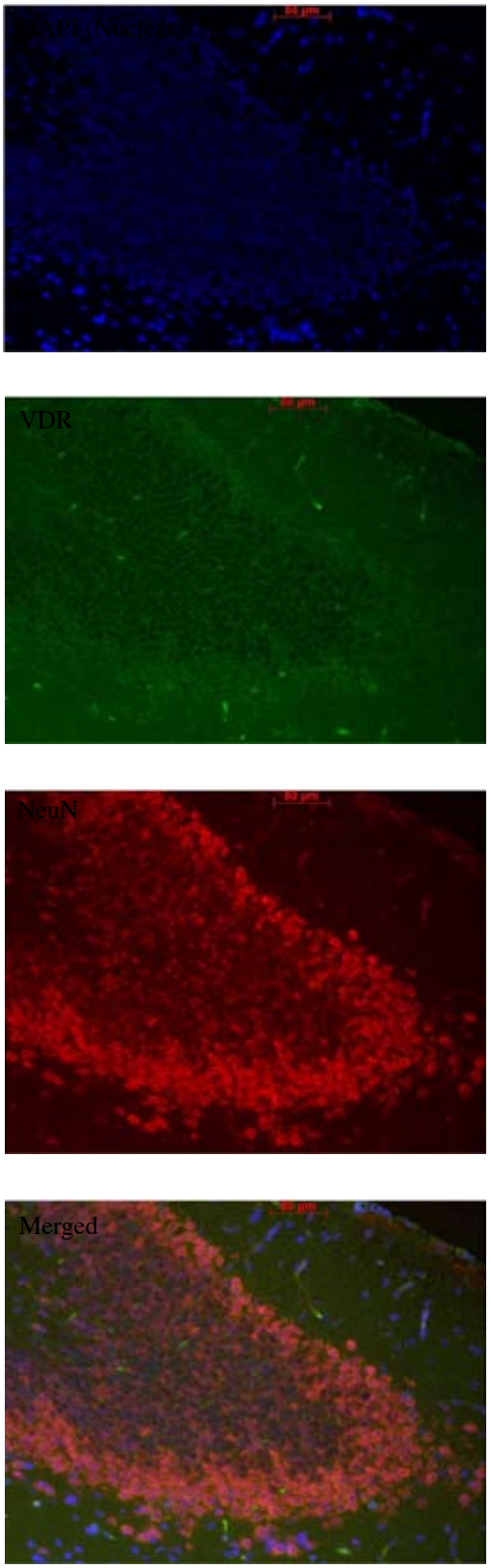


Figure 4. Immunohistochemistry of left hippocampus (ligated left common carotid) and Cell-specific VDR localization.(A)Nuclear-Dapi. VDR is expressed in neurons of the hippocampus, as indicated by NeuN- Cy3 (C), VDR- FITC (B), and merged (D).

require multiple experiments to further characterize VDR in a cell-specific fashion. We will propose to sort neural stem cells from control and injured brains to determine if there is a difference in viability. It will be important to determine if VDR has a detrimental or protective effect in these cells.

Conclusions

Based on the novel results of this investigation, we conclude that VDR may be directly involved in cell stress by responding to the conditions of excitotoxicity and hypoxia-ligation. Future studies must be conducted in order to understand cell and tissue response to these conditions in the presence of Vitamin D_3. Also, it is necessary to understand Vitamin D_3 mechanisms in damaged cell and tissue samples. Ultimately, after understanding the full neuroprotective potential of Vitamin D_3, drug therapy to animals and later to preterm infants may be completed, thus allowing for a preventative measure against brain injury in the neonate.

Acknowledgements

I would like to thank my mentor, Brian Sims MD, PhD for his guidance and support throughout this process. Thank you to everyone at the Sims Laboratory for your continued support and encouragement.

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faculty interview: biology

From Insects' Behavior to Extremophiles' Gene Regulation: An Interview with Dr. Asim Bej

Timmy Wang

When it comes to describing the work accomplished by the many great minds here at the University of Alabama at Birmingham, I am constantly reminded of a quote by Albert Szent-Gyorgyi: "Research is to see what everybody else has seen, and to think what nobody else has thought." As apt as this quote is in describing research, I see that the second part of the quote to be as fitting in describing the thought processes of the research faculty on campus. Recently, I had the privilege to interview Dr. Asim Bej, who many know as the Molecular Genetics professor but perhaps not as many know about his journey in science and his current research. From my time with Dr. Bej and hearing his passion for research, I find a growing story of interests in not only leading the discoveries of extremophiles but also helping young minds to become curious about research.

From the beginning, Dr. Bej has always expressed an interest in science. In fact, "rather than someone or something specifically turning me to science," Dr. Bej said he created his own personal experiments and tests of the information taught to him. From testing the pH of acid rain in middle school to personal examinations of moss growth in high

school, Dr. Bej would take what he "learned in the classroom or laboratory and go outside of the class to verify the facts." It is from these personal pursuits and the testing of different hypotheses that led Dr. Bej to engage in scientific research at the University of Calcutta. While working toward a B.S. in Zoology, Dr. Bej began his first research project after noticing a particular behavior exhibited by ants. While these ants are normally used as fish bait, Dr. Bej noticed how these ants would crawl over the eggs in the nest. From his viewpoint, these ants seemed to exhibit a type of parental care of spraying poison at potential threats to the eggs. This peculiar behavior of the ants peaked his curiosity, and he became "interested in what this poison gland was all about, what was the anatomical and histological structure, how it was sprayed, and what triggers the action." From his initial observations, Dr. Bej was able to enter into a lab and design his own project to understand the workings of the ants' poison glands, which he found to be a neuronal network and ganglia of the ant, allowing the quick spraying of poison at potential threats.

During his time in the zoology lab, Dr. Bej came to discover the lab as a fascinating place and that it would soon become his second home. Therefore, he decided to stay in the same laboratory at the university and to continue his work on insects as a Master's student. However, Dr. Bej decided to transform his previous project of the neural endocrine system to include a way for the research to have an application for human benefit. For his Master's degree, Dr. Bej worked on insect repellants, such as camphor, and their effects on the neurotransmitters and the nervous system. After his time working on the nervous system and it's neurotransmitters, Dr. Bej became interested in genetics, especially gene regulation and protein expression. This time, he decided to continue his career of research in America and was accepted into the PhD program in the Biology Department at the University of Louisville at Kentucky. However because insect research was limited at Louisville, he decided to study gene regulation at the microbial level. His project was to attempt to genetically modify microbes, a novel idea of the time. Specifically, he wanted to engineer microorganisms that would be able to eat up toxic material from their surrounding environment. Additionally, once these microorganisms had decontaminated their environment, they would then die to prevent an uncontrolled growth and spread of these novel microorganisms.

Yet, as stated previously, Dr. Bej found that there were some limitations into the kind of research that he wished to pursue, so he decided to come to the University of Alabama at Birmingham in 1991. He found UAB to be a "unique academic environment in which biology is so integrated that many disciplines can be combined together to answer a biological question." He felt that the academic setting, instead of the industrial setting, would mean more freedom for him to explore and research what he thinks to be fascinating and, at the same time, be able to "work with the great minds and the young minds." At UAB, Dr. Bej's current research focuses on microbial gene regulation and pathogen diagnostics from studying microorganisms that survive in extreme conditions, i.e. extremophiles. Looking especially at extremophiles that survive in cold environments, Dr. Bej regularly travels to Antarctica to gather different specimens and study the genetic pathways and regulations used by these organisms, enabling them to have freeze tolerance. From his studies, Dr. Bej hopes to achieve two goals. The first is to understand the "basic principles of biological systems of ancient organisms." These microorganisms live in a relatively undisturbed environment with few macroorganisms. This means that they have lived in an unperturbed and relatively slow environment. This gives researchers the window to see the gene structure, functions, and their relationship to different biological systems of organisms from many, many years ago. The second goal is to use this information to find a way for it to be used for human benefit. As a result of these organisms living in Anarctica, they must survive extremely low temperatures with limited food sources. Dr. Bej hopes to find the certain specialized products that protect the organisms and allow them to be competitively successful in obtaining nutrients. He anticipates being able to use these compounds as biomedical tools to help fight against human diseases. Already, Dr. Bej has found certain compounds and pigments, which have possible anticancer properties according to results, that show the ability to protect these microorganisms from solar radiation damage. Additionally, these products have the ability to create antimicrobial environments, allowing the microorganisms to successfully survive.

For Dr. Bej, biology is a broad area with multiple overlapping fields and in order to see the "big picture then you have to know the physiology background." He feels that this means that his progression through the years of research starting from behavior biology to insect biology and human benefits to the cellular level have eventually led to his current work in the genetics of extremophiles. At the same time, Dr. Bej hopes to encourage students to pursue research during their time at UAB. He believes that "UAB has many programs in science imbedded in one environment to the point that that you have no excuse to not find something that you want to do." Therefore, he wants to help students become interested in different subject matters after attending class. His personal advice for students desiring to get started in research is to simply ask professors if they would be willing for students to work in the lab and to become the "stars of tomorrow."

Use of a Modified Surface Killing Assay to Test the Killing of *S. pneumoniae* Serotype 3 Strain WU2 Using an Anti-PspA Monoclonal Antibody

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Abstract

Streptococcus pneumonia, *a gram-positive bacterium, causes meningitis, otitis media, and sepsis. Pneumococcal surface protein A (PspA) is a major protein virulence factor that contributes to colonization and invasion of* S. pneumoniae. *The development of PspA as a vaccine candidate requires a relevant* in vitro *surrogate assay of protection for antibody to PspA. However, such an assay is not available. We are developing an assay that can measure the ability of neutrophils to kill pneumococci opsonized with elicited antibody. In this assay, we measure the killing of* S. pneumoniae *by neutrophils on the surface of a blood-agar plate. We used a serotype 3 strain, WU2, and its PspA– mutant BR260.1. Our hypothesis is that the anti-PspA monoclonal antibodies (mAb) will facilitate the killing of WU2 by neutrophils. WU2 and its PspA– mutant BR260.1 were tested with anti-capsule 3 mAb 16.3 and anti-PspA mAbs 1b2.21, 8b2.19, and 5c6.1. When incubated with the mAb 16.3 both WU2 and BR260.1 were killed to a significant degree by neutrophils when compared to normal mouse serum alone. The mAbs 1b2.21 and 8b2.19 to PspA also facilitated significant increase in killing of WU2. However, when WU2 was incubated with the mAb 5c6.1, which is not protective in mice (unpublished data by K. Genschmer), no increase in killing by neutrophils was observed. Moreover, when mAb 1b2.21 was incubated with the PspA– mutant BR260.1 no enhancement of killing was detected. These findings indicated that our modified assay has the potential to serve as a surrogate assay for protection against pneumococcal infection.*

Introduction

Streptococcus pneumonia, a gram-positive bacterium, causes otitis media, pneumonia, meningitis, and sepsis. These diseases occur more frequently in young children, elderly people, and those whose immune systems are suppressed. According to Centers for Disease Control, each year 3,000 cases of meningitis, 500,000 cases of pneumonia, and 7,000,000 cases of otitis media were estimated in the United States (1). To prevent *S. pneumoniae* infections polysaccharide-containing pneumococcal vaccines are used. There is a non-conjugated polysaccharide vaccine that is used in adults, but it is not immunogenic in children (2). Pneumococcal Conjugate Vaccine, PCV7 vaccine, is one of the commonly used polysaccharide protein conjugated vaccines in children, which contains the 7 most common pneumococcal capsules that caused infections prior to 2000 (3). Conjugation of polysaccharides to protein increases immunogenicity of polysaccharides (2). Despite of this, the conjugate vaccine is not an improvement over the non-conjugated polysaccharide vaccine in adults (4). Moreover, since each capsular serotype has a unique polysaccharide structure, and there are over 90 capsular types (5), it is difficult to develop vaccines that target the polysaccharides of many pneumococci strains (6). Conjugated polysaccharide vaccines have greatly reduced pneumococcal diseases in children by strains of the 7 serotypes included in the vaccine (7). However, other serotypes that are not covered in the vaccine continue to cause diseases (8).

The majority of pneumococci share common immunogenic proteins on their surface including pneumococcal surface protein A (PspA), autolysin, pneumolysin, and neuraminidase (7, Figure 1). Because of this, a protein-based vaccine could have significant advantages over the present polysaccharide-based vaccines. PspA is present on all of the known pneumococcal strains that have been tested (7). One of the functions of PspA is inhibition of complement activation, which makes it difficult to clear pneumococci from its host (9). However, the binding of antibodies to PspA increases complement deposition, which facilitates killing of pneumococci by phagocytes (10 and 11). Furthermore, active and passive immunity to PspA have been shown to readily protect mice from infection (12). Because of its presence on the bacterial surface and its ability to cause immune response in a host, PspA became a strong protein vaccine candidate.

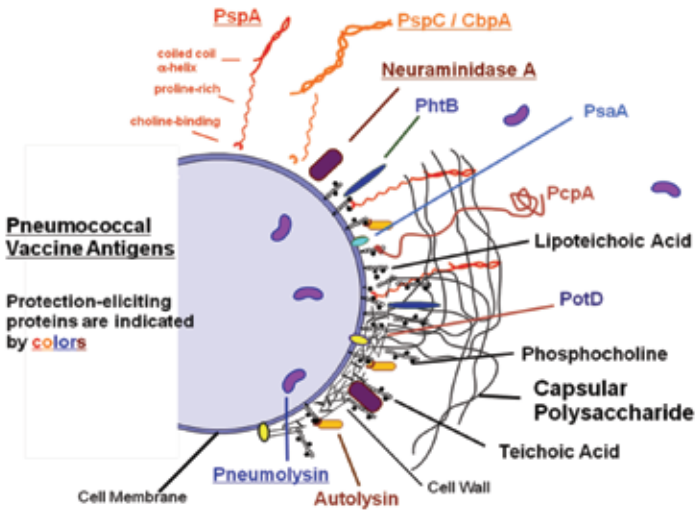

Figure 1. Virulent factors of S. pneumoniae *are shown in the picture. Part of PspA is covered by capsular polysaccharide (13).*

To be able to efficiently bridge from phase 1 vaccine trials to phase 3 efficacy trials, it is important to have an *in vitro* surrogate assay that can predict the protective capacity of the antibody in the serum of immunized humans. Vaccine trials include phase I safety trials, phase II immunogenicity trials, and phase III efficacy trials (14). Phase I trials of a vaccine are human safety trials that follow extensive animal safety studies. These trials involve immunization with relevant doses of FDA-approved antigen into a small number of human volunteers. Although the primary purpose of this trial is safety, the immune responses elicited can be examined to see if the immunized volunteers produced a protective response. In the case of pneumococci, the protective response is generally an antibody response (15), and the assay we are developing examines the protective effects of the elicited antibodies.

It is important to have a surrogate assay that can determine whether each individual made an antibody response that is expected to be protective. If evidence for protection is obtained in the phase I trial, it is likely that a phase II immunogenicity trial will follow. In the phase II trial, different antigen doses and immunization regimens are examined. The immune sera produced at this time must also be examined by a relevant surrogate assay to see if they are protective. At this stage, a reliable surrogate assay is especially critical because the information obtained will be essential for deciding whether a phase III trial will be conducted. The phase III trial will determine if the vaccine can protect against naturally-occurring disease. The phase III pneumococcal vaccine trials can cost between 50 and 100 million dollars and are never done unless there is strong evidence suggesting that the vaccine is able to elicit protective immunity.

At present, no such surrogate assay exists except for passive protection in mice. To do a quantitative passive protection assay in a statistically significant way requires between 100 and 140 mice per patient and would cost about $5,000 per patient. In these studies, we demonstrate an in vitro assay (Modified Surface Killing Assay [MSKA]) that measures antibody-dependent, complement-mediated killing of pneumococci by neutrophils.

With this assay, only capsular type 3 strain A66 had been tested so far in the Briles lab. To further investigate the effectiveness of the modified surface killing assay (4), we used a different serotype 3 strain, WU2, and its PspA deficient mutant, BR260.1. To test monoclonal antibody (mAb) reactivity to WU2 and BR260.1, we performed a dot blot assay with 5 different mAbs. The mAbs used are 16.3, an anti-capsule 3 mAb, and anti-PspA mAbs 1b2.21, 5c6.1, and 8b2.19.

My studies have shown that by using the MSKA, we observed complement-dependent and phagocyte-dependent killing of pneumococci that is facilitated by antibody to PspA. Because the protective activity of antibodies to PspA in vivo is thought to be largely complement dependent, our MSKA could provide the basis of a surrogate assay for protective human antibodies to a PspA vaccine. Such a surrogate might greatly facilitate PspA vaccine development, which could save 1 million lives per year.

Hypothesis

The MSKA can be useful to identify antibodies that can protect against pneumococcal challenge since anti-capsule mAb 16.3 will mediate killing of both WU2 and the WU2 PspA$^-$ mutant, BR260.1; anti-PspA mAb 1b2.21, 8b2.19 will facilitate killing of WU2 but will not increase killing of BR260.1. Also non-protective anti-PspA mAb 5c6.1 will not facilitate killing of WU2.

Materials and Methods

Bacteria and Cells:

Serotype 3 pneumococcal strain WU2 (PspA+) and its PspA- mutant, BR260.1, were grown in Todd Hewitt Broth with 0.5% yeast extract (THY) at 37°C with 5% CO_2. When an optical density (0.45) was reached at 600nm, the bacterial stocks were frozen at -80°C in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) (DB, Sparks, MD) containing 10% glycerol.

Neutrophils were isolated using Polymorphprep system (Axis-Shield, Oslo, Norway) following manufacturer's instructions. Neutrophils were washed and resuspended in Hanks' Balanced Salt Solution (HBSS) (Invitrogen, Auckland, NZ) with 1% bovine serum albumin (BSA) (ICN Biomedicals, Aurora, OH), then diluted to $2x10^6$ cells/ml in HBSS with 1% BSA.

Modified Surface Killing Assays:

Modified Surface Killing Assays were based on an assay done by Weinberger et al with several modifications (3). Anti-capsule type 3 mAb 16.3 was serially diluted (1:3 from 1:30) in HBSS with 1% BSA, and anti-PspA mAbs 1b2.21, 8b2.19, and 5c6.1 were serially diluted (1:4 or 1:8) in HBSS with 1% BSA. Strains WU2 and BR260.1 were diluted to $5x10^3$ CFU/ml and $1.5x10^4$ CFU/ml in HBSS with 1% BSA. WU2 (200 µl) or BR260.1 (200 µl of) was added to 80 µl of the mAb in HBSS + 1% BSA. A control tube had bacteria and HBSS with 1% BSA (80 µl) only. Each tube was incubated at 37°C and 5% of CO_2 for 30 minutes with shaking at 200 rpm. Normal mouse serum (NMS) was obtained from female BALB/cByJ mice (Jackson Laboratories, Bar Harbor, ME) and was added as a complement source (final concentration 6.7%). Heat-inactivated NMS (6.7%) was added for control. This mixture was incubated at 37°C and 5% of CO_2 for 30 minutes with shaking at 200 rpm. Six spots of 15 µl of the incubated mixtures were placed on dried sheep blood agar plates (THY II) (DB). After the 6 spots were absorbed into the agar, 20 µl of prepared neutrophil suspension was placed on top of 3 of the spots. When neutrophil suspension had absorbed into the agar, the plates were incubated in a candle jar at 37°C and 5% of CO_2 for 7 hours. CFUs in each spot were counted. Percent

killing was determined by comparing spots with neutrophils to spots without neutrophils and normalized to NMS sample. In other studies, we compared groups with complement to groups where the complement had been heat inactivated (HI). Analyses and graphing were performed in Graphpad Prism version 5.0.

Dot blot Assay:
WU2 (1x10^8 CFU/ml) and BR260.1 (1x10^8 CFU/ml) were diluted, and 1 µl of each bacteria was placed on a nitrocellulose membrane (Millipore, Billerica, MA), allowed to dry, and blocked in 1% Dulbecco's Phosphate Buffered Saline PBS (Mediatech, Manassas, VA) with BSA. The membrane was incubated with primary mAb 16.3, mAb 1b2.21, mAb 5c6.1, or mAb 8b2.19 for 1 hour at room temperature then washed 5 times for 5 minutes each with 0.1% tween20 (Fisher Scientific, Fair Lawn, NJ) + PBS. The membrane was then incubated with a solution containing Biotin-conjugated goat anti-mouse antibodies (SouthernBiotech, Birmingham, AL) and Strepavidin (SouthernBiotech) for 1 hour at room temperature and washed 5 times for 5 minutes each time with 0.1% tween20 + PBS. Dot blot was developed with BCIP+NBT tablet (Sigma-Aldrich, St. Louis, MO) per manufactures instruction.

Results and Conclusion
Growth curves (Fig. 2) were determined for WU2 and BR260.1. Our findings indicated that both strains were healthy when grown in vitro and helped us determine how long we would need to grow the bacteria for our experiments.

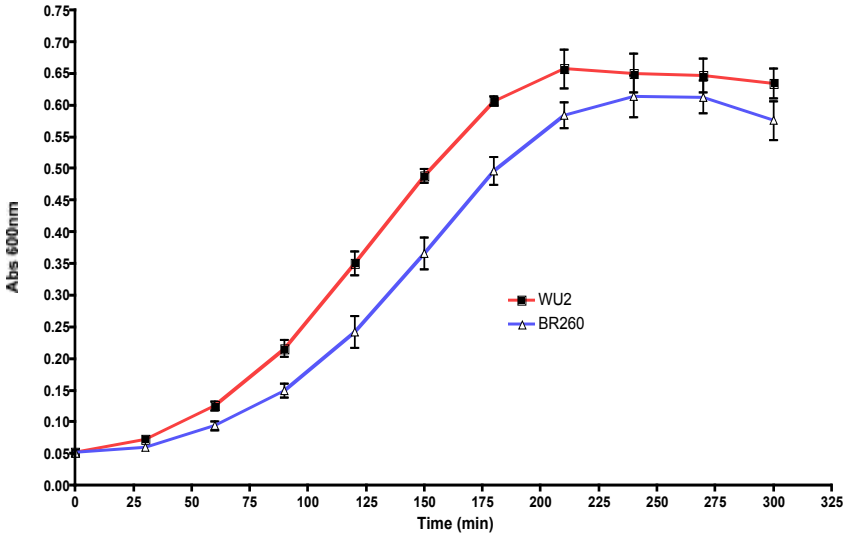

Figure 2. OD was taken at intervals of 30 minutes. Both strains reached their plateau after about 210 minutes. BR260.1 lagged slightly in growth compared to WU2, but the overall amount at stationary phase was comparable.

Dot blot with anti-capsule and anti-PspA monoclonal antibodies (mAbs) shows binding of the mAbs to WU2 and BR260.1.
To see the binding of mAbs to WU2 and BR260.1 before using the mAbs in the Modified Surface Killing Assay (MSKA), a dot blot was performed.

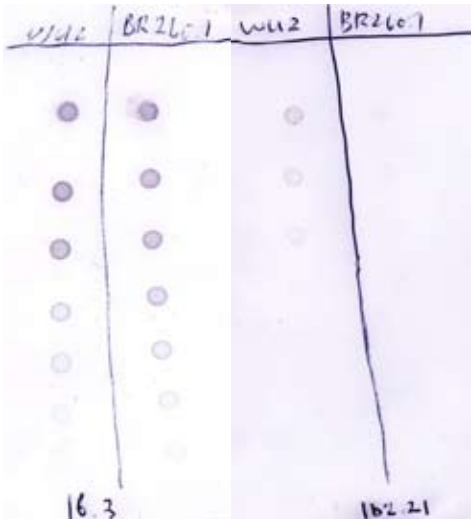

Figure 3. Dotblot was done with serotype 3 strain WU2 and its PspA deficient strain, BR260.1. Anti-capsule type 3mAb 16.3 was used as a positive control. Anti-PspA mAb 1b2.21 shows the strongest binding to WU2 among anti-PspA mAbs tested but does not bind to BR260.1.

The dot blot showed that mAb 16.3 has a strong binding to WU2 and BR260.1 since they are serotype 3 strain. Also, 1b2.21 had the strongest affinity to WU2 of the anti-PspA antibodies tested. Anti-PspA mAb 8b2.19 only weakly bound to the PspA expressed by strain WU2, and mAb 5c6.1 did not seem to bind. None of the anti-PspA antibodies reacted significantly with BR260.1 due to lack of PspA.

Anti-capsule monoclonal antibody 16.3 facilitates killing of both WU2 and BR260.1 in a Modified Surface Killing Assay (MSKA).
Due to lack of PspA on the surface of BR260.1, it was observed that a larger percent of the bacteria were killed in the NMS control compared to the amount of WU2 killed in its respective NMS control (Figure 4). This reflects the fact that PspA protects against deposition of complement. Anti-capsule type 3 mAb 16.3 enhanced killing of both WU2 and BR260.1 by neutrophils in the MSKA compared to NMS control (Figure 5 and 6). This reflects the fact that both bacteria have type 3 polysaccharide. The amount of killing of BR260.1 that was dependent on mAb 16.3 was less (Figure. 6) than was observed for WU2 (Figure 5). This observation is consistent with the fact that BR260.1 was already efficiently killed in the presence of complement in the absence of antibody (Figure 4).

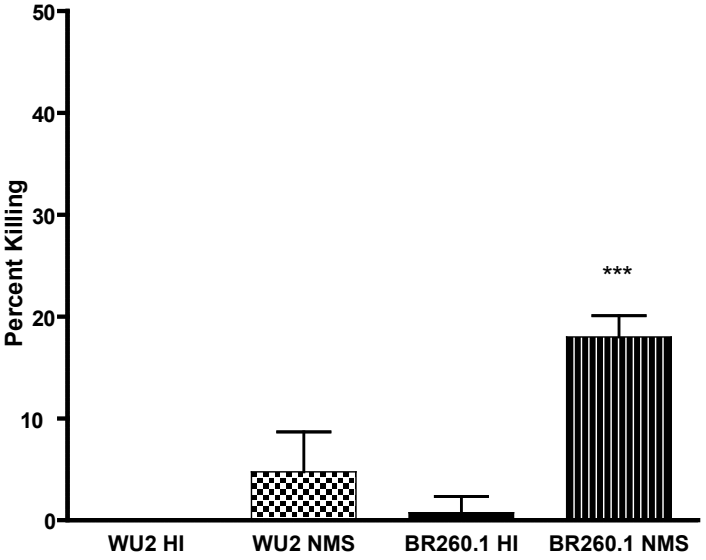

Figure 4. BR260.1 had significantly less survival in the presence of complement from NMS than WU2. Student's t-test was done comparing the results with Heat Inactivated (HI) to the results with Normal Mouse Serum (NMS).

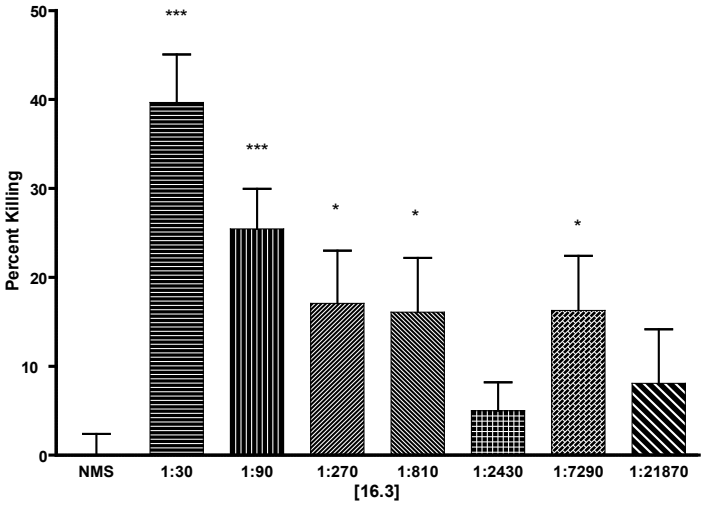

Figure 5. Effect of different dilutions of mAb 16.3 on WU2 is shown compared to its control by Modified Surface Killing Assay. Student's t-test was done comparing the results with NMS to the results of each mAb dilution.

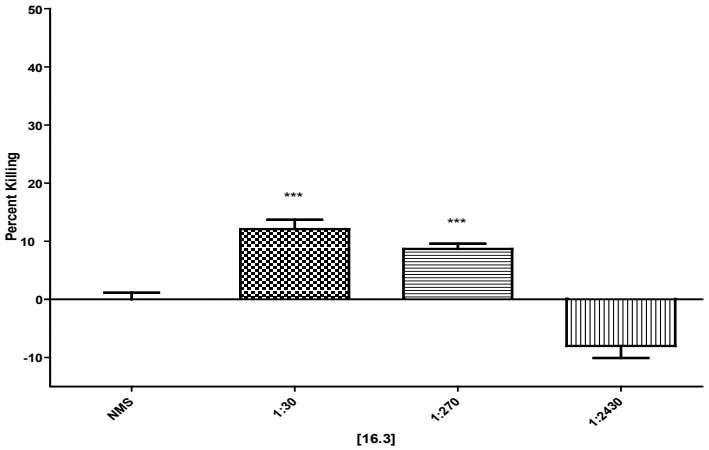

Figure 6. BR260.1 percent killing over control by serial dilutions of 16.3 in Modified Surface Killing Assay is shown. Student's t-test was done by comparing the results with NMS to the results of each mAb dilution.

Modified Surface Killing Assay can distinguish protective and non-protective monoclonal antibodies against pneumococcal infection.

Anti-PspA mAb 1b2.21 and 8b2.19 significantly increased killing of WU2 by neutrophils in the MSKA compared to NMS control (Figure 7 and 8). This shows the potential of this assay to identify protective antibodies and supports our hypothesis that MSKA can distinguish protective antibodies against pneumococcal challenge.

Anti-PspA mAb 1b2.21 did not have significant effect on killing of BR260.1 by neutrophils in the MSKA compared to NMS control. This reflects the lack of the PspA target on strain BR260.1 (data not shown).

Anti-PspA mAb 5c6.1, which did not show binding on dot blot and did not have significant effect on killing of WU2 by neutrophils in the MSKA compared to NMS control (data not shown). The failure of mAb 5c6.1to facilitate killing of WU2 is consistent with its inability to bind the PspA of WU2.

These results correlate with another data of serotype 3 strain A66.1 from K. Genschmer. The antibodies that facilitated killing of bacteria were also shown to be protective against infection in mice (unpublished result of K. Genschmer). Therefore, we infer than these antibodies will also protect mice against WU2 challenges. This supports our hypothesis that the MSKA can distinguish between protective and non-protective antibodies.

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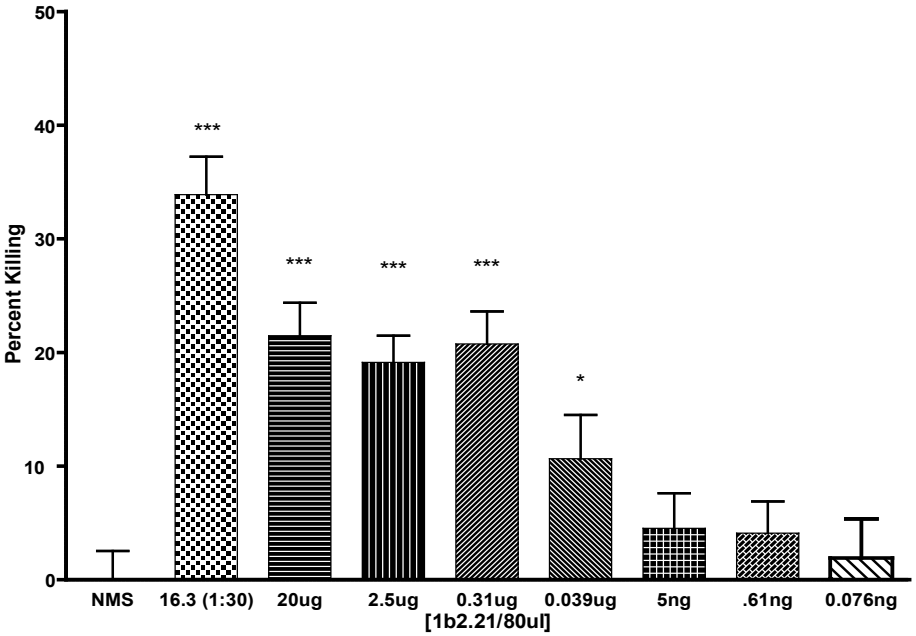

Figure 7. Percent of WU2 colonies killed by 16.3 and 1b2.21 compared to NMS is shown by Modified Surface Killing Assay. Both 16.3 and 1b2.21 greatly increase percent killing. Student's t-test was done by comparing the results with NMS to the results of each mAb dilution.

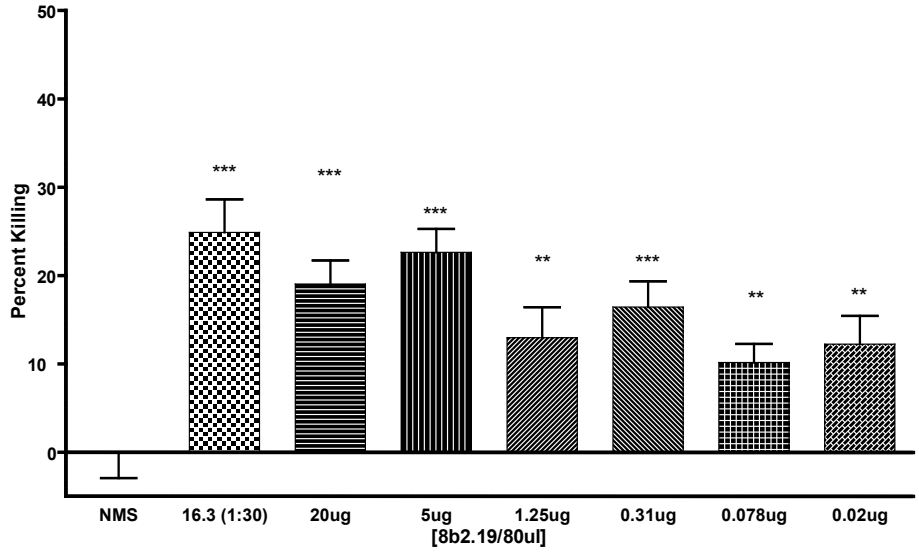

Figure 8. Percent of WU2 colonies killed by different concentrations of 8b2.19 compared to NMS is shown by Modified Surface Killing Assay. Student's t-test was done by comparing the results with NMS to the results of each mAb dilution.

In summary, the Modified Surface Killing Assay can identify monoclonal antibodies that are protective and non-protective against pneumococcal challenge. For future studies, the MSKA needs to be tested with additional mAb and additional challenge strains to validate the results and make it applicable to other strains. It would also be important to test the assay with human serum with and without human antibodies to PspA. Also, for each anti-PspA mAb, we will measure its ability to bind to PspA on the pneumococcal surface and to mediate complement deposition onto pneumococci. Lastly, using immortal cell line, HL-60, differentiated to neutrophil, could provide better consistency in the assay to overcome the variations in responses from neutrophils of different donors.

Acknowledgement

Thanks to Janice King for helping me obtain blood from volunteers from which we isolated the neutrophils. Thanks to Yvette Hale for helping with mouse procedures and acquisition of mouse serum. Thanks to Kristopher Genschmer for mentoring during the course of this project and for help in the preparation of human PMNs. This work was supported by National Institutes of Health grant AI-021548-26 (D.E.B.).

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Sex-ratio Predictions of *Eretmochelys imbricata* Nesting Beaches in Nicaragua and the Republic of Seychelles

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Abstract

The hawksbill sea turtle (Eretmochelys imbricata) occurs in tropical oceans throughout the world and is known for inhabiting coral reef environments. It is harvested for its "tortoise shell" which is used to make jewelry, and this species is considered "endangered" throughout its range. The hawksbill possesses temperature-dependent sex determination (TSD) in which temperatures during the middle third of incubation period determine the sex of the hatchling. The significance of understanding TSD is clear in a conservational sense, offering the possibility to monitor and optimize sex ratios in order to enhance the recovery of this endangered species. The current study addresses hatchling sex ratios produced by hawksbill turtles on major nesting beaches in the Republic of the Seychelles and in the Pearl Cays off the Caribbean coast of Nicaragua. Beach temperatures at nest depth and nest temperatures were monitored and used to predict sex ratios during the 2007-2008 nesting seasons. The average temperatures from the warmest and coolest sites were used to estimate the potential range of hatchling sex ratios from each site. The beach temperature data suggest that both of these major nesting locations may produce male biases. However, nest temperature data from a limited subsample of nests from Nicaragua suggest that nests in specific locations may produce female biases. These data will be used to evaluate and develop a conservation strategy for these endangered populations of the hawksbill turtle.

Introduction

The hawksbill (Figure 1) is a small to medium-sized sea turtle that has become endangered due to extensive harvesting for the "tortoise shell" trade (Mortimer, 2005). The turtle nests mostly in tropical regions of the Atlantic and Pacific Oceans and is often associated with coral reefs (Edelman, 2004).

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Figure 1. Hawksbill sea turtles are endangered due in part to the "tortoise shell" trade.

Sea turtles have temperature-dependent sex determination (TSD) in which incubation temperature determines the sex of the hatchling (Wibbels, 2003). This type of sex determination has the potential to produce highly biased sex ratios. Therefore, TSD has significant implications for the ecology and conservation of sea turtles. For example, extreme sex ratios could alter the reproductive output of an endangered population and thus affect its survival status. The significance of understanding TSD is clear in a conservational sense, serving as a method for predicting and optimizing sex ratios in endangered species to restore populations (Wibbels, Hillis-Starr & Phillips, 1999).

Hawksbills possess a male-female (MF) pattern of temperature-dependent sex determination in which warmer incubation temperatures produce females while cooler incubation temperatures produce males (Figure 2). A transitional range of temperatures (TRT) exists between the maximum temperature which produces 100% male hatchlings (28.5 °C) and the minimum temperature which produces 100% female hatchlings (30.3 °C) (Mrosovsky *et al.*, 1992). The pivotal temperature of 29.2 °C produces a 1:1 sex ratio (Wibbels, 2003; Mrosovsky *et al.*, 1992).

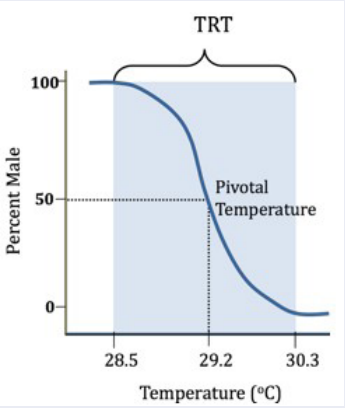

Figure 2. General pattern of TSD in the hawksbill sea turtle.

The current study addresses hatchling sex ratios in hawksbill turtles on major nesting beaches in the Pearl Cays off the Caribbean coast of Nicaragua (Figure 3) and in the Republic of Seychelles (Figure 4). Nesting beach temperatures and nest temperatures are evaluated in order to provide insight on the hatchling sex ratio produced on these nesting beaches. Such information is a prerequisite to developing effective conservation strategies for the recovery of these populations.

Figure 3. A representative island of the Pearl Cays in Nicaragua, where hawksbill nesting occurs.

Figure 4. An aerial view of the D'Arros and St. Joseph hawksbill nesting beaches in the Seychelles.

Materials and Methods

Small, battery-powered HOBO data loggers (Figure 5) from Onset Computer Corporation were used to record sand temperatures in beaches of Nicaragua and the Seychelles. The data loggers were programmed in the laboratory at the University of Alabama at Birmingham to record temperatures every 1 to 3 hours. They were heat-sealed in plastic bags with a container of desiccant. The microprocessor and temperature probe accurately recorded temperatures to approximately ±0.3 °C. The data loggers were placed in sand at a depth to approximate the center of a nest in areas that were representative of hawksbill nesting or in the center of the egg mass while the female turtle was nesting. In the Seychelles, 10 sites were examined in D'Arros Island, St. Joseph Atoll, and Bird Island in various habitat types. Approximately 6 data loggers were deployed at each site. In Nicaragua 9 sites, each with 1 to 10 data loggers, were monitored. Data loggers were also used to monitor 8 hawksbill nest temperatures in several locations in Nicaragua. Once the data loggers were collected,

they were sent back to the University of Alabama at Birmingham and downloaded using BoxCar Pro 3.51. Temperature analysis was done using Microsoft Excel. The average temperature corresponding to the middle third (20 days) of each lay week for the warmest and coolest sites were used for the Seychelles and Nicaragua beaches to predict sex ratios of the hatchlings.

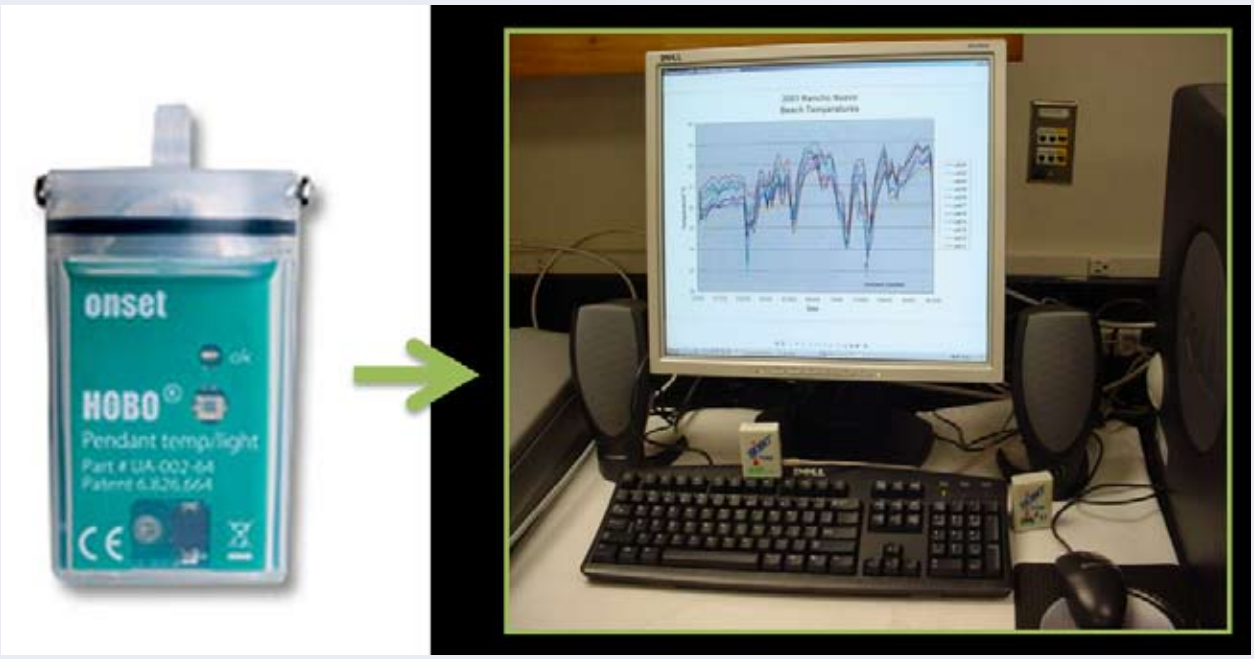

Figure 5. A data logger with a microprocessor and temperature probe. Data were analyzed using Microsoft Excel.

Results and Discussion

The results indicate that the sand temperature was frequently below the pivotal temperature of 29.2 °C (i.e. male-producing temperatures) for each nesting beach in Nicaragua and the Seychelles (Figures 6 and 7; Mrosovsky *et al.*, 1992). Peak nesting was estimated to occur during the months of October-January in the Seychelles and from June to September in Nicaragua. Based on these sand temperatures, Tables 1-4 show predicted sex ratios for nests laid each week during the peak portion of the nesting season (based on average temperature during the middle third of the incubation period). Overall, the observed average sand temperatures clearly indicate a strong male bias, with most lay dates indicating 100% male-producing temperatures (Tables 1-4). Water Beach, the warmest monitored beach in Nicaragua, produced one female-biased middle-third incubation period corresponding to a lay week of 08/10/08 (Table 1). Bird Island Site 1, the warmest monitored site in the Seychelles,

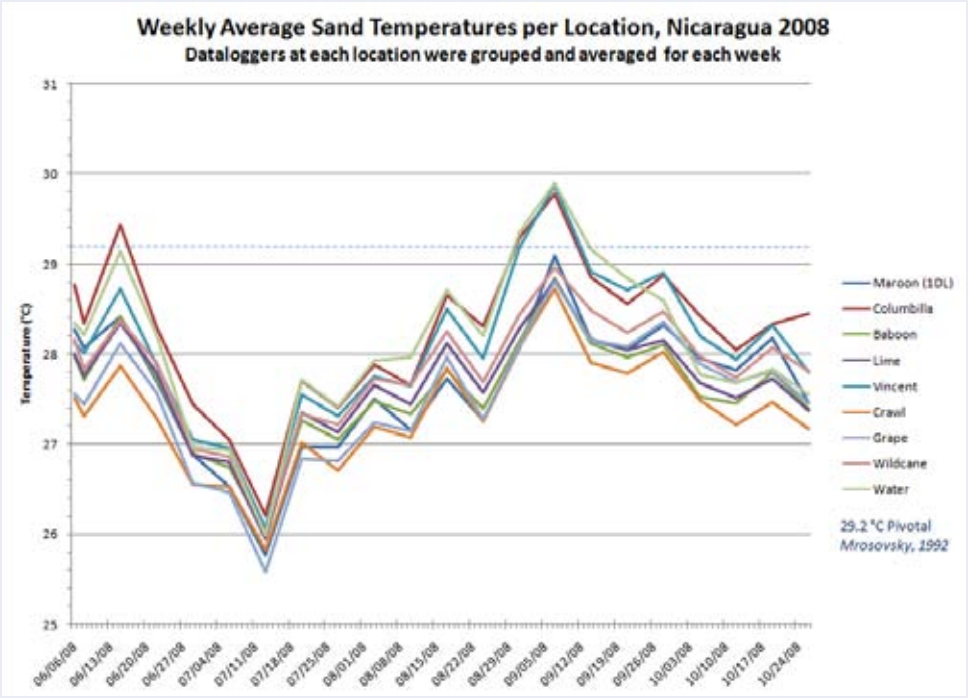

Figure 6. Average temperature of sites in Nicaragua (pivotal temperature is shown by dashed line).

produced a general male bias, with the 12/16/07, 12/23/07, and 12/30/07 lay weeks indicating 100% male-producing temperatures (Table 3). The coolest monitored beaches in both locations predict 100% male-producing temperatures during the entire duration of the peak nesting season (Tables 2 and 4).

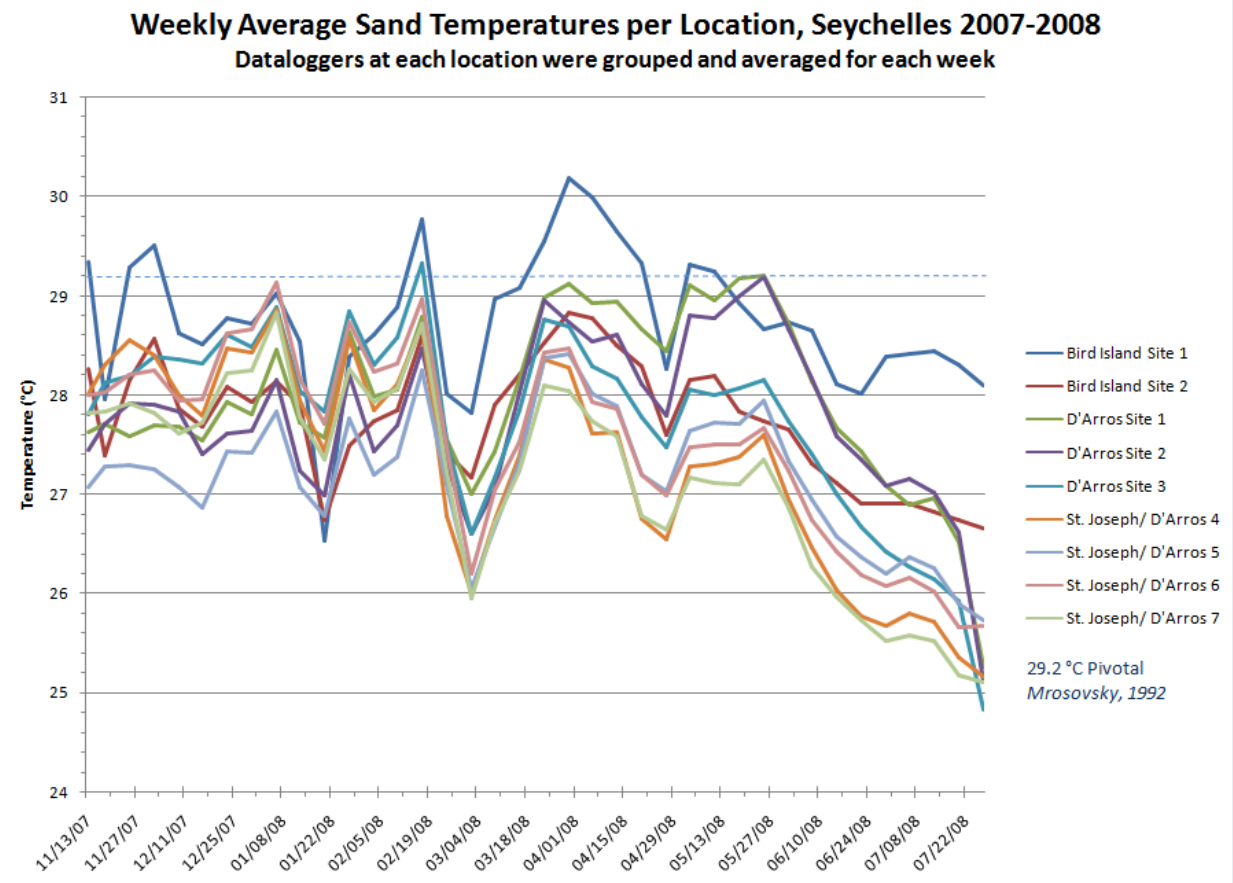

Figure 7. Average temperatures of sites in Seychelles (pivotal temperature is shown by dashed line).

Table 1. Sex-ratio predictions at Water Beach, the warmest monitored site in Nicaragua.

Lay Week	Middle Third	Temperature	Bias
06/08/08	06/28/08-07/17/08	26.730	100% male
06/15/08	07/05/08-07/24/08	26.779	100% male
06/22/08	07/12/08-07/31/08	26.970	100% male
06/29/08	07/19/08-08/07/08	27.584	100% male
07/06/08	07/26/08-08/14/08	27.730	100% male
07/13/08	08/02/08-08/21/08	28.128	100% male
07/20/08	08/09/08-08/28/08	28.225	100% male
07/27/08	08/16/08-09/04/08	28.681	male-bias
08/03/08	08/23/08-09/11/08	29.037	male-bias
08/10/08	08/30/08-09/18/08	29.540	female-bias
08/17/08	09/06/08-09/25/08	29.270	pivotal
08/24/08	09/13/08-10/02/08	28.975	male-bias
08/31/08	09/20/08-10/09/08	28.443	100% male
09/07/08	09/27/08-10/16/08	28.115	100% male
09/14/08	10/04/08-10/23/08	27.814	100% male
09/21/08	10/11/08-10/30/08	27.719	100% male
09/28/08	10/18/08-11/06/08	27.390	100% male

Table 2. Sex-ratio predictions at Crawl Beach, the coolest monitored site in Nicaragua.

Lay Week	Middle Third	Temperature	Bias
06/08/08	06/28/08-07/17/08	26.3737	100% male
06/15/08	07/05/08-07/24/08	26.3978	100% male
06/22/08	07/12/08-07/31/08	26.4713	100% male
06/29/08	07/19/08-08/07/08	26.9088	100% male
07/06/08	07/26/08-08/14/08	26.9872	100% male
07/13/08	08/02/08-08/21/08	27.3051	100% male
07/20/08	08/09/08-08/28/08	27.3627	100% male
07/27/08	08/16/08-09/04/08	27.6760	100% male
08/03/08	08/23/08-09/11/08	27.9206	100% male
08/10/08	08/30/08-09/18/08	28.3008	100% male
08/17/08	09/06/08-09/25/08	28.1029	100% male
08/24/08	09/13/08-10/02/08	27.9762	100% male
08/31/08	09/20/08-10/09/08	27.7831	100% male
09/07/08	09/27/08-10/16/08	27.6652	100% male
09/14/08	10/04/08-10/23/08	27.4770	100% male
09/21/08	10/11/08-10/30/08	27.3233	100% male
09/28/08	10/18/08-11/06/08	26.9984	100% male

Table 3. Sex-ratio predictions at Bird Island 1, the warmest monitored site in Seychelles.

Lay Week	Middle Third	Temperature	Bias
11/18/07	12/08/07-12/27/07	28.649	male-bias
11/25/07	12/15/07-01/03/08	28.694	male-bias
12/02/07	12/22/07-01/10/08	28.860	male-bias
12/09/07	12/29/07-01/17/08	28.856	male-bias
12/16/07	01/05/08-01/24/08	28.160	100% male
12/23/07	01/12/08-01/31/08	27.753	100% male
12/30/07	01/19/08-02/07/08	27.703	100% male
01/06/08	01/26/08-02/14/08	28.502	male-bias
01/13/08	02/02/08-02/21/08	28.999	male-bias
01/20/08	02/09/08-02/28/08	29.116	male-bias
01/27/08	02/16/08-03/06/08	28.583	male-bias

Table 4. Sex-ratio predictions at St. John/D'Arros 7, the coolest monitored site in Seychelles.

Lay Week	Middle Third	Temperature	Bias
11/18/07	12/08/07-12/27/07	27.667	100% male
11/25/07	12/15/07-01/03/08	27.796	100% male
12/02/07	12/22/07-01/10/08	27.930	100% male
12/09/07	12/29/07-01/17/08	27.844	100% male
12/16/07	01/05/08-01/24/08	27.627	100% male
12/23/07	01/12/08-01/31/08	27.428	100% male

12/30/07	01/19/08-02/07/08	27.459	100% male
01/06/08	01/26/08-02/14/08	27.502	100% male
01/13/08	02/02/08-02/21/08	27.457	100% male
01/20/08	02/09/08-02/28/08	26.813	100% male
01/27/08	02/16/08-03/06/08	25.659	100% male

In summary, the sand results show an overall male-biased hatchling sex ratio for the hawksbill sea turtle in both Nicaragua and the Seychelles for the 2007-2008 nesting season (Table 1-4). This bias is unusual since many sea turtle populations have been reported as having a female bias (Wibbels, 2003). For example, Godfrey et al. reported a >90% female bias in Brazil (1999). The beaches in the Seychelles and Nicaragua may be important for the recovery of the hawksbill population by producing a majority of males.

The sand temperature data are by far the most comprehensive data set collected in the current study. However, it is of particular interest that the temperature data from a sample of nests from Nicaragua (Figure 8) suggest that warmer temperatures are possible, with some nests predicted to produce female biases. These biases contrast the more comprehensive dataset collected from sand temperatures at nest depth which reported mostly male-producing temperatures. We are currently investigating the basis for this variation, but it may relate to factors such as specific locations and timing of the nests that were sampled. Hawksbill turtles are well known for selecting a wide variety of locations on a nesting beach ranging from those that are on open beach (i.e. direct sunlight) to those that are well up on the beach in the shaded vegetation zone. We are currently working with our collaborators to address this question. Regardless, the temperature data collected during the current study provide insight on hatchling sex ratios which can be used to evaluate and optimize current conservation strategies for these endangered populations of hawksbill turtles.

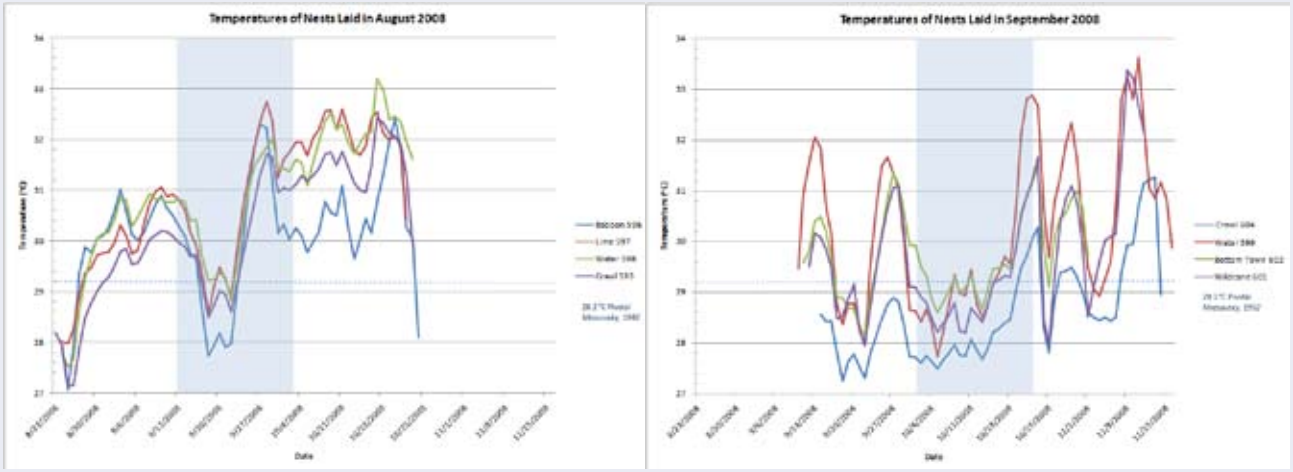

Figure 8. Average nest temperatures in Nicaragua. Shaded area represents temperature-sensitive period of TSD. Pivotal temperature is shown by dashed line.

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DNA Methylation Analysis of the Maspin Gene in the MCF10 Breast Cancer Progression Model

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University of Alabama at Birmingham

Abstract
Maspin (SERBINB5), a novel protease inhibitor, is a tumor-suppressor shown to inhibit tumor cell motility, invasion in cell culture, tumor growth and metastasis. High maspin expression is present in normal breast epithelial cells but is down-regulated during tumorigenesis in metastatic breast cancers, potentially leading to an increase in invasive potential and spread of metastatic disease. We examined the mRNA and protein expression of maspin as well as analyzed DNA methylation of the maspin promoter region in the genetically-related MCF10 cell lines, representing normal breast epithelial cells (MCF10A), pre-malignant breast disease (MCF10AT) and two separate metastatic carcinomas (MCF10CAa.1 and MCF10CAd.1α). Maspin expression was progressively down-regulated over the course of tumorigenesis in these cell lines. There was no evidence of overall DNA methylation in the maspin promoter in any of the cell lines studied. Thus, loss of maspin expression in these cell lines is not associated with aberrant cytosine methylation in the promoter region of the gene.

Introduction

Breast cancer is currently the second leading cause of cancer deaths in women today. One in eight women will be diagnosed with this disease in their lifetime (1). While evidence suggests that tumorigenesis is a distinctive multistep process involving the transformation of a normal human cell into a malignant cell, the mechanisms of tumor formation as well as acquisition of metastatic characteristics are not entirely known (2, 3).

Epigenetic modifications have been implicated in playing a role in the development of breast cancer. In particular, aberrant cytosine methylation at CpG dinucleotides in the promoters of tumor-suppressor genes is often associated with transcriptional silencing in carcinogenesis and metastasis (4, 5). DNA methylation changes are likely to occur during the transition from a non-metastatic neoplasia to a metastatic carcinoma.

The MCF10 isogenic breast cancer progression model mimics the multistep progression of breast cancer in humans and permits the analysis of specific gene expression at each distinctive stage of the disease (6-8). The MCF10 cell line was initiated as a mortal cell line from which the two immortal non-tumorigenic derivatives, MCF10A (attached cells) and MCF10F (floating cells), were derived. MCF10A cells were transformed with the addition of *c-Ha-Ras* to yield the tumorigenic, pre-metastatic MCF10AT cell line (9). The MCF10AT cells were injected via the tail vein into severe combined immunocompromised mice to generate two metastatic clones, MCF10CAa.1 and MCF10CAd.1α. The MCF10CAa.1 and MCF10CAd.1α cell lines are two separate variants that display all characteristics of a fully malignant breast cell type (10).

Maspin (*SERPINB5*) is a 42-kDA protein first identified in a screen for potential tumor suppressors that are lost in human breast cancer cells (11). Maspin is related to the serpin family of intracellular and extracellular protease inhibitors; however, maspin does not undergo the conformational change that is typical of members of the serpin family (12, 13). Down-regulation of maspin is an early event in breast tumorigenesis, making maspin expression a well-established diagnostic and prognostic indicator of cancer progression (14). Reintroduction of maspin into tumor cells inhibits growth, cell migration and invasion and angiogenesis. Loss of maspin expression typically leads to an increase in invasive potential and spread of metastatic disease (11). Recent studies suggest that maspin is restricted to an intracellular and possibly nuclear role in which it indirectly influences cell-matrix interactions (13). Thus, while the precise functions as well as cellular location of maspin have not been fully elucidated by researchers, its implications in breast cancer make it a target gene of interest.

Here, we report a study of gene expression and DNA methylation patterns in the maspin promoter of premalignant MCF10AT cells compared to the metastatic clones, MCF10CAa.1 and MCF10CAd.1α. This model allows us to identify discrete epigenetic modifications at each stage of breast tumorigenesis from premalignant breast cell to fully invasive breast cancer cell and attribute these epigenetic changes to phenotypic changes.

Methods

Generation of the MCF10 Progression Model and Cell Culture:

The cell lines studied in this experiment were acquired as a kind gift from Dr. Danny Welch (University of Alabama at Birmingham). Cell lines were generated as described by Miller (9, 15) and Santner (10) and were cultured as described by Welch (16).

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RNA, DNA and Protein Extraction:
RNA, DNA and protein were precipitated as per the manufacturer's protocol from MCF10AT, MCF10CAa.1 and MCF10CAd.1α cell pellets using *Trizol Reagent* (*Invitrogen; Carlsbad, CA*). A stepwise procedure was used to sequentially precipitate RNA, DNA and protein through a series of separatory layers (17). For each sample, four biological replicates were generated. RNA pellets were suspended in 100% deionized formamide and stored at -80°C. Following the final ethanol wash, DNA and protein pellets were stored in 100% ethanol at -20°C.

Quantitative-Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR):
qRT-PCR was used to assess the mRNA expression of maspin. RNA suspended in formamide from the MCF10AT, MCF10CAa.1 and MCF10CAd.1α cell lines was removed from -80°C and repurified using the RNA Cleanup Protocol in the RNeasy Kit (*Qiagen; Valencia, CA*) and then resuspended in RNase-free water. cDNA was generated using cDNA First Strand Synthesis Kit (*Invitrogen; Carlsbad, CA*) from 4 μg of RNA. cDNA was then purified using QIAprep PCR Reaction Cleanup (*Qiagen; Valenica, CA*). Real-Time PCR was carried out on 10 ng of cDNA. mRNA expression of maspin (Hs00985283_m1) was performed using Taqman Assays (*Applied Biosystems; Carlsbad, CA*) on a MiniOpticon Real-time Thermocycler (*Bio-Rad; Hercules, CA*). Specific assay ID is in parentheses. *GAPDH* served as the endogenous control. Expression values for MCF10CAa.1 and MCF10CAd.1α were calculated using the ΔΔCt relative to MCF10AT expression. The efficiency of target amplification was compared between the maspin expression in the MCF10CAa.1 and MCF10AT cells. This was compared to the efficiency of target amplification between the *GAPDH* expression in the MCF10CAa.1 and MCF10AT cells. The following formula was used:

$$\frac{E_{maspin}^{C_{t(MCF10AT)}-C_{t(MCF10CAa.1)}}}{E_{gapdh}^{C_{t(MCF10AT)}-C_{t(MCF10CAa.1)}}}$$

E=Efficiency C_t=Cycles to Threshold

This was also used to analyze the efficiency of target amplification in the MCF10CAd.1α and MCF10AT cells (18).

Western Blot:
Western blotting of protein extracts was used to determine protein expression of maspin. Protein concentration was determined with the 2D-DIGE Quant Kit (*Amersham Biosciences; San Francisco, CA*). Total protein extract (20 μg) was loaded onto a 4-15% Tris-HCl gel and separated by electrophoresis at 100 V until the dye ran off of the gel. The separated proteins were transferred to a nitrocellulose membrane and transferred at 100 V at 4°C. Membranes were blocked in 5% dry milk in Tris buffered saline solution with 0.1% Tween (TBST) overnight. Primary antibody incubations were completed overnight at 4°C using mouse monoclonal antibodies specific to maspin (sc-166260) and actin (sc-1616). The antibodies used in immunoblotting were obtained from Santa Cruz Biotechnology (*Santa Cruz, CA*).

Bisulfite Treatment and DNA Methylation Analysis:
Genomic DNA was prepared as described above using Trizol Reagent for the MCF10AT, MCF10CAd.1α and MCF10CAa.1 cell lines. Two μg of purified genomic DNA were bisulfite treated using the QIAgen Epitect Bisulfite Treatment Kit (*Qiagen; Valencia, CA*). Primers specific for the maspin gene region were used to amplify bisulfite-modified DNA via nested PCR (first round F: 5'-AAAAGAATGGAGATTAGAGTATTTTTT-GTG-3' and R: 5'-CCTAAAATCACAATTATCCTAAAAAA-TA-3') and (second round F: 5'-GAAATTTGTAGTGT-TATTATTATTATA-3' and R: 5'-AAAAACACAAAAAC-CTAAATATAAAAA-3'). PCR products were loaded on a 1.5% agarose gel; bands were purified and extracted using QIAquick Gel Extraction Kit (*Qiagen; Valencia; CA*). Purified promoter DNA generated as described above were cloned into a TOPO-TA Vector (*Invitrogen; Carlsbad, CA*). Plasmids were transformed into TOP10 Chemically Competent Cells (*Invitrogen; Carlsbad, CA*) and plated onto Luria-Bertani agar plates containing 50 μg/mL kanamyocin. Seven individual colonies were selected and used to inoculate 3 mL cultures of LB. Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (*Qiagen; Valencia, CA*). Plasmids containing the PCR products were sequenced using a T7 primer and a 3730 DNA Sequencer (*Applied Biosystems; Carlsbad, CA*).

Results

Maspin is down-regulated over the course of metastasis

Protein expression of maspin was qualitatively analyzed in the MCF10A, MCF10AT, MCF10CAa.1 and MCF10CAd.1α cell lines with a Western Blot (Figure 1). Maspin expression was less in the MCF10AT cell line compared to the MCF10A cell line. Maspin expression was undetectable in the metastatic carcinomas, MCF10CAa.1 and MCF10CAd.1α.

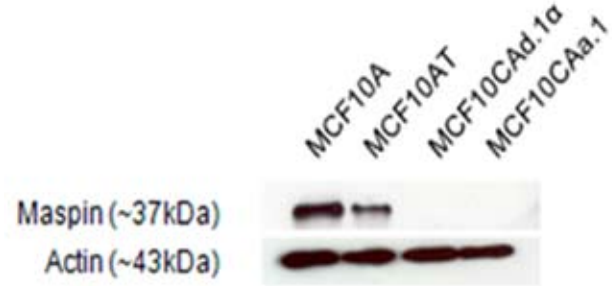

Figure 1. Western blot of maspin protein expression. Maspin expression was less in premalignant MCF10AT cells compared to immortalized MCF10A cells. Maspin expression was undetectable in the metastatic carcinomas (MCF10CAa.1 and MCF10CAd.1α cells). Actin served as the endogenous control.

In order to further validate the results generated from the Western Blot, we qualitatively assessed mRNA levels in the MCF10AT cell lines and the metastatic carcinomas, MCF10CAa.1 and MCF10CAd.1α (Figure 2). It was especially necessary to quantify the maspin mRNA expression of the metastatic carcinomas due to the lack of maspin protein band in each of those cell lines. Expression ratios for the MCF10CAa.1 and MCF10CAd.1a cells were calculated using the ΔΔCt method relative to the expression of MCF10AT cells. Maspin expression was nearly half in the metastatic carcinomas compared to the premalignant MCF10AT cells.

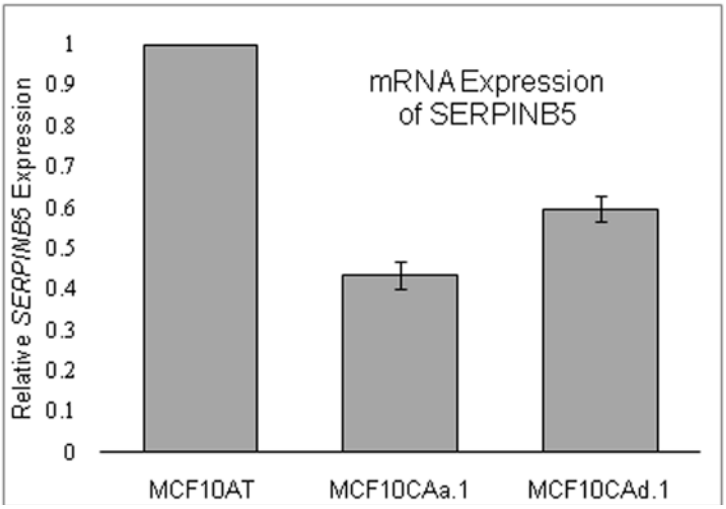

Figure 2. Real-Time PCR analysis of mRNA expression of maspin (SERPINB5) in metastatic progression model. Expression ratios for the MCF10CAa.1 cells and MCF10CAd.1α cells were calculated using the ΔΔCt method relative to the expression of MCF10AT cells (set to 1.0). Error bars represent SEM. GAPDH served as the endogenous control.

The down-regulation of maspin in both the Western Blot and real-time PCR indicate its association with a metastatic phenotype.

No overall DNA methylation in the maspin promoter

DNA from the MCF10AT cell lines and the metastatic carcinomas, MCF10CAa.1 and MCF10CAd.1α was bisulfite treated and DNA methylation was analyzed in the maspin promoter at CpG dinucleotides (Figure 3). Bisulfite sequencing indicated an overall lack of methylation in the maspin promoter of each of the cell lines, indicating that maspin down-regulation in the metastatic progression model is not attributed to DNA methylation.

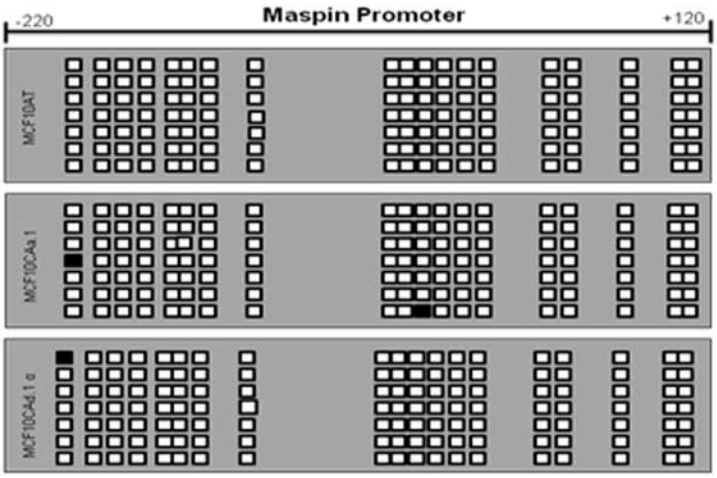

Figure 3. DNA methylation analysis of the promoter region of the maspin gene. Each box represents a CpG dinucleotide. Each column represents a CpG island in the maspin promoter. Individual rows correspond to the methylation pattern of a single colony.

Discussion

Maspin has been shown to inhibit tumor cell motility, invasion in cell culture, tumor growth and metastasis (11). High maspin expression is apparent in normal breast cells but is strongly down-regulated and lost in metastatic cells, indicating maspin's role as a tumor suppressor (21). Loss of maspin expression typically leads to an increase in invasive potential and spread of metastatic disease. Although maspin expression is silenced over the course of tumorigenesis, maspin gene deletions and mutations have not yet been found (11). Thus, a logical hypothesis is that the maspin gene may be silenced by epigenetic modifications, such as aberrant DNA methylation at CpG dinucleotides of the maspin promoter.

After confirming the down-regulation of maspin over the course of metastasis, we analyzed the cytosine methylation status of the 19 CpGs present in the maspin promoter in the MCF10AT cell lines and the metastatic carcinomas, MCF10CAa.1 and MCF10CAd.1α. For each cell line, 7 bacterial clones were bisulfite sequenced. Overall aberrant cytosine methylation was not detected in any of the cell lines studied. No CpGs were methylated in the MCF10AT cells while the MCF10CAd.1α and MCF10CAa.1 had two and one clone with a single methylated site, respectively. These findings indicate that the loss of maspin expression during the course of tumor progression and metastasis in the cell lines studied is not a result of aberrant cytosine methylation in the promoter region of the gene. It is interesting to note that DNA hypomethylation has been demonstrated to play a causal role in tumor formation, possibly by promoting chromosomal instability (22, 23). While this does not provide enough evidence to link the lack of methylation at the maspin promoter to breast cancer metastasis, it certainly provides a solid hypothetical foundation.

It is important to consider that loss of maspin expression has been shown to be closely linked with aberrant DNA methylation in the maspin promoter in other breast cell lines not used in this experiment (14). However, our findings are the first to indicate a lack of DNA methylation and the down-regulation of maspin expression in the MCF10 Breast Cancer Progression Model.

To fully understand other epigenetic modifications that may be associated with maspin gene silencing in breast cancer, it is also possible to assess chromatin accessibility of the maspin promoter of the cell lines in the metastatic progression model. However, the down-regulation of mRNA expression coupled with no protein expression and lack of DNA methylation changes suggests that RNA interference (RNAi) may be controlling the gene activity. Analyzing RNAi in the maspin promoter of the cell lines of the metastatic progression model is a more likely future research direction. In addition, due to complexity of metastasis, further experimentation is necessary to identify other gene targets involved in the malignant progression of breast cancer.

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paper: biology

Tear Component Interference in Cytometric Bead Based Assay of IL-8 and IP-10

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Abstract

Purpose: *To validate IL-8 and IP-10 concentrations in tears by comparing CBA and ELISA analyses to see if interference occurs.*
Method: *Three 6.5 μL samples of non-stimulated (NS) tears were collected using 10 μL microcapillary tubes from 20 subjects. Three 20 mL samples of stimulated (Stim) tears were also collected using 20 μL microcapillary tubes from the same subjects. Samples were assayed using CBA and ELISA. Results of analyses were quantified using ANOVA and regression analysis.*
Results: *Positive correlation between CBA and ELISA results for NS and Stim IL-8 indicate uniform interference effects in CBA. ELISA results for IL-8 were 2.7 times higher than CBA results. IP-10 levels were found to be higher in CBA for both NS and Stim tears. The correlation between ELISA and CBA was strong for Stim tears but weak for NS tears, suggesting interference in CBA.*
Conclusion: *The correlation between CBA and ELISA results for tear IL-8 indicates that CBA can predict the more accepted ELISA values. This contradicts the suggestion of other authors that tear IL-8 cannot be reliably measured by multiplexed assay. Correlated IP-10 results for Stim tears by both CBA and ELISA confirm that IP-10 is an important anti-angiogenic factor at the ocular surface. IP-10 CBA of NS tears does not correlate with ELISA data and should be treated with caution.*

Introduction

Cytokines and chemokines are inflammatory mediators that reflect the health of many tissues, including the ocular surface environment[1,2]. As part of an inflammatory response, chemokines attract leukocytes to sites of inflammation[3]. The concentrations of various chemokines can therefore provide useful information about the health of the "immune privileged" ocular surface. By bathing the ocular surface, tears provide a useful source of soluble ocular surface chemokines. However, tears will also contain cytokines secreted by the lacrimal gland.

Interleukin-8 (IL-8) and interferon gamma-inducible protein 10 (IP-10) are two chemokines that may contribute antagonistically to ocular immune privilege[4]. The role of IL-8 in inflammation is to bind (via a conserved glutamic acid-leucine-arginine sequence near the N-terminus) to CXCR1 or CXCR2 receptors, thus attracting neutrophils to the inflammatory site[1,5,6]. Because IL-8 is a pro-inflammatory and pro-angiogenic chemokine, its tear levels would be expected to be higher in disease states than in healthy eyes. Past reports of elevated tear IL-8 levels in dry eye patients confirm that it may be a useful dry eye biomarker[1]. Ocular sources of IL-8 include leukocytes, corneal and conjunctival epithelial cells, and keratocytes[1,7]. We would therefore expect higher levels of IL-8 in non-stimulated (NS) than stimulated (Stim) tears because increased tear flow dilutes the contribution of ocular surface components.

In contrast to IL-8, IP-10 (CXCL10) is an anti-angiogenic chemokine whose properties are consistent with inhibition of corneal neovascularization. For the ocular surface, IP-10 may be a key link between inflammation and angiogenesis. This is based on reports for other tissues that IP-10 promotes inflammation in such a way as to prevent angiogenesis due to shared receptor expression between leukocytes and endothelial cells8. Because IP-10 serves to prevent blood vessel growth, its presence at the ocular surface may relate to immune privilege. Tear concentrations of IP-10 would therefore presumably be relatively high under normal conditions. Previous studies in this laboratory support this assertion, finding IP-10 to be very active at the ocular surface. Using Cytometric Bead-Based Assay (CBA), LaFrance *et al*[2] measured tear IP-10 levels averaging 24,000 pg/mL. By contrast, serum IP-10 levels are typically reported to be in the 50 - 150 pg/mL range[8].

IP-10 is secreted by several cell types in response to interferon-gamma (IFN-). These cell types include monocytes, endothelial cells, fibroblasts and some epithelial cell types, including intestinal epithelium[9]. If IP-10 at the ocular surface is acting as an IL-8 antagonist to counteract its angiogenic effects, the high tear levels of IP-10 may serve to prevent the IL-8 activation of neutrophils[10,11].

Chemokines can be assayed using single-analyte ELISA or multi-analyte CBA. ELISA has been extensively used to quantify tear cytokines and remains the gold standard for antibody-based tear assay[12]. It is, however, limited to the assay of one cytokine per tear sample. Entire cytokine profiles can be assayed in a single tear sample using a multiplex assay format[2].

Based in part on other reports[13,14] that tear components may produce artifacts in multiplexed (multi-cytokine) assays, the current study was designed to compare tear levels of IL-8 and IP-10 measured by ELISA versus CBA. Comparative results of IP-10 analysis will help determine if the high CBA level is an artifact, and comparison of tear IL-8 levels measured by CBA and ELISA will determine if tear interference is present in CBA of NS and Stim tears as proposed by Sack[14]. If Sack is correct, we would expect IL-8 levels to be higher by CBA than ELISA

of matched tear samples. We would also expect a greater discrepancy in NS tears vs. Stim tears because higher levels of interfering agents have been reported in NS tears[15].

Methods

NS and Stim tears were collected noninvasively from 20 healthy subjects. Subjects collected three 6.5 µL samples of NS tears using 10 µL glass microcapillary tubes placed in the tear meniscus above the lower eyelid margin. Tear flow rate did not exceed 0.33 µL/min during collection. Matching Stim samples (same subjects) were collected using 20 µL microcapillary tubes following collection of an initial 20 µL washout. Inserting a cotton swab along the medial aspect of the nose induced the Stim tears[2]. All tear samples were diluted 1 in 10 with Bioplex Assay Buffer (Bio-Rad, Hercules, CA) and stored at -80°C until use. Tear samples were assayed using a Bio-Rad 27-Plex CBA Assay Kit and read on a Luminex 200 CBA System (Luminex, Austin, TX). Matching samples were assayed using Diaclone WHO-calibrated ELISAs for IL-8 and IP-10 (Cell Sciences, Canton MA). Results of CBA were compared to ELISA to determine the extent and linearity of interference. ANOVA and regression analysis were used to quantify the assay results for all NS and Stim tear comparisons.

Results

While mean NS tear IL-8 levels were 2.7 times higher by ELISA than CBA, there was a strong positive correlation between results for the two assays (Figure 1). The correlation indicates that, if assay interference is present in the NS tear CBA, it appears to be uniform for all measured tear samples. For Stim tear IL-8, a weaker, but still significant correlation between assay techniques was evident (Figure 2). A single data point appeared to skew the Stim tear regression rather than a general non-linear interference effect.

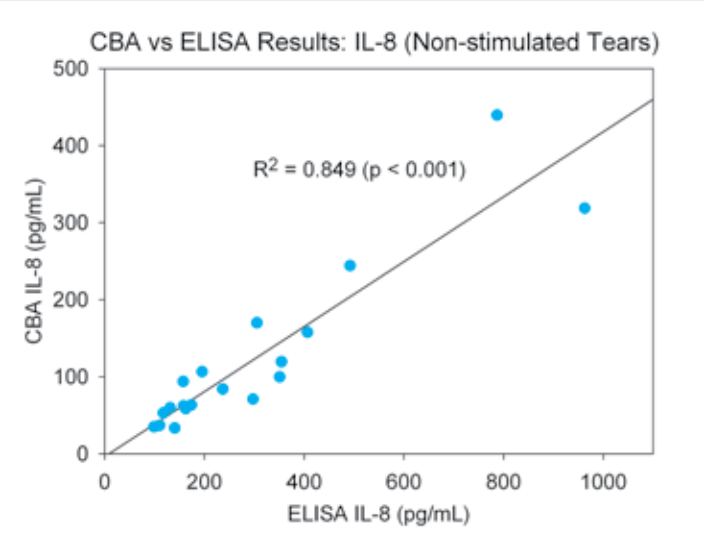

Figure 1. IL-8 in matched NS tear samples measured by CBA (y axis) and ELISA (x axis). ELISA results were 2.7x higher than CBA, but correlated strongly (p<0.001).

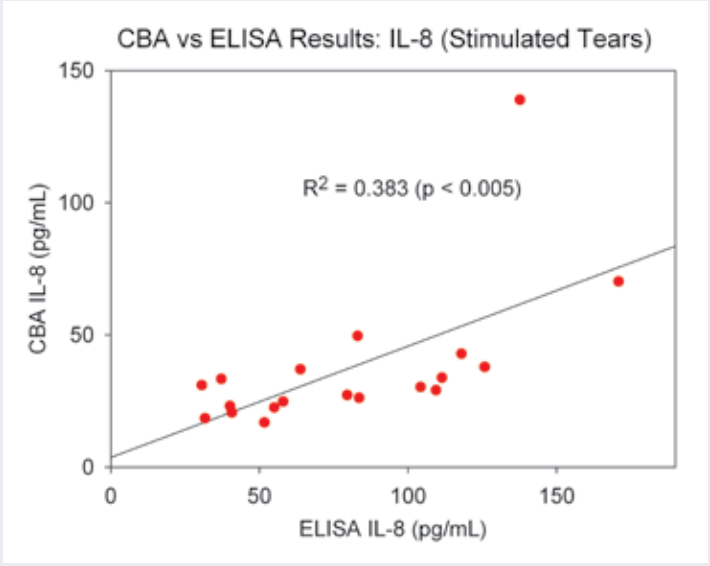

Figure 2. IL-8 in Stim tears measured by CBA (y axis) vs. ELISA (x axis) of matched tear samples. Mean Stim tear IL-8 levels were 2.4x higher by ELISA.

For IP-10, the two assay types show different trends than were evident with IL-8. For both NS (Figure 3) and Stim tears (Figure 4), CBA produced substantially higher IP-10 levels than ELISA. The correlation between assay types was stronger for Stim tears than NS, with a single data point again appearing to skew an otherwise uniform fit. NS tear IP-10 on the other hand showed considerable differences between assay types, suggesting non-linear interference in CBA.

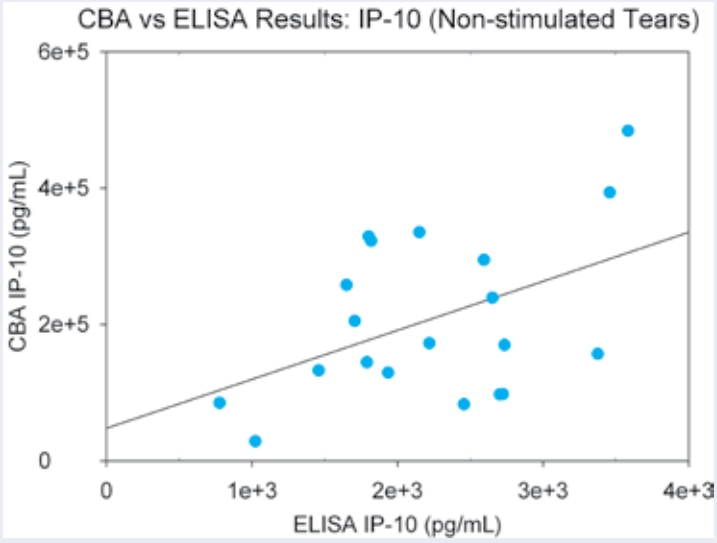

Figure 3. IP-10 in matched NS tear samples measured by CBA (y axis) and ELISA (x axis). CBA results were substantially higher than ELISA, but data is more scattered than for either IL-8 assay comparison.

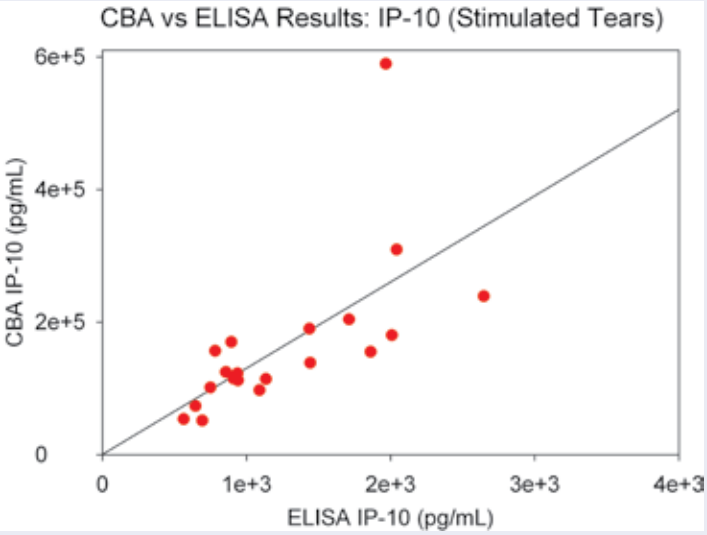

Figure 4. IP-10 in Stim tears measured by CBA (y axis) vs. ELISA (x axis) of matched tear samples. Mean Stim levels were substantially higher by CBA than ELISA. Assay techniques for tear IP-10 measurement correlate more strongly for Stim tears than NS.

Discussion

IL-8

Using ELISA as the standard, CBA results indicated moderate systematic interference effects. It is possible that partial IL-8 epitope blocking in the CBA[16] was responsible for the lower measured tear values. However, NS tear IL-8 levels correlated well between the two assay types. Ranking tests demonstrated that both assays produced essentially the same order of cytokine concentration among tear samples. These findings contradict those of Sack *et al*[14], who reported that multiplexed assay values were artificially high for tear IL-8.

Stimulated tears showed a less robust correlation between CBA and ELISA, suggesting that non-systematic interference may be a factor. With the multi-antibody/multi-antigen format of CBA, the chance for tear component-induced interference increases greatly[16]. Sample components have been reported to form primary to secondary antibody bridges, especially in multiplexed assays[16]. In tears, this would produce false positive artifacts. However, interfering agents may alternatively bind to the primary antibody and produce epitope shielding for the tear antigen, thus reducing measured tear levels. This may explain the lower levels of tear IL-8 measured by CBA, although epitope shielding would be expected to produce a non-linear dose-response curve for tear IL-8. A simpler explanation is that the differences were due to non-equivalent IL-8 standards for the two assays.

As expected, IL-8 levels were lower in Stim than NS tears. This confirms the prediction that ocular surface cells are the primary source of IL-8, and that reflex tears act simply as a diluting agent to decrease IL-8 concentration in the collected tear samples.

IP-10

Much higher levels of IP-10 by CBA than ELISA for NS and Stim tears indicate that CBA produces artificially elevated IP-10 values. This suggests assay interference and may explain the very high tear values found by LaFrance *et al*[2]. The poor correlation between CBA and ELISA values for NS tears indicates non-linear interference in CBA. Therefore CBA levels would not be able to reliably predict ELISA values through a simple calibration factor. Interference in Stim tear CBA appears more linear based on the significant correlation between assays. CBA levels of IP-10 in Stim tears could therefore be used to obtain a reasonable estimate of ELISA levels. Non-linear interference in NS tear IP-10 CBA is further supported by the lack of correlation between levels of tear IP-10 and its inducer, IFN-γ (also measured by CBA; data not shown). A strong correlation between Stim tear IP-10 and IFN-γ (an expected result) further supports the greater reliability of CBA Stim than NS tear IP-10 values. This is consistent with the finding of Shiozawa *et al*[11] report that IFN-γ is a potent inducer of IP-10 from many cell types. One possible explanation for the interference in NS tear IP-10 CBA may be binding of IP-10 to α_2-macroglobulin or another cytokine transport protein in NS tears. This would be less likely in freshly secreted Stim tears. Similar levels of tear IP-10 in NS and Stim tears (measured by ELISA) indicate that the lacrimal gland may be an important source of ocular surface IP-10.

Conclusion

Tear IL-8 levels measured by CBA are lower than ELISA, presumably due to interference effects in the former assay. Interference appears sufficiently linear to allow inter-conversion between ELISA and CBA results with a simple calibration factor. Results of the current study suggest that the membrane microarray assay artifact reported by Sack[14] to produce falsely elevated tear IL-8 levels does not affect CBA. CBA should therefore be a more reliable multiplex assay when studying tear cytokine profiles that include IL-8.

Tear IP-10 levels for both CBA and ELISA exceed reported serum values[10] supporting the theory that IP-10 is an important ocular surface anti-angiogenic factor. Non-linear interference in NS tear IP-10 CBA suggests that multiplex assay data for this chemokine in NS tears should be interpreted with caution. Stim tears show greater potential for providing reliable relative measures of IP-10 at the ocular surface. Studies of tear IP-10 in patients with corneal neovascularization may shed further light on the potential anti-angiogenic role of tear and ocular surface IP-10. Other studies could explore the nature of CBA interference by determining which blocking agents are most effective in reducing interference. Similarly, dissociation

studies could be used to determine if the IP-10 interference effect is due to protein binding *in vivo*, for example by α_2-macroglobulin. Finally, non-ELISA based assays (e.g. MALDI-TOF-MS or gene expression studies) could be used to confirm or refute the high ocular surface levels of IP-10 found in the current study.

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A Screen Designed to Identify MKS-like Genes Yields a Novel Allele of mks-5

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Abstract

Cilia are developmentally essential organelles projecting from most mammalian cells. Altered cilia function underlies a group of autosomal recessive disorders including Nephronophthisis (NPHP) and Meckel-Gruber syndrome (MKS). Although several genetic loci are linked to these diseases, causative mutations have not been identified in the majority of patients. Conservation of NPHP and MKS genes in C. elegans *allows its utilization for studying these disorders. Previously, our research revealed that the NPHP and MKS proteins interact and function as two distinct complexes in a region at the base of the cilium called the transition zone (TZ). Single mutations in* nphp *or* mks *genes in* C. elegans *alone have minimal affects on ciliogenesis; however, a combination of mutations in* nphp *with a mutation in any* mks *gene alters cilia formation. This relationship was utilized in a forward EMS mutagenesis screen in* nphp *mutants to identify novel candidate* mks*-like genes. At least nine novel loci were identified. Here using non-complementation and sequencing analysis, one of the alleles* (yhw91) *was identified as a new* mks-5 *mutation within an exon-intron splice site. The* yhw91 *mutation disrupted the localization of MKS-3, another protein involved in the MKS complex at the TZ. Hierarchy analysis was subsequently performed to determine that anchoring of all other known MKS complex proteins was dependent on MKS-5 function. Correspondingly, MKS-5 protein was detected at the TZ, and its localization was unaltered by* mks *and* nphp *mutations. This insinuates that MKS-5 may be the core anchoring protein in the MKS portion of the TZ complex. Analysis of other genes identified in the EMS screen is ongoing. Ultimately, MKS families will be screened for mutations in the homologs of genes identified from this screen.*

Introduction

The autosomally recessive Nephronophthisis (NPHP)-associated disorders are heterogenic and affect a variety of organs. Also termed ciliopathies, these genetic disorders result from mutations affecting proteins of largely unknown function that localize to the cilium or to cilia subdomains. In the purest form of NPHP, cysts will develop within the corticomedullary border of the kidney. Symptoms are isolated to the kidneys with renal interstitial infiltration in addition to fibrosis, and basement membrane disruption along with tubular atrophy. More severe forms of NPHP-related ciliopathies such as Meckel-Gruber syndrome (MKS) involve mutations in additional organs. MKS patients typically do not live past birth or are naturally aborted earlier. The additional symptoms of this autosomal recessive lethal disorder are central nervous system malformations, occipital encephalocele, post-axial polydactyly, bowing limbs, severe heart malformations, and hepatic developmental defects. [4] There is extensive genetic overlap between MKS and NPHP with distinct mutations identified in shared genes. This indicates that disease severity (and thus, clinical diagnosis) is influenced by which gene is affected, the nature of the mutation in that gene, and the genetic background of the patient. Unfortunately, the causative lesion in most MKS and NPHP patients remains unidentified. Identifying the missing genes involved in these disorders is critical to ultimately understanding the cellular and molecular basis of the disease and in turn developing possible genetic therapeutic strategies. [10]

Homologs of at least ten NPHP and MKS genes have been identified in the nematode *C. elegans*, and thus far, their encoded proteins localize to the transition zone (TZ) at the base of sensory cilia. [9, 10, 11] Whereas humans have primary cilia extending from the majority of their cells, *C. elegans* only have sensory cilia extending from a subset of their neurons in the head (amphids) and tail (phasmids).

Cilia are microtubule-based and membrane-bound organelles. They develop via nucleation of microtubules templated by the centriole, which can remain attached to the proximal end of mature cilia as the basal body. The basal body helps anchor the cilium axoneme to the plasma membrane and cytoskeleton. The basal bodies function in the assembly of proteins that are involved in intraflagellar transport (IFT) in addition to initializing ciliogenesis. IFT is a critical component of cilia formation and it mediates the trafficking of proteins along the cilia axoneme. Just distal to the basal body is a ciliary subdomain called the TZ in which the cell membrane transitions to the cilium membrane. In the TZ region, Y-shaped links of unknown molecular composition protrude from the ciliary microtubules and form attachments with the surrounding membrane. Although the utility of the TZ is currently unclear, its positioning at the base of the cilium implicates a role as a regulator of protein movement between the cell and ciliary membrane. (Figure 1) When mutations occur within genes encoding the basal body, TZ, and IFT components in mice, symptoms of the aforementioned ciliopathies (diseases associated with ciliary defects) will result. In contrast to the critical requirement of cilia for mammalian development, the cilia of *C. elegans* function primarily as sensory organs and are not essential for the viability of the organism. The nonessential nature of the sensory cilia along with the genetic malleability of *C. elegans* facilitates the analysis of interactions between the large number of NPHP and MKS gene orthologues with relation to cilia structure and/or function. [6,10]

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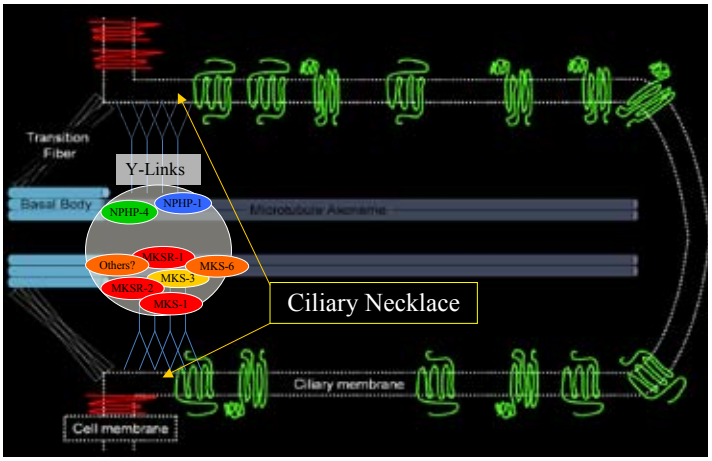


Figure 1. Anatomy of the cilium. The cilium features a membrane-encased microtubule backbone (axoneme) that protrudes from the cell surface. NPHP and MKS proteins are associated with the ciliary transition zone, which is a specialized region of the ciliary axoneme that connects to the basal body. The transition zone features Y-shaped links of unknown molecular composition that are attached to the transition zone microtubules and the surrounding ciliary membrane. This region of membrane is also known as the ciliary necklace as it features proteinacious decorations observable by electron microscopy. The transition zone is predicted to provide structural integrity to the cilium and to regulate protein trafficking into and out of the ciliary axoneme.

Genetic analyses have indicated the MKS and NPHP proteins form two distinct but potentially interacting functional complexes at the base of the cilia.[10, 11] Solitary mutations in genes in one complex will not cause a visible defect in cilia morphology in *C. elegans*; easily observable structural defects only arise when a combination of disruptions in both complexes is made. The disruption of cilia morphology in *mks;nphp* double mutant worms is most easily observed via a dye-filling assay in which the animals are exposed to a hydrophobic fluorescent dye. If cilia structure is normal, the dye is taken in through the cilia membrane and spreads throughout the sensory neurons. In the absence of properly formed cilia, the dye cannot stain the neurons; this phenomenon is referred to as a dye-filling defective (Dyf) phenotype. Any combination of mutations in the currently known *mks* genes with the *nphp-4(tm925)* mutation results in the Dyf phenotype (Figure 2).[9, 10, 11] Based on this phenomenon, we hypothesized that novel candidate MKS-like genes could be targeted in a mutagenesis screen for Dyf isolates in the context of the *nphp-4(tm925)* mutation. Once the mutants are identified from the *C. elegans* screen, an orthologue in the human genome might then be identified and screened in ciliopathy patients in whom a causative mutation has not been found. [10, 11]

Previously, an EMS (ethyl methane sulfate) mutagenesis screen was performed on *nphp-4(tm925)* mutant worms and ~200 Dyf F2 progeny were isolated (Svetlana Masyukova unpublished and[9]). A method of outcrossing with N2 Bristol males (wild-type) followed by subsequent phenotypic homozygous segregation analysis was utilized to filter new mutations as those independently causing the Dyf phenotype (1:3 Dyf versus wild-type segregation ratio) or those dependent on the presence of the *nphp-4(tm925)* allele for the Dyf phenotype (1:15 Dyf versus wild-type segregation ratio). (Figure 3) Through this outcrossing method, we uncovered ~40 new alleles (out of the original 200) that required the presence of the *nphp-4(tm925)* mutation to disrupt dye-filling.

Here, bulk segregate SNP analysis of the primary strain utilized in this study (YH972) showed linkage with chromosomes II (*novel mutation*) along with expected linkage with chromosome V at the *nphp-4* locus (Svetlana Masyukova unpublished).

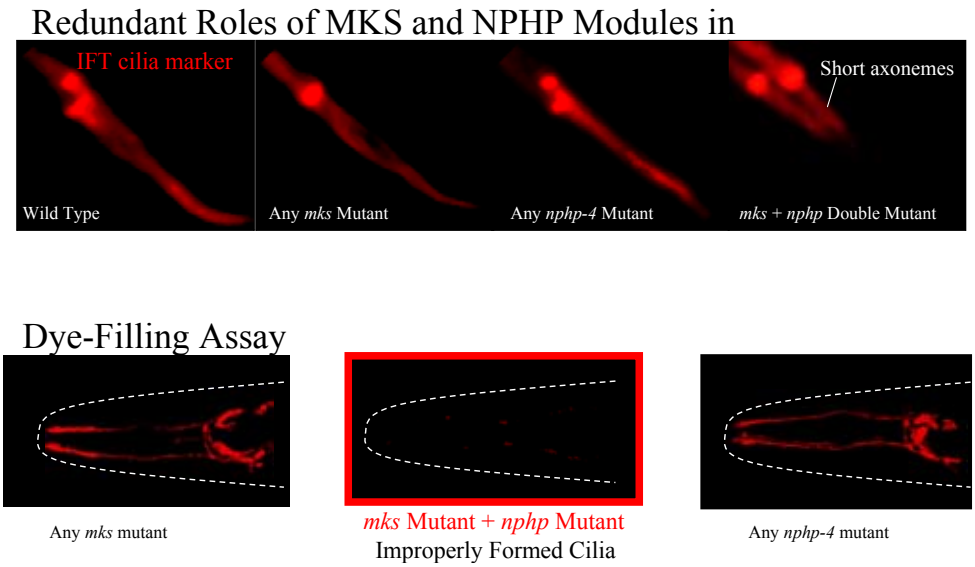


Figure 2. Redundant roles of MKS and NPHP modules in ciliogenesis

Figure 2a. Fluorescence images of worms expressing the cilia-specific IFT marker protein XBX-1::tdTomato in tail cilia pairs. Tail axoneme structure in mks and nphp single mutant worms is normal compared to wild type. In contrast, mks;nphp *double mutant worms have shortened cilia.*

Figure 2b. Fluorescence images of worms following exposure to DiI. mks *mutant worms and* nphp *mutant worms individually dye-fill normally, shown by the presence of DiI throughout the ciliated sensory neurons in the head. Combination of an* mks *mutation along with an* nphp *mutation in the same worm resulted in a failure to uptake dye due to alteration of cilia structure (see 2a).*

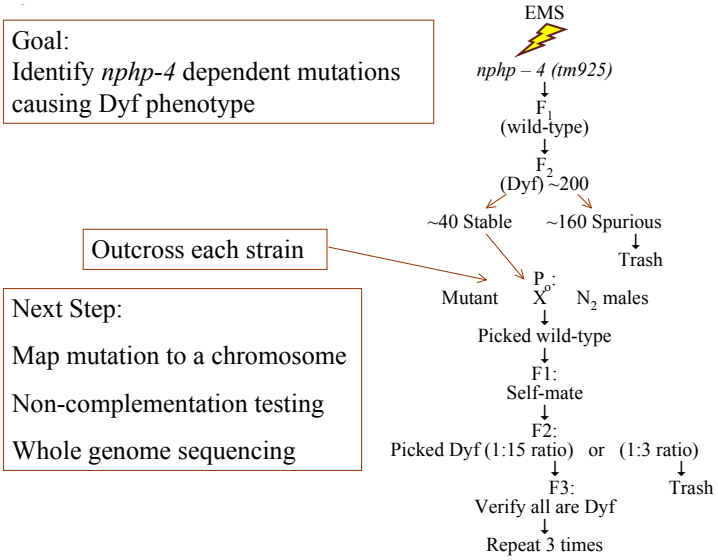


Figure 3. Outline of the mutagenesis screen performed on nphp-4 (tm925) *mutant worms.*

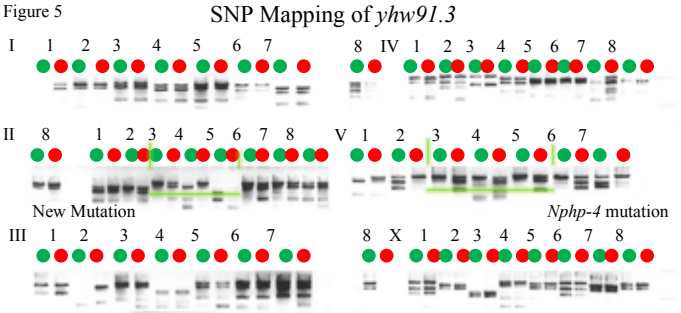
Non-complementation analysis was utilized to indicate that the YH972 mutation (*yhw91*) is a novel allele of the C09G5.8 gene, which is orthologous to human MKS-associated gene, *RPGRIP1L/MKS5*. [3] A base-pair mutation in the YH972 *mks-5*/C09G5.8 gene was detected by sequence analysis. Both the *yhw91* point mutation and an already characterized *mks-5(tm3100)* deletion mutation were utilized in mating schemes to visualize the phenotypic effect of the new point mutation obtained from the screen. Both *mks-5(yhw91)* and *mks-5(tm3100)* mutant animals were found to abrogate protein trafficking across the TZ . Additionally, the *yhw91* and *tm3100* mutations were both found to disrupt the localization of other MKS proteins at the TZ, which insinuates that the MKS-5 protein is required for normal localization of known MKS complex proteins. Interestingly, the localization of MKS-5 protein is unaltered by the loss of other MKS or NPHP proteins.

Procedure / Results

In previous studies chromosomal location was determined by bulk segregate analysis for each novel *nphp-4* dependent mutation generated in our EMS mutagenesis screen (Svetlana Masyukova unpublished, and summarized in Figure 4 and 5) [9]. As an attempt to validate whether the screen accurately targeted MKS-like genes we next determined whether any of these new mutations were in genes homologous to those already known to be associated with MKS in humans. This was accomplished by performing genetic non-complementation analysis between our newly mapped mutants and *mks-x;nphp-4* strains in which the corresponding *mks* gene was located on the same chromosome as the newly mapped mutation. Complementation is the term used to describe the phenomenon in which the mating between two animals with identical mutant phenotypes but with homozygous mutations in different genes produces offspring lacking the shared phenotype. This occurs because each parent will provide for its offspring one wild-type copy of the gene mutated in its mate. Alternatively, if both parents were homozygous mutant at the same locus, then their offspring would, as a rule, also be homozygous mutant and no phenotypic rescue would occur. This is referred to as non-complementation. This report focused on novel mutants that mapped to chromosome II. These include *yhw35, yhw36, yhw39, yhw91, yhw128,* and *yhw129*. Each strain was complementation tested against one another and against *mks-3* and *mks-5*, which both reside on chromosome II.

Allele	Linkage Group	Complementation Group
yhw35	Chr II	36, 39, 128, 129
yhw36	Chr II	35, 39, 128, 129
yhw128	Chr II	35, 36, 39, 129
yhw129	Chr II	35, 36, 39, 128
yhw91	**Chr II**	??????????
yhw65	Chr IV	66
yhw66	Chr IV	65
yhw3	Chr V	9, 15, 17, 19, 130, 131, 135
yhw9	Chr V	3, 5, 15, 17, 19, 130, 131, 135
yhw15	Chr V	3, 5, 9, 17, 19, 130, 131, 135
yhw17	Chr V	3, 5, 9, 15, 19, 130, 131, 135
yhw19	Chr V	3, 5, 9, 15, 17, 130, 131, 135
yhw130	Chr V	3, 5, 9, 15, 17, 19, 130, 135
yhw131	Chr V	3, 5, 9, 15, 17, 19, 130, 135
yhw135	Chr V	3, 5, 9, 15, 17, 19, 130, 131
yhw12	Chr V	68
yhw68	Chr V	12
yhw24	Chr X	26,71
yhw26	Chr X	24, 71
yhw71	Chr X	24,26

Figure 4. Novel nphp-4 *dependent mutations generated in the mutagenesis screen. Alleles are organized by linkage and complementation groups.*



C. elegans gene	Chromosome number
nphp-1	II
nphp-4	V
mks-1	III
mksr-1	X
mksr-2	IV
mks-3	II
mks-5	II
mks-6	I

Figure 5. SNP mapping of yhw91 *indicates its location on chromosome II.*

Figure 5a. DraI *digest profile of SNP regions amplified from F2 progeny of* yhw91;nphp-4 *crossed with a SNP mapping strain. The first column of each SNP shows Dyf F2 progeny digest profile, and the second column shows wild type F2 sibbling digest profile.*

Figure 5b. Genomic location of the MKS and NPHP module genes in C. elegans. yhw91 *is found on the same chromosome as* mks-3 *and* mks-5.

The non-complementation testing of novel chromosome II mutants went as follows: *mks-3;nphp-4* or *mks-5;nphp-4* double mutant hermaphrodites were crossed to *nphp-4* males to produce *mks-3*$^{-/+}$*;nphp-4*$^{-/-}$ or *mks-5*$^{-/+}$*;nphp-4*$^{-/-}$ males that are capable of mating (cilia morphology mutants are not adequate maters due to the involvement male sensory ray cilia in mating activities). These males were then crossed with each novel chromosome II mutant (Figure 6). A similar strategy was used to cross each novel chromosome II mutant with one another. The readout for this assay was the presence or absence of Dyf male progeny. If no Dyf males arise (complementation), it is indicative that the parent strains have mutations in separate genes. If some Dyf males arise (non-complementation), then the parents have mutations in the same gene. Remarkably, our analysis showed non-complementation between *yhw35, yhw36, yhw39, yhw128,* and *yhw129*, indicating the mutations in these strains all affect the same gene on chromosome II (Figure 6). All of these mutants were able to complement *mks-3(tm2547)*, *mks-5(tm3100)*, and *yhw91*. *yhw91*, on the other hand, complemented *mks-3(tm2547)* but showed non-complementation with *mks-5(tm3100)* (Figure 6), indicating that the *yhw91* molecular lesion likely affected the *mks-5* gene. Morphology analysis using fluorescently-tagged cilia proteins revealed *yhw91;nphp-4* double mutants have abnormally formed cilia and fail to properly localize MKS-3 (Figure 7).

Figure 6

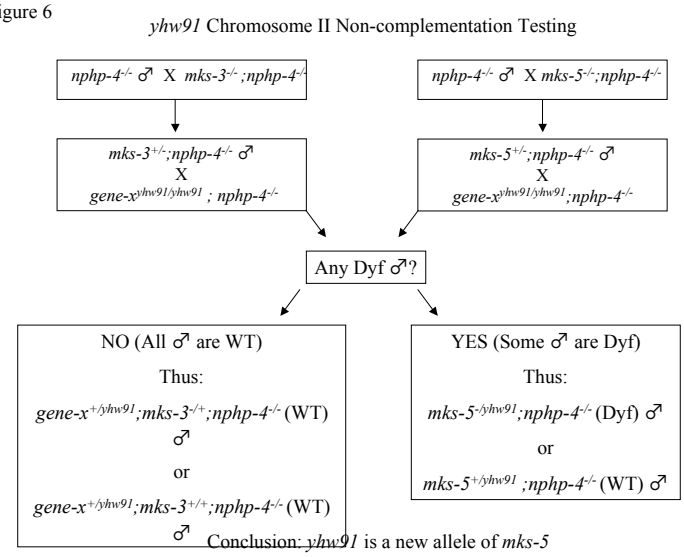


Figure 6. Non-complementation analysis of yhw91 *revealing its identity as a novel mks-5 allele.*

Figure 7

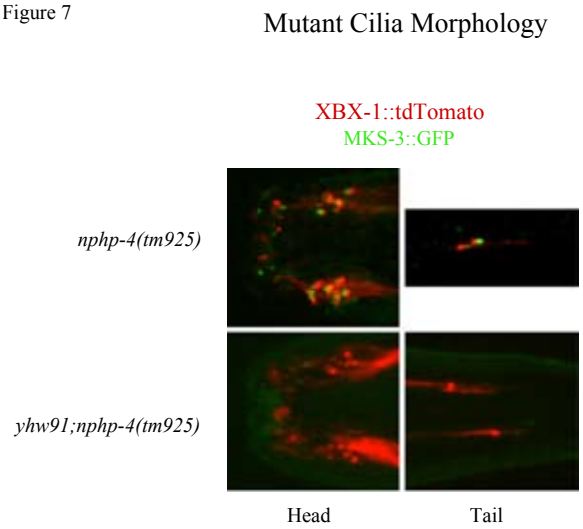


Figure 7. Fluorescence images of worms coexpressing XBX-1::tdtomato to mark cilia and MKS-3::GFP to mark transition zones. Compared to nphp-4(tm925) *single mutants,* yhw91;nphp-4 *double mutants have abnormally formed cilia and fail to properly localize MKS-3.*

We next wanted to sequence the *mks-5(yhw91)* genomic region to identify the location and nature of the mutation. First, a collection of primers were constructed in order to selectively sequence fragments of the roughly 12-kilobase gene. These primers were then used for PCR of genomic DNA isolated from *yhw91*, as well as N2 wild type controls. Through multiple primer sets, the entire genomic sequence of *mks-5* was obtained for *yhw91*, and we were able to identify a base-pair mutation exchanging a guanine for an adenine at the junction between exon and intron 20 (Figure 8). Through RT-PCR analysis of the mks-5 transcript in *yhw91* mutants, we confirmed this mutation was detrimental to splicing between exons 20 and 21 (Figure 9). The mutant transcript reads through the end of exon 20 into intron 20 and thereby introduces code for 17 new amino acids before reaching a stop codon (Figure 9). This indicates that the 84 C-terminal amino acids of MKS-5 will be absent in the *yhw91* mutant protein. Overall, our identification of a novel allele in a known *mks* gene indicates that the EMS screen was accurately designed to identify proteins that are potentially novel MKS proteins.

Apart from the observation that *mks-5;nphp-4* double mutants are synthetically Dyf, the function of MKS-5 in *C. elegans* has not been previously assessed. To examine the role of this protein, we utilized both the *yhw91* point mutation and the *tm3100* deletion mutation in assays for alterations in cilia function. For phenotypic analysis of the *yhw91* allele, we first outcrossed the *mks-5(yhw91);nphp-4(tm925)* double mutant with N2 worms to generate an F2 generation animal that was homozygous mutant at the *yhw91* locus and wild type at the *nphp-4* locus. This was accomplished by PCR and direct sequencing of the *yhw91*

locus in several F2 animals. Simultaneously, those animals were genotyped by PCR for the absence of the *nphp-4(tm925)* deletion mutation.

Figure 8

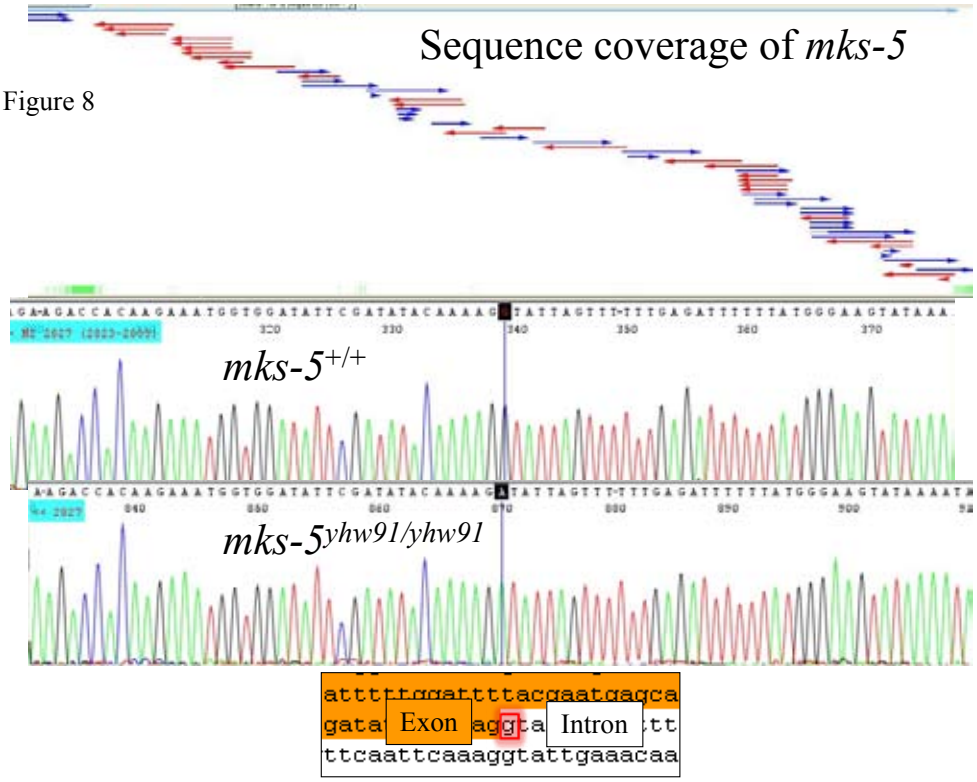


Figure 8. Sequence analysis of the mks-5(yhw91) *allele. Following whole gene sequencing analysis of* mks-5yhw91/yhw91 *mutant worms, a base pair mutation was identified at an intron-exon junction of exon 20.*

In a normal cell, some membrane-associated proteins are allowed to enter the ciliary axoneme, whereas others are kept out of the cilium. This barrier between the cilia and plasma membranes is hypothesized to the ciliary necklace, a portion of the membrane attached to TZ microtubules via Y-shaped links (Figure 1). In related studies, some *mks* mutations were found to disrupt cilia membrane composition by lack of regulation of protein trafficking across the TZ (Williams et al., in preparation). Specifically, upon disruption of MKS-associated and NPHP-associated genes, namely *nphp-1, nphp-4, mksr-1*, *mksr-2*, *mks-6*, and to a lesser extent *mks-1* and *mks-3*, restriction of the myristoylated membrane-associated protein RP2 from the ciliary membrane was markedly diminished. Thus, the TZ indeed appears to be responsible for keeping the protein content of the cilium membrane separate from the plasma membrane. Similar to what was observed in other *mks* mutants, the same defects were present in both *mks-5(yhw91)* and *mks-5(tm3100)* mutant animals (Figure 10), indicating that MKS-5 functions in a capacity similar to that of other MKS and NPHP proteins.

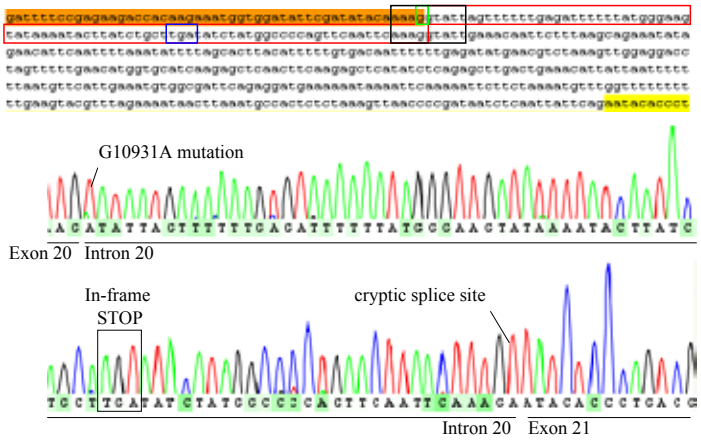


Figure 9. The yhw91 *mutation alters splicing of the* mks-5 *transcript. (Top) Sequence of* mks-5 *intron 20 region. A portion of exon 20 (orange), all of intron 20 (no coloration) and a portion of exon 21 (yellow) is shown. Nucleotide G10931 is boxed in green. (Bottom) Sequence trace of the* yhw91 *mutant transcript, showing readthrough at the mutated 10931 nucleotide into intron 20 (new sequence is highlighted in red above). Via a cryptic splice site in intron 21, splicing occurs with exon 21 downstream of an in-frame stop codon (blue box above). Interestingly, the cryptic splice site exactly matches the sequence at the end of exon 20 (black box).*

To further examine the potential role of MKS-5 and other MKS proteins in regulating ciliary membrane composition, we assessed the localization of the transmembrane-spanning TZ protein MKS-3. Normally, this protein is restricted at the TZ where it colocalizes with other MKS proteins. [10] However, in the background of either *mksr-1, mksr-2*, and our *mks-5* mutations, MKS-3 was no longer retained at the TZ and instead accumulated in the cilium membrane (Figures 10, 11, and 12) . This defect was not observed in either *nphp-1* or *nphp-4* mutants, which have altered RP2 levels in cilia, suggesting that the anchoring of MKS-3 at the TZ is MKS protein-specific (Williams *et al.*, in preparation).

MKS-3/TMEM67 Ciliary Accumulation is *mks* Mutant-Specific. RP2 Accumulation also Occurs in *mks* Mutants.

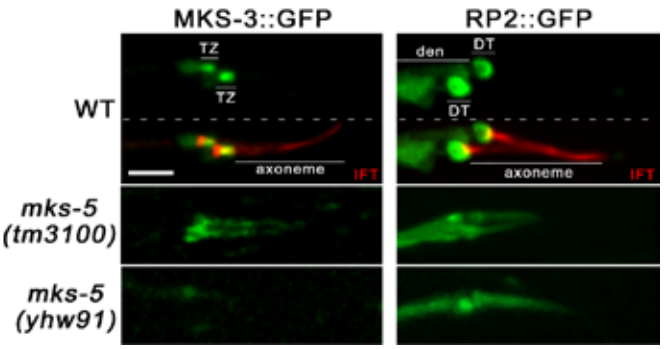


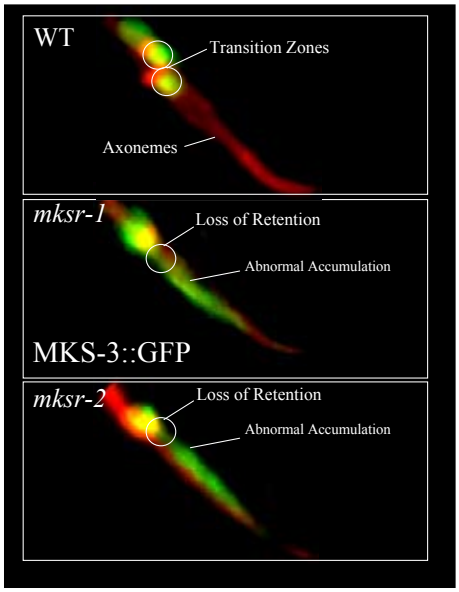
Figure 10. Cilia associated defects in mks-5(yhw91) *point mutants resemble those observed in* mks-5(tm3100) *deletion mutants. (left) Fluorescence images of worms expressing transmembrane MKS-3::GFP. Compared to wild type worms in which MKS-3 is enriched at the transition zone, MKS-3 was delocalized from the transition zone and accumulates in the ciliary axoneme when in the* mks-5(tm3100) *deletion mutant background. MKS-3::GFP was also delocalized from the transition zone in the* mks-5 (yhw91) *point mutant. This data shows that MKS-5 is essential for MKS-3 localization within the transition zone. (right) Similarly, the membrane-associated RP2::GFP protein also accumulated in cilia of both* mks-5(tm3100) *and* mks-5(yhw91) *mutants.*

Because MKS-3 TZ localization was lost in the background of *mks-5* mutations, we assessed whether other MKS proteins were dependent on MKS-5 for normal localization. A series of matings were set up to combine the *mks-5(tm3100)* mutation with each fluorophore-tagged MKS protein. These strains were then imaged along with control strains with the same transgenic markers in order to compare localization. Remarkably, *mks-5* was required for the localization of all other MKS proteins at the TZ (Figure 12). Since mammalian MKS5 (RPGRIP1L) was previously found to directly interact with NPHP4 [7], we also assessed whether disruption of *mks-5* in the worm would affect NPHP-4 TZ localization. Interestingly, upon loss of *mks-5* function, NPHP-4 (and NPHP-1) localization compared to wild type was restricted to a smaller region of the TZ (Figure 12). We were also interested in determining whether MKS-5 protein localized at the TZ along with the other MKS and NPHP proteins. By expressing tdTomato-tagged MKS-5 driven by the ciliated sensory neuron specific promoter of the *osm-5* gene, we were able to visualize MKS-5 localizing at the TZ at the base of cilia (Figure 12). Remarkably, the localization of MKS-5 was unaltered by the loss of other MKS or NPHP proteins (Figure 12). This observation along with the requirement of MKS-5 for the localization of all other known MKS proteins, suggests that MKS-5 is a major anchoring protein in a complex comprised specifically of MKS proteins, and to a lesser extent in the NPHP-1/4 complex (Figure 13).

Future endeavors

Based on this data, we have validated that the screen performed to identify novel genes functioning in similar fashion to known MKS genes is accurate in its intended goal. Additionally, we have discovered that MKS-5 is an essential component involved in the anchoring of the MKS and NPHP protein complexes. Further studies will involve the identification of molecular lesions in the additional genes resulting from the mutagenesis screen. Following their identification, these genes will be assessed for function in cilia and/or the TZ and their relationship to other MKS proteins. Ultimately, these novel MKS-like genes will be sequenced for mutations in human patients with cilia-related disorders. [5,8]

MKS Mutations Disrupt Ciliary Membrane Composition



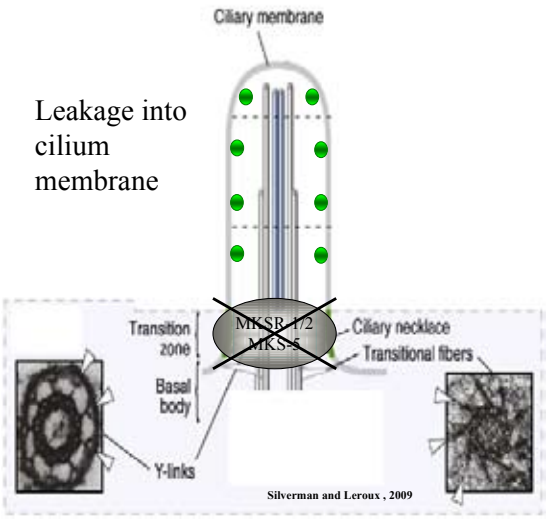
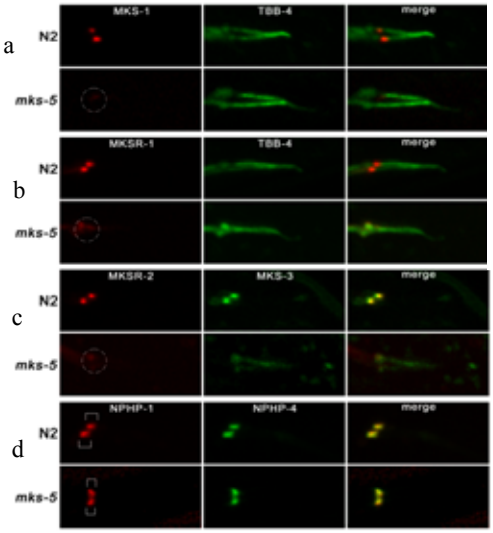
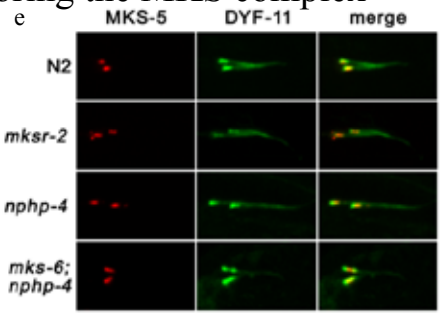


Figure 11. MKS-3 accumulates in the cilia of mksr-1 *and* mksr-2 *mutants. (left)Fluorescence images of worms coexpressing MKS-3::GFP and XBX-1::tdTomato (cilia marker). Similar to disruption of* mks-5 *(previous figure),* mksr-1 *and* mksr-2 *mutants also cause delocalization of* mks-3 *from the transition zone. (right) A model depicting accumulation of MKS-3 within the cilium membrane upon abrogation of MKSR-1, MKSR-2, or MKS-5 function.*

Figure 12

MKS-5 is essential for anchoring the MKS complex





f

Gene	Required for Correct Localization of:
mks-1	None
mksr-1	MKS-1 [7], MKS-3, MKS-6 (Williams et. al, in submission)
mksr-2	MKS-1 [7], MKSR-1 [7], MKS-3, MKS-6 (Williams et. al, in submission)
mks-3	None
mks-5 (RPGRIP1L)	**Any MKS**: MKS-1, MKS-3, MKSR-1, MKSR-2, MKS-3, MKS-6 (Williams et. al, in submission)
mks-6	None
nphp-1	None
nphp-4	NPHP-1 [9]

Figure 12. MKS-5 is found at the transition zone where it is essential for anchoring the NPHP and MKS modules. a. Fluorescence images of worms coexpressing MKS-1::tdTomato and TBB-4(β-tubulin)::GFP. Compared to wild type (N2), MKS-1 is delocalized from the transition zone in mks-5(tm3100) *mutants. b. Fluorescence images of worms coexpressing MKSR-1::tdTomato and TBB-4(β-tubulin)::GFP. Compared to wild type (N2), MKSR-1 is delocalized from the transition zone in* mks-5(tm3100) *mutants. c. Fluorescence images of worms coexpressing MKSR-2::tdTomato and MKS-3::GFP. Compared to wild type (N2), both MKSR-2 and MKS-3 are delocalized from the transition zone in* mks-5(tm3100) *mutants. d. Fluorescence images of worms coexpressing NPHP-1::CFP and NPHP-4::YFP (psuedocolored red and green, respectively). Compared to wild type (N2), both NPHP-1 and NPHP-4 occupy a smaller region of the transition zone in* mks-5(tm3100) *mutants. e. Fluorescence images of worms coexpressing MKS-5::tdTomato and DYF-11::GFP. In wild type (N2) worms, MKS-5 localizes to the transition zone and is unaffected in* mksr-2 *and* nphp-4 *mutants, and a combination* mks-6/nphp-4 *mutant. f. Localization requirement results from the hierarchy screen.*

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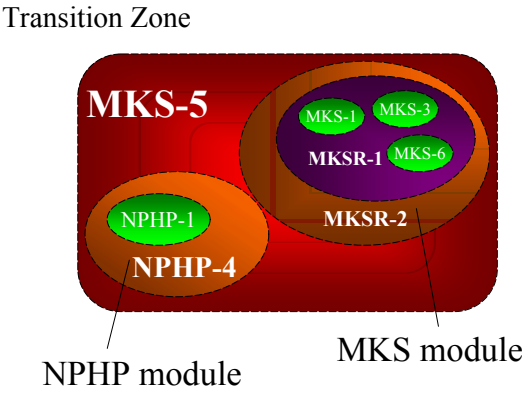

Figure 13. Hierarchical model for the ciliary transition zone functional modules. In the MKS module, MKS-1, MKS-3, and MKS-6 require MKSR-1 for transition zone localization, and MKSR-1 requires MKSR-2. MKS-5 is required for transition zone localization of each protein within the MKS module. In the NPHP module, NPHP-1 requires NPHP-4 for transition zone localization. NPHP-1 and NPHP-4 require MKS-5 for localization to a transition zone subdomain.

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paper: biology

A Pioneering Look at Current Trends in Nuclear Medicine Clinical Equipment in the State of Alabama

Findings from a 2010 Clinical Survey

Yvonne Flaherty, Norman Bolus, MPH

Background

Nuclear Medicine (NM) is a significant yet small component of the larger field of medical imaging. With the development of camera systems that are uniquely sensitive to radioactive emissions, the functional workings of the human body are now being revealed for diagnosis of pathology. NM has had a great influence on the diagnosis and treatment of a number of different illnesses and diseases through non-invasive means while providing information that cannot be obtained from other imaging modalities. Diagnostic imaging using NM has seen increasing specialization in the last few years into areas such as oncology and cardiology which is driving the evolution of cameras. Perhaps the most groundbreaking advancement in medical imaging has been the introduction of hybrid imaging modalities, in which anatomy merges with function to produce some of the most penetrating scans to date. Capturing anatomical as well as physiological information in a single exam, hybrid imaging automatically merges this data to form a composite image allowing us to see a much more comprehensive picture of what is going on beneath the surface. This research comprises the foundational attempt at looking into the current reality and locations of the gamut of nuclear medicine clinical equipment in the state of Alabama.

Before the advent of hybrid scanners, patient images were either compared side-by-side or manually "fused" with special software programs, using anatomical landmarks to align and create a single image. Although "fusion imaging" has become synonymous with "hybrid imaging" there remains a distinct difference. A fusion image is inherently altered to fit its counterpart image while a hybrid image is created when two images are acquired and processed simultaneously on one scanner to eliminate the unavoidable tweaking of images in the fusion process. Instigated by the emerging popularity of hybrid PET/CT and the continual development of new radiopharmaceuticals, an evolution from older equipment to newer innovations offers exciting change with a vital need for technologists that comprehend these new scanners.

Hybrid imaging continues to gain momentum, especially among researchers but remains a hard sell to imaging departments because of the need for expenditure. Diffusion of these multi-modality machines is slow even though the usage of nuclear medicine procedures is expanding (Frost and Sullivan). Despite the lagging distribution of hybridized scanners, such as positron emission tomography/computed tomography (PET/CT) and single photon emission computed tomography/computed tomography (SPECT/CT), their steady, even if slow, infiltration will continue improving the accuracy of detection, localization, and characterization of disease.

It was this idea, on "advancement of imaging tools", that prompted this survey and an interest in looking specifically at the current state of imaging in Alabama drove this project. This report summarizes the responses to a 2010 survey seeking to better understand the initial penetration of the newer technologies such as hybrid or fusion scanners in nuclear medicine departments across the state of Alabama. The report describes the demographic characteristics of scintillation cameras, licensure patterns of nuclear medicine technologists (NMT), the trends in NM patient volumes and, as an aside, the location of Geiger-Muller counters.

Survey analysis of department staff and equipment was necessary to understand first, what exists and second, where growing needs of the state of Alabama might exist with regard to hybrid imaging. Obtaining this data provides information for assessing the number of nuclear medicine facilities, the equipment currently in use, and the personnel staffing each facility. This survey is a first attempt at looking at the reality of nuclear medicine in Alabama. The poll was sent to 96 facilities within the state of Alabama with responses documented at 40 facilities. There were no incentives offered for answering the survey. It was expected that around 10% of surveys would be returned with recordable data. Surprisingly, 42% of the questionnaires were returned.

Design

The survey of nuclear medicine departments in the state of Alabama was designed with the help of faculty from the School of Health Professions at University of Alabama. Participating members were helpful in determining areas of interest to be included in the survey, framing questions and response options, and in advising about survey process and obtaining proper IRB approval. The sample was drawn from a list of hospitals and clinics in Alabama using public resources and verification of each facility's nuclear medicine department by phone contact. The survey questionnaire consisted of 11 questions with three main topics of concern including certification data, information about cameras and finally, services provided. Due to time constraints the survey was not field tested to identify any confusing or poorly stated questions/responses. As a result, some questions had no responses and were not included in the final results.

Sample

In April of 2010, paper surveys were mailed to a sample of nuclear medicine imaging facilities in Alabama. The sample was drawn from lists of all identified nuclear medicine imaging facilities, including hospitals, stand alone clinics, and mobile units as well. The goal was to identify and locate every nuclear medicine camera used for clinical purposes in the state. Facilities have been categorized by geographic location, quadrant, and county in Alabama. What follows is a simple segmentation of the state of Alabama. It is used simply for charting and classifying information from the survey in a coherent manner. (Lines are arbitrarily chosen for this study).

Figure 1. Region Segmentation

Geographic Region	# Hosp. in AL	% Resp. per region	# NMTs	% NMTs per region	# Cameras in AL	% Cameras per region
NW	43	44.8%	61	49.6%	43	42.2%
NE	20	20.8%	23	18.7%	16	15.7%
SW	17	17.7%	14	11.4%	16	15.7%
SE	16	16.7%	24	19.5%	26	25.5%
unknown			1	.8%	1	1%
Totals	96	100%	123	100%	102	100%

Table 1. Region Distribution of Hospitals, NMTs and Cameras in Alabama

Table 1 and Figure 1 together provide a description of the geographic representation of hospitals, NMTs, and cameras throughout the state. Information for this table and all other graphics and tables come strictly from information gathered by the survey.

Cameras

Survey of Nuclear Medicine Cameras

Demographics tell us a number of interesting facts. We draw insight into the number and type of cameras per region, location densities of scanners, and uncover underserved areas. Not surprisingly, we also become privy to the trends of advancing technology as well. Although the majority of cameras in Alabama are tomographic, signifying progress toward better and improved technology, planar cameras are still widely used. The advent of rotational capabilities in scanners has served to increase the specificity of many studies while also aiding in the diagnosis and specific location of pathology.

Camera accessibility is displayed in the following map. The map compares number of cameras in Alabama counties with the population of those areas. The northern parts of the state show proportionality between scanners and the population while the southern parts of the state, especially in the Mobile area, reveal a possible disproportionality. Each of the four quadrants that the map (figure 2) displays represents a well covered region as scintillation cameras go. Cameras located in each section are represented. Although diminished camera populations exist, patient access to cameras is within a reasonable distance to those from under-populated areas.

In figure 2 the map shows a large central concentration of cameras with dispersed concentrations coinciding with populations centers in Alabama.

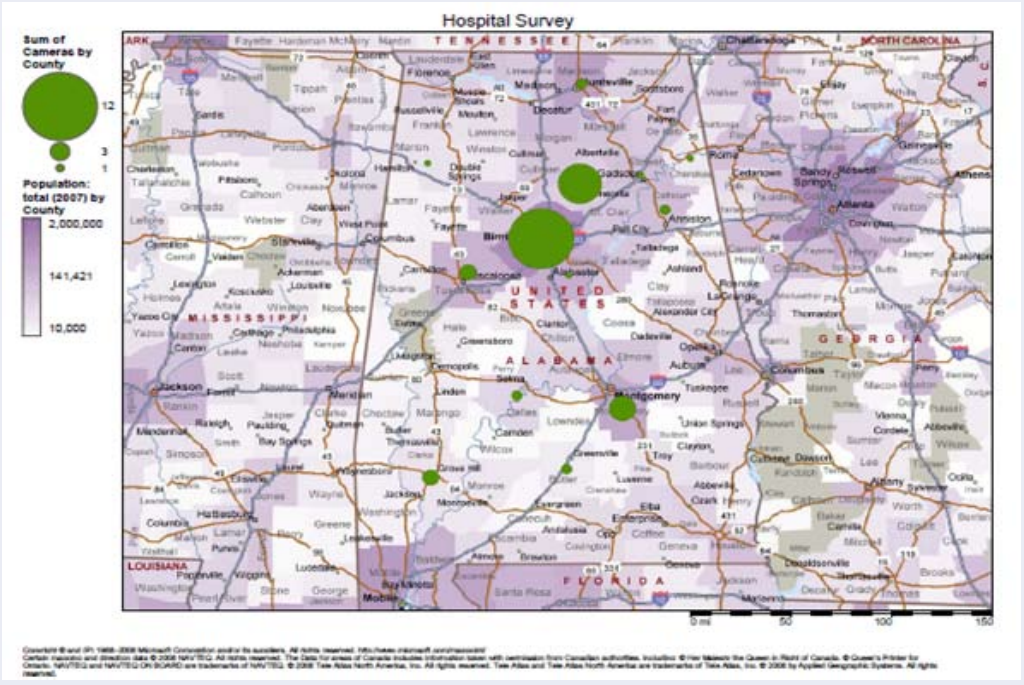

Figure 2. Camera Density by Location

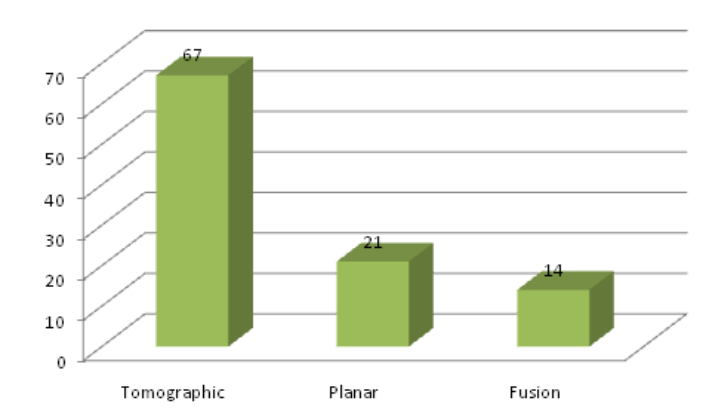

Figure 3. Camera Type and Count Demographics

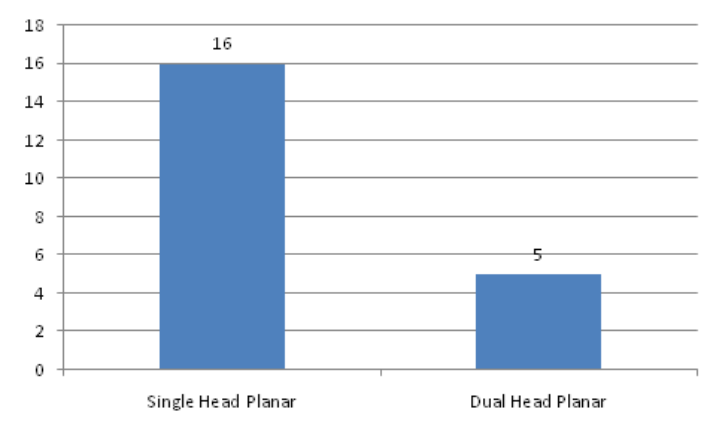

Figure 4. Planar Cameras Type and Count

The landscape is beginning to show that fusion or hybrid cameras have emerged but are not as prevalent as expected compared to the excitement they inspire. PET/CT and SPECT/CT are the most popular hybrid imaging systems found in Alabama. PET/CT studies are enjoying solid advancements due to the infrastructure of cyclotrons for PET radiopharmaceutical applications. In the series of figures 4-6 is information about cameras in use as well as the type of cameras. Hybrid cameras are creeping onto the scene which is especially depicted in figure 6.

SPECT/CT advancements are slow and difficult to comprehend. A variety of possibilities exist but without further information from a formal survey it would be irresponsible to speculate.

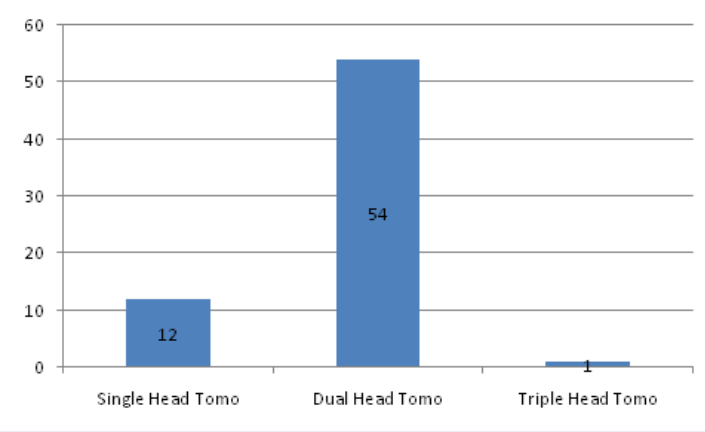

Figure 5. Tomographic Cameras Type and Count

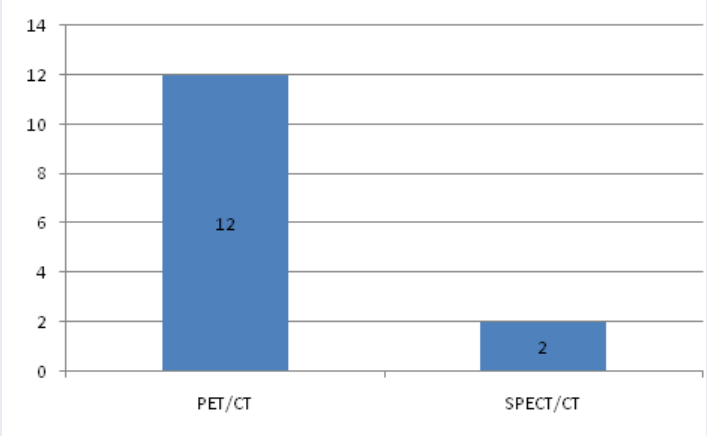

Figure 6. Hybrid Cameras Type and Count

The abundance of dual head tomography cameras illuminates one truth; at one time dual head cameras were the rage and the future of nuclear medicine. They have become the workhorse and continue to be an important acquisition for departments as shown in the statistics, since 33%, or 13 of 40, facilities responded that they have planned the future purchase of dual head or tomographic scanners.

Certification

NMTCB Certification

The certification background of nuclear medicine technology professionals helps in discerning the quality of technologists in the state. Even though the two primary certifying organizations are NMTCB and ARRT, for those seeking credentialing in nuclear medicine the survey focus was only on NMTCB certified technologists. One of the benefits of attaining the NMTCB certification is the level of recognition that it receives. According to the NMTCB, it is recognized not only by the profession of nuclear medicine, it is also recognized by state licensing agencies as well as by employers. All of these entities recognize the NMTCB certification as a premier, high-quality credential. The NMTCB is a strong supporter of federal legislation that would require basic educational and certification standards for health care workers who administer radiologic procedures in every state in the union (NMTCB). Although the ARRT is a nationally recognized certification board and highly respected throughout diagnostic imaging, it is the NMTCB that is nuclear medicine's standard certification.

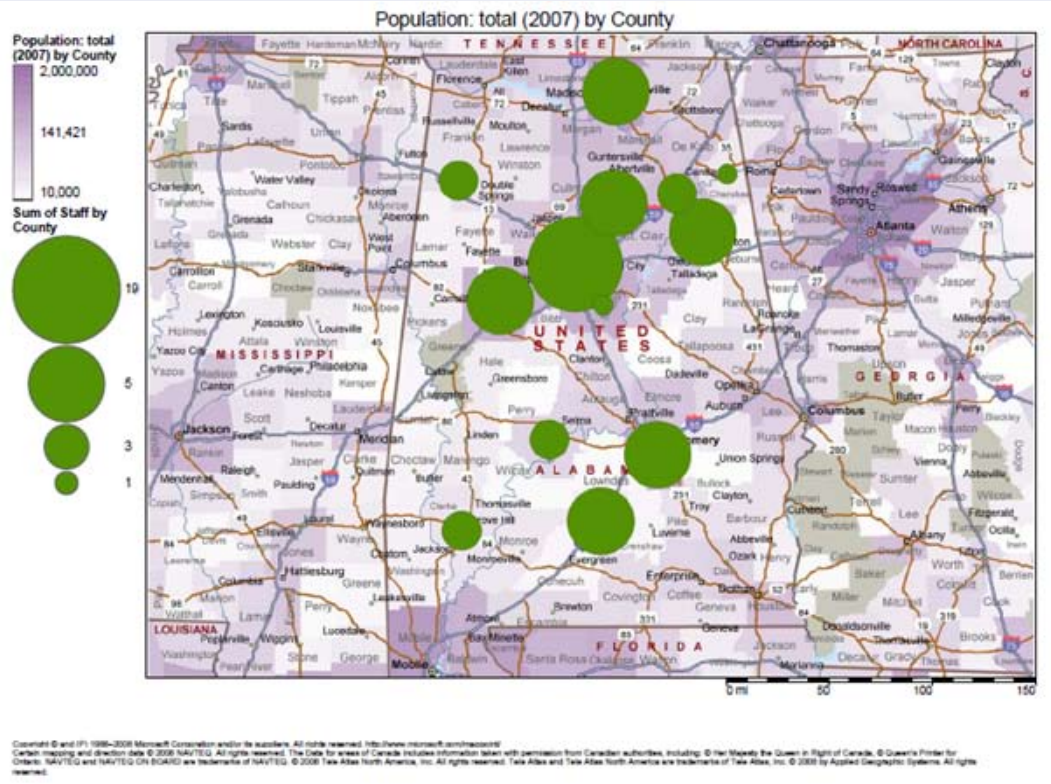

Figure 7. Population of NMTCB Certified vs. Non-Certified Techs in Alabama

The percentage of technologists in the sample with certification runs at 78%. There is a nearly 4 to 1 ratio of NMTCB certified techs to non-certified (figure 8), suggesting that the technologists in Alabama are

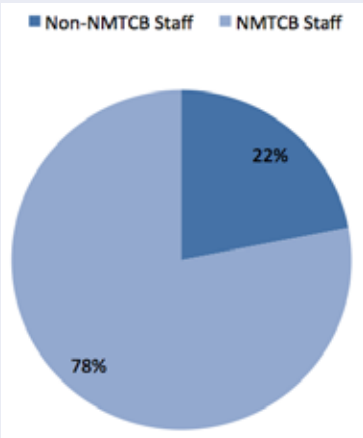

Figure 8. Percentage of NMTCB Certified vs. Non-Certified Techs in Alabama

well educated and experienced in nuclear medicine imaging. Indicators in addition to NMTCB certification are technologists that have been cross trained and even on the job trained. Although specific data was not tracked on other certifications many of NMTs with cross training and OJT training would have had other prior certifications in such modalities as x-ray, fluoroscopy, CT, MRI, suggesting a higher level of education also.

General vs. Specialized Staff

General NM studies represented the bulk of services provided by NMTs in Alabama. Only a small percentage of NMTs work in a specialized NM modality. As hybrid-type imaging moves into departments it is expected that staff will also undergo a type of merge following the scanners' model. As the future comes and changes arrive, departments may be forced to do away with staff trained in a single modality. It will become much more cost effective to pay one employee who has dual/triple certifications rather than hire three staff people to run one camera.

As hybrid imaging, such as PET/CT, has become more prevalent, employees are learning multiple modalities in advance and coming into the hybrid area with more experience and often with dual certification. As this trend continues, facilities such as clinics or hospitals will be able to conserve their funds by having one staff operating both aspects of the machine.

The survey questionnaire brought to light that many certified employees are either cross-trained in NM or on-the-job-trained, declaring evidence that a semi-merging of technologist's training has begun. See figure 11 in the next section for delineation of this. NMTs also must be aware of the shift in the locus of NM services beginning to take place. As is true for many imaging modalities, NM is shifting out of hospitals into ambulatory facilities. It is also shifting away from NM physicians toward cardiologists, oncologists, and other specialists who are becoming increasingly reliant on NM for both diagnostic and therapeutic purposes (SNM).

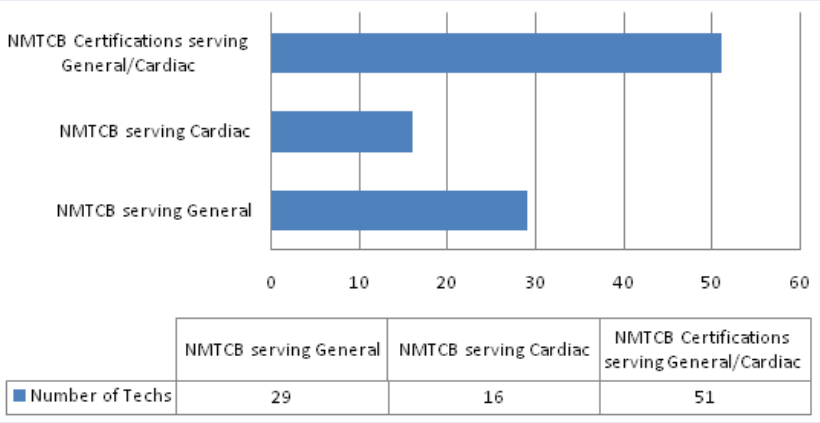

Figure 9. Facility Type and Number of NMTCB Certified Techs at Each Facility in Alabama

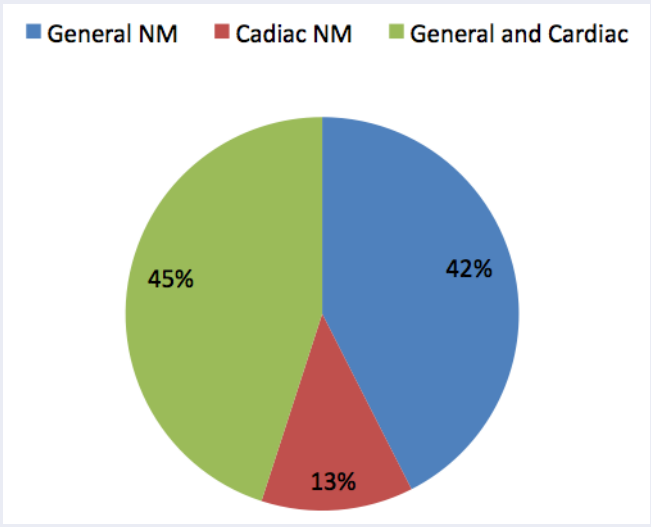

Figure 10. Percentage of Facility Type in Alabama

Cross Trained and On-the-Job Trained

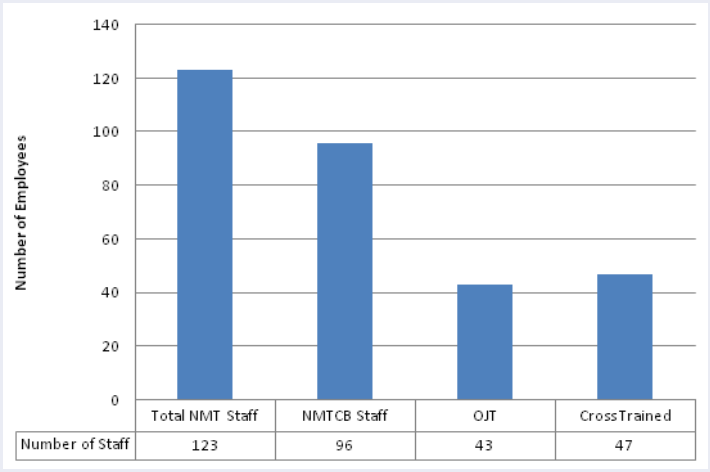

Figure 11. Diversity of NMT Training of Techs in Alabama

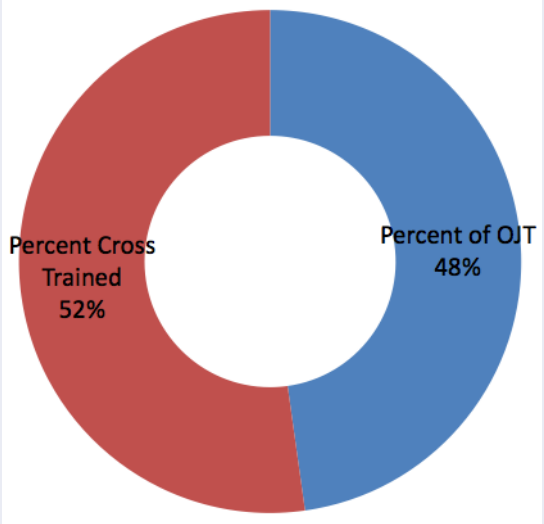

Figure 12. Comparison of NMT Backgrounds/ Alternative Training of Techs in Alabama

The background training of nuclear medicine technologists holds interesting information that lends itself to an interesting future study. For the purposes of this survey suffice to say there is roughly the same amount of cross trained techs as there are on-the-job-trained (OJT). Out of the 40 facilities that returned surveys statistics showed:

- 23 of the 123 employees are not NMTCB certified
- 4 facilities had all employees cross trained and OJT
- 12 facilities with no employees cross trained or OJT

Patient Trends

Patient Volume Increases or Decreases

Without actual statistical data from each department it is impossible to know the exact data concerning the patient volume. Polling the chief technologist at each facility as to whether there was an increase or decrease in services rendered was an exercise in speculation but it has yielded some interesting content. Of the facilities surveyed roughly 50% felt their departments had seen an increase in patient load while 45% said they were experiencing a decrease in patients.

Trend	Increase	Decrease
General facility	6	11
Cardiac facility	3	2
General/Cardiac facility	11	5
Totals	20	18
Percentages	50%	45%

Figure 13. Trends in Patient Volume

The questionnaire asked chief technologists if they were experiencing an increase or decrease in patient load. The data above shows the simple facts. Several imaging managers felt an onus to add information to the survey, stating they felt the decrease they were experiencing was only due to the profound and lasting ^{99m}Tc shortage gripping the industry. [The shortage was triggered by the shutdown of two reactors – one in Chalk River, Ontario and the other in Petten, The Netherlands.] Many additional closures followed due to scheduled maintenances. Closures exacerbated the preexisting crisis of aged reactors and brought their plight to the forefront. The confluence of dose shortages with the demise of reactors, a lack of reimbursement for PET studies and financial hardships have served to increase the pressure felt in the nuclear medicine imaging community.

Reactions to Decreased Patient Load

Facilities have resorted to a variety of measures in order to compensate for the shortages by switching to alternative imaging agents, switching to PET or CT scans when appropriate and finally, reducing dosages or even canceling procedures if needed. Cardiac facilities are relying on Thallium more for their studies. The most complex problems that arise are the financial ones impacting the facilities, the hospitals, the patients, and the pharmacies who supply radiopharmaceuticals, as they are all impacted by the reductions.

In an article in *Pharmacy Practice News* in December 2009 Bronstein and Jakubiak stated, "The Society of Nuclear Medicine recently released the results of a survey designed to assess the impact of the ^{99m}Tc shortage on several practice sites, including hospitals and outpatient imaging facilities. More than 90% of the respondents said they were affected by the shortages of the isotope. About one-third said they were only able to operate at 52% to 75% of their normal testing capacity. And 92% said they had to either cancel or postpone imaging procedures" (Bronstein and Jakubiak). It is reasonable to say that during the time the SNM survey was sent out, nuclear medicine in general was experiencing dire circumstances concerning isotope production. Cascading events undoubtedly impacted the results logged by department personnel who answered the inquiries to the SNM survey as well as the survey in Alabama. Since a direct relationship exists, between isotope production and nuclear medicine, without radiopharmaceuticals there is no nuclear medicine practice going on.

As odd as it may seem, there were plenty of facilities who responded to the survey that claimed an increase in nuclear medicine studies. Of the respondents, 50% said they saw an increase while 45% said they experienced a decrease in patient load. Without having exact information to substantiate the data it is again an educated guess as to the reason for these responses. Some have speculated that the increased supply in some departments had to do with the different radiopharmacy suppliers to each facility; some had supplies while others didn't. Some of the bigger hospitals had to go without radiopharmaceutical supply, while smaller hospitals were able to maintain and even increase their patients due to parceling by the pharmacies. Rationing most surely had a large part to play in the allotment of ^{99m}Tc, and it could have been the reason for shifting of patients to lesser used sites. Whatever the driving force for increased throughput of patients it turned out to be an exciting turn of events for Alabama. Any increase during a time of such tumult is a very positive sign.

Geiger-Muller Counters

One interesting side note to the distribution of gamma cameras is the fact that they are ideal screening tools for detecting the presence of fission products and therefore contamination from nuclear fallout. The scanners could be used to provide gross sensitivities that land between a whole body dose counter and a Geiger-Muller (GM) detector. GM detectors are portable and very sensitive to changes in radioactivity. It was a nuclear medicine radiologist that suggested the need to identify the location and count of GM detectors in the United States. In the event of a radiation emergency, response teams would know the logistics of these devices. As for Alabama, the

statistics tallied from the respondents look like this:

- 77 GM counters are located in the 40 facilities
- An average of 1.95 GM counters per facility
- There should be at least 56 more in the state

Conclusion

A sturdy foundation for burgeoning growth in nuclear medicine is vital. With the onslaught of troubles facing medicine in general and the specific troubles plaguing nuclear medicine it is imperative to keep hope and efforts alive. Surveying departments in Alabama has been helpful to see how resilient our state is in coping with crises and adjusting to the many troubling setbacks. The recession, the reactor shut downs, the government's healthcare overhaul, reimbursement cutbacks, and no passage of *American Medical Isotopes Production Act of 2009* (H.R. 3276) have served to buffet the nuclear medicine industry in Alabama. As these storms blow through the landscape some have come and gone leaving more damage than others. Amazingly, some of the misfortunes have yielded opportunity for change and improvement.
It became clear that the state of Alabama, although well equipped with a variety of scintillation cameras, is not a hub for hybrid scanners. Alabama proved to be more of a nursery for new technology; the prospect for growth and expansion is rife. New cameras are planned for and anticipated for the present and future.

Alabama's nuclear medicine industry is showing a glimmer of preparedness with the existing sites that are purchasing, have plans to purchase, or have already purchased fusion scanners. Of the 40 responses, 4 sites are buying a PET/CT camera while another 4 of the 40 are looking to a future with additional SPECT/CT cameras. Many sites are planning to buy a dual head camera or cameras that have tomographic capabilities. There is evidence that there is a movement toward new technology which means certification in these new modalities is an important aspect of job training/skill.

The greatest measure of the future for hybrid imaging is attitude. Twenty-seven (68%) chief technologists felt that there was a very strong need for fusion imaging in nuclear medicine. Only 33% opined there was not a need to move into new technology (most of these came from facilities in more remote areas). Comments in support of fusion imaging were varied and informative. Here are a few examples: "better attenuation correction", "need new PET agents to grow NM Fusion", "it is the future of all modalities", "it will cut down on false-positives in cardiac studies", "MR/NM will help with pacemaker problems", "better resolution and versatility", and finally, "tumor localizing is on the increase so more fusion is needed". These statements show without doubt that a warm welcome awaits the future. The technologists are key components in the acquisition process of new scanners and equipment. Their opinions matter and can often be prime movers since their skills and knowledge of the equipment and scans are the foundation on which the future of nuclear medicine is built.

Fusion imaging offers a panorama of possibilities like increased patient appointments, faster, more accurate images, and highly trained and skilled technologist all the while contributing to the development of more elaborate hybrid scanners, advances in molecular imaging, proliferating cyclotrons, PET/CT, SPECT/CT, PET/MRs, all adding onto the existing foundation.

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The Effects of Apolipoprotein Mimetic Peptide 4F on Paraoxonase-1 Activity and Levels

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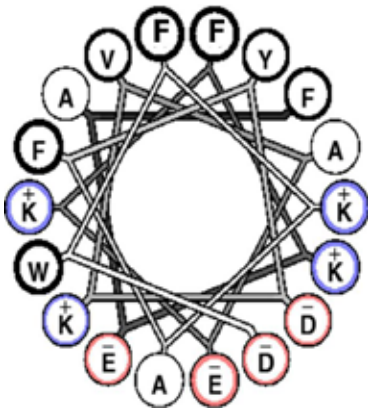
Abstract

Background- *Although there have been many advances in the diagnosis and treatment of coronary artery disease (CAD), CAD remains the major cause of deaths in the U.S. Atherosclerosis, the formation of arterial plaques, is a factor in CAD. Peptide 4F, an apolipoprotein A-I mimetic peptide, has been found to inhibit atherosclerosis in atherosclerosis-susceptible mouse models. Paraoxonase-1 (PON-1) is an enzyme to which many of the anti-oxidative properties of HDL have been credited. Even though peptide 4F is in phase 2 clinical trials, the mechanism in which it operates to increase PON-1 activity and decrease atherosclerosis is not known. We hypothesize that 4F affects PON-1 by increasing PON-1 activity in the plasma or by enhancing plasma levels of the enzyme (or both).* **Methods**-*Female apoE null mice (6 week old) were obtained from Jackson laboratories. Baseline cholesterol levels and PON-1 activity were measured in these animals and animals were divided into 2 groups, group 1 received saline and group 2 received peptide L-4F (50µg/day). Mice were injected intraperitoneally every day for 4 weeks. Animals were euthanized after 2 weeks. Plasma was analyzed for PON-1 activity and PON-1 levels. Livers were analyzed for PON-1 levels by Western blotting and gene expression by quantitative PCR.* **Results**-*There was a significant increase in plasma PON-1 activity in the group treated with L-4F as compared to the group treated with saline ($p<0.05$) and there was an increase of plasma PON-1 levels ($p<0.05$). Hepatic tissue samples showed no change in PON-1 mRNA or protein levels but did exhibit a significant decrease of tubulin levels in the group treated with L-4F compared to the group treated with saline ($p<0.05$), suggesting reduced hepatic inflammation.* **Conclusion**- *Peptide L-4F significantly decreased PON-1 activity and increased PON-1 levels in the plasma but showed no change in paraoxonase-1 levels in the liver. The information obtained from this study will provide insight into potential mechanisms by which peptide 4F decreases atherosclerosis through the regulation of PON-1.*

Introduction

Coronary artery disease (CAD) in humans is the major cause of death in the United States and the leading cause of mortality worldwide. CAD is associated with low levels of high-density lipoprotein (HDL) cholesterol and high levels of low-density lipoprotein (LDL) cholesterol (Parthasarathy, 2008). The major protein component of HDL, apolipoprotein (apo) A-I, is thought to be responsible for the atheroprotective qualities of HDL. ApoA-I has been postulated to possess ten α-helical sequences. The majority of these sequences form class A structures that can be mimicked by several 18-residue peptide analogues. One such peptide, peptide 4F, mimics many of the properties of apo A-I (Figure 1). A close relationship has been observed between paraoxonase-1 (PON-1) deficiency and accelerated progression of atherosclerosis in animals models, such as the apoE null mouse model. The apoE null mouse is a gene knockout of apoE, a ligand for the LDL receptor, and develops atherosclerosis spontaneously.



Paraoxonase-1 is an enzyme synthesized in the liver and secreted into the blood stream, where it is primarily located on HDL and associated with apo ApoA-I (Mackness, 2001) (Figure 2). Many of the anti-oxidative properties of HDL have been credited to PON-1. Previous research has shown that PON-1 destroys lipid hydroperoxides (LOOH), degrades oxidized LDL phospholipids, reduces accumulation of oxidized lipids in LDL, hydrolyzes oxidized LDL-associated compounds, and inhibits both LDL and HDL oxidation (Florentin. 2008). Administration of the apolipoprotein A-I mimetic peptide 4F increased paraoxonase activity and apo A-I, especially in the HDL subfraction containing pre-β HDL (Navab, 2004). Increased levels of PON-1 in the plasma can result either from increased synthesis of the enzyme in the liver or increased efflux from the liver into the plasma. Although peptide 4F is in clinical trial phase 2 studies, the mechanism in which it operates to increase PON-1 activity and decrease atherosclerosis is not known; however, we hypothesize that 4F affects PON-1 by increasing PON-1 activity or by increasing PON-1 levels in the plasma (or both) (Figure 3). To provide insight into the mechanism of peptide 4F's affects on PON-1, we have studied the changes in

Ac-AspTrpPheLysAlaPheTyrAspLysValAlaGluLysPheLysGluAlaPhe-NH_2
D----W----F----K----A----F----Y----D----K----V----A----E----K----F----K----E----A----F

Figure 1. The Class A structure of peptide 4F

PON-1 activity, PON-1 levels, and genetic expression of the enzyme with administration of peptide 4F in apoE null mice.

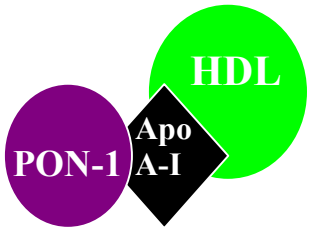


Figure 2. HDL complex with paraoxonase-1

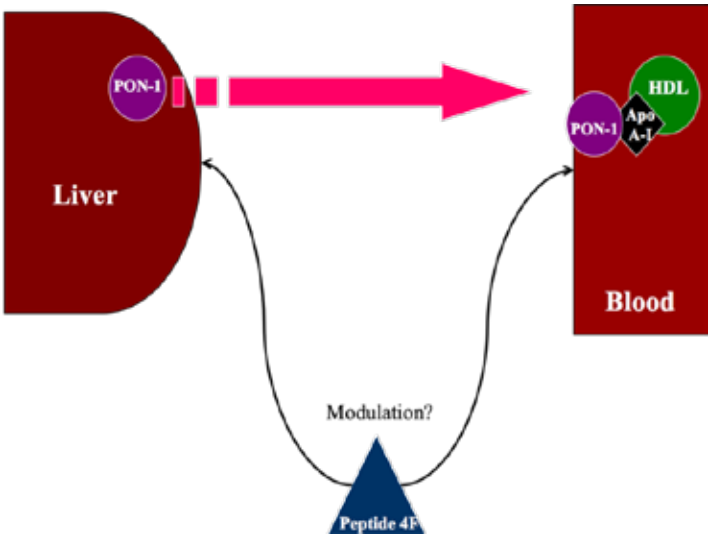


Figure 3. Hypothesized mechanisms of the effect of peptide 4F on PON-1

Methods

Samples

Samples from a previous experiment, with 20 female apoE null mice at 12 weeks of age, were provided by a doctoral candidate, Gaurav Nayyar, for analysis for PON-1 activity and PON-1 levels in the plasma. Animals were separated into 2 groups: vehicle, treated with saline, and peptide, treated with L-4F. Treatment lasted 2 weeks. At 14 weeks of age, animals were euthanized and plasma was collected in heparinized tubes.

Subjects

Female apoE null mice were purchased at 6-weeks of age from Jackson Laboratory (Bar Harbor, ME). They were fed normal chow. Animals were not studied until they had reached at least 7 weeks of age. All procedures were reviewed and approved by the Institutional Animal Use and Care Committee of the University of Alabama at Birmingham.

Peptide synthesis

Peptide L-4F (i.e. Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH_2) was synthesized by solid-phase peptide synthesis. The purity of synthetic peptides was established by analytical HPLC and mass spectrometry. The peptide was dissolved in sterile saline for injection studies.

Injection Protocols

Animals were anesthetized and blood samples were taken from the retro-orbital sinus under isoflurane anesthesia immediately before injection at 7 weeks of age. Peptide was injected intraperitoneally at a dose of 50 µg/day/mouse (0.5 mg/ml concentration; 100 µl volume). Saline (100 µl) was injected into control animals. Blood samples were collected into heparinized capillary tubes, then placed in microcentrifuge tubes; the plasma was separated by centrifugation.

At the time of euthanasia (11 weeks of age), whole blood samples were obtained under xylazine-ketamine anesthesia from cardiac puncture and placed in heparinized microcentrifuge tubes. Portion of livers were immediate placed in RNAlater (Qiagen; Valencia, CA) and stored at -80°.

Paraoxonase-1 activity

Three µl of whole plasma was mixed with 200 µl of PON buffer (100 mmol/L Tris containing 2 mmol/L CaCl2, pH 8.0) containing paraoxon (1 mmol/L O,O-diethyl-O-p nitrophenylphosphate; Sigma; St. Louis, MO), and the rate of formation of 4-nitrophenol over a period of twenty minutes was determined spectrophotometrically at 405 nm. Blanks were included to correct for the spontaneous hydrolysis of paraoxon. The assay was performed in a 96-well plate (Costar, Fischer; Pittsburg, PA), and readings were taken every 2 minutes. The quantity of 4-nitrophenol formed was calculated using the molar extinction coefficient of 17,100 $mol/L^{-1}cm^{-1}$. One unit of PON activity was defined as 1 nmol of 4-nitrophenol formed per minute (Navab, 2004).

Western Blotting on whole plasma

Five µl of whole plasma was separated on a pre-made 10% Novex gel. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer apparatus. The membrane was incubated with primary antibody anti-PON-1 (gift from Dr. Srinivasa Reddy, UCLA) in 5% milk in phosphate buffered saline (pH 7.4) and 0.05% Tween-20 (PBST) buffer at 4°C overnight with constant shaking. The membrane was then washed and incubated with secondary antibody (CAT 2305; Santa Cruz; CA) for an hour and washed. Western blots were developed with enhanced chemiluminescence (ECL) and exposed to X-ray film. ApoA-I levels were determined after the membrane was stripped at 50°C for 30min. The membrane was then incubated for an hour with primary antibody for A-I (Biotin Anti-mouse apo A-I) in 1% gelatin and TTBS (1% Tween-20 in tris buffered saline). Secondary antibody (Bio-Rad; CA) was then used. Membranes were developed with alkaline phosphatase conjugate substrate (Bio-Rad; CA). Immunoreactivity was quantified by densitometry and Sigma Plot 11.0 software was used for statistical analysis.

Western Blotting of Hepatic tissue

Thirty mg of hepatic tissue was homogenized with a mortar and

pestle in 1.0 ml of tris-buffered saline and protease inhibitor cocktail. Ten µl of the supernatant was separated on a pre-made 10% Novex gel. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer apparatus. The membrane was incubated with primary antibody anti-PON-1 (Srinivasa Reddy; gift, UCLA, CA) in 5% milk phosphate buffered saline (pH 7.4) and 0.05% Tween-20 (PBST) buffer at 4°C overnight with constant shaking. The membrane was then washed and incubated with secondary antibody (CAT 2305; Santa Cruz; CA) for one hour and washed. Western blots were developed with enhanced chemiluminescence (ECL) and exposed to X-ray film. Tubulin levels were determined after the membrane was stripped at 50°C for 30min. The membrane was then incubated for one hour with primary antibody for Tubulin (CAT 5168; Sigma; St. Louis, MO) in 5% milk block. Secondary antibody (CAT 2304; Santa Cruz; CA) was then applied and membranes were developed with ECL. Immunoreactivity was quantified by densitometry and Sigma plot 11.0 software was used for statistical analysis.

Immunohistochemistry

Immunohistochemistry was performed on unstained, unfixed frozen histological sections of livers. Primary PON 1 antibody (N20 Cat SC21146; Santa Cruz; CA) and secondary Donkey Antigoat HRP antibody (Cat-SC-2304; Santa Cruz; CA) were used with a hemotoxin counter-stain. The secondary antibody was biotinylated and contained horseradish peroxidase to observe PON-1 by color.

Quantitative Real-Time PCR

Hepatic tissues (30mg) were placed in RNAlater (Qiagen; CA) and RNA was isolated using the RNA isolation kit according to manufacturer's instruction. Five µg RNA was used and reverse-transcribed into complementary DNA (cDNA) using a SuperScript First Strand Synthesis system (Invitrogen; CA) according to the manufacturer's instructions. A total of 2 µl cDNA was used and amplification was performed in duplicate in a MyiQ Real-Time PCR Detection System (Bio-Rad; CA) using PCR primers for mouse PON1 (forward: GAT TGG CAC TGT GTT CCA C, reverse: ATC ACT GTG GTA GGC ACC TT) (Bradshaw, 2005). Real-Time PCR efficiencies were calculated on average efficiencies received from each sample.

Statistics

Control and experimental groups were compared for significant differences by two-tailed t-tests. Differences were considered significant at $p<0.05$. Regression analysis was considered significant based on R values greater than 0.6 and $p<0.05$.

Results

Cholesterol Levels

An increased cholesterol level is a risk factor for atherosclerotic plaque deposition. ApoE null mice were injected daily for 4weeks with saline or L-4F (50 µg/mouse/day). Peptide 4F significantly affected cholesterol levels by decreasing the amount of cholesterol in plasma of peptide treated apoE null mice (Figure 4; *p=0.002 compared with saline controls).

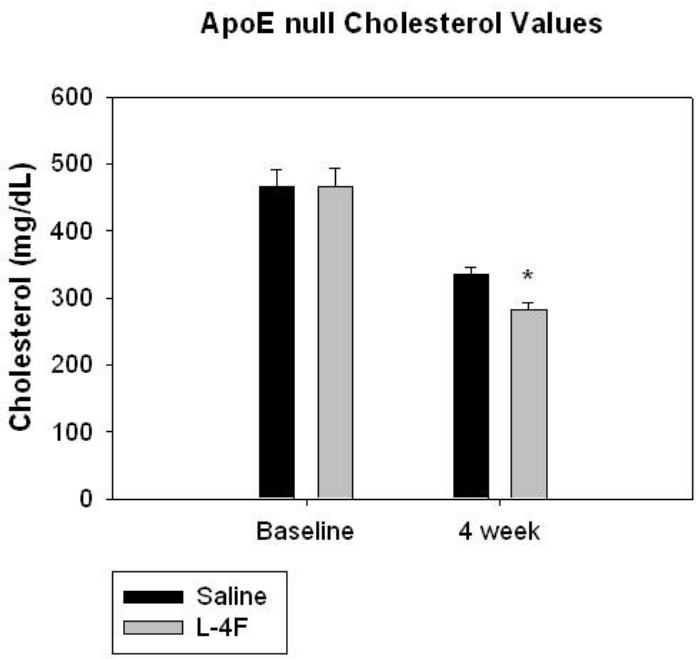


*Figure 4. Cholesterol levels were significantly lower in L-4F treated mice compared to control mice. ApoE- mice were injected daily for 4 weeks. (n=15 in each group; *p=0.002 vs saline).*

PON-1 activity in plasma

The activity of PON-1 in the plasma was measured in both control and experimental groups treated with saline or 4F. PON-1 activity, in the 20 samples provided, showed a significant increase in animals treated with peptide L-4F (Figure 5, *p=0.018). With the 30 apoE null mice, the activity of PON-1 decreased significantly in peptide treated apoE null mice (Figure 6; **p<0.017 between vehicle and L-4F groups at baseline. †p<0.001 between vehicle and L-4F groups at 4weeks.).

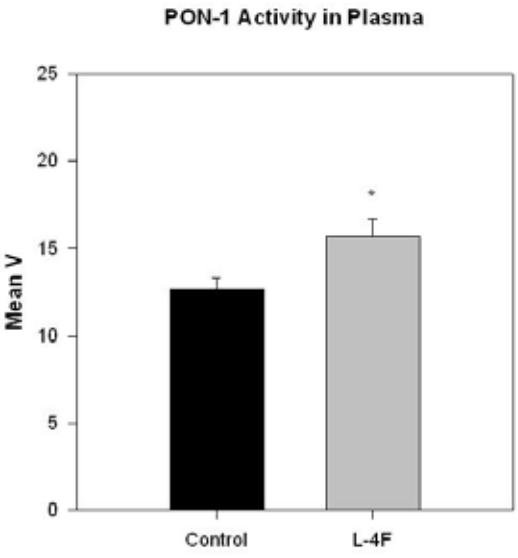


*Figure 5. PON-1 activity in plasma was significantly higher after 2 weeks of administration of the peptide L-4F in 14 week old female apoE- mice. Saline (black bar, n=10) or L-4F (grey bar, n= 10). *p=0.018*

PON-1 levels in plasma

In order to determine if the amount of PON-1 in the plasma correlated with its activity, PON-1 levels were measured through western blotting on 4-20% gels. The peptide-associated increase of PON-1 was barely significant (Figure 7; *p=0.049). PON-1 activity correlation with PON-1 levels in the plasma was measured through regression in both peptide treated and vehicle treated mice (Figure 8). No correlation was detected in peptide treated mice (R=0.140) or control mice (R=0.0525). This was different from the PON-1 activity and PON-1 levels correlation seen previously in the samples provided (Figure 9, R=0.690). Corresponding apoA-I levels were determined to observe if apoA-I levels also increased within the plasma. ApoA-I levels showed no change (Figure 10; p>0.05).

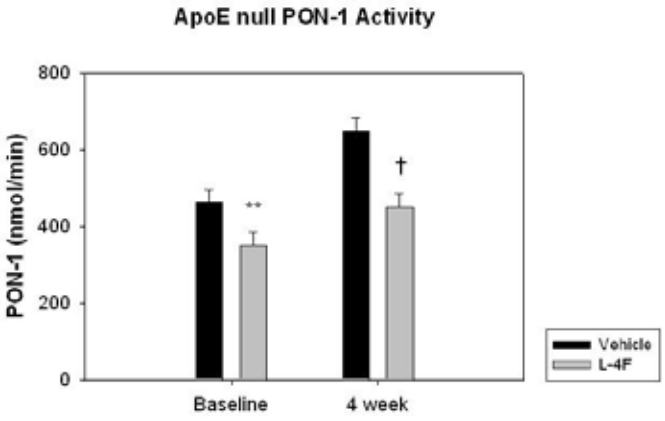


*Figure 6. Chronic administration of the peptide L-4F decreased PON-1 activity. Female apoE- were administered with saline (black bar, n=15) or L-4F (grey bar, n= 15) for 4 weeks. **p<0.017 between vehicle and L-4F groups at baseline (7 weeks old). †p<0.001 between vehicle and L-4F groups at 4weeks (11 weeks of age).*

PON-1 in the liver

To determine if the changes within the amount of PON-1 in the plasma was due to an increased secretion of PON-1 from the liver, histology specific to PON-1 was done (Figure 11). No change in PON-1 within hepatic tissue of both control (saline-treated) and experimental (peptide-treated) mice was seen. PON-1 was found consistently dispersed throughout the hepatic tissues.

Since PON-1 was present in large amounts, and changes in PON-1 levels were not apparent in immunohistochemistry, we decided to blot for PON-1 specifically. Ten µl of protein supernatant (0.5 µg/µl), from 5 animals per group, was separated on 4-20% gels (Figure 12). No significant changes in hepatic PON-1 levels between groups were found (Figure 12; p>0.05). Tubulin was used as a normalizing protein to determine the relative expression of PON-1 within the liver (Figure 13). Blotting for tubulin showed a significant decrease of tubulin levels in the mice treated with peptide 4F (Figure 13; *p=0.002).

Although there was no change in the amount of PON-1 within

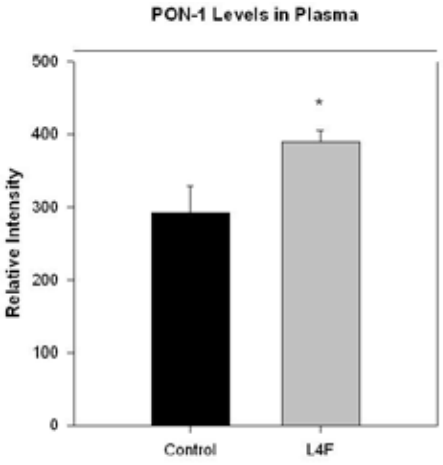
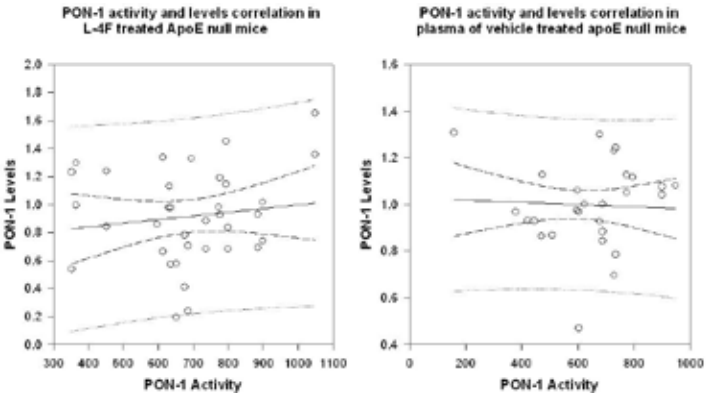
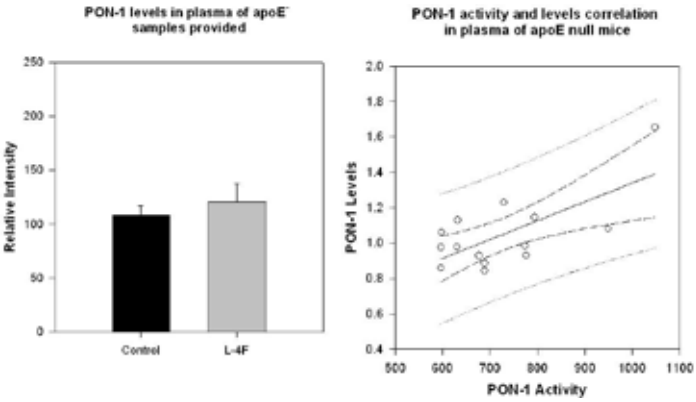


*Figure 7. PON-1 plasma levels increased in peptide L-4F treated apoE- mice. *p=0.049*



A) *B)*

Figure 8. No correlation seen between PON-1 activity and levels in plasma of A) peptide 4F treated apoE- mice R=0.140 or B) vehicle treated apoE- mice R=0.0525



A) *B)*

Figure 9. A) No significant change in PON-1 levels of apoE- mice samples detected B)Correlation seen between PON-1 activity and PON-1 levels in plasma of apoE- mice samples provided R=0.690

the liver, quantitative real time (RT-PCR) was done on PON-1 mRNA levels to decide if peptide 4F affected PON-1 genetic expression. RT-PCR showed fold changes less than 1 (data not shown), representing no detectible change in the genetic expression of PON-1.

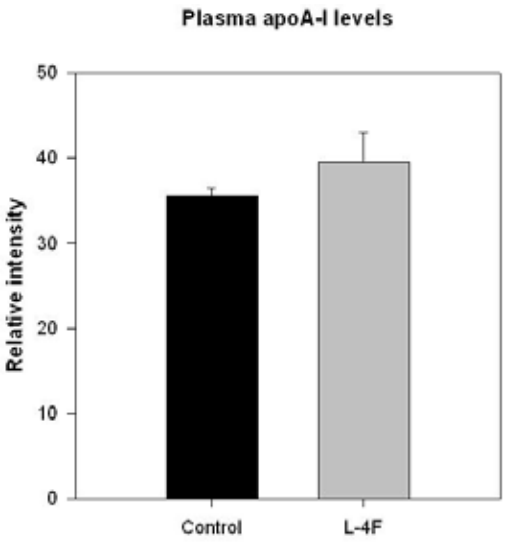
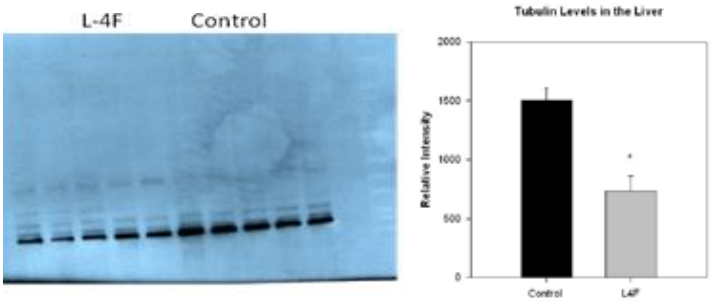


Figure 10. ApoA-1 plasma levels showed no change in peptide L-4F treated apoE- mice at 11 weeks of age.



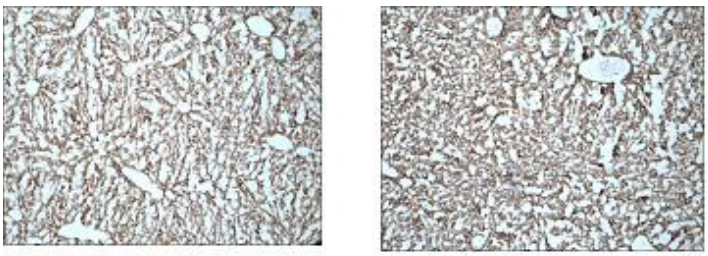
changes plasma PON-1 levels

A) *B)*

*Figure 13. Corresponding tubulin levels in the liver of apoE- mice. A) Tubulin levels are visibly decreased in apoE- mice treated with peptide 4F compared to control at 11 weeks of age. B) Tubulin levels were significantly decreased in peptide treated mice *p=0.002*

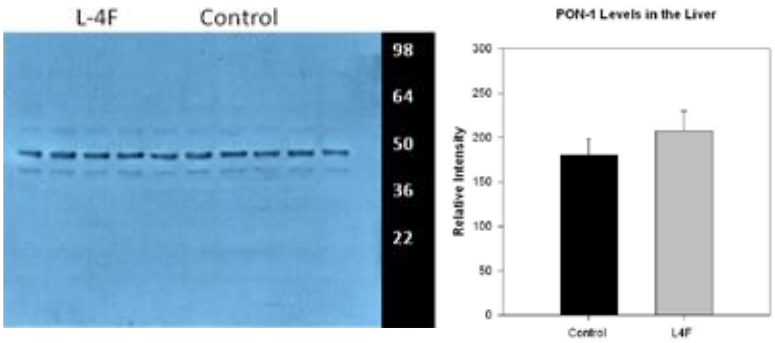
Discussion

Previous studies, including the samples provided, showed that peptide L-4F increased PON-1 activity and reduced atherosclerosis in atherosclerosis susceptible mice models. The general interpretation of this finding was the peptide 4F had antioxidative properties by increasing the activity of antioxidative enzymes. Here, we do not support previous findings by showing that peptide 4F does not increase PON-1 activity within the plasma but without changing apparent hepatic expression or levels. This may be because PON-1 activity was not measured in isolated HDL but in whole plasma samples.



A) *B)*

Figure 11. Immunohistochemical section of hepatic tissue from apoE- mice, using PON-1 antibody A) injected with saline B) injected with L-4F



A) *B)*

Figure 12. PON-1 liver levels A) western blot showed no visible change in PON-1 levels from peptide L-4F treated apoE- mice compared to control mice at 11 weeks of age. B) Quantitave results from western blot.

While decreasing PON-1 activity, peptide L-4F also decreased total cholesterol in these animals. This reduction of cholesterol decreases the accumulation of cholesterol within the artery walls and therefore, reduces plaque burden. Our findings are not consistent with previously reported studies of peptide L-4F in apoE null mice, which showed no change in plasma cholesterol levels.

In order to determine the mechanism in which peptide 4F modulates PON-1 and its activity, we hypothesized that peptide 4F increases PON-1 levels within the plasma and then compensates for this increase within the plasma by secreting PON-1 from the liver and decreasing the amounts of PON-1 within hepatic tissue or by increasing the genetic expression of PON-1 within the liver or both. In the samples provided, we did not find a significant increase of PON-1 levels within the plasma. However, in the apoE null mice treated with peptide, we found a slight increase in PON-1 levels within the plasma. With this finding, we are unsure if the change in PON-1 levels is responsible for the change in PON-1 activity seen in peptide treated mice, or if the peptide increased the activity by remodeling HDL particles or directly activating PON-1. A correlation between PON-1 activity and PON-1 levels was detected in the samples provided by the doctoral candidate. This suggested that peptide L-4F did not activate PON-1 directly but activated PON-1 in the plasma by increasing the PON-1 levels. However, no correlation was detected in this experiment, with 30 apoE null mice, between PON-1 activity and levels in the plasma. These paradoxical results may have been due to group of animals or other factors unknown. ApoA-I levels in the plasma, however, did not change. In order to determine the role

of PON-1 and apoA-I in peptide 4F function, mouse models such as PON-1 null and apoA-I null would need to be used. We hypothesized that an increase in PON-1 levels in the plasma may have been due to an increase of PON-1 secretion from the liver or an increase in genetic expression. No change in PON-1 levels within the liver were detected by immunohistochemistry or immunoblotting in peptide treated mice even though PON-1 levels slightly increased in the plasma, suggesting that, if more PON-1 had been secreted from the liver, the levels were low compared to hepatic PON-1 content, or that the genetic expression of PON-1 within the liver was increased. However, we did not detect increased PON-1 genetic expression, suggesting that peptide 4F does not induce PON-1 genetic expression.

While determining the relative expression of PON-1 levels in the liver, we found in a single experiment that tubulin was decreased in the livers of peptide treated mice. Tubulin, a structural protein commonly used as the normalizing gene in many tissues, has not previously been reported to decrease in atherosclerosis models. Tubulin is used as a normalizing gene to measure the levels of other proteins presented in several tissues including brain and liver. Tubulin levels are usually consistent within tissue levels. In this case, hepatic tissue showed a significant decrease in tubulin levels in mice treated with peptide L-4F. Previous literature has reported increased levels of tubulin as a pro-inflammatory response in the liver (Maurice, 1980). Our data may suggest that peptide 4F decreases inflammatory responses within the liver and therefore tubulin levels in the liver. Future studies using better established inflammation markers within the liver will be needed to confirm a peptide-mediated reduction in hepatic inflammation. If this change in tubulin levels is confirmed, for relative expression of PON-1 other housekeeping genes and proteins, such as β-actin, will be used instead of tubulin.

Conclusion

Peptide L-4F has been shown to act as an anti-inflammatory and anti-oxidant in the plasma in part by increasing the activity of PON-1 in the plasma (Navab, 1980). This increase in PON-1 levels and activity may result in the increased anti-oxidative properties of HDL seen in the plasma of animals treated with peptide L-4F; however, our results did not support this. No highly significant change in hepatic PON-1 levels or genetic expression suggests that peptide 4F does not interact with the PON-1 gene. The mechanism of peptide modulation of PON-1 remains unknown but the results presented here provide insight into the possibilities of peptide 4F activating PON-1 within the plasma through PON-1 levels.

ACKNOWLEDGEMENTS

We would like to thank the National Institutes of Health and McNair Scholars Program for funding. We would like to thank the Science and Technology Honors Program for support. Also, would like to thank Srinivasa T. Reddy of UCLA for the PON-1 antibody.

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faculty interview: biomedical engineering

An Interview with Dr. Alan Eberhardt, Department of Biomedical Engineering

Interviewed by: Jake Fletcher

This interview was conducted with Dr. Alan Eberhardt, currently a professor of bio-medical engineering and one of the associate directors of the science and technology honors program. His research deals with the impact of automotive collisions on the pelvis, as well as implant tissue biomechanics. I also had the opportunity to ask what suggestions he had for students considering a future career in research.

How and where did you become interested in research?

I guess it all started when I was doing my masters project in civil engineering at the University of Delaware. I had stayed in school basically because I had done well in school, and I had an offer for a fellowship. I was doing my Masters, when I got interested in Orthopedic Biomechanics, which is what I eventually ended up doing for my PhD.

So your interests weren't always related in Biomedical Engineering?

No, actually I started in Geology, and I quickly switched out of that because I found it to be pure memorization, which is not one of my fortes. I found myself more interested in problem solving, applied mathematics, and mechanics, which is why I went into civil engineering. After I had done civil engineering for my Masters, I started wanting to do something that was more related to helping people. I guess the call of biomechanics got me because it offered the opportunity to apply what I was doing in research to help people.

At what time did you realize what research field you wanted to specialize in?

Really, I think it was during my Masters in civil engineering. I realized I didn't want to build bridges; I wanted to build things that would help people that had been injured, or would help people that had some kind of disease or condition.

When did you know what your career goals were?

I guess it was when I was doing my PhD, and I began to understand the idea of what it was like to do research and to stay in academia, teaching and working with students – a combination of things.

Where did you do your undergraduate and graduate work?

My undergraduate and Master's Degrees came from the University of Delaware, and my PhD was at Northwestern and the University of Minnesota.

So did you split time between the University of Northwestern and the University of Minnesota?

My PhD is in theoretical and applied mechanics which was at Northwestern, but when I got there Dr. Jack Lewis, who was doing orthopedic Biomechanics, had just moved to Minnesota and was working collaboratively with Northwestern. After the first year of study of my PhD I took my qualifying exam, and then went to the University of Minnesota and worked there for about a year and a half. I then came back to Northwestern to essentially defend my thesis and wrap everything up.

How long have you been at UAB and what persuaded you to come here?

I have been at UAB since 1991, so 19 years. UAB offered me a position that was what I was looking for. I had a couple of other offers that were more outside of academia. I had an offer at UC-San Francisco that was working in a gate analysis lab, and it wasn't really what I wanted to do. I came to UAB, and they presented a very favorable environment. It was really open to doing research. I was hired into Mechanical Engineering at the time, and it was an up and coming program that was interested in expanding into the realm of biomechanics. It was really a perfect fit. Plus I was born in Alabama, so it was kind of like coming home.

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How did you become interested in injury biomechanics specifically?

My Phd was in applied mechanics, which was essentially structural mechanics. I was turned on to the idea of orthopedic biomechanics when I saw that structural engineering was being applied to the body, and I thought that was interesting. My whole area of pelvic injury biomechanics was an area that I essentially inherited. I started working with Ken Mann, who was running the program at the time. When he left, he left this funded area of Pelvic Side-Impact Biomechanics open, and I jumped onto the opportunity.

Can you please describe your current research projects and current research interests?

We are still interested in Orthopedic Biomechanics and are now working with some different orthopedic surgeons. Whereas, in the past, it was primarily hip and knee, we have been working a lot with Dr. Brent Ponce in shoulder biomechanics. We are also still working closely with Dr. Jack Lemons, in implant tissue biomechanics, essentially studying the biomechanics of bone ingrowth and ongrowth in non-cemented devices. We are also still active in the biomechanics of the pelvis. I am part of the Global Human Body Models Consortium (GHBMC), which is a worldwide effort to create full-body finite element models. Since our area is the pelvis, I have been the investigator on the pelvic component for this full-body model.

Who else works with you on the Global Human Body Models Consortium?

Our project is a sub-contract with the University of Virginia, and the other universities that are involved are Wayne State University, University of Waterloo, University of Virginia, and a group called Inrets, which is from France.

What kind of impact will these projects have on the scientific world, and how might these projects impact everyday life?

The models promise to be the most biofidelic finite element models to date and should be the most accurate models of the human body's response to high speed impact conditions. The goal is to use them in the development of safer vehicles. So in addition to crash testing, where you smash a $40,000 vehicle and damage a $100,000 crash-test dummy, employ 5 engineers and spend $300,000 per test, you would be able to have the computational simulations going on that would complement these crash tests to give you more information about your new car design. The whole idea with the GHBMC is that there are a number of car companies - General Motors, Nissan, Toyota and others – involved, and the idea is that those models would be shared so that all of the car companies would be using the same models, which would be the best models to date. Hopefully, the long-term impact will be safer vehicles. Right now we are building models for 50th percentile males, so average guys. The next step of this project will be to move into 5th percentile females, or petite females, and 95th percentile males or large guys. Then we will also want to look into pedestrian impacts; we will want to create these models so that they can be both in a car and outside of a car. Hopefully, this will bring in a new decade of improved automotive safety.

How many students currently work in your lab?

Right now, I have two graduate students working in my lab - I have no undergraduates working in my lab. I'm looking increase those numbers probably within the next few months. The funding for my lab has gotten a little low so I have been hesitant to get any new students in, but I am working on a few grant proposals that should get some more funding, so those number should hopefully change pretty soon.

What advice would you give to undergraduates considering research activity when they are first starting out?

I would say that the most important thing would be to find something that interests you. Also find something that plays to your strengths. If you are hands on kind of person and you want to do experiments, get in a lab that is more hands on. If you work well with computers and you want to sit in front of a computer all day, find a lab that does that type of work. The other thing is you would want to find a lab mentor that suits you. If you are the kind of person kind of wants to work on your own unsupervised, that is great. If you want to work in a lab with thirteen people and 5 post-docs, then you need to find that type of a lab.

What advice would you give to soon-to-be graduates who are planning on doing research as a career?

Try and get involved in an area that is likely to be funded. Particularly these days in biomedical engineering, there are certain areas that are hot, there are certain areas that are promising to hold secure funding in the future, and I think as we move forward, especially in biomedical engineering, where there are more and more biomedical engineering students hitting the workforce, that money will get tighter and tighter. You need to find a niche that you can hopefully stay funded in.

Can you briefly describe your involvement in the Science and Technology Honors Program?

I am one of two associate directors, and my involvement varies from teaching courses. I team-teach the introductory seminar class and lead the introductory Research Approaches I Engineering class. I do advising for all of the biomedical engineering students in Sci/Tech. I also work with Dr. Tucker and Dr. March to help maintain and grow the program. I also try to bring in money in terms of grant support that will help the program.

Simultaneous Mapping of Membrane Potential, Calcium Transient, and Wall Motion in an Isolated Heart

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Department of Biomedical Engineering
The University of Alabama at Birmingham

Abstract

Optical technology has been incorporated in cardiac studies, utilizing intracellular dyes and photo detectors to image physiological parameters. However, one major challenge in optical mapping is motion artifact, produced by the physical beating of the heart. To this end, it is known that deformation of the heart can be captured and quantified to eliminate motion artifact without using chemical agents. However, we have planned to develop this technique further to simultaneously quantify membrane potential, calcium transients, and mechanical contractions in isolated hearts. Hearts are double stained with voltage-sensitive dye di-4-ANEPPS and calcium-sensitive dye x-rhod-1. Ring markers are attached to the epicardium and tracked in software, quantifying wall motion concurrently with membrane potential and calcium transient signals. Emitted fluorescence is elicited on alternating frames by 450, 505, and 580 nm LEDs, captured by a camera integrated with a 605/25 nm emission filter. Interlaced signals from 450, 505 and 580 nm fluorescence are collected from pixels encircled by the ring marker. De-interlacing of the raw signal results in 3 individual signals: 450 nm signal contains non-voltage sensitive and non-calcium sensitive signal, consisting of only motion artifact. 505 nm signal contains V_m with motion artifact, while the 580 nm signal contains C_{am} with motion artifact. A ratio of the 505 and 580 nm to the 450 nm signal will estimate V_m and C_{am} respectively. This instrumentation design will be the first to simultaneously map three fundamental parameters of heart contraction.

Introduction

Ventricular fibrillation (VF) is a lethal heart condition characterized by the uncoordinated contraction of the ventricles. Despite successful defibrillation, the heart may still exhibit post-resuscitation contractile dysfunction - a depressed contractile motion of heart which prevents the heart from perfusing sufficient blood to sustain life.[1] Little progress has been made in the understanding of the complex phenomenon of VF and its mechanical and chemical behavior. Previous studies have approached the understanding of VF by focusing on various stages of the cardiac contraction mechanism (See Figure 1). Because the electrical and mechanical functions of the heart are coupled, researchers have performed qualitative studies to evaluate each stage. Spatiotemporal mapping of electrical activity in the heart is widely performed and is traditionally accomplished by attaching multiple electrodes to a specific region of the myocardium.[2]

A more current approach is to employ optical techniques with intracellular dyes. To optically map membrane potential, pig hearts are isolated using a Langendorff preparation and stained with voltage-sensitive fluorescent dye. When the heart is illuminated with excitation light, the emitted fluorescence from the heart transmits a signal proportional to the transmembrane potential.[3] The same method can be used to measure intracellular calcium transient using an ester form of a calcium indicator dye. As the dye enters the cell, intracellular esterase enzymes will cleave the esters allowing for the Ca^{+2} to bind, thus trapping the dye. Calcium concentration is then measured by recording the intensity of the emitted fluorescence.[4] One method to optically map membrane potential and calcium transients is to stain the heart with both di-4-ANEPPS voltage sensitive dye and x-rhod-2 calcium sensitive dye. Both dyes absorb excitation light of different wavelength, but emit at the same wavelengths such that the same detectors can capture the alternating signal.[5] Previous studies have recorded calcium transients and membrane potential and epicardial wall motion sequentially 6,but spatiotemporal mapping of these three parameters has not been performed. The primary advantage of optical mapping is the ability to map multiple regions of the heart without attaching various electrodes to the regions of interest as is done with the traditional method of electrical mapping. As a result, the lack of electrical stimulation will also leave the acquired signals free from its associated artifact.

While optical mapping may appear seemingly sound, it has a major disadvantage when imaging beating hearts. The contraction of the myocardium causes physical displacement of heart, resulting in a loss of correspondence between the photodetector pixel and the imaged tissue. This movement produces a data set of the desired signal combined with motion artifact. Conventional studies have utilized electromechanical uncoupling agents (butanedione monoxime) to chemically arrest heart motion. However, recent investigations have dealt with this problem that by taking an additional dataset containing only artifact signals, removing motion artifact by taking a ratio of the two datasets.[7]

In this study, we want to expand and implement optical technology to improve the understanding of contractile dysfunction following defibrillation, with the goal of improving success rates for resuscitation due to prolonged VF. The development process will specifically investigate the usage of optical mapping techniques to simultaneously map membrane potential, calcium transient, and wall motion of the myocardium. This instrumentation design will

be the first of its kind, capable of measuring these three essential parameters of contraction.

Aims

To implement a new optical mapping method designed to capture membrane potential, intracellular calcium, and epicardial wall motion simultaneously from multiple sites in an isolated heart preparation. Using swine hearts, we plan to investigate the feasibility of using optical methods to simultaneously characterize the electrical and mechanical processes from selected sites on the myocardium.

Methods

Preparation of Heart

Domestic swine hearts of either sex was used. An isolated heart (Langendorff) preparation was performed to allow optimal access to the heart. The aortic root was cannulated and perfused with 2 liters of warm (37°C) Tyrode solution to wash out blood and metabolites. The heart was then connected to a constant-flow (200 ml/min) Langendorff apparatus. Two 22 gauge needles were inserted into the ventricles to minimize the beating heart motion. During perfusion, droplets of perfusate were collected within the ring markers, diffracting emitted fluorescence and ultimately affect the acquired signal of interest. To minimize this effect, the heart was blotted with absorbent gauze immediately before each optical recording.

Material Point Markers

4-mm diameter plastic rings with 2.5 mm diameter holes composed of polyethylene plastic material were used. The markers were placed on the epicardial surface with black cyanoacrylic gel (Loctite #426) to serve as a reference point for recording various signals. Multiple markers was placed on the epicardial surface of the left ventricle and tracked by software.

Dual Staining

x-rhod-1 and di-4-ANEPPS were fluorescent dyes that are calcium and voltage sensitive respectively. The dyes have separate excitation bands, but overlapping emission bands (See Figure 2). The entire heart was stained with di-4-ANEPPS through the perfusion system while x-rhod-1 dye will be applied by locally injecting small amounts in the center of the applied markers. 2 LED lights were used to stimulate each respective dye: cyan (di-4-ANEPPS), and amber (x-rhod-1). A third color, royal blue, elicits fluorescence that is insensitive to both membrane potential and calcium transients. This LED served as a reference signal used to remove motion artifact by employing a ratiometry technique. 5

Software Integration of LED Lights

A counter board was used to integrate the delivery of the 5-V pulses to the LEDs and the camera capturing software. The heart was illuminated by the three different excitation colors on successive camera frames (See Figure 3). Signals was captured by the same CCD camera set to 86 x 128 pixel resolution and fitted with a 605/25 bandpass filter positioned 10 cm from the heart. The resulting signal was interlaced in time. Fluorescence elicited by cyan, amber, and blue are deinterlaced in software after data acquisition.

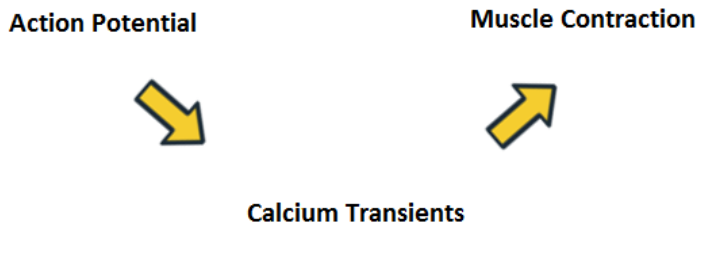


Figure 1. Cardiac Contraction Model. Each stage is dependent on the previous stage to ultimately elicit muscle contraction.

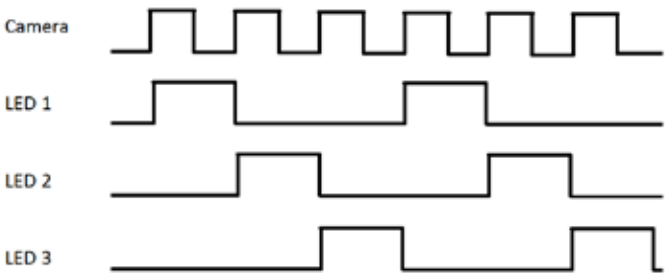


Figure 3. Pulse train configuration interlacing camera control and LED activation in time. Each high edge represents a 5V pulse which activates the camera to capture a frame and the LED to flash.

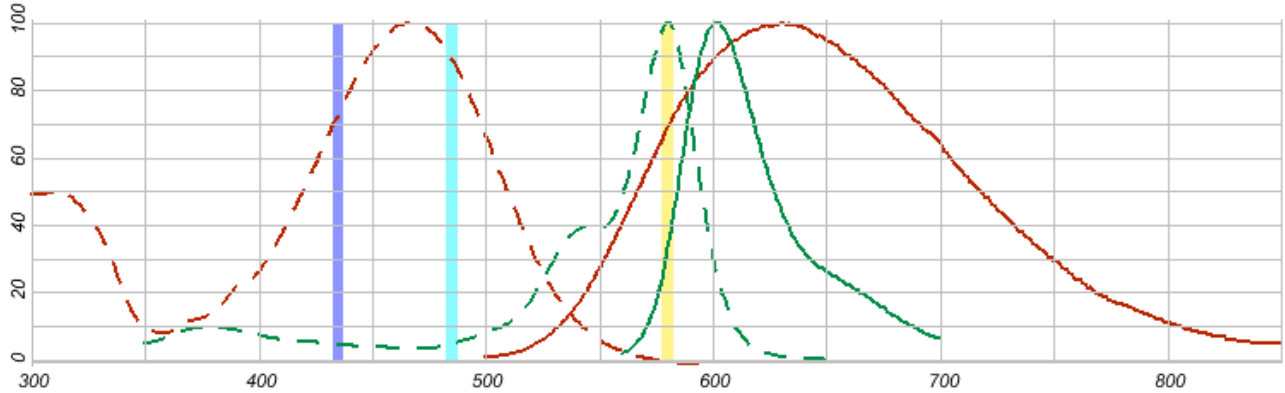


Figure 2. Excitation (Dotted) and Emission Spectra of x-rhod-1 (Green) and di-4-ANEPPS (Red)

Validation Studies and Cross-Talk Analysis

Separate hearts was stained with di-4-ANEPPS, x-rhod-1 alone to ensure that only the signal emitted by each respective dye was present. Membrane potential, calcium transient, and epicardial wall motion will be modulated with drugs to test the sensitivity of the mapping system.

Results and Conclusion

We successfully mapped each of the three fundamental parameters individually. By collecting signals from the marked surface, we were able to obtain inverted raw signals of calcium transient, membrane potential and motion from pilot studies (See Figure 4). These signals were all replicated utilizing the equipment available in our laboratory. To test instrumentation simultaneity, we effectively produced an interlaced signal of membrane potential and its corresponding motion artifact (See Figure 5). Cross-talk analysis between signals acquired from di-4-ANEPPS and x-rhod-1 were performed to further ensure signal quality. Though the dyes may have separate excitation bands, they still possess an overlapping region. As a result, it is possible that the signal from one parameter can be detected by the stimulation of the non-corresponding LED. Although this may produce additional artifact, the effect is greatly minimized by equipping LEDs with filters to narrow the wavelength of the excitation light.

We are currently investigating the feasibility of examining a double stained heart and employing ratiometry techniques to optimally eliminate motion artifact from the raw signal. Ultimately, this developmental study will show the interaction of various mechanisms during cardiac contraction. When applied to different cardiac conditions such as VF and post resuscitation cardiac dysfunction, it will allow a better understanding of the underlying mechanisms behind cardiac diseases.

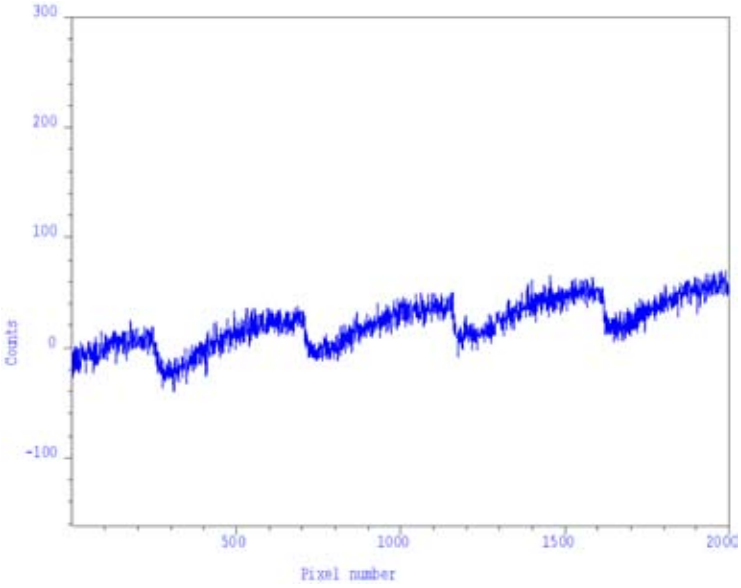
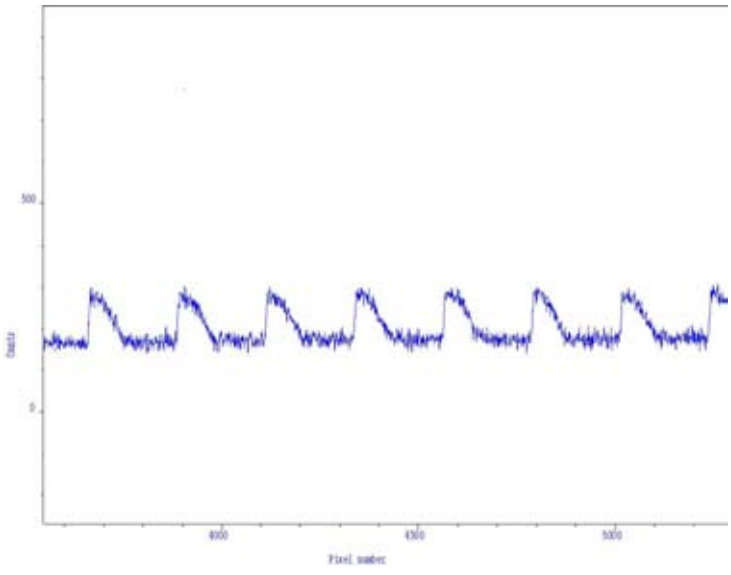
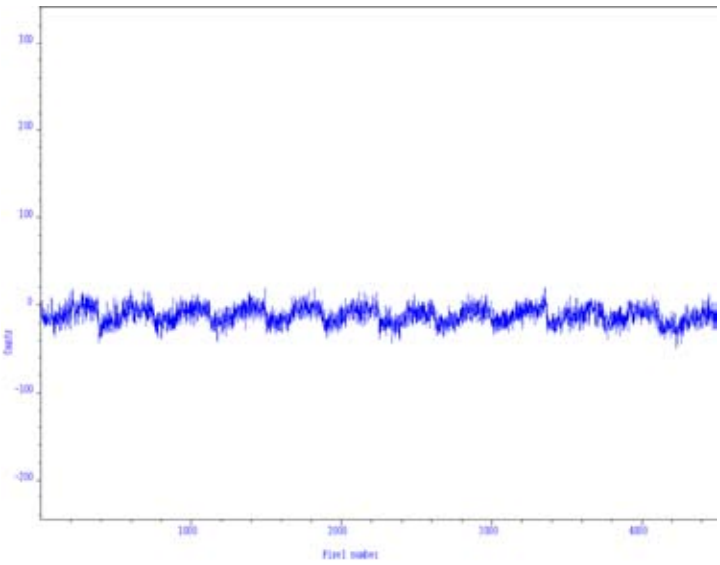


Figure 4. a) Fluorescent calcium transient signal containing motion artifact.



4.b) Fluorescent action potential signal containing motion artifact.



4.c) Signal containing only motion artifact.

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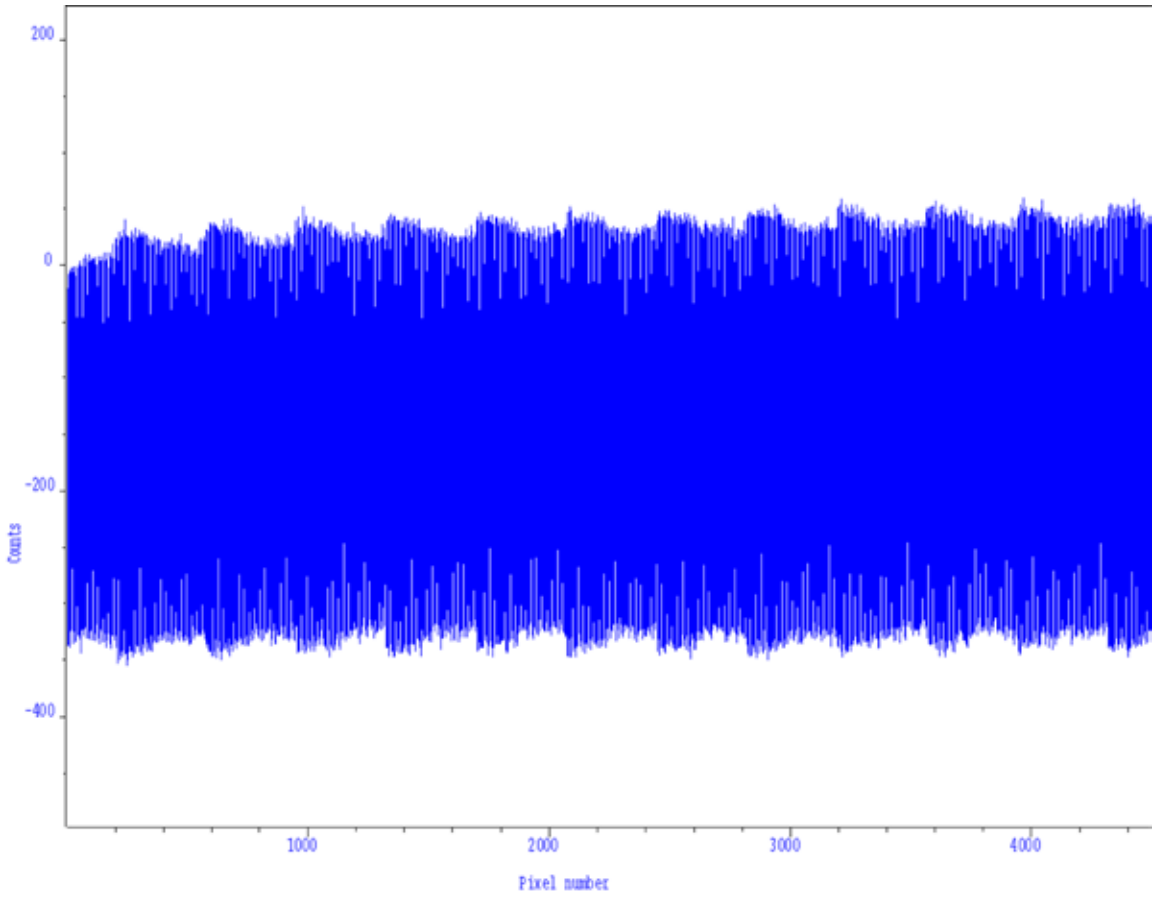

Figure 5. Interlaced signal of membrane potential dataset with its corresponding motion artifact only data set. The data points on top of the graph represent the dataset of membrane potential polluted with motion artifact. The data points on the bottom are the corresponding motion artifact only dataset.

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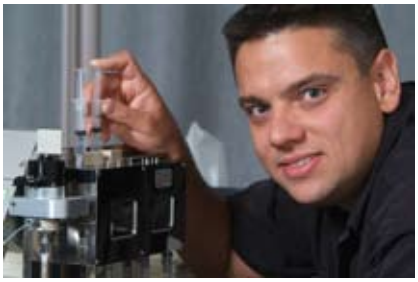
faculty interview: chemistry

An Interview with Dr. Aaron Lucius

Andrew Buie



"Undergraduates should be in the research laboratory. They aid in creating a positive environment with their enthusiasm where new and refreshing ideas can be brought to the table. Students should get into the lab early during their undergraduate education and determine if is a potential future career. Don't wait until you are in graduate school to decide if research is right for you."

Dr. Aaron Lucius started his undergraduate education at Boise State University in Boise, Idaho with the intention of obtaining an MD/PhD dual degree. After experiencing a lack of research at the university, he transferred to Oregon State University in Corvallis, Oregon for the remainder of his college career, where a solid program in biochemistry and biophysics could be found. Starting out in a new setting, Dr. Lucius found a place in the laboratory of Dr. Isaac Wong, researching HIV integrase protein and its DNA integration in the host. When the time came for the MD application process, he made the career decision to pursue a PhD degree instead. With the help of his mentor, applications were sent to the graduate schools of Penn State, Duke, and Washington University in St. Lois, MO. Upon interviewing at these three programs, Dr. Lucius decided on the world-renowned research facility of Washington University as being right for him. Under mentor Dr. Timothy Lohman, a researcher in the field of kinetics and thermodynamics of helicases, he spent the next five and a half years developing methods to study and examine DNA unwinding. After completing his Post Doc at the University of Texas Medical Branch in Galveston, Dr. Lucius began looking for faculty positions at undergraduate universities. His search led him to UAB in June 2006.

Coming to UAB was not a hard decision to make. Dr. Lucius found not only the faculty interactions here to be enjoyable, but also the chemistry program itself to be appealing. Dr. David Graves, as the Department Chair, offered an incredible mentoring and learning experience for Dr. Lucius, leaving him enthusiastic about his future at the university. Supplied with materials and graduate students, there was little lacking to create the perfect research environment. In addition, UAB offered to Dr. Lucius an abundant supply of undergraduates that could become involved in his laboratory. Having had his career start as an undergraduate in a lab, Dr. Lucius appreciated this fact. Since his arrival, there has been one Post Doc, 7 graduate students, and 6 undergraduates that have spent time in his lab working on research projects.

Currently, the research being done in Dr. Lucius' lab is a continuation of projects he experienced as a graduate student and postdoctoral fellow, centered on the translocation of helicases on DNA. The motor proteins they examine are similar to helicases, but, instead of translocating on nucleic acids, they translocate on other proteins, unfolding them to remove them from the cell. These proteins must be removed due to their potential to aggregate as a result of heat shock or stress. Using helicases as models, Dr. Lucius has adapted a way to examine these motor proteins translocating at the molecular level. This breakthrough method, a study in kinetics, led to a published paper in the *Journal of Molecular Biology*, a big accomplishment for the new addition to UAB. In addition to such kinetic activities on the molecular level, Dr. Lucius also studies molecular thermodynamics. In particular, he studies ligand binding and protein assembly. Of great interest to his lab are the thermodynamic and hydrodynamic properties resulting from the formation of hexomeric rings by motor proteins. In all, his current research has led to the publishing of 5 official papers with two more in the works.

The work of Dr. Lucius stands to benefit the global community on many levels. One way in particular is the use of proteins as a drug, specifically in neurodegenerative diseases like Huntington's, Alzheimer's, and Mad Cow Disease. Through his research, Dr. Lucius is examining proteins that are able to disrupt protein aggregates that form in these disease states. By taking a homolog of ClpA like ClpB (a disrupting protein) and inserting it into mouse models of Huntington's, it has been shown that ClpB can disrupt the aggregates *in vivo*, indicating a potential avenue for a successful cure. Also, these homologs can be found in bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, serving them during times of shock and stress. It has been further shown that by removing these proteins in various gram-positive bacteria, they have reduced virulence. Thus, these proteins may serve as antibiotic targets. A final benefit from his research is to the basic concepts of science. Through his research, he is developing methods

and strategies for studying extraordinarily complicated systems at the molecular level.

When asked about having undergraduates in his laboratory, Dr. Lucius responded in a positive manner. He desires that everyone receive the opportunity he had during his undergraduate career. Being able to work in a lab at such a young age allowed him perspective regarding his future. It was his experience in a lab that enabled him to pick a profession as a professor and researcher. In this way, he opens his lab to undergraduates, allowing them to bring their ideas and enthusiasms to the table. The importance of knowing what you want to do with your life is something that holds great value with Dr. Lucius. In his opinion, there is no need to waste time by applying to graduate school, only to find you do not enjoy the atmosphere once you are placed into a lab. Therefore, he suggests undergraduates try to find their niche in the world, be it in the lab or not, before making any career choices.

paper: chemistry

Stability Studies of Novel Oligodeoxynucleotides in TLR-9 Mediated Cellular Invasion

Elise M. Ottenfeld, Jason S. Hudson, Dr. David E. Graves
Department of Chemistry

Abstract

While the primary structure of DNA studied is that of the double helix, new technologies have lead to the discovery of an array of DNA structures such as the quadruplex or hairpin. These oligodeoxynucleotides (ODNs) have been the focus of research studies for their possible therapeutic effects in chemotherapy treatments. Here, a novel 9 nucleotide sequence 5'-CGC-GAA-GCC-3'is studied alongside 3 mutants (Figure 1) in order to determine the basis of its high stability and resistance to nuclease degradation. Differential Scanning Calorimetry (DSC) was used to compare the thermodynamic stability of the ODN mutants to the original wild type 9mer sequence. The results found that removing the terminal loop adenine significantly decreases the melting temperature of the ODN indicating it is essential in maintaining hairpin stability. Further studies using NMR and modeling techniques are planned to fully understand the basis of this novel result.

Introduction

In recent years, cancer biology studies have focused on determining the underlying mechanisms of cancer development in order to improve anti-cancer pharmaceuticals and chemotherapy techniques. Especially of interest is apoptosis or programmed cell death, a process essential to maintaining healthy tissue development which tumor cells do not undergo. During apoptosis, a cascade of nucleases is released to degrade cellular DNA, the most important nuclease being the DNA fragmentation factor, commonly abbreviated as DFF. Release of the DFF enzyme effectively arrests cell replication and allows phagocytosis of the cell to occur. Since several studies have shown certain ODNs produce an increased cellular immunological response through the TLR9 receptor thought to trigger metastasis in tumor cells, studies have increasingly sought to determine which types of ODNs are most invasive and the point of apoptosis at which these invasive fragments are produced. This research seeks to study variations of a invasive ODN 9mer, which were thermodynamically designed using Origin™ software, in order to determine the relationship between ODN stability and invasive characteristics. Figure 2 illustrates the anticipated hairpin structure of the wild type 9mer sequence as modeled by the Origin™ program, though additional NMR studies will be performed to confirm this result. The technique applied will include differential scanning calorimetry.

Differential scanning calorimetry (DSC) is an experimental technique primarily used to determine the phase transitions of a given sample; the technique is implemented in this experiment in order to determine the melting temperature (T_m) for the above mentioned ODNs. In DSC, a constant amount of heat is applied to both a reference cell containing buffer as well as a sample cell, both of Tantalum Alloy™. As the sample undergoes a phase transition, its heat capacity changes, thereby, an additional amount of heat must be applied in order to maintain a temperature constant to that of the reference cell. Differences in applied heat are measured and used to calculate the heat capacity of the sample from which an array of other biophysical properties such as entropy and enthalpy can be calculated.

5'-CGCGAAGCG-3'
5'-CGCGATGCG-3'
5'-CGCGTAGCG-3'
5'-CGCGTTCGC-3'

Figure 1. ODN Sequences: Figure 1 shows the original 9mer sequence, first listed, as well as the individual nucleotide variations made for each mutant which are highlighted in red.

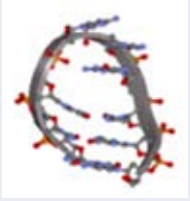


Figure 2. 9mer Hairpin –OriginTM Model: Figure 2 shows the anticipated hairpin structure for the wild type 9mer sequence as obtained from the Origin™ program to be later confirmed through NMR experiments.

Methods

Differential Scanning Calorimetry: DSC studies were performed using the Microcal VP-DSC. Oligos were solvated in 100 mM NaCl and 10 mM BPES buffer with a final concentration of 400 µM for all DSC samples. Scans of the wild type and GTA loop mutant were performed over a temperature range of 20-120 °C while the GAT and GTT loop mutants were scanned over a range of 15-115 °C. All scans were performed at a rate of 1.5 °C/min.

Results and Conclusions

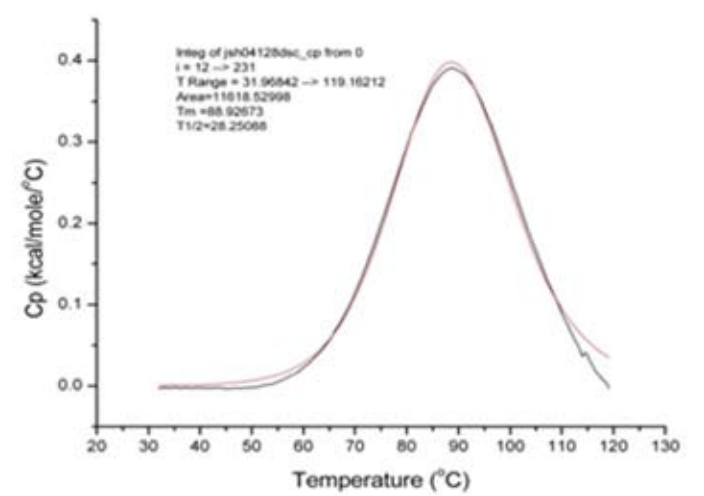


Figure 3. 9mer Wild Type –GAA Loop: Figure 3 shows the DSC scan obtained for the wild type 9mer sequence.

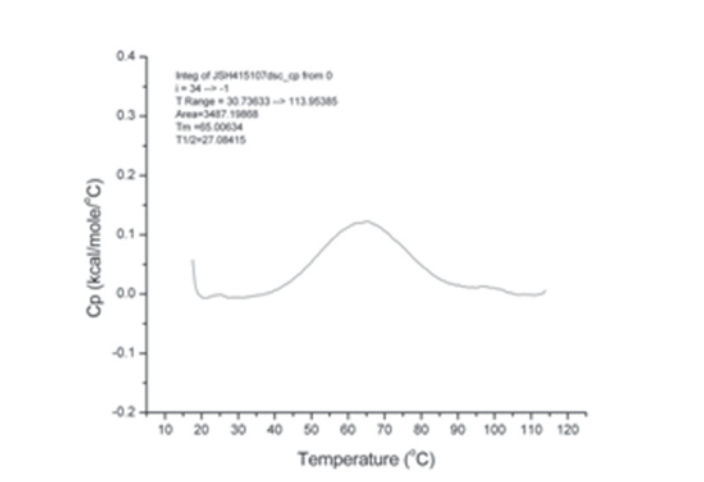


Figure 4. 9mer GAT Loop: Figure 4 shows the DSC scan obtained for the first mutated sequence which included the mutation of the terminal loop adenine to a thymine.

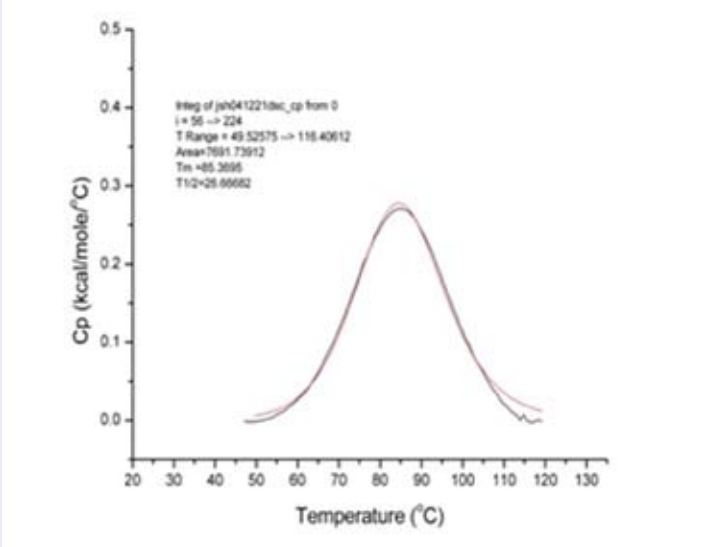


Figure 5. 9mer GTA Loop: Figure 5 shows the DSC scan obtained for the second mutant which included the mutation of the internal loop adenine to a thymine.

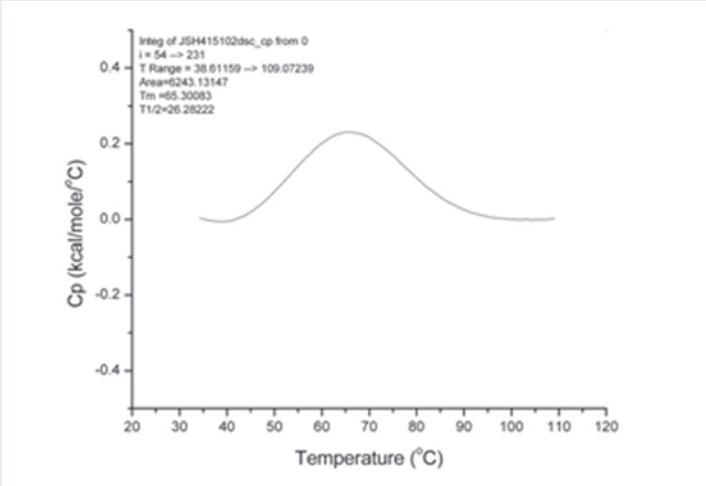


Figure 6. 9mer GTT Loop: Figure 6 illustrates the DSC scan obtained for the final mutation which included the modification of both the internal and terminal loop adenines to thymines.

Loop	T_m (°C)	ΔH (kcal/mol)
GAA	88.93	11.62
GAT	65.00	3.49
GTA	85.37	7.69
GTT	65.30	6.24

Table 1. Summarization of Melting Temperatures and Enthalpies Obtained for Sequences

Figures 3-6 show the DSC scans obtained for the wild type 9mer and its mutants while the results for the melting temperatures and enthalpies are summarized in Table 1. Our results indicate that the terminal loop adenine is essential for the unprecedented stability of the wild type 9mer oligonucle-

otide. As expected, the wild type remained the most stable oliognucleotide structure with a T_m of 88.93°C (Figure 3), though the GTA loop mutant showed impressive stability with 85.37°C (Figure 5). Shown in the melting profiles presented here, the GAT and GTT loop mutants, Figures 4 and 6 respectively, show significant destabilization, nearly a 25°C drop in T_m, while the GTA loop mutant presents only enthalpic changes. Such a change is most likely due to the ability of the loop guanine and terminal adenine to undergo base stacking due to their similar purine structure. Thymine, as a pyrimidine, cannot participate in such interactions, thereby reducing the amount of available bonding spaces which destabilizes the overall structure. In terms of enthalpic effects, the wild type mutant offered the highest enthalpy with 11.62 kcal/mol. Interestingly, the GTA and GTT loop mutants showed similar enthalpic changes though their respective melting temperatures vary greatly. Additional NMR studies will be performed in order to determine possible explanations for the differences seen here.

Experimental Outlook

Additionally, NMR and ionic studies will be performed in order to determine specific interactions which contribute to stability such as entropic effects. These results will be paired to cellular invasion assay studies performed in conjunction with the Comprehensive Cancer. Cellular invasion assays serve to mimic the process of metastasis. Through these experiments, we hope to determine the relationship between ODN stability and invasive character. Overall, the goal of the study is to identify potential targets for therapeutic cancer treatments.

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faculty interview: computer science

The Computer Science Field: *An Interview with Dr. Barrett Bryant*

Helen Lin

This interview was conducted with Dr. Barrett Bryant, the Associate Chair of the Computer and Information Sciences Department. He has been part of the UAB family for more than twenty years. This exchange discusses computer science and his research at UAB.

Dr. Bryant became interested in computer science by accident. During his senior year of high school, Dr. Bryant took a math elective computer science course. It seemed like a cool course to take in the 70's as computers were a relatively new concept. He attended the University of Arkansas at Little Rock, receiving his Bachelor of Science in Computer science. Bryant then decided to pursue graduate school at Northwestern University, where he graduated with a Masters Degree and a Doctoral Degree in Computer science. He is now a professor and the Associate Chair of Computer and Information Sciences here at UAB. His research interests range from theory and implementation of programming languages to formal specification of software systems to component-based software engineering. He has authored and co-authored 120 papers and received numerous grants.

Looking back on how he entered the field, he notes a counterintuitive trend. As technology and the prevalence of computer science has grown, the number of high school computer classes offered has decreased. Schools have to emphasis the core subjects, and unfortunately, computer science is not rec-

ognized as a core subject. Part of the problem is that many classes are called computer science that are not really computer sciences like ones focused on learning to use word processor or spreadsheet. While everyone needs to know how to use those, classes like those should never be called computer science. In Alabama, there are about 460 high schools; only 10 offer computer science. Presently, there is a shortage of students in both the undergraduate and graduate programs. There are even less students pursuing research in computer science.

In a university setting, you are following your interests – being more independent – rather than working for a company where you are not pursuing your own interests but being directed…

In choosing where to do his research Dr. Bryant decided on an academic environment rather than a corporate one. I just really like the University environment, having enjoyed it as an undergraduate and graduate student too. At the time, and I think it's even true today, when you look at academia and industry, they are compatible in different respects. In a university setting, you are following your interests – being more independent – rather than working for a company where you are not pursuing your own interests but being directed (of course they are providing the funding). It is a toss-up between the two worlds, but I just like the university environment better. It gives more flexibility and more opportunities to pursue my interests.

While he serves as the Associate Chair of Computer science here at UAB, Dr. Bryant still finds time to research and teach. He directs the Software Composition and Modeling Laboratory, examining software engineering and domain specific programming languages. He is seeking to find better ways of building software – particularly how to automate the software building process. On his recent sabbatical at Tsinghua University in Beijing, China, he looked at security, privacy and how to specify those aspects for software. Software research looks at how to engineer better software engines. Software controls almost everything that we do now from cell phones to airplanes to cars. Engineering better software systems is good for all those things. It gives us better reliability and safety, and it provides us with more opportunities for technological growth. It is useful in the critical areas where people's lives depend on it and where people can improve their quality of life. While hardware is a part of that, software is what controls hardware. **His research mainly focuses on building better software; particularly how to better automate the software development process.** One of his active projects delves into software evolution. Like any engineering disciple, we need to build models. Unlike a model of a bridge, for example, which is static, a software model is dynamic. Civil engineers design a bridge, build it, and it stays there for a hundred years. Once you build a software system and deploy it, it constantly changes – new technologies develop or the industry changes. The banking system is a perfect example. When ATMs were introduced, it required a huge change in how banking software was used and then internet banking was introduced, etc. Developments require a major change in the underlying software and, thus, the models of the software must also change – model evolution. I am trying to find a way to automate model evolution so that it reflects the new model of the software system; it evolves with the needs of the software system. We are investigating two approaches: can you completely regenerate the model given new core principles or can we evolve the current model with changes? The idea is to tell the system what changes are needed and have the software evolve rather than the current method of changing the system manually.

In fact, Dr. Bryant recently returned from a sabbatical in at Tsinghua University in Beijing, where he researched various aspects of modeling for Requirement Engineering. He looked at security, privacy and how you specify those aspects for software. His findings were published by the Association of Computing Machinery international conferences on Software Engineering. There are many opportunities to travel in Computer science, including many worldwide conferences. **He recently presented his findings from his sabbatical and presented at the Inforum Simpósio Informática this past September at the University of Minho in Portugal.** There are more travel opportunities than there are students to take advantage of them. Recently, I had a travel grant to send students working in a particular area to a conference in Slovenia. One of the requirements when you get funding from the federal government is that they should be U.S. students. Computer science at the graduate level is heavily populated by foreign students. We did not have any U.S. students working in this area. Even though I was the principal investigator, I could not send a single student from UAB. But fortunately, the grant was really open in that it could fund any students from anywhere in the U.S. It was a pity I could not find any UAB students to send. Part of this is because the job market is really good. We have a lot of U.S. students at the undergraduate level. When these students graduate, they get a job rather than going to graduate school. As such, the number of U.S. students doing research is less than we would like to see.

For students interested in doing research as an undergraduate, Dr. Bryant recommends that they should approach faculty members. If you took a course that you really liked or completed a project that you really liked, approach the professor and ask if that project can be extended into a research project. When ap-

proaching faculty, students should, ideally, have a more specific interest rather than a broad and general topic. In this way, the faculty member can know where the student is coming from and can better find a match for the student. The key thing is students can't wait too long so that they can finish the project. At the same time, students need to have some background. The best time would be as junior or early senior year because the background is there and interests are more developed. Students can also do Honors research as part of the Computer science curriculum.

Dr. Bryant also had suggestions for potential and current computer science students. Students best fitted to computer science majors are those that have good problem solving skills and enjoy solving life problems. This is a subject less suited to memorization. Computer science is a field where there are basic foundations rather than methods and concepts to be memorized. Students should also be patient because many computer science problems take time to solve. New developments in technology are constantly creating increased areas of research and growth.

Computer science is a growing field with many opportunities. In fact, a lot of students get jobs before they graduate, which I do not recommend. They should concentrate on finishing their degrees. While jobs bring in money, it takes time away from doing school work. We have had unfortunate situations where students have taken jobs and not graduated because the job was a time sink. My advice is not to hurry; the job opportunities are great. **Computer science is a field that will continue to grow.** The field will continue to grow because every technological advancement requires some form of computer science. Scientific developments like cell phones and iPads all require computer technology; without computer science underneath, those things would not work. Right now, we almost have the computational power of a computer in your hand held phone. That is not going to change. You will probably get more and more power. This is a developing field as new things keep coming out. The opportunities will be better than if you took a job without your degree.

paper: physics

Near- and Mid-Infrared Spectra of Cosmic Ice Analogues Subject to Ultraviolet Photolysis

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Abstract: *Infrared spectroscopy has played an instrumental role in the understanding of the effects of solar UV bombardment on ices consisting of CO_2 in mixture with other molecules on the surfaces of outer solar system bodies. Utilizing the infrared features of the vibrational modes of the ice constituents, we can determine the photochemical products produced, their concentration, and their rate of production. Included here are the results of experiments involving the UV-photolyzed ice analogs with CO_2 in mixture with N_2 and H_2O on an IR transparent substrate held at a constant temperature of 6 K. Similarly seen in previous experiments, the photochemical products include N_2O, CO, CO_3 and O_3, observed in both the mid and near infrared (400–10000 cm^{-1}).*

Introduction

The mid-infrared features of many molecules have been well-studied and thoroughly documented due to their relative strength when compared to signature bands in the near infrared (Gerakines *et al*. 1995). Because instruments obtaining spectra in the mid-infrared are more difficult to maintain, the atmosphere is opaque to the mid-IR in all but the highest of elevations, and the highest flux of the sun's IR is near-visible, most instruments for observation within the solar system are equipped with only near-infrared equipment. It is thus easy to see why studying these compounds in the near-IR could be useful. The goal of our experiments was to obtain a better understanding of the evolution of outer solar system ices due to UV photolysis from the sun's exposure, through obtaining spectra with photochemically produced features in the NIR spectral range.

Infrared spectroscopy is useful for studying these molecules due to their vibrational energy level transitions that each correspond to a unique wavelength. The quantized frequencies of vibrations in the compound can only transition to lower or higher frequencies, or energy levels, at prescribed energies, the wavelengths of which are determined by the equation:

$$E = [(n + ½) \cdot h \cdot c] / \lambda$$

where E is the energy of the transition and resultant photon, h is the Planck constant, n is the quantum number and λ is the wavelength of the photon. The energy transitions are

especially strong in the MIR (400-4500 cm^{-1}), where these molecules absorb photons more readily.

The moons of the outer solar system planets, such as Triton, as well as Pluto, contain ices rich in molecular nitrogen as well as other trace molecules (Hudson et al. 2001); as such, experiments involving a precise mixture of N_2:CO_2:H_2O at 5:1:0.65 concentration have yielded valuable insight into the evolution of the surfaces of these bodies.

Methods

The methods by which UV-photolyzed experiments have been done previously at UAB have been explained in detail by Mares *et al*. (2007) and Richey *et al*. (2003), but a brief summary is included here. Figure 1 is a schematic of the experimental setup in the astrophysics lab at UAB

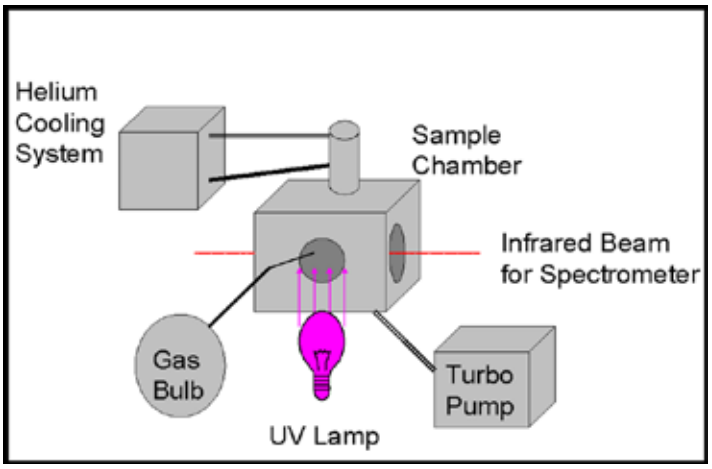

Figure 1. A diagram of the apparatus used in these experiments. The windows allowing the FTIR to measure the sample are made of KBr, and the window to the UV lamp is comprised of MgF_2. The helium cooling unit brings the substrate down to 6 K after the turbo pump creates the 3x10-6 torr vacuum. Gasses are prepared in a bulb attached to the setup and then deposited via an adjustable line.

The apparatus used for the experiment consists of a chamber which can be cooled to 6 K by a helium refrigerator unit, and the temperature can be maintained to up to 300 K by a heating element located near the substrate chamber. The chamber is situated so that it can be rotated into three positions for portions of the experiment: deposition, scanning, and photolysis. The chamber has three windows; two opposite, IR-transparent windows of KBr are situated such that they face the detector and light source of the spectrometer, and the third, a UV-transparent window made of MgF_2, allows the sample to be irradiated by UV photons (100-250 nm) when in the "UV" position.

Deposits are made via a needle-valve system attached to a thin line leading to the sample chamber 1 centimeter in front of the substrate from a bulb containing the premixed sample gasses (which were mixed on a separate gas manifold line and then attached to the system), and are applied in timed intervals between UV exposures. Utilizing results of past experiments, the nozzle is adjusted via the needle-valve system in order to determine the proper rate of gas application. The system is changed to compensate for a decrease in pressure in the sample bulb, in order to achieve the desired ice growth rate of 1-5 μm per hour. A turbo pump is used to reduce the pressure to an approximate vacuum of $3.0x10^{-6}$ torr before deposition. A chemical trap is attached to the turbo pump to remove excess water, a typical contaminant, as well as prevent pump oil from entering the system. The chamber pressure is monitored using a sensor attached to the turbo pump consisting of a filament lamp.

For UV photolysis, hydrogen flows through an Opthos Instruments quartz lamp at a pressure between 500-600 microns (about 0.5-0.6 torr); excitement of the hydrogen is maintained by a McCarroll microwave cavity, after initial excitement via tazing. The result is a lamp emitting in the Lyman series (10.2eV-13.6eV, λ= 121.6-91.15nm), which is adjacent to the UV-transparent window in the substrate chamber. Due to UV photons only being able to penetrate 0.1-1 μm deep into the ice, a layering method was implemented to produce a sufficiently thick UV-photolyzed ice such that near-IR features could be detected.

Scans are taken in both the mid-infrared (4,000-400 cm^{-1} or 2.5-25 μm) and the near-infrared (10,000-4,000 cm^{-1} or 1-2.5 μm) via a Thermo Mattson Infinity Gold Fourier transform infrared spectrometer when the substrate chamber is moved to the "Scan" position. After each 0.1-1 μm-thick layer and session of photolysis, scans are taken throughout the experiment, which totaled 45 layers. Scans are ratioed with their background scans to create viable absorption spectra. Due to the lengthiness of the experiment, requiring it to be left overnight, overnight scans are taken to ensure contamination is not significant. The finalized scans are then compared to pure ice samples (samples of the same ice mixtures that are not subjected to UV) to verify changes to the original mixture and the creation of any photochemical products. From these data, useful comparisons can be made to determine the effectiveness of the photolysis to model ice spectra taken observationally. In addition, the signature modes can be integrated to determine the rate of production of the photochemical molecules.

Results

Figures 2 and 3 are the mid- and near-infrared plots of N_2:CO_2:H_2O in 5:1:0.65 ratio at 6 K after 45 layer of photolysis (for a total time of 2,880 minutes of exposure). Shown in Figure 2 is a progression of development of the features of these compounds over 45 layers in the mid-infrared. Seen in similar experiments (Gerakines *et al.* 2004, Brucato *et al.*, 1997) several photochemically produced compounds are readily apparent in the mid-infrared, including N_2O, CO, CO_3 and O_3. Most of these are seen between the signature asymmetric stretching mode of CO_2 at 3711 cm^{-1} and its bending mode at 660 cm^{-1}.

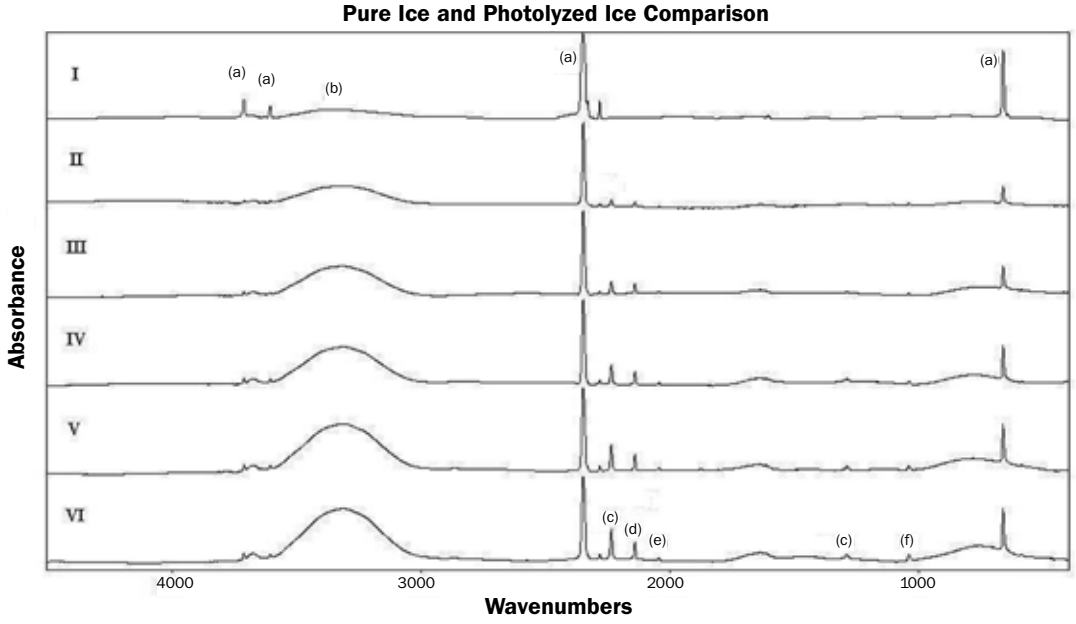

Figure 2. From top to bottom; I: pure ice sample (at 5:1: .65 concentration) of N_2:CO_2:H_2O after a 40 minute deposit; II: after 10 layers of photolyzed ice; III: after 20; IV: after 30; V: after 40; VI: after 45. (a) Features at 3711, 3600, 2343, and 660 cm^{-1} are due to two distinct combination, asymmetric stretching, and bending modes of CO_2 respectively. The absorption peak to the right of the asymmetric stretching mode is due to the isotope C13 in CO_2, causing a different asymmetric mode feature (b) a dominant water feature that can be seen to grow over the course of the experiment. Photochemical products are most easily seen on plot VI, and are as follows: (c) 2235 cm^{-1} and 1290 cm^{-1} features of N2O (d) a CO line at 2137 cm^{-1} (e) this is a CO_3 mode at 2044 cm^{-1} (f) ozone at 1038 cm^{-1}

Molecule	Peak Position cm^{-1}	Peak Position μm
CO_2	3711	2.69
	3600	2.78
	2343	4.26
	660	15.15
$C^{13}O_2$	2280	4.39
CO	2137	4.70
N_2O	2235	4.47
	1290	7.75
O_3	1038	9.63
CO_3	2044	4.89
NO	1874	5.34

Characteristic Features of MIR Vibrational Modes

Table 1. These are the wavenumbers and wavelengths of CO_2 and the products of photolysis. These are most easily seen in spectra VI of figure 2, and can be observed sequentially beginning at the CO_2 feature at 2343 cm^{-1}.

The most prominent of the photochemical products shown is the N_2O vibrational transition at 2235 cm^{-1}, which can be seen to grow very rapidly during the experiment. In addition, the characteristic N_2O line at 1290 cm^{-1} is visible, though less drastically. In figure 4, these two features are presented to demonstrate the high linear correlation of their growth. Another product is CO, which is of particular interest due to its abundance in Kuiper belt objects, Pluto, Charon, Hale-Bopp, and Triton (Hudson *et al.* 2001); Figure 5 depicts the strongly correlated linear growth of the 2137 cm^{-1} CO feature over the nearly 2900 minutes of photolysis.

Figure 6 shows the characteristic feature at 2044 cm^{-1} of CO_3, but does not seem to be as closely dependent to UV exposure time when compared to figures 4 and 5. CO_3 itself is an unstable molecule, which could explain its slightly erratic behavior. The ozone peak growth at 1038 cm^{-1} is evident in figure 7.

NIR graphs are not conclusive when attempting to review peaks of all of the photochemical products save CO, but a distinct line at 4253 cm^{-1} is clear upon inspection of figure 3.

For each product, the characteristic MIR peak of was integrated with respect to the continuum. Data points represent integrations for every other layer's graph used to obtain figures 4-7.

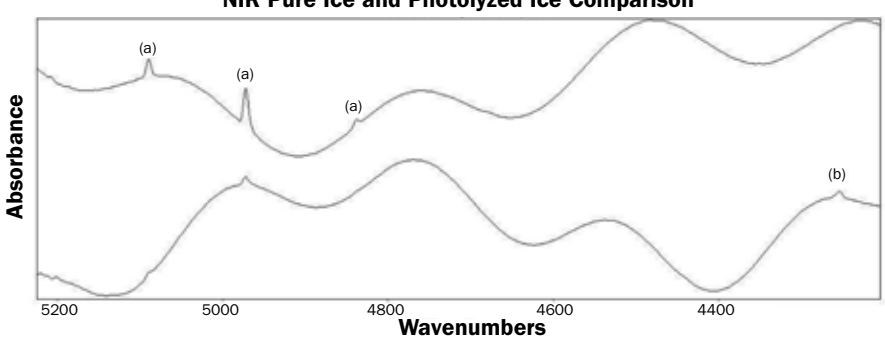

Figure 3. Top; a magnified NIR scan of a pure ice sample. Bottom; magnified near-infrared scan of photolyzed ice after 45 layers. (a) three lines can be attributed to CO_2 at 5087 cm^{-1}, 4971 cm^{-1}, and a weaker one only apparent in the pure ice sample at 4832 cm^{-1}; all three are combination modes. (b) this peak at 4252 cm^{-1} corresponds to a signature mode of CO, and is the particularly interesting feature in the photolyzed ice as it was produced via photolysis

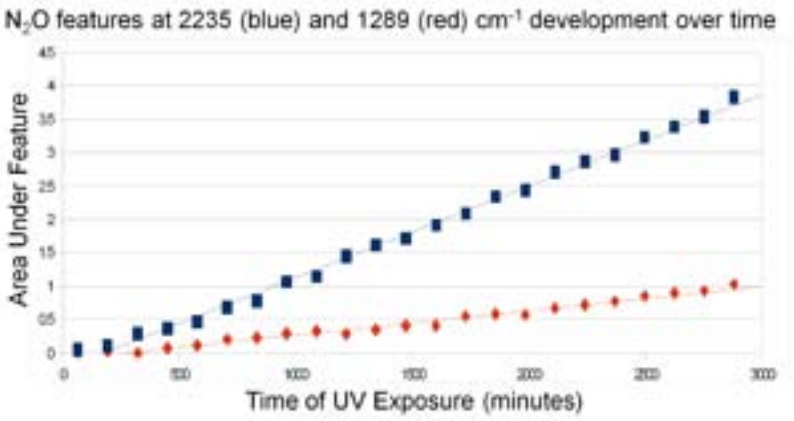

Figure 4. This is the N_2O feature at 2235 cm^{-1} (in blue) versus the UV exposure in minutes, which can be seen to increase linearly. The data in orange come from a second feature, at 1289 cm^{-1}. Nitrous oxide is a big contributor to the photochemical products in this experiment. This is an indicator of the effectiveness of the experiment in modeling potential sources of N_2O in cosmic ice mantles and outer solar system bodies.

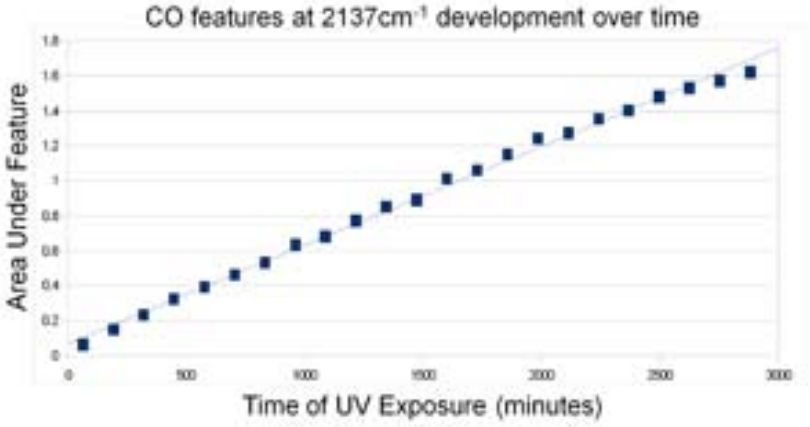

Figure 5. Shown is the CO stretching mode at 2137 cm^{-1} feature's area versus the time of UV exposure. The high linear correlation of the feature's growth is to be expected if each layer contributes the same amount of CO to the ice mixture, which would be a direct result of the limit of the ultraviolet penetration of the ice.

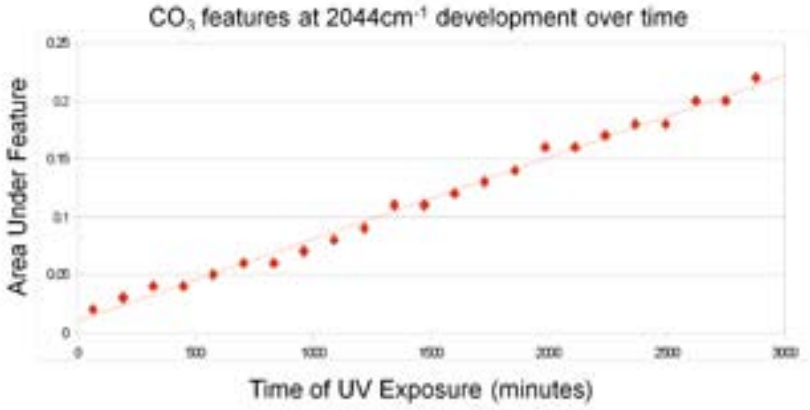

Figure 6. The integrated CO_3 peak at 2044 cm^{-1} vs. time of UV exposure. The amount of CO_3 seems to increase linearly, but the data is not as strongly correlated, perhaps due to the instability of the molecule.

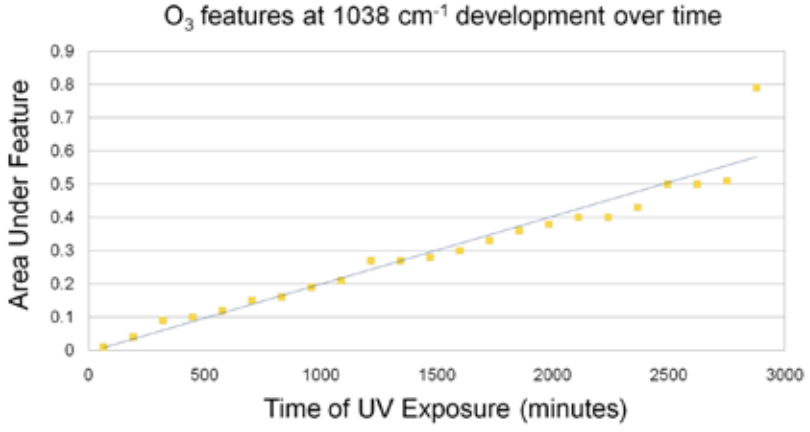

Figure 7. Depicted here is the O_3 feature at 1038 cm^{-1} integrated, versus time of UV exposure. Ozone is a by-product of photolysis, but the molecule itself has an affinity for UV light absorption, though generally of longer wavelengths than the Lyman series.

Discussion

The importance of the results of the experiment lies in the ability to both explain the existence of all of the photo-dissociated and recombined molecules in observational data, as well as provide an important goal in future work: to verify the existence of these molecules in photolyzed ices of similar composition and in appropriate environments in which UV light is incident, such as Triton and Pluto (Hudson et al. 2001). These two bodies would be the best place to begin looking, due to the specificity of the experiment parameters; Triton contains all three of the initial ice constituents, and Pluto contains H_2O and N_2 (Hudson et al. 2001). N_2O could be a particularly useful indicator, as its prominence in the MIR spectra (at 2235 cm^{-1} specifically) could provide an easily recognizable feature with which to verify these results.

Additionally, the CO peak at 4253 cm^{-1} is especially encouraging, as it represents something that is able to be seen via NIR measurement. Many instruments on observational equipment today utilize the NIR spectrum to determine chemical compositions, and any features of photochemical products in this region are evidence that these results could be verified via this equipment.

Summary

Our experiments implemented a high vacuum chamber with a substrate reduced to a temperature of 6 K to create ices from a prepared mixture of gasses in an attached bulb. Ices were then subjected to 64 minute exposures, per layer, to the Lyman series (UV, 10.2eV-13.6eV, λ= 121.6-91.15nm) in order to photolyze the ices. Spectra taken after deposition and photolysis were compared to spectra acquired prior to the experiment of non-photolyzed ice of the same mixture to determine any photochemically produced molecules.

Signature features from N_2O, CO, CO_3 and O_3 were observed, which are indicative of a source of these compounds in astronomical settings where UV radiation occurs. These lines were integrated over the bases of their features, and their growth is set against the time of the ice's exposure to the ultraviolet lamp.

Additionally, a characteristic CO peak was measured in the near-infrared, which is extremely important due to observations of icy solar system bodies typically being performed in the near-infrared. The ability to observe this feature in the laboratory begs the question as to the possibility of it being seen in spectra of celestial bodies such as Triton and Pluto, given enough sensitivity.

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ACKNOWLEDGEMENTS

I'd like to acknowledge support from the National Aeronautics and Space Administration (NASA)-Alabama Space Grant Consortium – Research Experiences for Undergraduates (REU) award to UAB.

I'd also like to thank Dr. Perry Gerakines, my mentor who took the time to patiently walk me through the lab and answer my many, many questions, Chrissy Richey, who made my REU at UAB a possibility, by spending so many hours teaching me the ropes, all the while taking on her own projects and finally, to the University of Alabama at Birmingham, which gave me the opportunity to undertake astrophysics research in the first place.

faculty interview: physics

The Development of Nanotechnology: *An Interview with Dr. Andrei Stanishevsky*

By Kavita Nadendla

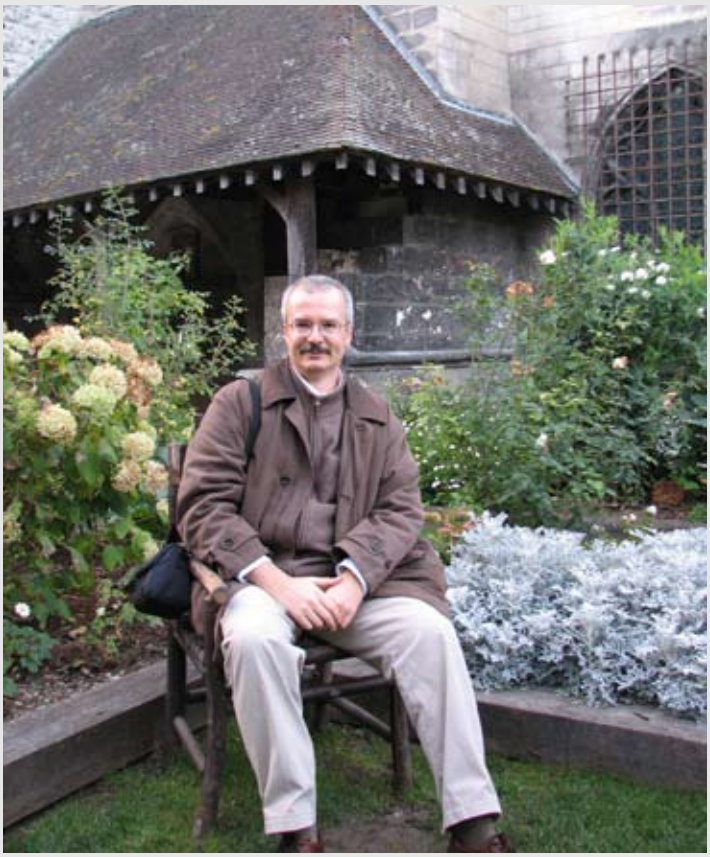
After taking Dr. Stanishevsky's Physics 201 course last year, I thought I would have no trouble interviewing him. As I made my way past the doors of faculty offices in Campbell Hall, I found my hands shaking as I neared room number 342. Students refer to him as "that Russian scientist who studies nanotechnology." Of course I would be intimidated to talk to him, in fear of sounding dumb or not understanding his work. However, I was glad to find Dr. Stanishevsky a welcoming man with a great sense of humor.

Dr. Andrei Stanishevsky has been at UAB for eight years as a professor in the Department of Physics in the College of Arts and Sciences. Coming from Belarus, Dr. Stanishevsky's educational and career path were different from the traditional path taken by students in the United States. His interest in academic teaching and research is what he would consider "genetic." Because both of his parents were university professors, he was exposed to the laboratory environment at a very young age. Even his middle school and high school curricula were geared toward the sciences. When explaining the rigor of his education, Stanishevsky said, "I was in a school that emphasized physics and mathematics. We had mandatory 4 years of chemistry and 5 years of physics, algebra in 4th grade, trigonometry in 8th, and calculus in 9th and 10th grades."

Because their school system does not have the equivalent of an undergraduate bachelor's degree, Stanishevsky entered a master's program right out of high school in a university in Minsk, Belarus, with a focus in electrical engineering, an extremely popular area of research at the time. Dr. Stanishevsky stressed the importance early lab experience had on his scientific endeavours. "I knew even before graduating from college that I wanted to do research. I started working in a lab my first year at college. Research experience gives students an upper hand when applying to graduate schools."

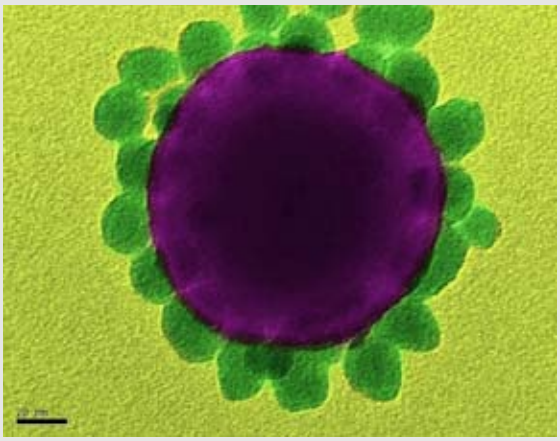
While completing his engineering degree, he became interested in microscopic systems research in materials and thin films, which is why he "decided to do research after graduation in an academic research center. It was mandatory to have two years of research or industrial experience before entering graduate school." At a research center of the Belarus Academy of Sciences, Dr. Stanishevsky finished his PhD in solid state physics.

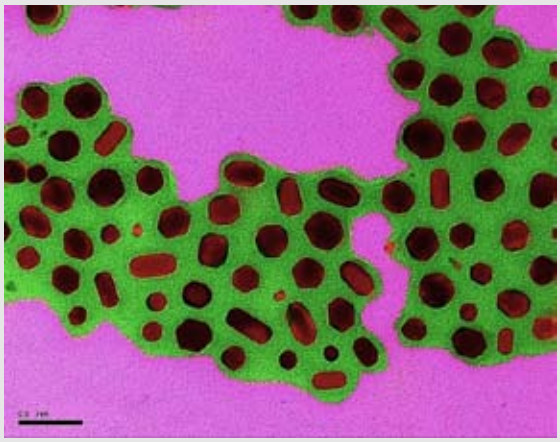
During his PhD research, Dr. Stanishevsky teamed with many researchers in Europe, Japan, and the U.S. In 1996 he came to the U.S. after receiving a grant from the United States National Research Council to do post-doctoral studies at Penn State



University. He worked later as a research scientist at the University of Maryland where he was exposed to nanoscience and nanotechnology. He eventually joined the UAB Department of Physics in 2002. When asked what made him choose UAB, he answered, "UAB is a relatively young university that is aggressively growing. It offers a lot of opportunities and I saw that I could do something new here. The facilities and fellow faculty are very good, so I saw a chance for good collaboration, which is an important part of research because you are expected to build a team to broaden your research interests."

Dr. Stanishevsky's current work focuses on two major directions related to the physics of materials – hard thin film coatings and nanoparticles. The study of hard thin film coatings is essentially the evaluation of new materials that simultaneously possess extreme hardness and toughness. This makes them attractive for cutting tools, biomedical implants, or parts of engines as examples. They are intended to survive extreme conditions in which harsh environments could destroy them. Stanishevsky's team produces thin film coatings using nitrides and carbides of different metals. "These materials can approach the hardness of diamond – the hardest material currently known. But they also possess properties diamonds don't, such as high thermal resistance, making them more ad-





Left: Transmission Electron Microscopy (TEM) image of a polymer nanosphere decorated with smaller (approximately 10 nm) silica particles. Right: Transmission Electron Microscopy (TEM) image of gold nanocrystals with various shapes.

vanced than diamond in some applications." For this research focus, he has started various partnerships in Europe, including ParisTech (Cluny Center), France, Aalto University School of Science and Technology in Finland, and Technical University of Lodz in Poland.

His second major project deals with nanoparticle research – entities that range from 1-100 nm in size. There are special properties associated with the extremely small size of nanoparticles. Dr. Stanishevsky notes, "When the size of a particle is small enough, new electronic, optical, or chemical properties arise. This is attractive for numerous applications – composite materials, biomedical research as biomarkers, biosensors, drug delivery systems, and for monitoring and tracking states of living systems." His team designs the manufacturing of such particles from different semiconductors, metals, or ceramics with different shapes and dimensions to control unique particle properties in a broad range. An example being nanoparticles of calcium phosphate called hydroxyapatite, the major constituent of human hard tissues. He enlightened me that "up to 70% of our bones consist of nanoscale particles of hydroxyapatite held together by a collagen matrix. A potential application of synthetic hydroxyapatite nanoceramic-based composite material would be to build artificial bones or substitutes for repair of natural tissue."

In all areas of his research, Stanishevsky stresses the importance of research collaborations and partnerships on campus as well as abroad. Because scientific research has become an interdisciplinary effort, it's important to get many people from different backgrounds involved. Dr. Stanishevsky focuses on the material itself and its properties and his colleagues focus on how the particles behave in a certain environment, whether *in vitro* or *in vivo*. For example, researchers in UAB Medical School evaluate toxicity of artificially engineered nanomaterials and their effects on human organs. The Department of Biology tests the effects of the nanomaterials in the natural environment. Collaboration with the Chemistry Department and the Department of Materials Science Engineering is important for producing composite materials and nanoparticle systems for biological and chemical sensors. Several of Dr. Stanishevsky's international activities, funded by the National Science Foundation (NSF), initiated cooperation in new research ventures with several universities in France, Poland, and Finland. Through this cooperation, nineteen UAB undergraduate and graduate student exchange programs have been organized, as well as, scientific meetings in Europe, such as nanodiamond workshops in Poland and the Czech Republic, and a dual-PhD program with ParisTech (France).

Stanishevsky's ultimate goal is to "design new materials for improving the life of people and to bring those materials to production." He hopes to build nanoscale systems and nanoparticles useful in enhancing the service life of biomedical implants, processing tools, and biosensors. Essentially, the new nanomaterials can be used to make pure water out of waste through ultrafiltration. But for those expecting immediate results, he cautions "many projects are fundamental. They are not meant to develop a specific product, but to answer the question 'Why'. Why is the material toxic, what type of effect does it have on living systems, and how can we make it more reliable and long-lasting?"

With over sixty publications and all of his collaborative efforts, Dr. Stanishevsky helps motivate undergraduate students to pursue their goals in the scientific field. He challenges them to take on demanding projects in synthesizing nanoparticles and studying their biomedical applications. He encourages them to author articles in peer-reviewed journals and present at esteemed conferences in Europe. He has three key suggestions for undergraduate students considering research: 1) start early, 2) read a lot, and 3) keep a balance. Starting early gives students time to engage in different opportunities and to develop a primary interest. Because most fields are interdisciplinary, it is important for students to read both within and outside their major. Quoting Niels Bohr, Stanishevsky said that it's useful to work with people from different areas of research because they are not bound by the frames of that one subject. They could actually make a discovery faster than those who have worked in that one area for years because they are able to think outside the box.

The Effects of Fatigue and Difficulty on Cardiovascular Response to Aerobic Exercise Challenge: An Obesity Simulation

Jin Joo Shim and Rex A. Wright
University of Alabama at Birmingham

Abstract

Two studies simulated the effect of obesity on fatigue and its later influence on effort-related cardiovascular responses to an aerobic challenge. Study 1 involved two fatigue and difficulty levels. Participants first walked on a treadmill for 10 minutes while wearing a vest fitted with 5- or 25 pounds of weight. Later, they mounted a recumbent (i.e., reclined) stationary bicycle and were asked to pedal for 10 minutes with the chance to earn a modest incentive if they attained a low (40 rpm) or high (60rpm) cycling standard. As expected, analysis of cardiovascular responses during the cycling period indicated interactions for systolic blood pressure and heart rate. Whereas responses were stronger for the heavy vest (i.e., high fatigue) group when the standard was low, they were weaker for this group when the standard was high. Study 2 evaluated an interpretation alternative to fatigue. It involved the same procedure as Study 1, but included no treadmill period and required all participants to pedal while wearing the heavy vest. Investigators reasoned that if the original findings for the heavy vest group were due to fatigue, then they should not *replicate. Instead, results should show stronger responses under high- than low standard conditions. As expected, findings supported the fatigue view.*

Introduction

A recent conceptual analysis addresses the role that fatigue may play in determining effort and associated cardiovascular (CV) responses in people confronted with performance challenges (Wright & Stewart, 2011). Central assumptions of the analysis are threefold. One is that CV response varies with effort, that is, the degree to which performers apply themselves in action circumstances (Light, 1981; Obrist, 1981). A second is that effort varies nonmonotonically with the difficulty of a performance challenge, first rising and then falling sharply with the fall occurring where success appear impossible or excessively difficult given the benefit that it is likely to yield (Brehm & Self, 1989). The third is that perceptions of difficulty increase with fatigue (e.g., Hockey, 1997).

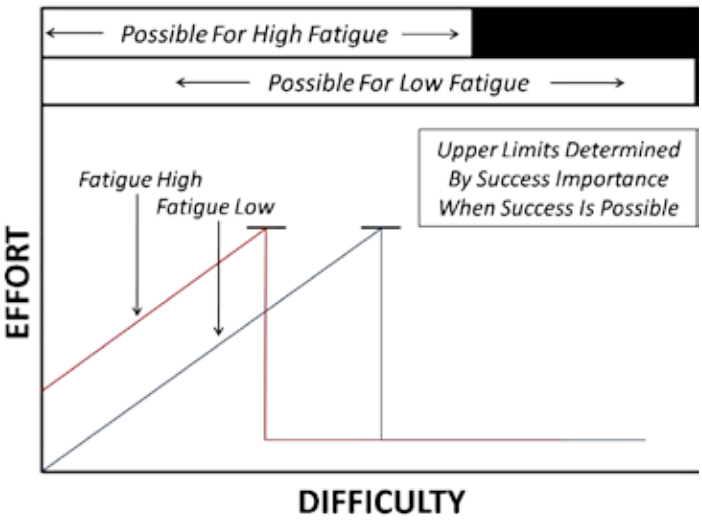


Figure 1. Effort as a function of fatigue and difficulty.

Collectively, the assumptions above suggest that fatigue should have different effects on effort and CV responses, depending on the difficulty of the challenge at hand (Figure 1). When fatigue leaves unchanged a perception that success is possible and worthwhile, it should lead performers to exert compensatory effort and experience heightened CV arousal. When fatigue causes success to appear impossible or excessively difficult, it should lead performers to withhold effort and display minimal CV arousal. When fatigue reinforces a perception that success is impossible or excessively difficult, it should confirm performers' inclination to exert low effort and experience minimal CV arousal.

Support

Numerous studies have yielded support for the preceding analysis. An example is an experiment that examined the influence of muscular fatigue under different task conditions (Wright & Penacerrada, 2002). Participants initially performed a series of easy (low fatigue) or difficult (high fatigue) dynamometer grips with their left or right hand. They then made a modest grip with their right hand while CV responses were measured. For Right-Hand participants, initial performance of the difficult grips was expected to reduce the ability to perform the follow-up grip. However, for Left-Hand participants, initial performance of the difficult grips was not expected to reduce the ability to perform the follow-up grip. Therefore, it was predicted that effort and associated CV responses during the second grip would be greater under high- than low fatigue conditions for the Right-Hand participants, but not for Left-Hand participants. Systolic blood pressure (SBP) results confirmed this prediction.

A further example is a study that examined mental fatigue influence at low and high levels of challenge difficulty (Wright, Martin & Bland, 2003). Participants first performed a minimally demanding (fatigue low) or highly demanding (fatigue high) counting task. Shortly thereafter, they were presented arithmetic problems with the chance to earn a prize by attaining a low or high performance standard. Analysis of data collected during the arithmetic period indicated fatigue x difficulty interactions for

SBP, diastolic blood pressure (DBP), and mean arterial pressure (MAP). Whereas Low Fatigue participants displayed relatively stronger responses when the standard was high than when it was low, the High Fatigue participants displayed the reverse. Comparisons at each standard level indicated that responses were or tended to be greater for High Fatigue participants when the standard was low, but were or tended to be greater for Low Fatigue participants when it was high.

The Present Research

The first study described below was initiated with two central purposes: (1) to show the interactional effects of fatigue and difficulty in a physical (aerobic) performance context, and (2) to do so using a protocol that induced fatigue by having participants perform a preliminary treadmill task wearing a vest loaded with different amounts of weight. It was reasoned that this protocol would simulate the experience of being more or less overweight and shed light on fatigue effects associated with obesity (e.g., Browning, Baker, Herron & Kram, 2006).

Central predictions were twofold. First, it was expected that Light Vest (i.e., Low Fatigue) participants would display relatively greater effort and CV responsiveness under high- than low standard condition. Second, it was expected that Heavy Vest (i.e., High Fatigue) participants would display the reverse, greater effort and CV responsiveness under low- than high standard conditions, with their low standard responses being stronger than those of the Light Vest participants and their high standard responses being weaker than those of the Light Vest participants. These predictions were based on the assumptions (1) that Heavy Vest participants would perceive the cycling challenge as harder than would the Light Vest participants, and (2) that the Light Vest participants would perceive success as possible and worthwhile in both standard conditions, whereas Heavy Vest participants would perceive success as possible and worthwhile only in the low standard condition. There is reason to believe that SBP and HR responses should be especially sensitive to effort influence in a cycling situation (Brownley, Hurwitz & Schneiderman, 2000). Consequently, we focused on those measures in evaluating the predicted crossover response pattern.

Method: Study 1

Participants

Participants were 75 normal weight female undergraduate volunteers, most of Western European or African heritage. They received class credit for participation and were assigned randomly to one of four experimental groups: light-vest/low-standard, light-vest /high-standard, heavy-vest /low-standard, and heavy-vest/high-standard.

Measurement of CV Responses

CV measurements were taken with a Dinamap intermittent monitor, which utilizes a conventional arm inflation cuff. Samples were taken every 2 minutes during a 10 minute baseline and the 10 minute pedaling period.

Procedure

Participants were met by a female experimenter, escorted to the experimenter chamber and asked to sit at a table that contained an informed consent statement and – off to the side – a basket containing full size Snickers bars. Once seated, participants signed a consent form and completed an initial mood checklist that included items assessing feelings of tiredness, energy and (physical) fatigue. Responses were made on 11-point scales with endpoints of 0 (not at all) and 10 (extremely).

After participants completed the forms, the experiment escorted participants to a hallway and measured their weight and height. As she returned to the experimental chamber, the experimenter (1) briefly provided an overview of study, (2) asked participants to be seated on a recumbent bicycle (Fitnex R70 Recumbent Exercise Bike) with their feet on pedals, and (3) attached the Dinamap arm cuff to participants' right arm. Participants were asked to rest for the next 10 minutes while CV measures were taken every 2 minutes. The experimenter returned to control room and determined the load (vest) condition of participants by turning a load (light versus heavy) condition index card.

After baseline, the experimenter returned, removed the arm cuff and fitted participants with a light or heavy vest. The light vest contained 5 pounds of weight; the heavy vest contained 25 pounds of weight. Once the vest was fitted, the experimenter directed participants to step on a treadmill (Smooth 6.45M Treadmill - Smooth Fitness) and start the 10 minute treadmill period. During the treadmill period, participants walked at a modest (2 mile per hour) pace on a flat conveying belt. After the treadmill period, the experimenter returned and asked participants to move back to the recumbent bicycle. Once participants were seated, the experimenter asked them to complete a second affect check list. The checklist included the same items as the first, but asked participants to indicate their current – instead of their earlier – feelings. After participants completed the checklist, the experimenter attached the Dinamap cuff to the participants' right arm and gave general instructions on how to use bicycle. Special attention was paid to showing participants how to operate the display board, which conveyed information in repetitions per minute (RPMs) about cycling speed.

When the cycling instructions were clear, the experimenter returned to the control room, telling participants that further instructions would be conveyed over a public address system. Once in the control room, the experimenter determined the standard condition by turning a standard condition index card. Depending on the standard displayed, the experimenter read one of the two messages below. In the first stages of the study, the experimenter read this information over the public address system. In later stages, the experimenter opened the door to the experimental chamber and presented the information in person. The change in

procedure occurred because a second – ongoing – experiment created distracting noise in the control room.

> *For Low Standard*: "Ok. Your pedaling exercise will be to pedal constantly for 10 minutes. Pedaling speed is not critical; however, some consistency in speed among participants is desirable. Therefore, the professor in charge of this study will award a full size Snickers bar sitting in the basket on the table in front of you to everyone who averages a pedaling rate of 40 RPMs."

> *For High Standard*: "Ok. Your pedaling exercise will be to pedal constantly for 10 minutes. Pedaling speed is not critical; however, some consistency in speed among participants is desirable. Therefore, the professor in charge of this study will award a full size Snickers bar sitting in the basket on the table in front of you to everyone who averages a pedaling rate of 60 RPMs."

Participants were asked to raise their left arm if they understood the instructions and were ready to begin. Once they did, the experimenter directed the participants to start pedaling. During the pedaling period, the experimenter recorded CV measures every 2 minutes. After the pedaling period, the experimenter returned for debriefing. Following the debriefing, the experimenter awarded all participants a Snicker's bar.

Results

Tiredness, Energy and Fatigue

Two (Vest: light versus heavy) x 2 (Standard: low versus high) analyses of variance (ANOVAs) on base tiredness, energy and fatigue scores yielded no effects. Change scores were computed by subtracting base values from task values and examined with ANOVAs. The ANOVAs yielded Vest effects for tiredness and energy ($ps \leq .05$). For tiredness, the Vest effect reflected higher values for Heavy Vest participants (low standard M = -.50, high standard M = 1.59) than Light Vest participants (low standard M = -1.19, high standard M = -.56). For energy, it reflected lower values for Heavy Vest participants (low standard M = -.94, high standard M = -1.59) than Light Vest participants (low standard M = .19, high standard M = -.33).

Cardiovascular Measures

Two way ANOVAs on CV measures taken during the final 2 minutes of the baseline period produced no effects. CV change scores were computed by subtracting base values from values obtained during the performance period. In examining the CV data, investigators focused on responses obtained at the 1st, 3rd and 5th minutes, averaging across the samples. The reason for focusing on samples from these time periods was because they were periods in which there was near complete data for all participants. By the end of minute 5, considerable data were lost because participants stopped cycling. Although the focus was on the early samples, it is of note that CV responses were, in fact, largely consistent across sampling periods.

ANOVAs on the change scores revealed Vest x Standard interactions for SBP and HR ($ps < .007$). As expected, responses rose from the low- to the high standard condition among Light Vest participants ($ps < .05$, see Figure 2). By contrast, they tended to fall from the low- to the high standard conditions for Heavy Vest participants. Comparisons of the vest (fatigue) groups at each difficulty level indicated that responses tended to be higher for Heavy Vest participants when the standard was low, but were higher for Light Vest participants when the standard was high ($ps < .05$).

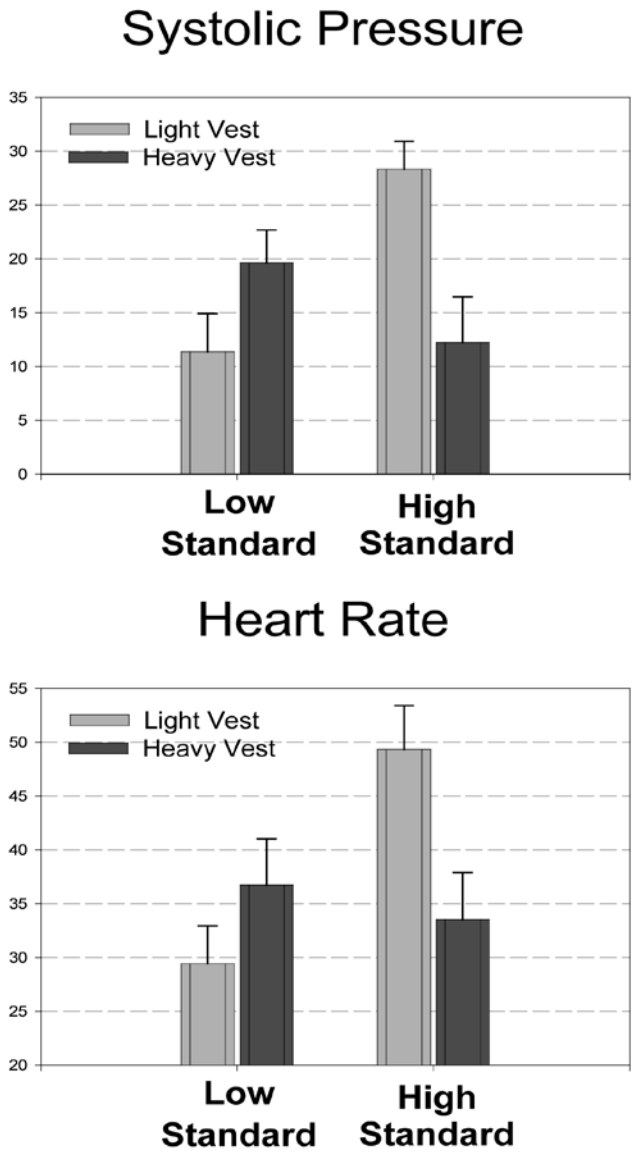


Figure 2. Cardiovascular responses (with standard errors) for Light- and Heavy Vest (i.e., Low- and High Fatigue) participants at different standard levels during the cycling period (Study 1).

Discussion

The subjective energy and tiredness ratings supported our assumption that participants walking with the heavier vest would fatigue more quickly. Surprisingly, fatigue ratings did not, although it is of note that means were ordered as expected.

The SBP and HR response data comported with predictions and the interactional analysis of fatigue influence insofar as they showed different (opposing) effects of fatigue at low- and high levels of task difficulty. However, their interpretation was clouded by the fact that participants wore their vest while they were cycling. This aspect of the procedure maximized the extension of findings to obesity effects that might be found in real life, but also allowed for the possibility that the observed CV effects were due not to the fatigue generated by the treadmill exercise, but rather to the weight of the vest being worn while cycling. That is, it could be that Heavy Vest participants exerted more effort in the low standard condition and less effort in the high standard condition simply because they were more weighted down. This alternative possibility seemed unlikely because participants were in a recumbent sitting position when they cycled, which should have minimized or eliminated the effect of vest weight. Nevertheless, it could not be ruled out and called for additional research.

Study 2 was designed to address the alternative interpretation above. It involved a procedure virtually identical to that in Study 1, with two crucial exceptions. First, it included no treadmill period. Second, it included only heavy vest conditions. Specifically, all participants were assigned the heavy vest when they arrived and cycled wearing that vest. Participants also completed only the initial affect checklist.

The logic associated with Study 2 was twofold. First, if vest weight during cycling was the key determinant of participants' CV responses in Study 1, then the CV results for Study 2 should closely resemble the CV results obtained in the heavy vest conditions of that study. Specifically, one should find in Study 2 a reduction in CV response moving from the low- to the high standard condition. Second, if treadmill fatigue was the key determinant of participants' CV responses in Study 1, then CV results for Study 2 should be different from those obtained in the heavy vest conditions of that study. Specifically, one should find that without the fatigue generated by the treadmill activity, CV responses increase moving from the low- to the high standard condition.

A working assumption in Study 2 was that recumbent positioning during the cycling period in fact minimized the influence of vest weight. Thus, the prediction was that SBP and HR responses would rise, rather than fall, from the low- to the high standard condition.

Method: Study 2

Participants

Participants were 36 normal weight female undergraduate volunteers, most of Western European or African heritage. They received class credit for participation and were assigned randomly to either a low- or high standard condition.

Measurement of CV Responses and Procedure

CV samples were obtained using the same equipment that was used in Study 1. Sampling periods (baseline and cycling) also were the same as in Study 1. The procedure was the same as that Study 1 with three exceptions. First, all participants were assigned the heavy vest following the point at which their height and weight were measured. Second, the treadmill period was omitted. All participants proceeded to the cycling period when the baseline period was completed. Third, the affect checklist was administered only at baseline. It also is of note that all standard instructions were delivered through the laboratory public address system.

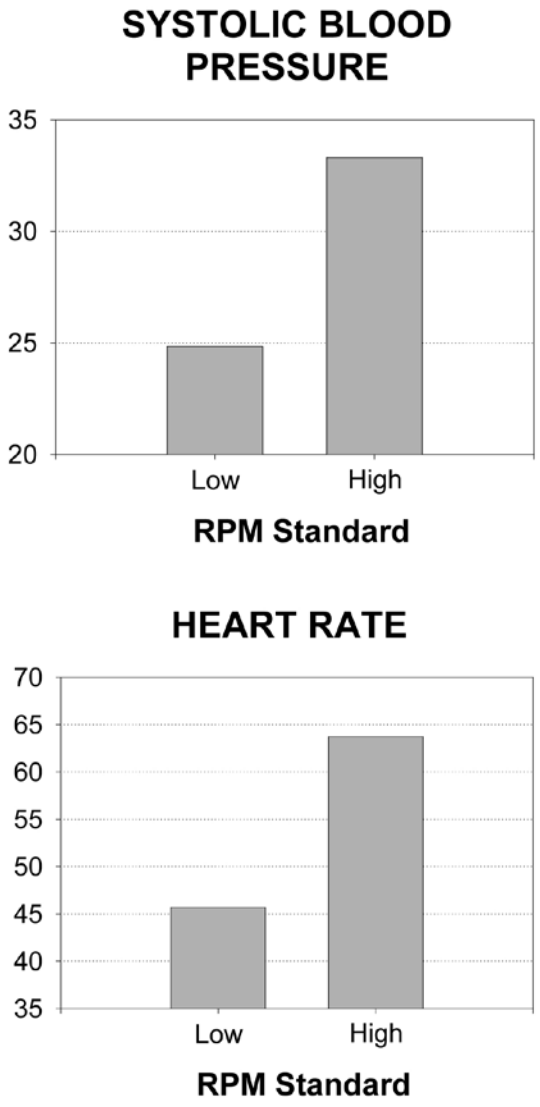


Figure 3. Cardiovascular responses for Heavy Vest participants at different standard levels during the cycling period (Study 2).

Results

Tiredness, Energy and Fatigue

Base tiredness, energy and fatigue scores were analyzed with one-way (standard) ANOVAs, which produced no effects.

Cardiovascular Measures

One way ANOVAs on the base CV data yielded no effects. CV change scores were computed as they were in Study 1, with analyses focusing again on values obtained in the 1st, 3rd and 5th measurement periods. ANOVAs yielded a standard effect for HR (p = .01), and a marginally reliable standard effect for SBP (p = .10). In both cases, the effect reflected stronger responses for High Standard participants (Figure 3).

Discussion

In Study 1, CV values for Heavy Vest participants tended to decline moving from the low- to the high-standard condition. By contrast, CV responses in Study 2 rose from the low- to the high-standard condition – just as they did in the light vest conditions of Study 1. This suggests that vest weight during cycling was not sufficient to produce the response pattern observed for Heavy Vest participants in Study 1. Instead, it appears that treadmill fatigue was the key determining factor.

Conclusions and Comments

Together, the present studies provide fresh and persuasive support for the interactional fatigue analysis. Study 1 showed the crossover response pattern implied across the different fatigue and difficulty conditions. Study 2 provided evidence that the crossover pattern was produced by fatigue rather than vest weight during the cycling period.

Insofar as our vest weight procedure approximates the experience of carrying more and less weight in real life, these findings have implications for effort and CV responses of obese and non-obese people. Specifically, they imply that obese people might have special fatigue trajectories that would cause them to display increasingly unique CV responses over the course of a typical day. So long fatigued obese people view success as possible and worthwhile, they should evince stronger effort and CV responses, with the latter having potentially pathological consequences (e.g., Glass, 1977). One also would expect fatigued obese people to be especially prone to disengagement in the face of certain challenges, which presumably would have an adverse impact on performance.

Naturally, inferences about obesity influence on fatigue, effort and CV response should be made guardedly. Wearing a weighted vest is not the same as being overweight. One can imagine adaptive mechanisms that would attenuate or circumvent entirely the unfavorable outcomes mentioned above. For example, it could be that heavier people develop strength in relevant muscle systems that allow them to carry their weight more easily. Clearly, additional non-simulation research will be needed before confident conclusions can be drawn.

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2010 Barry M. Goldwater Scholarship Recipients

Timothy Fernandez

If UAB students Tamara Burleson, Atbin Doroodchi, and Shweta Patel had one lesson to share with future undergraduate researchers, it would be the importance of perseverance. "Don't give up! Whatever your goal is – Don't take no for an answer! Don't be disappointed if it doesn't seem like you're doing anything. The process is gradual. Don't give up when you make your first mistake! The best things you learn come from them." Throughout their research careers, they have achieved high levels of academic merit through their strong work-ethic, self-drive, and perseverance: the qualities that exemplify Goldwater Scholars.

The Barry M. Goldwater Scholarship is given to highly qualified undergraduate researchers who excel in academics and want to pursue a research career in the fields of mathematics, science, engineering, or computer science. For the 2010-2011 academic year, 278 of these scholars, 5 from Alabama, were selected from 1,111 applicants to receive the award, and Tamara, Atbin, and Shweta are the tenth, eleventh, and twelfth UAB undergraduates to be given the prestige. For each of them, the award symbolizes more than just the honor.

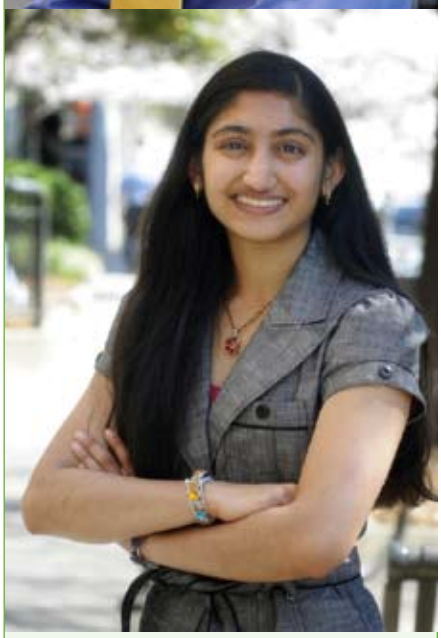
Tamara Burleson, a supplementation instructor for Organic Chemistry from Red Bay, Alabama, is a senior pursing a degree in Chemistry and a minor in Biology. Currently, her career plan is to get her MD/PhD and become a research physician specializing in medical oncology. She is working in the laboratory of Christopher Willey at UAB's Radiation Oncology Center and is investigating the links between specific proteins and radiation treatment effectiveness. Since a very young age, the value of hard labor and independence has been instilled in her, and these characteristics translated into the desire for academic research. Tamara says that winning the award still feels surreal, yet she believes that the sincere effort she put into the process was just as rewarding and beneficial. For her, the opportunity to go out and teach herself independently is an experience she will value for the rest of her career.

Atbin Doroodchi, a sophomore in the Science and Technology Honors Program from Birmingham, Alabama, works in the lab of Yuqing Li, Ph.D., investigating the function of the homolog of *BTBD9* gene in a roundworm *Caenorhabditis elegans*. The *BTBD9* gene has been implicated to be a susceptibility gene for restless legs syndrome and Tourette syndrome. He is pursuing his dual bachelor degrees in Molecular Biology and Mathematics. He hopes to be a trained neurologist with a PhD in Cellular Biology and conduct neurobiology research for the National Institutes of Health or a leading research university specifically on the pathophysiology of movement disorders. For Doroodchi, the Goldwater Scholarship manifested from his own drive and determination. Even though lab work does not produce results every time, he always finds the joy and the ambition to form a new hypothesis. He believes this honor will set off a domino effect to open his future to greater scientific endeavors. Atbin is grateful for the opportunity to work in such cutting-edge research, and he encourages students to apply if they feel eligible.

Shweta Patel, a member of the University Honors Program from Birmingham, Alabama, is a senior in the lab of Trygve Tollefsbol, Ph.D., investigating the role of natural compounds in breast cancer prevention. She is pursuing a degree in Molecular Biology and in the process of applying to medical schools and MD/PhD programs to hopefully pursue a career in academic medicine. Whether in the classroom or one on one instruction, Patel hopes to establish a career epitomizing the mentor/student dynamic. Upon winning this award, she emphasized that the entire journey and accomplishments should be regarded as "a group effort – more than just an individual achievement." For her, the award should be seen as the product of great mentorship, from family to faculty, throughout her entire education.

...the importance of perseverance. "Don't give up! Whatever your goal is – Don't take no for an answer! Don't be disappointed if it doesn't seem like you're doing anything. The process is gradual. Don't give up when you make your first mistake! The best things you learn come from them."

Undergraduates like Tamara, Atbin, and Shweta add to the prominence that is UAB research. Students with extensive research experience should consider applying for the Barry M. Goldwater Scholarship. It is a prestigious honor, and the opportunities and facilities available here at UAB make students highly competitive. More information can be found at http://www.act.org/goldwater/ as well as https://star.uab.edu/bitx/star/live/.



Top to bottom: Tamara Burleson, Atbin Doroodchi, Shweta Patel

2011 Inquiro Submission Guidelines

Any student participating in scientific research at UAB is invited to submit a research paper for submission in the 2011 issue of Inquiro. Papers will be subject to student and faculty review.

The deadline for submissions in May 13, 2011; however, students participating in summer research at UAB or another institution are encouraged to submit by August 31, 2011.

Initial submissions should follow these guidelines:

1) 12 point font, double spaced, pages numbered with the author's name appearing in a header on every page (further formatting will be required upon acceptance).

2) Figures, tables, and graphs should be submitted in their original formats in the highest resolution possible as separate files. A .tiff file at 300 dpi is ideal.

3) All research papers should be submitted with the Inquiro Permission to Publish Form.

Staff also invites students to submit research narratives, interviews with faculty members, and science related editorials.

Short Reports: These reports are short papers derived from the text of science posters. Please convert the original poster to a Word document which includes all text, figures, tables, and images from the poster. As above, images should be submitted additionally as a separate file. The suggested length is 2,500 words.

Research Narratives/Other: If students would like to submit editorial or narrative pieces related to scientific research, they may certainly do so. The journal staff will review the article and consider it based on relevance and quality. The suggested length is 900 words.

Anyone who wishes to join the Inquiro staff should fill out the application on our website.

Pleases send submission or questions to
sciencejournal.inquiro@gmail.com

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inquiro staff

Chief Editors

Andrew Buie

Andrew Buie is a senior Chemistry major with a Spanish minor at UAB. He is a member of the Chemistry Fellowship, the University Honors Program, and the newly created Spanish for Specific Purposes Certificate program. Deeply immersed in the Student Affiliates of the American Chemical Society (SAACS), Andrew enjoys serving as a teaching assistant in the UAB Chemistry Department and leading Chemistry demonstrations within the Birmingham community. Currently, he is involved with UAB Opera's spring production of Purcell's *Dido and Aeneas*. In Fall 2011, he plans to attend Osteopathic Medical College to obtain his D.O. degree.

Shweta Patel

Shweta Patel is a senior Biology major with a concentration in Molecular and a Chemistry and Spanish minor. She is a member of the University Honors Program and the pre-health honor society Alpha Epsilon Delta. Currently, she is conducting breast cancer research in Dr. Trygve Tollefsbol's lab. Her professional career goal is to practice medicine in an academic setting and to teach and conduct research at a leading research university where scientific projects and clinical projects complement each other. Outside of academics, Shweta has an unyielding passion for intramural sports.

Assistant Editors

Khushboo Jhala

Khushboo Jhala is a junior in the Early Medial Acceptance Program at UAB. She is majoring in International Studies and minoring in Chemistry with the intent of pursuing a degree in medicine and using a global background to address international medicine. Her research interests include analyzing stage transitions of different types of cancers as well as preventative medicine in Neuropsychology. Her favorite food is mac-and-cheese.

Rachael Rosales

Rachael Rosales is a sophomore studying Biology. She is a member of the University Honors Program (UHP) and the Early Medical School Acceptance Program (EMSAP). In addition to her scientific interests, Rachael has done research in the UAB Philosophy Department and participates in UAB Ethics Bowl. She plans on pursuing a career in the medical field after her undergraduate career.

Staff Writers

Ashruta Patel

Ashruta Patel is a senior Chemistry major and a Biology and Mathematics minor. She is currently working on her honors thesis research in the Department of Psychiatry and Behavioral Neurobiology with Dr. Rita Cowell. On her free time, she likes to travel, meet new people, volunteer, and play soccer.

Atbin Doroodchi

Atbin is a junior Molecular Biology and Mathematics double major. Atbin is a member of Science and Technology Honors Program, Math Fast-Track program. Atbin currently works on his honors thesis in the Department of Neurology under the direction of Dr. Yuqing Li for which Atbin received 2010 Barry M. Goldwater Scholarship. Outside of school, he volunteers in Greenbrier at the Altamont, and he enjoys running.

Helen Lin

Helen Lin is a junior Molecular Biology major and a Chemistry/Linguistics double minor. She is a member of the Early Medical School Acceptance Program and president of the Phi Sigma Biological Honor Society. Currently, she is researching the characteristics of membrane targeting of the protein Golgi-specific brefeldin-A sensitive binding factor 1 (GBF1), which is important in cellular trafficking. With a combined MD/PhD degree she plans to practice medicine and conduct research at a leading research university. When not in lab or in class, she volunteers her time with Scrollworks teaching piano and clarinet.

Kavita Nadendla

Kavita Nadendla is a junior double major in Biology and Neuroscience with a minor in Chemistry. She is a member of the University Honors Program and the Early Medical School Acceptance Program. She has been performing research for nearly two years on the genetics of schizophrenia in Dr. James Meador-Woodruff's laboratory for the Honors in Biology program. Kavita is also an officer for the Asian American Organization and a tutor in Biology and Chemistry. She volunteers with the after-school program at Glen Iris Elementary to tutor children. Upon graduation, Kavita will attend medical school in the Fall of 2012.

Natalie Mitchell

Natalie Mitchell is a senior Molecular Biology major. She is a member of the Science and Technology Honors Program and 5th Year Masters in Biology Program. Natalie is conducting her thesis research in the Department of Biology with Dr. Trygve Tollefsbol. In addition, she is a member of the UAB softball team. Following graduation in 2011, Natalie plans to attend medical school.

Timmy Wang

Timmy Wang is a senior Biology major with a Chemistry minor. He is a member of the University Honors Program, Alpha Lambda Delta, and the Early Medical School Acceptance Program. His hobbies are reading, watching movies, and playing tennis. After graduation, he plans on attending medical school.

Timothy Fernandez

Timothy Fernandez is a sophomore Chemistry major with a concentration in Biochemistry. He is a member of the University Honors Program and is the UHP volunteer coordinator for Habitat for Humanity. He is a Chemistry Scholar as well as the President of the Undergraduate Research Association. His hobbies include playing music and working out. Currently, he is working under Dr. Jamil Saad in the Department of Microbiology. He hopes to pursue an MD/PhD after graduation.

Toral Patel

Toral Patel is a senior Molecular Biology major with a Chemistry minor. She is a member of the Science and Technology Honors Program, and chair of its Peer Mentor Program, and the Phi Sigma Biology Honors Society. She is also the historian for the pre-health honors society Alpha Epsilon Delta. Her interests include traveling, painting, and reading. Currently she is working on her Master's thesis in the Department of Medicine with Dr. David Garber. After completing her degree in 2011, Toral plans to attend medical school.

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acknowledgements

Without the help and support of UAB faculty and staff, the vision of Inquiro could not have been made a reality. Many thanks to the following individuals:

2010 Faculty Reviewers:
Dr. Charles Amsler
Dr. Aaron Catledge
Dr. Anne Cusic
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Dr. Shahid Mukhtar
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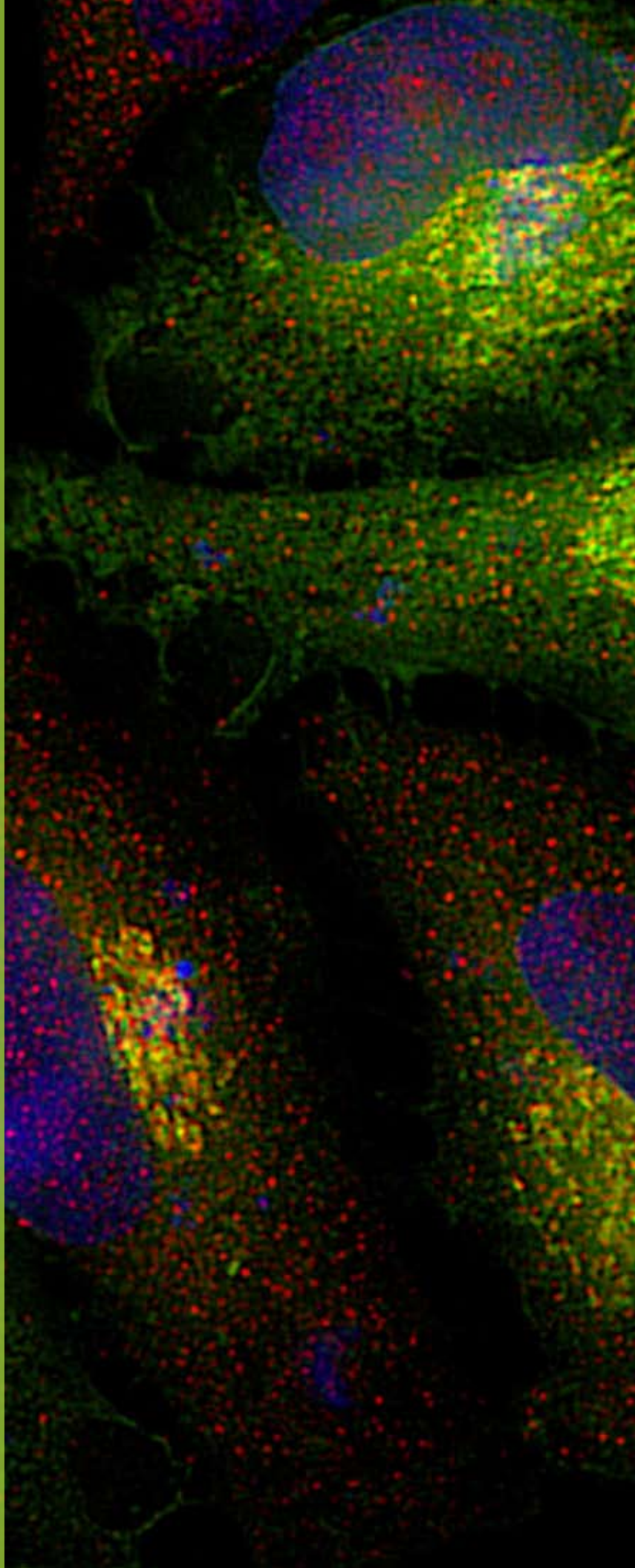
Dr. Mickie Powell
Dr. Cynthia Ryan
Dr. Mike Sloane
Dr. Thane Wibbels

Dr. Mike Sloane and the University Honors Program
Dr. Diane Tucker and the Science and Technology Honors Program
Dr. Chris Reaves and the Office of Undergraduate Research
Amanda Childress, UAB Printing Solutions (production and printing)

A special thanks to Dr. Thomas DiLorenzo, Dean of the College of Arts and Sciences, for his support of this endeavor.

The production and publication of this journal was made possible through the funding supplied by the College of Arts and Sciences of the University of Alabama at Birmingham.



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Volume 4 • 2010

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