NEW JERSEY MEDICAL SCHOOL
OF UMDNJ

Funded by NCI Cancer Education Program Grant R25 CA019536-29

CANCER SUMMER STUDENT
RESEARCH ABSTRACTS

2008

Harvey L. Ozer, MD, Principal Investigator and Director
Gwendolyn M. Mahon, PhD, Co-Director
Lorie-Anne Phillips, M.S., Coordinator
The Cancer Summer Student Research Program has been in existence at NJMS since 1969, is supported through an NCI Cancer Education Program Grant (Principal Investigator, Harvey L. Ozer, MD, R25CA019536) and is one of only eight of its kind currently funded by the NCI. This program, which has been continuously funded by the NCI for 36 years, provides a unique eight-week research experience for New Jersey Medical School's first and second-year medical students as well as undergraduate students enrolled in our combined BS/MD seven-year program. This year 23 medical students and 11 undergraduates participated in biomedical research activities in both laboratory and clinical settings at either the NJMS-UH Cancer Center, or the broader cancer center research community on the Newark Campus, while developing a close working relationship with their faculty mentors.

With the recent opening of the new NJMS-UH Cancer Center and the expansion of oncology programs on the Newark campus, the administration of the summer program is now focused at the Cancer Center where it is a part of the center's broader training mission in cancer education for both PhD and MD scientists. The Program Directors, Drs. Ozer and Mahon, and the Program Coordinator, Ms. Lorie-Anne Philips, expanded the program this year to include student attendance at the NJMS-UH Cancer Center multi-disciplinary tumor boards, a workshop on scientific poster presentation, the generation of a group website that allows participants to interact with each other over the internet, pod-casting of the weekly seminar series, and a new on-line survey used to evaluate the program. All students were required to present their research at a poster session during the concluding symposium, one of the program highlights. Movie clips of these presentations, as well as other information about the program, are available for viewing at the Cancer Center website under the Training tab at the following URL: http://njmsuhcc.umdnj.edu/home/index.php

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FACULTY EXECUTIVE ADVISORY COMMITTEE

The faculty executive advisory committee is responsible for carefully reviewing and selecting the projects submitted by faculty, as well as review the structure of the forthcoming Cancer Summer Program. We appreciate the time they took out of their busy schedules to evaluate the program and help make it a success.

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CANCER EDUCATION PROGRAM FACULTY

While the Cancer Center building currently houses 12 laboratories, the broader Cancer Center Community (CCC) is composed of 96 investigators from several departments of the NJMS, the New Jersey Dental School and the School of Public Health. It is a truly interdisciplinary and interdepartmental group that embraces research enterprises extending from basic science programs in tumor immunology, signal transduction, cancer stem cells, tumor virology, cell biology, animal models, and imaging, to health disparities and clinical trials. The faculty mentors that participate in this program are devoted to training and education and have played a pivotal role at NJMS in cancer education for over 30 years. The 2008 participating faculty and their respective departments are listed below.

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Wen-Ching Liu, PhD  Department of Radiology
W. Clark Lambert, MD, PhD  Department of Pathology
1. **RON AVRAHAM (NJMS 2011)**

**VIEWS ON MEDICARE AND CANCER IN THE ELDERLY LATINO COMMUNITY**

Mentor: Ana Natale-Pereira, MD, MPH, (Division of Academic Medicine, Geriatrics, and Community Programs)

**Objective:**

To identify trusted sources of cancer information used by members of the elderly Latino community and to assess their understanding of Medicare policy, especially in regards to cancer. This study was part of a larger parent project, the New Jersey Medical School – Cancer Prevention and Treatment Demonstration (CPTD), whose overall goal is to assist Latino seniors as they navigate the health care system and facilitate screening, diagnosis, and treatment of breast, cervical, colorectal, and prostate cancer. This project is funded by the Center for Medicare and Medicaid Services (CMS) and allows for implementation of the Multilevel Approach to Community Health (MATCH) planning program. The MATCH planning model is organized into five phases: 1) Health Goals Selection, 2) Intervention Planning, 3) Development, 4) Implementation, 5) Evaluation.

A baseline questionnaire was developed to assess the status of various cancer screenings among prospective participants and to gather pertinent medical history, demographic background information and data particular to the Latino community. An Addendum questionnaire was then created in collaboration with Johns Hopkins University and MD Anderson Cancer Center to explore a range of healthcare issues that were specific to minority communities, and then adapted to the Latino community in the NJMS-CPTD project. This summer project analyzes a portion of the data collected from the Addendum questionnaire administered during the baseline interview in order to:

1) Assess baseline knowledge of Medicare benefits with regards to cancer screenings and treatments.
2) Determine what information sources people in this community trust to educate themselves about cancer.
3) Explore the level of understanding of Medicare policy to allow for proficient utilization of its prevention and screening coverage.

**Background:**

The Latino community is the fastest-growing minority population in the United States,¹ expecting to triple in size and accounting for the majority of the nation’s population growth from 2005 through 2050, at which point it will make up 29% of the U.S. population². Within the Latino community in the U.S., cancer ranks as the second leading cause of death after heart disease³. Considering the rapid growth of the elderly population in the United States, especially within the Latino community, it is concerning to note that Latinos are disproportionately less likely to have

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visited a doctor within the past year when compared to groups of similar income\textsuperscript{4}. This becomes a critical factor in regards to cancer, since it is a disease of older age, with the risk of disease nearly doubling at age 65.

As a minority group, Latinos are unique in that they remain the only ethnic group in the U.S. whose rates of not having a usual source of medical care have risen\textsuperscript{5}. In addition, Latinos have been shown to have poorer quality of care than non-Latino whites for around 40\% of quality measures, including receiving screening for cancer\textsuperscript{6}. The lack of consistent and appropriate healthcare care for members of the Latino community combined with the community’s own explosive growth has led to the use of cancer prevention and control strategies such as education, empowerment, and screening for this national health care priority.

The Centers for Medicare and Medicaid Services--Cancer Prevention and Treatment Demonstration (CMS-CPTD) Project is a Congressional Mandate aimed at reducing disparities in cancer care for minority populations. The NJMS-CPTD project was established to institute an innovative cancer care facilitation program for Latinos in Newark, New Jersey. The aim is to reduce the disparities observed in screening, time to diagnosis, and treatment services, by implementing the MATCH planning model to incorporate community outreach, education, access to screening, and patient assistance in every aspect of the cancer care continuum. This reduction in disparities is achieved by the structure of the project which provides assistance to Latino seniors as they navigate the health care system and facilitates screening, diagnosis, and treatment of breast, cervical and prostate cancer.

**Methods:**

This community-based project is a randomized intervention control study. Currently, a total of 644 cancer-free/screening and 55 cancer positive/treatment participants are enrolled. Both groups complete a baseline questionnaire provided by CMS to assess prior cancer screenings, pertinent medical history and demographic background information. In an effort to capture important information regarding the minority population view of Medicare, their trust of the health care system, their view of discrimination and acculturation issues, an addendum questionnaire was also administered. For this summer project preliminary data from 100 completed Addendum surveys was analyzed. Sets of data from two sections of the Addendum were selected for review. The first set of questions assessed the participants’ understanding of Medicare policy, especially in regards to cancer treatment and screening. The questions were designed to reflect the participant's own perspective on their knowledge of Medicare policy and benefits. The second set of questions was intended to assess the trust-worthiness of a number of various sources of cancer information.

**Summary:**

\textsuperscript{4} Marsha Lillie-Blanton et al., op. cit.
\textsuperscript{5} Marsha Lillie-Blanton, Osula Evadne Rushing, and Sonia Ruiz, Key Facts: Race, Ethnicity & Medical Care, Update June 2003 (Menlo Park, CA: Kaiser Family Foundation, June 2003), available online at \url{http://www.kff.org/minorityhealth/upload/Key-Facts-Race-Ethnicity-Medical-Care-Chartbook.pdf}.
Medicare Knowledge:
A striking finding of this study was the lack of overall knowledge that many of the participants expressed in regards to Medicare policy and benefits. A disproportionate number (41%) claimed to seriously lack knowledge about the program, while an even higher number (45%) felt that they did not know what they thought they should in regards to payment for medical services. More than half of the participants (52%) did not understand Medicare coverage for screening tests. In addition, an even higher number (57%) felt that they knew almost nothing about Medicare coverage for cancer treatment.

Sources of Cancer Information:
An overwhelming number of participants (78%) trusted their doctor other healthcare professional for information regarding cancer. The second most trusted source for information was the Television (30%). Other sources of media such as newspapers, radio, Internet and magazines were not significantly trusted sources of information regarding cancer. Of all the sources, the Internet was the least trusted for information regarding cancer.

Conclusion:
The Latino community in the United States is currently grappling with the growing epidemic of cancer. Though the Latino population has lower rates for incidence and mortality for most cancers, they unfortunately also have lower rates of cancer screening, advanced stage of diseases at diagnosis, and overall poor cancer outcomes. A number of the participants showed a limited level of understanding of Medicare policy. This is especially concerning in light of the fact that 91% of elderly Latinos in the continental U.S. are covered under Medicare. In addition, Latinos utilize Medicare benefits to a significantly lesser degree than do non-Latinos. This lack of familiarity with Medicare coverage and its associated benefits appears to encompass all aspects of the program and as a result deprives certain members of the Latino community from undergoing effective and necessary cancer screening exams, and, if needed, cancer treatments. To combat this growing trend it seems that effective steps should be taken to educate Latinos about both the importance of cancer screenings and instruction of Medicare policies, the latter of which has been shown to increase adherence to screenings, such as mammograms, by as much as 6-fold.

Cancer education has been shown to be a significant predictor of cancer screening among members of the Latino community. As shown in this study, the strong expression of trust in Doctors and healthcare professionals provides compelling evidence to support the notion that the most effective cancer education can be done in a Doctor’s office or other healthcare setting. A possible avenue for further research might be to unite this finding with an appropriate cancer-screening pamphlet for Latinos in order to maximize the effectiveness of education done in the physician’s office. In addition, the poor showing of trust in the Internet suggests that efforts to educate the elderly Latino community through this venue might not be an effective approach.

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2. PRIYA BOLIKAL (NJMS 2012)

NUCLEAR DISTRIBUTION OF BCR AND ITS ASSOCIATION WITH XPB CHANGES IN RESPONSE TO UVC IRRADIATION

Mentor: Ian Whitehead, PhD, (Microbiology and Molecular Genetics)

Objective:

It is known that nearly all patients with chronic myelogenous leukemia (CML) express the fusion protein BCR-ABL. It has also been shown that BCR-ABL interacts with the DNA helicase, xeroderma pigmentosum B (XPB) that is involved in both transcription and nucleotide excision repair (NER). NER is a process by which cells repair DNA damage caused by UV irradiation. This process is disrupted by the interaction between BCR-ABL and XPB. Since the interaction takes place on the BCR portion of BCR-ABL, and XPB has been shown to interact with BCR alone, it is important to understand the role of the BCR/XPB interaction in NER before we can understand how BCR-ABL is affecting it. Thus, the aim of this project was to investigate the interaction between BCR and XPB in response to UVC irradiation.

Methods:

Three irradiation experiments were done in order to observe the interaction between BCR and XPB.

First, human HaCaT cells (epithelial cells) were irradiated using increasing levels of UVC light and compared with controls using immunofluorescence. A total of six plates of cells were used: two plates received 30J/m² of UVC irradiation, two received 100J/m², and two received no irradiation. The cells were left to incubate for 3.5 hours at 37°C and were then fixed in 3.7% formaldehyde. Cells were imaged according to immunofluorescence imaging protocol.

In the second experiment, human 293T cells (embryonic kidney cells) were irradiated at 30J/m² and harvested at four different time points after irradiation. The time points were 15 minutes, 45 minutes, 2 hours, and 3.5 hours after irradiation. These cells were compared with control cells that received no irradiation. After harvesting the cells, an immunoprecipitation was done. First, the IP was done with XPB and then probed with BCR to show BCR/XPB interaction. Then an IP with XPB and an IB with XPB were done to show total XPB at each time point.

The third experiment was done by irradiating human HaCaT cells at 30J/m² and doing a cell fractionation followed by an immunoprecipitation to examine the BCR/XPB interaction in the nucleus and cytoplasm after irradiation. Six plates of cells were used- three were irradiated and three were controls. The cells were fractionated following protocol. 40 µl of each lysate was taken out and two immunoprecipitations were done, each using 20 µl of lysate and a Lamin A/C antibody (nuclear marker) or a Rock1 antibody (cytoplasmic marker). These immunoprecipitations were done in order to confirm that the cells were separated into nuclear and cytoplasmic fractions without contamination. An immunoprecipitation followed by Western blotting was done on the remaining volume of lysates. First, the IP was done with XPB and then probed with BCR. Then an IP with XPB and an IB with XPB were done.
Summary:

The immunofluorescence images from the first experiment revealed a large concentration of BCR within the nuclei of control cells and a scattered staining of BCR after irradiation. Consistent with previous studies, XPB was concentrated mostly in the nucleus with some in the cytoplasm. After quantifying the number of cells that contained the large concentration of BCR in the nucleus, it was found that 82% of control cells had the concentration whereas only 7% of irradiated cells still had it. This data showed a change in distribution of BCR after irradiation, but did not elucidate the effect of irradiation on the BCR/XPB interaction. (Figure 1)

![Percent of Cells with Nuclear Foci Staining of BCR](image)

The second experiment showed that in the 293T cells, the total BCR/XPB interaction did not change in the 3.5 hours after irradiation. The slight variation in XPB/BCR interaction is mirrored in the total XPB, so this variation can be attributed to total protein rather than an effect of irradiation. (Figure 2)

![Western Blots (Figure 2)](image)

The cell fractionation revealed that, although there was no change in the overall BCR/XPB interaction, there was a dramatic increase in interaction within the nuclear portion of the cell. In the cytoplasm, there is a slight increase, but it is not significant. The total XPB does not vary much between control cells and irradiated cells, so here the change is attributable to the irradiation. The third Western shows Lamin A/C, confirming that the cytoplasmic portion is pure. The fourth Western shows Rock 1 in the cytoplasmic fraction as well as some in the nuclear fraction. This is because the fractionation protocol results in any unlysed cells being pelleted with the nuclear fraction. This is normal in this protocol as we enriched for the nuclear portion. (Figure 3)
Conclusion:

It has previously been shown that BCR, a part of the BCR-ABL fusion protein found in CML patients, interacts with the repair protein XPB. It has also been shown that cells expressing BCR-ABL have a disruption in NER, a process involving XPB that repairs DNA damage caused by UV irradiation. To better understand the disruption in repair, a series of irradiation experiments was done on normal, BCR expressing cells to better understand how the interaction between BCR and XPB is affected by UVC light.

The immunofluorescence data showed that BCR changed in distribution after irradiation. The two immunoprecipitations showed that although the total BCR/XPB interaction remained unchanged after irradiation, the interaction within the nucleus increased considerably. We speculate that in non-irradiated cells, BCR is being held in a ready state in a concentrated form within the nucleus, and after irradiation it is being released and is able to interact with XPB. This may mean that BCR plays some role in regulating XPB after irradiation, but further study is needed to determine how the change in interaction is related to NER.

References:

3. STEVEN CARVALHO (NJMS 2011)

MUTATIONAL ANALYSIS OF PHD DOMAINS IN THE GROWTH SUPPRESSOR PHF10

Mentors: Satnam S. Banga, PhD, Harvey L. Ozer, MD (Microbiology and Molecular Genetics)

Objective:

Previous studies suggested the presence of a suppressor gene on human chromosome 6q27. Further investigation of this region showed that overexpression of PHF10 suppresses growth of both normal and immortalized human cell lines. PHF10 is a multidomain protein that has homologs in a number of different species. It contains two PHD domains (referred to as PHD1 and PHD2) towards its C-terminal end, which are typically associated with chromatin modification and regulation of transcription. PHD domains contain a characteristic Cys4-Hys-Cys3 sequence. The cysteine residues interact with Zinc to create PHD fingers and may be important for the protein function. Overexpression causes immortalized cells to arrest in the G1/G0 phase of the cell cycle, causes morphological changes to cells, and induces a senescence-like phenotype. These characteristics imply a possible role in tumor suppression.

Previous data suggest that the PHD fingers are important for growth inhibition and that the absence of PHD domains from PHF10 is growth stimulatory. It has been shown to interact with SETDB1 and KAP1 in vitro, two proteins which are known to methylate histones to suppress transcription. It was our goal to test the importance of the PHD domains in PHF10.

To test the hypothesis that PHD finger sequences are important to the PHF10 function, we designed 4 oligonucleotide primers that contained point mutations aimed to inhibit the function of the PHD domains, by targeting the cysteine residues. Oligo1 inserted Cys292>Gly in Cys1 of PHD1. Oligo 2 inserted Cys313>Gly in Cys2 of PHD1. Oligo 3 inserted Glu357>Stop, and should remove PHD2 from the protein. Oligo 4 inserted Cys363>Gly in C3 of PHD2. We created mutant plasmids using our Oligos as primers for a thermal cycling reaction with the pLPCX retroviral vector, which already contained the gene for PHF10 protein with a hemagglutinin (HA) epitope tag at the C-terminus. DpnI digestion of each thermal cycling reaction product was conducted to digest methylated and hemi-methylated template DNA and leave single stranded plasmids containing our directed mutations. Ultracompetent E. Coli cells were transformed with plasmids containing single mutations, named Mutants 1, 2, 3 and 4 for their respective oligos. Cells were also transformed with plasmids containing Oligos 1, 2 and 4, named Mutant 5. Mutant plasmids from individual bacterial colonies were screened for the presence of the PHF10 gene by BamH1 digestion, since the restriction enzyme cuts once within the gene, and nowhere in the vector. The presence of the directed mutations was then confirmed by sequencing. Mutated plasmids from clones 5-5 and 3-4 were transfected into human 293T cells. Protein expression was tested by Western Blot. Changes in the interaction of Mutant 5-5 with both KAP1 and SETDB1 were tested for by coimmunoprecipitation and Western Blot.

Methods:

Inserting mutations into PHF10 using mutagenic oligonucleotides and the Stratagene QuikChange Multi Site-Directed Mutagenesis Kit

Stock oligonucleotide solutions of 1ug/ul were diluted to 100ng/ul. Reaction mixtures to create Mutant Plasmids 1, 2, 3 and 4 each contained 2.5ul Reaction Buffer, 17ul HzO, 2ul of pLPCX-PHF10HA plasmid (50ng/ul), 1ul of the respective oligo, 1ul of dNTP mix, and 1ul of QuikChange Multi enzyme blend. The Mutant Plasmid 5 reaction mix contained equal amounts
of Reaction Buffer, pLPCX-PHF10HA plasmid, dNTP mix and enzyme blend, but contained 15uL H2O and 1uL each of Oligos 1, 2 and 4. Each reaction mixture was subjected to thermal cycling with an initial segment of 95°C for one minute, followed by 30 cycles of 95°C for 1 minute, 55°C for 1 minute, and 65°C for 15 min (2min/kb of plasmid length). Reaction mixtures were held at 4°C until Dpn1 digestion. 1uL of Dpn1 enzyme was added to each reaction mixture, and incubated at 37°C for 1 hour.

Transformation into XL10-Gold Ultracompetent Cells

45uL of ultracompetent cells and 2uL of Beta-Mercaptoethanol were mixed and, put on ice for 10 minutes and swirled every 2 minutes. 1uL of Dpn1 treated DNA from each of the 5 reaction mixture was added to separate tubes, and put on ice for 30 minutes. Samples were heat shocked at 42°C for 30 seconds, and put on ice for 2 minutes. 500uL of SOC broth was added to each tube, and incubated at 37°C for 1 hour, while shaking at 225 rpm. 100uL and 200uL from each tube was then plated on separate dishes containing LB with carbenicillin and incubated for 16 hours at 37°C.

Screening for colonies containing the PHF10 gene

Individual colonies were picked for each Mutant and grown in 10mL LB with 20uL carbenicillin, and incubated at 37°C for 16 hours, while shaking at 225 rpm. DNA was isolated from each culture using the Qiagen QIAprep Spin Miniprep Kit. 1uL of each isolated DNA was digested by BamH1 in a 15uL reaction, incubated at 37°C for 2 hours, and then subjected to electrophoresis on a 1% agarose gel.

Expression analysis of PHF10 mutants by Western Blot

5ug of DNA from mutants 2-13, 3-4, 3-14, 4-6, 5-5, 5-11 and the pLPCX-PHF10HA plasmid as a positive control, were transfected into 293T cells in serum free media, using 12.5uL of Lipofectamine 2000 (2.5uL/ug DNA). Cells were washed with PBS with 10mM N-ethyl maleimide (NEM). 200uL of extraction buffer with protease inhibitors was added. Extraction buffer was composed of 100mM NaCl, 2mM MgCl2, 1% NP40, 50mM Tris-HCl, pH 7.4 and 10mM NEM. Plates were rocked at 4°C for 10 minutes, before being transferred to microcentrifuge tubes and vortexed to disrupt cells. Contents were centrifuged for 20 minutes at 14000 rpm at 4°C. 30ug of cell lysate from each sample was then subjected to electrophoresis on a 7.5% polyacrylimide gel. Proteins underwent an overnight transfer to a membrane, which was then blocked in 5% milk for 1 hour, probed with primary anti-HA antibody for 2 hours, washed 3 times for 10 min with PBST, probed with secondary anti-rat IgG HRP for 1 hour, and then washed 3 more times with PBST for 10 min.

Analysis of PHF10 Mutant 5-5 Protein-Interaction with KAP1 and SETDB1 by Immunoprecipitation and Western Blot

5ug DNA from Mutant 5-5 or PHF10HA was transfected into 293T cells in serum free media, together with 5ug of Flag-tagged SETDB1 or with 5ug Myc-tagged KAP1, using 25uL Lipofectamine 2000 for each plate. Soluble proteins were isolated in the lysate as described above. 2mg total protein was used for immunoprecipitation experiments. The mix with SETDB1 was immunoprecipitated with anti-Flag antibody, and the KAP1 mix was immunoprecipitated with anti-Myc antibody. Both IPs were then subjected to electrophoresis on a 7.5% polyaclrylimide gel and probed with anti-HA antibody for the presence of PHF10 as described above.

Results:
Screening with BamH1 digestion suggested that mutations were contained in 5 out of 31 clones screened for Mutant 1, 3 out of 20 clones screened for Mutant 2, 4 out of 17 clones screened for Mutant 3, 5 out of 16 clones screened for Mutant 4, and 2 out of 13 clones screened for Mutant 5. Direct sequencing of those clones, by the MRF of NJMS, showed both the directed mutation, as well as the proper PHF10 sequence for 1 clone containing Mutation 1, 2 clones for Mutation 2, 3 clones for Mutation 3, 4 clones for Mutant 4, and 2 clones for Mutation 5. Of the 2 mutant 5 clones, Mutant 5-5 showed mutations for Oligos 1 and 4, whereas Mutant 5-11 contained all three desired mutations (Oligos 1, 2 and 4). Expression analysis showed similar expression of Mutants 2-13, 4-6, 5-11, 5-5 and our positive control, as seen in Figure 1. Expression of Mutants 3-14 and 3-4 could not be evaluated due to the lack of an HA tag on the C-terminus. Immunoprecipitation analysis of Mutant 5-5 showed no change in PHF10 interaction with SETDB1, but appeared to show diminished interaction with KAP1, as seen in Figure 2.

**Figure 1:** Expression analysis of mutant PHF10 proteins as well as wild-type PHF10-HA protein.

**Figure 2:** Analysis of Mutant5-5 Protein-Interaction with KAP1 and SETDB1 by Immunoprecipitation and Western Blot. The Supernatant corresponds to the cell lysate after IP.

**Conclusions:**

We successfully generated mutations in both PHD domains of PHF10, as confirmed by nucleotide sequencing. Mutants containing mutations in either of, or both of these domains showed no change in protein expression after transfection into 293T cells. The negative results for Mutants containing Mutation 3, a stop codon, were expected since the stop codon removes the HA tag, as well as PHD2. Therefore Mutants 3-4 and 3-14 can be thought of as negative controls for this experiment. PHF10 with mutations in both PHD domains appears to show diminished PHF10 interaction with KAP1, but not with SETDB1, as demonstrated by immunoprecipitation and Western blot analysis of Mutant 5-5.
It must be noted that these results are preliminary and must be repeated several times to confirm this change in function. In the future, all mutants should be tested for their interaction with KAP1 and SETDB1. Most importantly, the mutants will need to be tested for their effect on the growth suppression property of PHF10. An anti-PHF10 antibody should also be obtained to test for any effects due to Mutation 3. If none of the mutations created show an effect on protein interactions, other residues in the PHD domains, as well as in other areas of the protein could be mutated to test for their importance in PHF10 function.
4. NORMAN CHAN (NJMS 2011)

DETERMINATION OF THE FIXATIVE FOR THE OPTIMUM PRESERVATION OF DNA STRUCTURE AND FUNCTION IN NORMAL AND XPC FIBROBLAST CELLS

Mentors: W.C. Lambert, MD PhD, (Pathology) and Claude E. Gagna PhD, (New York Institute of Technology, Old Westbury, NY 11568)

Objective:

Patients with xeroderma pigmentosum (XP) are susceptible to skin cancer induced by ultraviolet radiation and must avoid sunlight. XP is an autosomally inherited disease that is associated with defects in nucleotide excision repair (NER). This disease can involve eight different complementation groups (XP-A through XP-G and XP-V) that when mutated lead to the accumulation of mutations in the genome of affected individuals. XPC is the gene that encodes the complementation group protein that is involved in the first step of a subtype of NER known as global genome repair (GGR), which can occur anywhere in the genome. The other type of NER is known as transcription coupled repair, and it differs from GGR because it occurs only at the transcribed strands of active genes. The XPC protein is responsible for recognizing damaged DNA and allowing GGR to occur (1). DNA and RNA molecules have many different structural conformations such as right-handed B-DNA and left-handed Z-DNA (2). Mutations in XPC, a majority of which are nonsense and frameshift mutations, are one of the most frequent causes of XP and are associated with many different types of solid tumors, such as those seen in bladder cancer (3,4).

The purpose of this study was to determine which of six fixatives tested served best to preserve the structure and function of nucleic acids in normal and XPC fibroblast cells. The six fixatives that were evaluated were Carnoy’s solution, Bouin’s solution, 10% neutral buffered formalin (NBF), zinc formalin, 70% ethanol/100% methanol (3:1), and an acetone-containing fixative. The effectiveness of the fixatives was evaluated with H&E staining and observation with a compound light microscope. The next step will involve the use of anti-ds-B-DNA, anti-ss-DNA and anti-Z-DNA polyclonal antibody staining. Our group will determine which of these fixatives is best for immunohistochemistry.

Methods:

Normal fibroblasts and those that possessed mutated XPC genes were grown in separate tissue culture flasks for a couple of weeks before being used for the procedures of this study. Twenty-four eight-chambered slides were used. They were of three different types: 8 Lab-Tek™ II - CC² ™, 14 Lab-Tek™ II and 2 Labtek™ I. Four of the Lab-Tek™ II - CC² ™ chamber slides and all of the Lab-Tek™ I and II chamber slides were pretreated with poly-L-lysine (PLL). On the following day, all of the chamber slides were seeded with the two types of fibroblasts. The cells were detached from the flask by treatment with trypsin and then placed in eppendorf tubes to be counted using a hemacytometer and a compound light microscope. After obtaining satisfactory counts, it was determined that in order to make suspensions of each type of cell that contained 60,000 cells/ml 5.3 ml of the XPC suspension and 3.9 ml of the normal suspension were needed. These amounts of cellular suspension were centrifuged. The resulting cell pellet was used to make the 60,000 cells/ml suspensions. Dilutions were made to create 15 ml of a 30,000 cells/ml and a 15,000 cells/ml suspension and 30 ml of a 7,500 cells/ml suspension of each cell type. Following creation of these four dilutions of the normal and XPC fibroblast suspensions, 0.5 ml of these suspensions were added to each of the wells of the chamber slides. After setting all twenty-four slides in this way, they were put into the incubator
for a day. On the next day, the media in the wells of all twenty-four slides was replaced with fresh media.

After another day had passed, the fibroblasts were treated with the six fixatives tested in this study. The six fixatives employed were Carnoy’s solution, Bouin’s solution, 10% neutral buffered formalin (NBF), zinc formalin, 70% ethanol/100% methanol (3:1), and an acetone-containing fixative. The actual formula of the acetone-containing fixative is: 250 ml 37% formaldehyde, 200 mg Na₂HPO₄, 1000 mg KH₂PO₄, 200 ml of distilled water, and 450 ml of acetone. The procedure used in applying each fixative was slightly different. Four slides were fixed with each of the fixatives and the type of slide used with each fixative was chosen at random. All steps were performed under a ventilated hood. The first few steps in fixing all of the chamber slides was the removal of the media using vacuum suction, rinsing of each well, and removal of the chambers using the key device included in the chamber slide kit.

Using Carnoy’s solution, 1 Lab-Tek™ II - CC²™ pretreated with PLL, 1 Lab-Tek™ II - CC² without pretreatment, and 2 Lab-Tek™ II were fixed. These slides were placed into a staining jar filled with Carnoy’s solution for 3 min. They were then placed into 100% ethanol four times, and the slides were in the ethanol for 10 min prior to each change.

A Lab-Tek™ II - CC²™ pretreated with PLL, one Lab-Tek™ II - CC²™ without pretreatment, 1 Labtek™ I, and 1 Labtek™ II were fixed with Bouin’s solution. The slides were immersed in Bouin’s solution for 3 min and subsequently washed with distilled water 10 times. The slides were then washed twice with 70% ethanol. For the first wash, the slides were immersed in the ethanol for 1.5 hrs. The second wash lasted 30 min. All slides were air dried.

Four Labtek™ II slides were fixed with 10% NBF. The slides were immersed in 10% NBF for 5 min. The slides were then washed with distilled water three times for 3 min each dipping in the water.

A Lab-Tek™ II - CC²™ pretreated with PLL, one Lab-Tek™ II - CC²™ without pretreatment, and 2 Labtek™ II were fixed with zinc formalin. The slides were immersed in zinc formalin for 15 min. The slides were then washed with distilled water three times for 3 min each. The slides were air dried.

For the 70% ethanol/100% methanol (3:1) fixative procedure, 3 Labtek™ II chamber slides were fixed by immersion in the fixative for 5 min and then washed with 70% ethanol twice for 3 min each. For the fourth chamber slide that was designated to be fixed with this particular fixative, the procedure differed because the slide had cracked into three pieces during removal of the chambers with the key device. One of the three pieces of this slide (the piece with the label) was salvaged. However, it had been left under the hood to dry for 30 min prior to its fixation with the ethanol/methanol fixative. It was immersed in the fixative for 10 min.

The last fixative used was the acetone-containing fixative. It was used to fix 1 Lab-Tek™ II - CC²™ pretreated with PLL, one Lab-Tek™ II - CC²™ without pretreatment, 1 Labtek™ I, and 1 Labtek™ II. The slides were immersed in the fixative for 1 min and then washed with distilled water three times for 3 min each.

All slides were air dried for three days. Some of the slides were stained with hematoxylin and eosin (H&E). Six slides were chosen at random to be stained with H&E (all were Lab-Tek™ II chamber slides). Each of these slides had been treated with a different fixative. To H&E stain these slides, they were first subjected to a xylene substitute three times for 2 min each. The chamber slides were then washed in absolute alcohol 10 times. Next, they were dipped 10 times each in two changes of 95% alcohol. Distilled water was used to wash the slides until the water ran off the slides evenly. The slides were immersed in Harris hematoxylin with acetic acid for 2 min and then dipped 10 times each in two changes of distilled water. Bluing agent was applied to the slides until the cells appeared blue. The slides were then washed again in two changes of distilled water by dipping 10 times each. Following that, the slides were dipped in eosin 15 times then washed in two changes of 95% alcohol (13 dips). The slides were subsequently washed in
absolute alcohol then xylene substitute, with three changes of each. While still wet with the xylene substitute, the slides had Permount mounting medium applied to them and then immediately had a coverslip placed on them. The slides were air dried for two days before being observed with a compound light microscope. Photographs of all slides were taken with a Cannon digital camera.

Summary:
After reviewing all the photographs taken of the slides, it was determined that the slide that best served the purpose of preserving the structure and function of DNA in the normal and XPC fibroblast cells for H&E staining was Bouin’s solution (Figure 1). The nuclei and the entities within it appeared to be the clearest while viewed under microscope. Also, more of the internal nuclear structures could be seen. In addition, the slide fixed with Bouin’s solution was able to best retain the eosin dye. The other slides did not seem to stain well, if at all, with eosin. The worst of the fixatives seems to be the non-cross-linking Carnoy’s solution (Figure 2). The other four fixatives were intermediate in their level of effectiveness in preparing the cells for H&E staining (Figure 3).

Conclusion:
Of the six fixatives that were tested, the Bouin’s solution was determined to be the fixative that best prepared the normal and XPC fibroblast cells for H&E staining. With the completion of this task, the next step in attaining the goal involves the determination of the distribution of nucleic acids in XP cells. This will be achieved by immunostaining fibroblasts prepared with the same six fixatives used in this study. Using several fixatives (i.e., cross-linking, non-cross-linking) to preserve the cultured cells allows characterization of different nuclear factors, viz., protein-free nucleic acids (Carnoy’s solution), DNA-protein-formaldehyde complexes (e.g., 10 % NBF), zinc formalin (DNA-protein complexes) (5). This characterization will involve histochemical (e.g., PicoGreen, Ribogreen) and immunohistochemical studies using anti-B-DNA, anti-Z-DNA and anti-single-stranded DNA polyclonal and monoclonal antibody probes (5). These probes will provide valuable insight into the molecular biology of XP and may provide novel target sites for therapy. Looking further ahead, we hope to examine cells with mutations (UV light induced) in all of the other genes that cause XP. Heavy emphasis will be placed on comparing genes with and without segments of left-handed Z-DNA.

Literature cited:
Figure 1. H&E stained normal and XPC fibroblasts fixed with Bouin’s solution are shown on the left (A) and right (B), respectively. Bouin’s solution was determined to be the best fixative for preparing the cells for H&E staining. Slides prepared with the different fixatives showed different abilities to retain the eosin dye (pink color). The slide fixed with the Bouin’s fixative was the one most effectively stained with eosin (most pink).

Figure 2. H&E stained slides of normal and XPC fibroblasts fixed with Carnoy’s solution are shown on the left (A) and right (B), respectively. This fixative was the worst choice in preparing the cells for H&E staining and little eosin (pink color), if any, is seen in the cells.

Figure 3. Normal and XPC fibroblasts fixed with 10% neutral buffered formalin (NBF) and subsequently stained with H&E are shown on the left (A) and right (B), respectively. The 10% NBF appears to have an intermediate level of effectiveness in preparing the cells for H&E staining when compared to Carnoy’s solution, which is the worst fixative for this purpose, and Bouin’s solution, which was found to be the best. Three other fixatives tested (zinc formalin, 70% ethanol/100% methanol (3:1), and an esterase fixative) were similar to 10% NBF in their level of effectiveness in preparing the cells for H&E staining (data not shown).
5. DEBRA EHRLICH (NJMS 2011)

ANTI-HUD IMMUNOTOXIN THERAPY FOR NEUROBLASTOMA

Mentor: RuiRong Yuan, MD/PhD (Department of Neurosciences & Neurology, VA Medical Center-UMDNJ)

Objective:

Neuroblastoma ranks as the third most common pediatric cancer, and despite several available treatment options, the long-term survival for patients when diagnosed after 1 year of age still remains quite low (1). Therefore, new and more effective treatment options for neuroblastoma are still needed. The HuD-antigen is a neuronal RNA-binding protein that is expressed in 80% of all human neuroblastomas (2). It is speculated that anti-HuD antibodies bind to these HuD cell surface antigens and then are internalized and transported to the nucleus.

Immunotoxins are proteins that are produced by attaching a toxin to an antibody. Immunotoxins are currently being investigated as promising options in the treatment of cancer because cancer cells have been found to express unique antigens which normally replicating cells lack. Therefore, if antibodies can be developed to target specific antigens that are expressed only by cancer cells, then it may be possible to guide the toxins to kill cancer cells while leaving other cells unharmed (3,4).

Saporin is a plant-derived toxin that irreversibly blocks protein synthesis by damaging the eukaryotic ribosome at the elongation factor 2 (EF2) binding site (5). In addition to its irreversible inactivation of ribosomes, saporin is also an excellent immunotoxin candidate because saporin is highly resistant to denaturation and proteolytic attack, indicating that a saporin based immunotoxin would confer a good degree of stability in vivo (6).

In this study, we explore the use of a novel immunotoxin called BW-2, which is constructed through the conjugation of a biotinylated anti-HuD monoclonal antibody onto a streptavidin-saporin complex. We hypothesize that the BW-2 immunotoxin will kill neuroblastoma cells in vitro and reduce tumor size and increase survival times of neuroblastoma allografted mice in vivo.

Methods:

Cell lines
Neuro-2a (mouse neuroblastoma) and IMR-32 (human neuroblastoma) (American Type Culture Collection) cell lines were cultured according to manufacturer’s instructions.

Western blot analysis
Neuro-2a and IMR-32 cells were lysed and total protein content was determined by measuring its absorbance. Aliquots of 30 μg total protein from each sample were subjected to SDS-polyacrylamide gel electrophoresis on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) followed by transfer to a PVDF membrane. The membrane was probed with the primary antibody anti-HuC/D (5 μg/mL, Molecular Probes) followed by incubation with appropriate HRP-conjugated secondary antibodies (GE healthcare). The signal was visualized through the use of the ECL Plus Western Blotting Detection System (GE Healthcare) and exposure to ECL Hyperfilm. The membrane was stripped with Re-Blot Plus Strong between probing.
Cytotoxicity assays
Neuro-2a and IMR-32 cells were plated in 24-well plates at a concentration of $5 \times 10^4$/mL. 300μL of culture medium was placed in each well and doses of 0.5 and 5μg/mL of BW-2 were added to the cell culture wells, while equivalent molar concentrations of saporin alone and filtered PBS served as controls. Cells were collected at intervals of 48 and 72 hours and cell viability and cell death counts were obtained using trypan blue staining (Gibco) and a hemocytometer. Percentages of live and dead cells from each condition and time point were calculated.

Neuroblastoma tumor allograft and treatment protocol
Neuro-2a cells (1x10^7/100μL) were injected subcutaneously into the flank of female A/J mice at 6-8 weeks of age (Jackson Laboratory). Tumor size was measured and final tumor volumes were calculated using the formula: length x width x height x 1/6 x π (7). When primary tumors reached approximately 150-200mm^3, mice received two intratumoral injections at weekly intervals of either BW-2 at a dose of 1mg/kg or equivalent doses of PBS. Mice were monitored following treatments 5 times per week for tumor growth, general condition, and survival.

Statistical analysis
Statistical analysis was performed using Instat Software 3.0 (Graphpad Software). The composite data was analyzed by the Kruskal-Wallis one-way analysis of variance and the Mann-Whitney U test (two-tailed) was used to determine the significance of intergroup clinical response differences after treatment. A value of $P<0.05$ was considered significant.

Summary:
1. Western blot analysis of protein extracts from Neuro-2a and IMR-32 cells revealed strong signals at ~40kDa when the membrane was probed with a HuD antibody. Our results confirmed that both cell lines express the HuD antigen.

2. Cytotoxicity assays demonstrated BW-2 killed much greater percentages of Neuro-2a cells than PBS alone or saporin alone (Fig.1 (I &II)).

3. Immunotoxin BW-2 profoundly inhibited tumor progression in tumor implanted mice and
prolonged animal survival compared to PBS controls (Fig.2)

Figure 2. Two weekly doses of intratumoral BW-2 treatment in Neuro-2a allografted A/J mice significantly inhibited local tumor growth as compared to PBS treated controls. BW-2 treatment was well tolerated and the only side effect observed was a temporary loss of fur at the site of injection.

Conclusion:

Since the HuD antigen has been found to be expressed by the majority of human neuroblastoma tumors, we predicted that the BW-2 immunotoxin, which is composed of monoclonal antibodies to HuD, might kill neuroblastoma cells effectively both in vitro and in vivo. Our western blot analysis confirmed that both the mouse neuroblastoma (Neuro2a) and human neuroblastoma (IMR-32) cell lines express the HuD antigen. Cytotoxicity assays demonstrated that BW-2 treatment killed more neuroblastoma cells than when compared to PBS or saporin alone. Our in vivo experiments showed that when the BW-2 immunotoxin was injected directly into neuroblastoma tumor sites, local tumor progression was profoundly inhibited and animal survival was substantially prolonged as compared to PBS sham treatment. While much further testing is still needed, our data suggest that BW-2 may be an effective treatment option for patients with neuroblastoma. In addition to the mouse model of neuroblastoma described here, the same immunotoxin treatment strategy could be applied to animal models of other cancers by switching to other tumor/antigen specific immunotoxin compounds. In the future, immunotoxin therapies may offer clinicians a novel alternative treatment option for patients with solid tumors.
References:

6. KATHERINE GALLAGHER (NJMS 2011)

EFFECT OF PLANT ANTIOXIDANT SILIBININ ON 1,25D-INDUCED MONOCYTIC DIFFERENTIATION

Mentor: George P. Studzinski, MD,PhD (Pathology)

Objective:

Investigate the effect of the antioxidant silibinin and G-CSF on potentiation of Vitamin D-induced monocytic differentiation of human leukemia cells.

Methods:

Cell Culture
Experiments were conducted on HL60-G, HL60-ATCC and U937 cells, lines of human leukemia cells derived from AML, grown at 37°C in RPMI 1640 medium with 10% iron-supplemented bovine calf serum. Cells were suspended in 1.5x10⁵ cells in the growth medium and the appropriate groups were treated with 60μM silibinin. After incubation with CO₂ for 30 minutes some groups were treated with 25 ng/ml G-CSF and incubated for 30 minutes. Cells were then exposed to varying concentrations of 1,25D and deltanoids. After 24 and 48 hours of incubation with CO₂ at 37°C, the cells were harvested and viability was determined by 0.5% trypan blue.

Determination of Cell Differentiation Markers
Cells were washed once with 1xPBS and centrifuged for 5 minutes at 2000 rpm and stained with MY4-RD-1 (anti-CD14) and M01-FITC (anti-CD11b) antibodies. A Coulter flow cytometer and Cell Quest software were used to perform two-parameter analysis. Slides were made to determine monocytic phenotype by non-specific esterase (NSE) activity.

Summary:

Induction of monocytic differentiation of myeloid leukemia cells is a promising therapy for AML and other forms of cancer. The activated form of vitamin D, 1-alpha, 25-dihydroxyvitamin D (1,25D), induces neoplastic cells to terminally differentiate and has anti-proliferative effects. However, vitamin D’s effects on differentiation are limited by its hypercalcemic effects. For this reason, potentiation of 1,25D’s effects and its analogs by antioxidants have become of interest.

This project first investigates the effect of silibinin on 1,25D-induced differentiation in 3 leukemia cell lines. Next, the effects of 1,25D analogs Bxl-01-0062 and Bxl-01-0143 (a metabolite of 0062), which are more potent than 1,25D, are examined. These analogs (deltanoids) differ from 1,25D in a cyclopropyl group that prevents its normal metabolism. The aim is to identify the concentration at which these analogs can be used with antioxidants, specifically silibinin (SiL), and G-CSF (used to optimize in vitro conditions) in order to achieve maximum differentiation.
Expression of differentiation markers in HL60-G cells after 48 hour exposure to 1,25D. HL60-G cells were treated with 1,25D, SiL and/or G-CSF and incubated at 37°C for 48 hours. Cell surface differentiation makers CD11b and CD14 were analyzed using flow cytometry. At 24 hours (not shown) and 48 hours, there was an increase in differentiation markers when SiL and G-CSF were added to cells exposed to 1,25D. There was also an increase in differentiation markers when analogs were used alone compared to 1,25D.

Expression of differentiation markers after 48 hour exposure to 1,25D analogs. HL60-G cells were treated with 1,25D, SiL and/or G-CSF and incubated at 37°C for 48 hours. Cell surface differentiation markers CD11b and CD14 were analyzed using flow
cytometry. At 24 hours (not shown) and 48 hours, there was an observed increase in differentiation markers when SiL and G-CSF were added to cells exposed to 1,25D analogs. There was no observable increase in differentiation markers when analogs were used compared to 1,25D.

Figure 3.

**Differentiation Markers-48hours**

![Graph showing differentiation markers](image)

**Expression of differentiation markers in 3 cell lines after 48 hour exposure to 1,25D and/or SiL.** HL60-G, ATCC and U-937 cells were treated with 1 nm 1,25D and or 60uM SiL and incubated at 37°C for 48 hours. Cell surface differentiation makers CD11b and CD14 were analyzed using flow cytometry and slides were made to determine cell differentiation via NSE stain. At 48 hours, there was an observed increase in differentiation markers when SiL was added to cells exposed to 1,25D in HL60 cells. U-937 has a less marked increase in differentiation markers in response to 1,25D and silibinin appears to inhibit 1,25D induced differentiation.

**Conclusion:**

These experiments demonstrate that 1,25D induces monocytic differentiation in HL60 and U-937 cells. In HL60 cells, 1,25D's effects appear to be potentiated by 60 uM silibinin. However, silibinin does not appear to have this effect on U-937 cells. In these experiments, silibinin appears to slightly inhibit 1,25D’s effect on differentiation. In all of the cell lines cell viability was not compromised by exposure to any of these compounds. NSE data confirms the findings for these experiments.

These preliminary data show that Bxl-01-0062 and Bxl-01-0143 may be promising treatments for AML and possibly leukemia prevention. At near physiological levels (100 pM) of 1,25D, silibinin and G-CSF have supra-additive effects on differentiation when used with 1,25D after 48 hours. At this concentration, the synthetic analogs appear to be more potent than 1,25D. At very low concentrations (10 pM), synthetic analogs show increased differentiation in cells exposed to these compounds in combination with silibinin and G-CSF. These results, when confirmed in more extensive studies, could be important in the treatment of leukemias because they show that exposure to low concentrations of synthetic analogs can cause cells to terminally differentiate without causing hypercalcemia.
Future experiments should include replicates of all experiments to determine reproducibility. In addition, the mechanism underlying the action of these analogs will be studied. Also, NSE stained slides from these experiments will be counted to analyze cytoplasmic markers of monocytic differentiation.

References:


7. SONAL GANDHI (TCNJ 2010)

THE RELATIONSHIP BETWEEN AGE AND CHRONIC ILLNESS ON CANCER TREATMENT ADHERENCE

Mentors: Denise C. Fyffe, Ph.D., (Institute for the Elimination of Health Disparities), Charles Cathcart, M.D., (Radiation Oncology)

Objective:

Adherence to a prescribed cancer treatment regimen is critical to patient survival or relief of symptoms. However, research has indicated that there is a prevalence of non-adherence to cancer treatments. Although there may not be a single factor responsible for this problem, identifying the causes might lead to improvements in alleviating this behavior (Cathcart et al., 1997).

Chronic illnesses are seen in patients of all ages. However, higher rates of chronic illness have been observed among older individuals (Hoffman et al, 1996). Elderly patients have been found to take a passive approach to treatment; whereas, younger individuals more actively seek information about the disease and its treatment (Felton & Revenson, 1987). The presence of comorbidities has been shown to decrease adherence to a prescribed treatment regimen for pneumonia (Menendez et al., 2005).

Therefore, the objective of this study was to determine the relationship between age and chronic illnesses on cancer treatment adherence. It was hypothesized that there will be a negative relationship between age and treatment adherence as well as between the presence of chronic illness and treatment adherence.

Methods:

The data for this study was collected through retrospective chart review of the patients treated by the Radiation Oncology Department of University Hospital from January 2006 to November 2006. Patients under the age of 18 at start of treatment, patients treated for a recurrence at the same site of cancer, patients receiving treatment for heterotopic bone, patients receiving less than five fractions of radiation, and patients receiving exclusively brachytherapy were excluded from this study. Of the eligible patients, patients lost to follow up or those who expired before the end of treatment were omitted from current analysis.

Demographics, cancer treatment, and cancer risk factors and history information, along with medical diagnoses were abstracted from the patient’s chart and the medical systems: Logician, Epic Hyperspace, and Access. Adherence was measured using the adherence ratio (AR). This was calculated using the equation:

\[ AR = \frac{\text{number of fractions completed}}{\text{number of days elapsed}} \]

The relative frequency of patient characteristics and mean adherence to prescribed treatment is presented in Table 1. After abstraction, the data was compiled and entered into a Microsoft Access database. 290 patients were used for the analysis. Statistical analysis was done using SPSS to determine the correlation between a variety of socioeconomic factors and the patient’s adherence to treatment. Univariate analyses were run to examine the correlation between patients’ age and their adherence and the correlation between adherence and the most prevalent chronic illnesses observed among this patient group.
Table 1. Relative frequency of patient characteristics and mean adherence to Radiation Treatment

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n (%)</th>
<th>Mean Adherence (SD)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Patients</td>
<td>290 (100)</td>
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<td></td>
</tr>
<tr>
<td>Gender (n=290)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>149 (51.4)</td>
<td>0.87 (0.77)</td>
<td>0.038</td>
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<tr>
<td>Female</td>
<td>141 (48.6)</td>
<td>0.73 (0.20)</td>
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</tr>
<tr>
<td>Age (n=290)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 60</td>
<td>169 (58.3)</td>
<td>0.76 (0.38)</td>
<td>0.122</td>
</tr>
<tr>
<td>≥ 60</td>
<td>121 (41.7)</td>
<td>0.87 (0.76)</td>
<td></td>
</tr>
<tr>
<td>Race (n=290)</td>
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</tr>
<tr>
<td>Black</td>
<td>120 (41.4)</td>
<td>0.76 (0.44)</td>
<td>0.595</td>
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<td>Hispanic</td>
<td>84 (29.0 )</td>
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<tr>
<td>White</td>
<td>74 (25.5 )</td>
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<tr>
<td>Other</td>
<td>12 (4.1 )</td>
<td>0.71 (0.05)</td>
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<td>Marital Status (n=287)</td>
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<td>Married</td>
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<td>0.556</td>
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<td>Single</td>
<td>96 (33.4 )</td>
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<tr>
<td>Separated/ Widowed</td>
<td>49 (17.1 )</td>
<td>0.80 (0.45)</td>
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<tr>
<td>Divorced</td>
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<td>77 (34.8 )</td>
<td>0.85 (0.77)</td>
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<td>0.78 (0.41)</td>
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<td>24 (9.3 )</td>
<td>0.69 (0.07)</td>
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<td>2</td>
<td>54 (20.8 )</td>
<td>0.76 (0.36)</td>
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<tr>
<td>3</td>
<td>60 (23.2 )</td>
<td>0.70 (0.07)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>115 (44.4)</td>
<td>0.90 (0.84)</td>
<td></td>
</tr>
<tr>
<td>Chronic Illness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substance Abuse</td>
<td>29 (10 )</td>
<td>0.82 (0.51)</td>
<td>0.872</td>
</tr>
<tr>
<td>Hypertension</td>
<td>128 (44.1)</td>
<td>0.85 (0.77)</td>
<td>0.268</td>
</tr>
<tr>
<td>Diabetes</td>
<td>50 (17.2 )</td>
<td>0.90 (0.89)</td>
<td>0.220</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>31 (10.7 )</td>
<td>0.89 (0.78)</td>
<td>0.405</td>
</tr>
<tr>
<td>Type of Radiation (n=290)</td>
<td></td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>Curative</td>
<td>203 (70.0)</td>
<td>0.73 (0.24)</td>
<td></td>
</tr>
<tr>
<td>Palliative</td>
<td>87 (30.0 )</td>
<td>0.98 (0.96)</td>
<td></td>
</tr>
<tr>
<td>Side Effects (n=290)</td>
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<td></td>
<td>0.000</td>
</tr>
<tr>
<td>Reported</td>
<td>238 (82.1)</td>
<td>0.72 (0.18)</td>
<td></td>
</tr>
<tr>
<td>None Reported</td>
<td>52 (17.9 )</td>
<td>1.20 (1.23)</td>
<td></td>
</tr>
<tr>
<td>Smoking History (n=281)</td>
<td></td>
<td></td>
<td>0.111</td>
</tr>
<tr>
<td>No</td>
<td>102 (36.3)</td>
<td>0.73 (0.11)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>179 (63.7)</td>
<td>0.84 (0.70)</td>
<td></td>
</tr>
<tr>
<td>History of Cancer (n=290)</td>
<td></td>
<td></td>
<td>0.83</td>
</tr>
<tr>
<td>Yes</td>
<td>23 (7.9 )</td>
<td>1.00 (1.24)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>267 (92.1)</td>
<td>0.79 (0.47)</td>
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<td>Family Hx of Cancer (n=281)</td>
<td></td>
<td></td>
<td>0.596</td>
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<tr>
<td>Yes</td>
<td>142 (49.0)</td>
<td>0.78 (0.44)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>139 (47.9)</td>
<td>0.82 (0.67)</td>
<td></td>
</tr>
</tbody>
</table>
Summary:

Overall, most patients had an adherence ratio of 0.70 to 0.79 (Figure 1). Additionally, there is a positive correlation between age and treatment adherence ($r=0.82$); however, these results are not significant ($p=0.161$). There is also a positive correlation between the presence of a chronic illness and treatment adherence ($r=0.39$); however, these results are also not significant ($p=0.510$).

Male patients were found to be significantly more adherent on average ($\text{mean}=0.87$, $\text{SD}=0.77$) than female patients ($\text{mean}=0.73$, $\text{SD}=0.20$). Retired patients were significantly more adherent on average ($\text{mean}=0.94$, $\text{SD}=0.93$) than unemployed ($\text{mean}=0.72$, $\text{SD}=0.13$) or currently employed patients ($\text{mean}=0.73$, $\text{SD}=0.09$). Patients who reported no side effects were significantly more adherent on average ($\text{mean}=1.20$, $\text{SD}=1.23$) than those who reported side effects ($\text{mean}=0.72$, $\text{SD}=0.18$), and patients treated with palliative radiation therapy were also significantly more adherent on average ($\text{mean}=0.98$, $\text{SD}=0.96$) than patients treated with curative therapy ($\text{mean}=0.73$, $\text{SD}=0.24$).

Figure 1.
Conclusion:

Most patients were adherent to their prescribed course of radiation therapy. Neither increased age nor presence of chronic illness was proven to have a negative correlation to treatment adherence. Instead, both were shown to have a positive, albeit not significant relationship with adherence.

The study did reveal that men were more adherent than women to treatment, retired individuals were more adherent than unemployed or currently employed individuals, individuals treated with palliative radiation were more adherent than those treated with curative intent, and individuals reporting no side effects were more adherent than individuals reporting one or more side effects as a result of their treatment.

Future studies should be conducted with a larger sample size in other hospital settings to increasing generalizability. Additionally, future research should investigate why these sociodemographic and clinical characteristics affects treatment adherence and whether the presence of multiple chronic illnesses and their joint affect on an individual influences adherence.

Literature Cited:


8. ZACH GOLDSTEIN (TCNJ 2010)

SILIBININ POTENTIATION OF VITAMIN D₃-INDUCED DIFFERENTIATION AND HUMAN KINASE SUPPRESSOR OF RAS-2.

Mentor: George P. Studzinski, MD, PhD, (Pathology)

Objective:
This work investigated the role of hKSR-2 in the regulation of silibinin-potentiation of vitamin D-induced differentiation in HL60-G cells.

Methods:
Cells were initially split into two groups. One was exposed to 20 μL endoporter – an endocytosis-inducing agent – and 20 μL hKSR-2 antisense, and the other to 20 μL endoporter and 20 μL scrambled hKSR-2 oligonucleotide. These two of groups are referred to as “AS KSR” and “Scrambled,” respectively. hKSR-2 antisense is a strand of DNA complementary to hKSR-2 mRNA, that binds to the mRNA in the cytoplasm to prevent translation. Scrambled oligonucleotide was a scrambled code that was not complementary to any known mRNA – this served as a control. Cells were harvested after 24 hours and 48 hours exposure to 1α,25-dihydroxyvitamin D₃ (1,25D) and silibinin (SiL) and analyzed for extent and intensity of differentiation.

Extent and intensity of differentiation were determined through CD11b/CD14 analysis via flow cytometry (Coulter) and α-naphthyl acetate esterase stain (Sigma). Samples were incubated in CD11b and CD14 antibodies for 45 minutes then analyzed via flow cytometry. Expression of CD11b, a protein subunit of integrins, indicates cell clumping typical of late-stage differentiation. CD14, on the other hand, is a receptor that recognizes lipopolysaccharides in bacteria and induces inflammatory response; this is a characteristic marker of early-stage differentiation. The data produced through this test was then re-gated with WINMIDI to include only the most intensely stained double-positive cells – those which have matured the furthest. Nonspecific esterase (α-naphthyl acetate esterase) is a cytoplasmic marker of differentiation that accumulates in the cell during the process of differentiation. Cells were smeared onto slides, stained, and examined microscopically.

hKSR-2 knockdown was established through western blotting. Whole-cell proteins were extracted from cells and separated in a 10% SDS polyacrylamide gel then transferred to a nitrocellulose membrane. The membranes were probed with an hKSR-2 antibody (Abcam) and then Crk-L (Santa Cruz) as a loading control. Optical densities were determined with Adobe Photoshop. Each experiment was done at least three times and the results were expressed as percentages (mean ± SD). Significance of the difference between mean values was assessed by a two-tailed Student’s t test. All computations were done with an IBM-compatible personal computer using Microsoft Excel.

Experimental Design:

<table>
<thead>
<tr>
<th>Group Contents</th>
<th>Group Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scrambled</td>
<td>AS hKSR2</td>
</tr>
<tr>
<td>1 Untreated Control</td>
<td>5 Untreated Control</td>
</tr>
<tr>
<td>2 1 nM 1,25D</td>
<td>6 1 nM 1,25D</td>
</tr>
<tr>
<td>3 60 μM SiL</td>
<td>7 60 μM SiL</td>
</tr>
<tr>
<td>4 SiL + 1,25D</td>
<td>8 SiL + 1,25D</td>
</tr>
</tbody>
</table>
Summary:
The active form of vitamin D – 1α,25-dihydroxyvitamin D3 (1,25D) – is currently under investigation as a differentiating agent in the treatment of leukemia. 1,25D has been found to have an antineoplastic effect that induces immature cancerous myeloid cells into terminal differentiation. This is a promising field of research that may provide new methods of chemotherapy and chemoprevention unassociated with the typical harmful side effects of cytotoxic drugs such as cytosine arabinoside. In order to overcome the barriers to a vitamin D-based therapy, the Studzinski lab is currently investigating the use of vitamin D analogs (deltanoids) that do not induce hypocalcaemia and combinations of plant-derived antioxidants that enhance the differentiating effects of 1,25D.

Vitamin D, which is generated in the body primarily through sunlight exposure, is converted into its active form in the liver and kidney. A lipophilic molecule, 1,25D enters the cells directly through the plasma membrane and activates Vitamin D Receptor (VDR) in the cytoplasm, which binds to retinoid X receptor; the resulting heterodimer acts as a nuclear transcription factor that activates the expression of proteins and kinase cascades associated with maturation and cell survival. hKSR-2, a novel protein closely related to the scaffold protein KSR-1 that facilitates Ras signaling, has been found to be directly regulated by 1,25D and required for advanced cellular maturation and cell survival. Although its role in the cell and mechanisms of action are not totally clear, there is evidence that hKSR-2 increases cell survival during the later states of differentiation.¹ There is also evidence that the effects of 1,25D are potentiated by a number of plant-derived antioxidants, such as carnosic acid and silibinin.² However, the mechanisms through which many of these compounds work have yet to be determined.

Figure 1

HL60-G cells harvested at 24 hours were exposed to CD11b and CD14 antibodies to determine the amount of cells expressing both surface membrane proteins. Cells exposed to 1,25D alone, SiL alone, and 1,25D+SiL exhibited a significant decrease in both CD11b and CD14 expression when exposed to AS hKSR-2 (p<0.05).
HL60-G cells harvested at 48 hours were exposed to CD11b and CD14 antibodies to determine the amount of cells expressing both surface membrane proteins. Cells exposed to 1,25D alone and 1,25D+SiL exhibited a significant decrease in both CD11b and CD14 expression when exposed to AS hKSR-2 (p<0.05). Cells exposed to SiL alone showed a significant decrease only in CD14 expression (p<0.05).

Flow cytometry data was analyzed through WINMIDI and manually re-gated to compare the percent of intensely stained cells between groups. This allowed determination of full versus partial differentiation. At 24 hours, there was no significant decrease in any group exposed to hKSR-2 (p>0.05). At 48 hours, there was a significant decrease in every group exposed to hKSR-2, including the control (p<0.03).
Summary of Western Blots showing hKSR-2 knockdown in HL60-G cells. The above graph represents expression of hKSR-2 as a percent of the untreated scrambled control. All values are adjusted for their individual loading controls. The ninth group was a control group of protein taken from untreated HL60-40AF cells, a line of cells resistant to 1,25D-induced differentiation. There was an unusual drop in hKSR-2 in the 1,25D-exposed Scrambled group. Knockdown of hKSR-2 produced a noticeable decrease in hKSR-2 expression in all groups.

**Conclusion:** These data reflect the conclusion made by Dr. Xuening Wang and Dr. George P. Studzinski in 2006 that hKSR-2 is necessary for optimal late-stage 1,25D-induced differentiation. The most noticeable result of hKSR-2 knockdown was the reduction in the percent of highly differentiated cells. However, AS hKSR-2 did not significantly reduce differentiation in groups exposed SiL+1,25D more than it did in groups treated with 1,25D alone. In other words, differentiation was not reduced in 1,25D + SiL-exposed groups more than it would have been in groups exposed to 1,25D alone. This indicates that SiL-potentiation of 1,25D-induced differentiation is not directly regulated by hKSR-2. Rather, the significant drop in differentiation in SiL-exposed groups may be due to the fact that hKSR-2 is required for optimal differentiation in general. In this case, the effect of any differentiating agent would be decreased by hKSR-2 knockdown. Not only does this evidence narrow the possible mechanisms of SiL-potentiation of vitamin D-induced differentiation, it helps elucidate the role of hKSR-2 in the process of cell maturation. It is important to note that this work is still preliminary, and it is necessary to repeat several of the tests, including the western blot and α-napthyl acetate esterase stains in order to determine reproducibility.

Research was supported by NIH grants R01-CA-44722 and R01-CA117942

**References:**

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9. MARGARET GORACY (NJMS 2011)

DOES THE TUMOR BOLD fMRI SIGNAL HAVE UNIQUE FREQUENCIES?

Mentors: Wen-Ching Liu, PhD and Susan C. Feldman, PhD (Radiology)

Objective:

BOLD fMRI is a method for detecting hemodynamic changes in the brain that correlate with neural activity. The BOLD fMRI signal has been used to map functionally related areas of the brain. We have shown that the BOLD signal can be used to identify brain tumors and to distinguish them from normal brain¹. In this study, we explored the frequency fluctuation, a fundamental characteristic of the BOLD signal, in different types of brain tumors. By converting the time domain to the frequency domain using the Fast Fourier Transform option within AFNI, we were able to obtain the BOLD signal's range of frequencies and their associated amplitudes. We hypothesized that the range of frequencies and associated amplitudes of the BOLD signal from brain tumors should be distinct from that in the normal, non-tumor regions. Understanding the differences between the BOLD signal from tumors versus that from normal tissues could aid in determining the borders of brain tumors. This information is critical in neurosurgery to allow for resection of maximum tumor tissue.

Methods:

Subjects and Tasks
This was a retrospective study of twelve neurosurgery patients with cerebral meningiomas or gliomas who were scanned at University Hospital using an IRB-approved protocol. The patient population consisted of 8 males and 4 females (age range 23-71). All patients performed a motor task of bilateral, sequential thumb-to-digits opposition while in the fMRI scanner. Patients #1, 7 and 8 were also scanned in the resting state; these patients remained in the scanner for the same length of time without any movement or stimulation with their eyes closed.

fMRI Acquisition and Processing
BOLD fMR images were acquired on a 1.5 T GE Echo speed Horizon scanner. Following fMRI, a set of gadolinium-enhanced images was acquired for anatomical information (Fig. 2, top row). Data was pre-processed with the neuroimaging software Statistical Parametric Mapping (SPM99, Friston 2003)²; functional analysis was performed with Analysis of Functional Neuroimages (AFNI, Cox 1996)³. The tumor area was defined on fMRI by choosing a ‘seed point’, or voxel of interest. This activated all voxels in the section with a similar signal at a particular correlation value (tumor r-value). The r-value for each seed was adjusted in order to maximize the amount of tumor area highlighted (area B on Fig. 2) and minimize the amount of normal brain tissue highlighted.

To obtain the range and strength of frequencies of the BOLD signals from the tumors and control areas, the BOLD curves were converted from the time domain to the frequency domain using the Fast Fourier Transform (FFT) option within AFNI; this generated a power spectrum of frequency vs. amplitude (Fig. 3).

Data Analysis
The two power spectra from the tumor and the contralateral normal area were analyzed to determine differences of frequencies and their associated amplitudes. Ten points (Fig. 1) were sampled per slice of tumor tissue as seen on fMR axial images. Points of interest were selected according to the following formula: four points located along the tumor's periphery, four points between the periphery and the center of the tumor, and two points located centrally within the tumor. For the normal tissue we sampled five points (Fig. 1): four points on the periphery of the normal tissue area, and one point in
the center of this area. For each point sampled within tumor or control areas, we analyzed the frequencies and amplitudes of the three tallest peaks from each power spectrum (Fig. 3) (Tables 2 & 3). Paired Student's T-tests were used to determine if there was a significant difference between the data from the tumor area versus that from normal control tissue.

**Summary:**

Signal Frequencies: Within the tumor, the average frequencies ranged from 0.010Hz – 0.110Hz; within the control area the range was 0.010Hz - 0.189Hz. The only significant difference between the frequencies from the tumors and normal tissue was seen in the third peak; the others were not significantly different (Table 2).

Signal Amplitude: There was a non-significant trend of higher average amplitudes for control area versus tumor area for all three peaks (Table 3). On a patient-by-patient basis, higher average amplitudes were observed for normal brain tissue versus tumor tissue in 75% of cases (4 meningioma and 5 glioma cases out of 12 total cases).

**Figure 1. Points Sampled in Control Area and in Tumor Area**
Each number represents a sampled point; for every brain slice containing tumor, five points were selected for analysis from the control tissue (area contralateral to tumor area) and ten points were selected for analysis from the tumor tissue (highlighted area).

**Figure 2. Glioma, Patient 4, Anatomical images (top row), fMR images (bottom row)**
All patients were scanned to generate 2 types of images: 1) T1 weighted post-contrast images (conventional anatomical images, top row), used to identify the tumor area, and 2) functional images (bottom row). Functional imaging allowed for acquisition of the BOLD signal, whose characteristics (frequency and amplitude) were analyzed within the control area (below, box A) and tumor area (below, box B).
Figure 3. Power Spectrum graphs, for control area (top) and tumor area (bottom)
For each point sampled from tumor and control tissues, a power spectrum graph was generated; highest three amplitudes (Peak #1, Peak #2, Peak #3) were recorded, along with frequencies represented by the peaks.

Table 2. Mean Top 3 Peak Amplitudes, Patients 1-12
*Peak Frequency #3** data set is statistically significant.

<table>
<thead>
<tr>
<th>Patient (Tumor Type*)</th>
<th>Peak Amplitude #1</th>
<th>Peak Amplitude #2</th>
<th>Peak Amplitude #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Tumor</td>
<td>Control</td>
<td>Tumor</td>
</tr>
<tr>
<td>1 (G)</td>
<td>1801</td>
<td>1035</td>
<td>799</td>
</tr>
<tr>
<td>2 (G)</td>
<td>4092</td>
<td>5041</td>
<td>1740</td>
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<tr>
<td>3 (G)</td>
<td>3737</td>
<td>1801</td>
<td>2644</td>
</tr>
<tr>
<td>4 (G)</td>
<td>5237</td>
<td>4899</td>
<td>1510</td>
</tr>
<tr>
<td>5 (G)</td>
<td>35280</td>
<td>12272</td>
<td>23126</td>
</tr>
<tr>
<td>6 (G)</td>
<td>1112</td>
<td>1047</td>
<td>864</td>
</tr>
<tr>
<td>7 (M)</td>
<td>4956</td>
<td>3810</td>
<td>2920</td>
</tr>
<tr>
<td>8 (M)</td>
<td>8903</td>
<td>31538</td>
<td>3573</td>
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<tr>
<td>9 (M)</td>
<td>374</td>
<td>981</td>
<td>231</td>
</tr>
<tr>
<td>10 (M)</td>
<td>1083</td>
<td>747</td>
<td>697</td>
</tr>
<tr>
<td>11 (M)</td>
<td>4749</td>
<td>3812</td>
<td>1426</td>
</tr>
<tr>
<td>12 (M)</td>
<td>3673</td>
<td>1987</td>
<td>2199</td>
</tr>
<tr>
<td>Mean</td>
<td>6250</td>
<td>5747</td>
<td>3477</td>
</tr>
<tr>
<td>StDev</td>
<td>9438</td>
<td>8729</td>
<td>6268</td>
</tr>
</tbody>
</table>

*Tumor Type: G = Glioma; M= Meningioma

Table 3. Mean Top 3 Peak Frequencies (Hz), Patients 1-12

<table>
<thead>
<tr>
<th>Patient (Tumor Type*)</th>
<th>Peak Frequency #1 (Hz)</th>
<th>Peak Frequency #2 (Hz)</th>
<th>Peak Frequency #3 (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Tumor</td>
<td>Control</td>
<td>Tumor</td>
</tr>
<tr>
<td>1 (G)</td>
<td>0.026</td>
<td>0.031</td>
<td>0.055</td>
</tr>
<tr>
<td>2 (G)</td>
<td>0.031</td>
<td>0.027</td>
<td>0.041</td>
</tr>
<tr>
<td>3 (G)</td>
<td>0.033</td>
<td>0.100</td>
<td>0.049</td>
</tr>
<tr>
<td>4 (G)</td>
<td>0.055</td>
<td>0.189</td>
<td>0.072</td>
</tr>
<tr>
<td>5 (G)</td>
<td>0.010</td>
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<tr>
<td>6 (G)</td>
<td>0.084</td>
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</tr>
<tr>
<td>7 (M)</td>
<td>0.022</td>
<td>0.017</td>
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</tr>
<tr>
<td>8 (M)</td>
<td>0.029</td>
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<tr>
<td>12 (M)</td>
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<td>0.058</td>
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<tr>
<td>Mean</td>
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<td>0.058</td>
<td>0.059</td>
</tr>
<tr>
<td>StDev</td>
<td>0.028</td>
<td>0.058</td>
<td>0.033</td>
</tr>
</tbody>
</table>

**Results are statistically significant
Conclusion:

This is part of a larger study designed to demonstrate and analyze the BOLD signal from brain tumors. In this study, the range of frequencies and the range of amplitudes observed within the tumors were slightly lower than those seen in the control areas; however, the differences were not significant. While tumor and normal brain tissues differ anatomically and physiologically, frequency analysis did not demonstrate significant difference between their respective BOLD signals. The strength of our conclusions was limited by several factors including a small patient cohort and limitations of MRI imaging itself, especially problems of tissue edema and hemorrhage. Future studies should address the issue of what factors are responsible for the BOLD signal.

References:


Acknowledgements: We would like to thank Dr. Maureen Barry for her help with the radiology.
10. SAMUEL GREENSTEIN (NJMS 2011), MEENA ALADDIN (NJIT 2010)

ASSESSING PALLIATIVE CARE USING THE ESAS IN A HOSPITAL SETTING

Mentors: Marilyn Pierre-Louis, MD (Family Medicine, HUMC), Steven Keller, PhD (Family Medicine)

Objective:
Palliative care, in its simplest form, aims to improve the quality of the patient's life. However, there have been many misconceptions over the years regarding what kind of patient is eligible for palliative care. While terminally ill or end-of-life patients certainly deserve and should be receiving palliative care, it does not have to be restricted to these patients. The World Health Organization (WHO) states that a few of the goals of palliative care are to provide relief from pain and other symptoms, combine the psychological and spiritual aspects into patient care, and neither hasten nor delay death.¹ The Center to Advance Palliative Care sponsors a website which informs the general public about palliative care and addresses some of the misconceptions regarding palliative care.² The site states that palliative care can be administered at any time during a serious illness and that it can be provided at the same time as other curative therapies.

There are many tools used in palliative care settings that assess the patient. The Edmonton Symptom Assessment Scale (ESAS), is a tool that assesses nine symptoms that are common in palliative care patients (pain, tiredness, nausea, depression, anxiety, drowsiness, appetite, feeling of well being, and shortness of breath).³

In 2007, Hoboken University Medical Center launched a pain and palliative care service. In March 2008, the hospital expanded the service for patients experiencing any kind of pain. The program focuses on addressing patient comfort, as well as treating symptoms such as pain and anxiety. The service is available to all patients during their stay at the hospital.⁴

The purpose of this pilot QA/QI study is to evaluate the palliative care program in family medicine in-patients at the Hoboken University Medical Center. Specifically, the study will assess pain management as well as the other 8 symptoms that the ESAS assesses.

Methods:
This pilot QA/QI study assessed the family medicine in-patients’ symptoms and symptom management. The sampling time lasted four weeks. Based on the list of the family medicine in-patients, new patient encounters occurred between Monday and Thursday during those four weeks. Patients were assessed using the ESAS and based on the patients' length of stay, a follow up assessment occurred the following day as well as two days after the first initial assessment. A positive encounter is defined as a patient who received a follow up assessment on the following day. Symptoms were assessed both quantitatively, using the ESAS as well as qualitatively, through a chart review. Prospectively assessing the symptoms eliminates the recall bias that patients may have regarding their symptoms the day before. At the time of assessment, the severity of each symptom is rated from 0 to 10. This study included English, Spanish, and Arabic speaking patients. Patients were also assessed for time (day of the week, month, day, but not the year) as well as place.

After every positive encounter, a chart review was performed. The chart review aimed to see which symptoms that the ESAS assesses, were also assessed by the physicians, nurses, and
other hospital staff members. Furthermore, the chart review noted which medications were prescribed for the treatment of specific symptoms (pain, anxiety, nausea etc.) the day before the second ESAS assessment.

Summary:
After four weeks of recruitment there was a total of 47 initial encounters. Four of the encounters were excluded from the study because they were non-adherent. Of the remaining 43 encounters that took an initial ESAS, 34 completed the ESAS the following day. The remaining 9 were discharged. Of the 34 encounters that took the ESAS twice, 22 of them completed the ESAS a third time once again on the following day. The remaining 12 were discharged. With regard to the 34 encounters which took the ESAS twice, the chart's Admission Database revealed the pain upon admission. The mean pain upon admission score was 5.00 with a standard error of 0.73. The mean score for pain upon the first ESAS assessment was 3.37 with a standard error of 0.62. The mean score for pain upon the second interview was 3.78 with a standard error of 0.69. Figure 1. shows the remaining means and standard errors for the other symptoms that the ESAS assesses.

Figure 2. shows the paired sampled T tests for each ESAS symptom score. When the pain scores from the first ESAS encounter were paired with pain upon admission scores the p value was significant at 0.016. The paired sample T test for pain upon admission and pain upon the second assessment showed a trend with a p value 0.151. When the pain scores from the first ESAS encounter were paired with the pain scores from the second ESAS encounter the p value was 0.484. The pain score from the first and second ESAS assessments had a correlation value of 0.614 (Figure 3.). When the tiredness scores from the first ESAS encounter were paired with the tiredness scores from the second ESAS encounter, the p value was 0.058. The paired T sample statistic for depression also showed a trend with a p value of 0.131. The paired sample T tests for the remaining symptoms can be found in Figure 2.

The medication records from the charts revealed that 6 of the encounters (after reviewing 31 medication records out of the 34) did not receive pain medication in between the first and second ESAS assessment. These six encounters had a mean pain score based on the first ESAS assessment of 1.00. In this scenario, the maximum pain was 4 and the minimum pain was 0 which can potentially explain why these patients did not receive treatment.

Figure 1. ESAS Mean Scores: 2 Consecutive Encounters (n=34*)

*Depression 1, n=33; Appetite 1, n=32
Conclusion:
This project aimed to assess palliative care in a clinical hospital setting. The paired sample T statistic determines whether there is significant change in scores from the first assessment and second assessment for each symptom. The paired T sample statistic for tiredness showed a trend from T1-T2 (p<0.06). This may be due to the fact that the patients have been able to adjust their sleep cycle while staying in the hospital. The paired T sample statistic for depression also showed a trend with a p value of 0.131. This may be due to the fact that the patient became more comfortable with their overall situation. Further investigation needs to be done regarding depression.

The study focused on pain management which is one of the main components of palliative care. Pain can affect many symptoms that patients are experiencing and being able to minimize or stabilize pain certainly improves the quality of the patients’ life as well as the family at large. Looking directly at the results regarding the 34 encounters and pain, the paired sample T statistic revealed that there was a significant change in mean pain score upon admission and the mean pain score based on the first ESAS assessment. Furthermore, there was a trend in significance regarding mean pain score upon admission and the mean pain score upon the second assessment. There was no significant change in pain from the first and second assessment, however the mean pain was higher by 0.3 in the second assessment (Figure 1).
This finding is not necessarily surprising. One would hope that once the pain has been addressed upon admission, the pain is being managed appropriately and either continues to decrease towards 0 or remains the same. Upon first glance this may explain the high correlation value of 0.614 between the first and second ESAS pain scores. However, upon conducting the chart review, the “Nurses Notes” revealed that many patients are having their pain fluctuate during the course of the day. The “Nurses Notes” showed that there were essentially 2 groups of patients, those with 0 pain and those with non-zero pain. The non-zero pain group showed considerable pain fluctuation during the course of the day. This poses two potential problems with the results, and warrants further investigation. Since the ESAS is supposed to assess symptoms at the time of the administration, certainly it is possible that the scores could have changed if the ESAS was administered a few hours earlier or later that same day. Pain variance may be related to the disease as well as the treatment. Thus, the results from the study may have been different based on the time that the ESAS was administered and/or the group of patients assessed.

Pain management continues to be an integral component of palliative care and patient’s overall well being. The original goal of this study was to evaluate the palliative care program in family medicine in-patients at the Hoboken University Medical Center. The study used the ESAS as the tool to assess the symptoms. Although the mean symptom scores (except for pain and appetite) did decrease, the majority of the symptoms did not change at a significant level after one day. However, scores for pain, nausea and shortness of breath were below 4, 1.5, and 2, respectively. Through the chart review, the study found that the ESAS administered once a day may not be the most reliable assessment for pain in this scenario. Further investigation needs to be done regarding the other 8 symptoms that the ESAS assesses. With regard to pain, this QA/QI recommends that future studies with family medicine patients consider administering the ESAS multiple times the same day in conjunction with the “Nurses Notes,” to evaluate pain variance within one day as well as from day to day. By taking multiple pain assessments throughout the day, a time-series can be generated which may present a more accurate picture of the patient’s pain. Furthermore, a detailed investigation regarding whether pain fluctuation is related to treatment plan needs to be performed. Lastly, this study recommends that in the future the sample size be significantly increased.

References:
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11. ODETTE HABIB, (DREW UNIVERSITY 2010)

INCREASED TRANSFER OF MITOCHONDRIAL DNA TO THE NUCLEUS DURING THE CHRONOLOGICAL AGING PROCESS IN BAKERS YEAST

Mentor: Andreas Ivessa, Ph.D. (Cellular Biology and Molecular Medicine)

Objective: The main objective of Dr. Ivessa’s lab is to understand how mitochondrial DNA (mtDNA) is inherited and maintained using baker’s yeast (S. cerevisiae) as a model system. Mutations in mtDNA correlate with numerous different types of cancers. Surprisingly, mtDNA sequence elements can also be found scattered throughout various eukaryotic genomes including the human genome. Although it has been believed that these sequence elements were generated on an evolutionary scale, recently it was shown in yeast that mtDNA fragments can migrate actively to the nucleus where they possibly perturb nuclear genomic function. Given this process, we hypothesize that the transfer frequency of the mtDNA fragments to the nucleus increases during the aging process. In particular, we measured the transfer frequency during the chronological aging process of baker’s yeast. Chronological aging is used to see how long cells can live when their nutrients are depleted over time (see figure below). Yeast cells are grown to the stationary growth phase, then we determined both the survival and the transfer rates during this phase. We used two yeast strains named PTY 28 ϱ+, and PTY 33 ϱ+ which had the nuclear TRP1 gene directly inserted into the mitochondrial genome. Once the TRP1 gene has migrated to the nucleus, it will be expressed and allows growth on medium lacking the amino acid tryptophan. Since cancer is the main focus of this program, as well as the main focus of Dr. Ivessa’s research, I observed how aging affects the PTY 33 ϱ+ ability to transfer the TRP1 gene to the nucleus. We predicted to see a decrease of survival as the cells age with a concomitant increase of mtDNA transfer to the nucleus.

Methods: We used two yeast strains: PTY 28 ϱ+, and PTY 33 TRP1 ϱ+. PTY 33 had the TRP1 gene removed from the nucleus and inserted in the mtDNA, while the PTY 28 strain lacks completely the TRP1 gene. Both strains were separately grown on a YEP-Glycerol plate and then transferred to tubes containing YEP-Dextrose. The strains were grown to stationary phase and then the optical density was measured at a 1:100 and a dilution was calculated so that there would be approximately 300 cells being plated on YEP-Dextrose plates as well as 5 x 10⁷ cells being plated on YC-TRP (Glucose) plates. Optical density was measured every other day and the same number of cells was being plated every other day as well. Plating on YEP-Dextrose was done in order to see a grand perspective of the survival rate of the strains. Plating on YC-TRP (Glucose) was used to determine the transfer rate of the TRP1 gene to the nucleus from mtDNA. Every other day, pictures were taken of the YC-TRP (Glucose) plates. Also, the
Summary: Various single colonies of the PTY 33 TRP1 strain were able to grow in the absence of TRP while the PTY 28 strain was not showing any growth. Pictures of the YEPD plates were used as a way to see on a bigger scale the growth of the colonies and were not needed to be shown since it was known beforehand that the strains were able to grow in the presence of Glucose.

When it comes to counting the colonies on the plates, the idea was that by having both strains undergo chronological aging that the number of colonies that would be observed would start to decrease after 1 or 2 weeks.

The YEPD plates allowed us to observe the frequency of colonies on a larger scale since both strains were able to grow in the presence of Glucose.

<table>
<thead>
<tr>
<th>Day</th>
<th>PTY 28 YEPD</th>
<th>PTY 33 YEPD</th>
<th>PTY 28 YC-TRP</th>
<th>PTY 33 YC-TRP</th>
<th>Ratio: [PTY 33 YC-TRP] [PTY 33 YEPD]</th>
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</table>

Conclusion: By observing how the YC-TRP (Glucose) plates differed between the PTY 28 and the PTY 33 TRP1 strains we were able to conclude that in the PTY 33 strains where the nuclear gene TRP1 gene was injected directly into the nucleus, that there was migration of the gene to the nucleus. The presence of colonies confirmed the hypothesis of TRP1 migration because if the gene had remained in the mitochondria, there would not have been any growth in the absence of TRP since the gene is unable to function unless it is in the nucleus. A further assay for this would be to test to see if the TRP1 gene was integrated into the nuclear genome or if it just existed in the nucleus as a circular molecule.

By observing the numbers in table 1, one can see that there was an instance when there were two colonies of the PTY 28 strain that lacked TRP1. The possible thoughts as to why that is, is that maybe there is a suppressor or a mutation that was able to bypass the TRP1 deletion mutation.

We were able to observe that both the PTY28 and the PYY33 strains lose viability over time. In contrast, the transfer frequency increased during the chronological aging process almost 18-fold. The mtDNA fragments that migrate to the nucleus in aged cells may contribute to the aging process itself, what might be a new mechanism of aging that has not yet been described.

HISTONE SIDE-CHAIN MODIFICATIONS RELATED TO THE INDUCTION OF DIFFERENTIATION MARKERS IN COLON CANCER CELLS

Mentor: Michael A. Lea, PhD, (Biochemistry and Molecular Biology)

Objective:

The objective of this investigation was to identify mechanisms by which differentiation of colon cancer cells can be induced. Differentiation can be monitored by the induction of marker enzymes. The inductions of alkaline phosphatase and dipeptidyl peptidase activities have been used as markers of colon cancer cell differentiation. Previous work in our laboratory has shown that in Caco-2 human colon cancer cells, there can be additive effects of histone deacetylase (HDAC) inhibitors and some polyphenolic molecules that occur in fruits and vegetables. In the colon cancer cell lines that have been examined, variability has been observed in the response to different HDAC inhibitors and the combinations with polyphenolic molecules.

Since histone side-chain modifications can be associated with the regulation of gene expression, we have examined such modifications, including histone H3 acetylation and dimethylation at lysine 9, to determine if they correlate with the responses of the different colon cancer cell lines. Acetylation of histones is often associated with increased gene expression while methylation at lysine 9 of H3 histone has generally been associated with down-regulation of gene expression. We have studied valproate as an HDAC inhibitor that is presently under clinical investigation and the polyphenolic molecules gallate, ellagate, and resveratrol. It was established in previous work that cell lines differ in the induction of differentiation markers in response to valproate. We wished to determine if this reflected differences in the effects on histone side-chain modification.

Methods:

Caco-2, HT-29, SW1116, and NCM460 human colon cancer cells were incubated at 37°C in RPMI 1640 medium with 5% fetal calf serum. These cells were treated with the histone deacetylase inhibitor, valproate, and several polyphenolic compounds, including ellagate, gallate, and resveratrol, either separately or in combination with valproate. Histones were isolated from the four different cell lines by extraction of nuclei with 0.25 N HCl and precipitation with acetone. Protein was assayed by the Bradford procedure. Once the histone concentration in each sample was determined, gel electrophoresis was performed on urea-acetic acid polyacrylamide gels. Upon transfer of the gel to a nitrocellulose membrane, Western blot analysis was carried out using an antibody either for the acetylated H3 histone or for the dimethylated H3 histone at lysine 9. The relative levels of acetylated H3 histone and dimethylated H3 histone at lysine 9 were quantified by densitometry analyses of the Western blots.

Summary:

Gel electrophoresis and subsequent Coomassie Blue staining of Caco-2, HT-29, SW1116, and NCM460 cells suggested that valproate causes an increase in acetylation of H4 histones. This increased acetylation is suggested by the retarded migration of the H4 histone bands in lanes 4, 5, 8, and 9 and is illustrated for HT29 cells in Fig. 1.
Using an antibody for the acetylated H3 histone, Western blot analyses were conducted on Caco-2, HT-29, SW1116, and NCM460 (Figure 2) cells. Quantitative analyses of the blots indicated that 24-hour and 72-hour treatments with 1mM valproate, when used alone or in combination with 25uM ellagate, 25uM gallate, or 50uM resveratrol, induces acetylation of H3 histones in all cell lines studied. Figure 3 shows the relative densities of the Western blot in Figure 2.

Figure 2. Western blot of acetylated H3 histone in NCM460 cells after 72-hour treatment

Lanes 1-3: NCM460 control
Lanes 4 and 5: Treatment with 1mM valproate
Lanes 5 and 6: Treatment with 25uM gallate
Lanes 7 and 8: Treatment with 1mM valproate + 25uM gallate

Figure 3

Western blots of acetylated H3 histone in NCM460 cells after 72 hours

Using an antibody for the H3 histone dimethylated on the lysine 9 residue, Western blot analyses were conducted on Caco-2, HT-29, SW1116, and NCM460 cells. Quantitative analyses of the blots indicated that 24-hour and 72-hour treatments with 1mM valproate, when used alone or in combination with 25uM ellagate, 25uM gallate, or 50uM...
resveratrol, have little, if any, effect on the dimethylation of lysine 9 on the H3 histone. Figure 5 shows the relative densities of the Western blot in Figure 4.

Figure 4. Western blot of H3 histone dimethylated on lysine 9 in HT-29 cells after 24-hour treatment

Lanes 1-3: HT29 control
Lanes 4 and 5: Treatment with 1mM valproate
Lanes 5 and 6: Treatment with 50µM resveratrol
Lanes 7 and 8: Treatment with 1mM valproate + 50µM resveratrol

Figure 5

Western blot of H3 histone dimethylated on K9 in HT29 cells after 24hr

**Conclusion:**

Valproate caused an increased acetylation of H3 and H4 histones in the four colon cancer cell lines that were examined (Caco-2, HT-29, SW1116, and NCM460). Increased acetylation was similar in the different cell lines and was prolonged, being similar after 24- or 72-hour incubations. In contrast to the effect on acetylation, there was little, if any, effect on the dimethylation at lysine 9 of H3 histones.

Under the conditions examined, histone side-chain modifications were not greatly affected by the polyphenols gallate, ellagate, and resveratrol, either as single agents or in combination with valproate, relative to valproate as a single agent.

Although NCM460 cells do not show marker enzyme induction when treated with valproate, the increase in histone acetylation was similar to cells in which there is a large increase in enzyme activity.

The data suggested that although valproate caused increased histone acetylation in all cell lines examined, that effect may not be sufficient to cause an induction of differentiation markers in some cells.
13. ZAIN HUSAIN (NJMS 2011)

STATUS OF LARGE ALLOGRAFTS FOLLOWING LIMB SALVAGE RESECTION OF TUMORS

Mentors: Sagar K. Shah, Anthony D. Uglialoro MD*, Joseph Benevenia MD, Francis R. Patterson MD, Kathleen S. Beebe MD* (Orthopaedic Surgery; Division of Musculoskeletal Oncology)

* Co-mentors

Introduction:

Allograft bone is often used in oncological and trauma limb salvage procedures. Approximately 975,000 musculoskeletal allograft procedures are performed each year in the United States [2]. With recent advances in diagnostic technology and multidisciplinary treatment approaches in musculoskeletal oncology, limb sparing surgery with allograft reconstruction has emerged as a favorable treatment option when compared to amputative surgery. Limb salvage is being performed more frequently because it allows the surgeon to reconstruct segment skeletal defects following tumor resection, while preserving the functional integrity of the limb.

Prior to transplant into patients, allografts must be harvested and treated according to stringent guidelines set forth by the American Association of Tissue Banks and the US Food and Drug Administration. Patient safety is of primary concern for tissue banks, which process allografts. They often employ comprehensive screening tests, lavage washing, alcohol and antibiotic treatment, freezing, and irradiation to ensure graft safety. One of the largest banks is the Musculoskeletal Transplant Foundation (MTF), which uses validated aseptic tissue processing methods in environmentally controlled clean rooms to ensure graft safety. Such methods include exposing unprocessed frozen tissues to gamma irradiation and controlled exposure of tissues to chemical disinfectants or antibiotics [8]. There are four principal types of bone allograft based on treatment protocols used: 1) fresh 2) fresh-frozen 3) freeze-dried and 4) demineralized allografts. Each type of graft possesses different properties, enabling the orthopaedic surgeon to choose particular grafts based on mechanical strength, histocompatibility, and resorption rate, which is important for optimal clinical outcomes [11].

Once the allograft has undergone complete treatment, it is ready for surgical use. There are three main uses for allografts in clinical settings: 1) bone for grafting, 2) soft tissue for ligament reconstruction, and 3) synthetics or bone substitutes for grafting. In each of these cases, the primary goal is to restore skeletal integrity by enabling normal physiologic and structural bone functions. Thus, graft incorporation and healing must occur. Allografts and bone graft substitutes, which are biologics used to facilitate graft incorporation, are used to promote the natural healing of bone. They fill a defect following tumor removal or trauma, or to promote healing of a non-union of fusion. There are three main processes involved in bone healing: 1) osteoconduction, which is providing a biologic scaffold upon which new bone can develop 2) osteoinduction, which is the process whereby local and distant mesenchymal stem cells are induced into the graft area by local and systemic proteins and 3) osteogenesis, which is the process by which the mesenchymal cells differentiate into bone tissue. These processes must occur in a predictable histologic manner and specific sequence for bone graft or bone graft substitutes to be incorporated and eventually converted into normal bone [8].
The purpose of this ongoing study is to investigate outcomes following limb salvage surgery using large allograft tissue distributed by one organization, Musculoskeletal Transplant Foundation (MTF). Although long-term results are satisfactory in the majority of patients receiving large segmental allografts, there are several complications commonly associated with their use. Primary causes of allograft failure are infection, fracture, and non-union. Each of these major complications has been investigated individually in large retrospective studies. However, no study to date has collectively investigated all complications encountered in patients receiving large segmental allografts distributed by a single tissue bank. New developments in biologics, therapies and technology lent the need to investigate the clinical outcomes of massive allograft transplants used in limb salvage surgeries for patients with tumors or major trauma.

Materials and Methods:

This study was approved by the Institutional Review Board of UMDNJ and supported by the MTF through the Foundation of UMDNJ. The data reviewed was collected between 2005 and 2008 on large graft recipients seen at University Hospital-UMDNJ between 1999 through 2008. Data collected includes subject demographics, surgical summaries, allograft integration through pre- and post-operative radiographic and follow-up assessment of up to 5 years post-operative, as available. Patient privacy is maintained through use of the MTF unique identification system for graft tracking. The surgeon/investigator is apprised of, and uses the allograft identifier to review historical clinical and radiographic records for the recipient of the graft. The surgeon supplies the data on forms designed for documentation of the initial patient assessment, pre- and post-operative therapeutic regimen, graft integration and complications for consecutive post-operative visits up to and including the post-operative fifth year or until patient death or loss to follow up when occurring within the five year post-operative period.

Results:

Forty-one patients, 15 males and 26 females, ages 6-70 years old with a mean age of 31.3 years old at time of transplant were retrospectively evaluated. The mean length of follow-up was 33.1 months. In total, 42 operative procedures were performed for 32 malignant, 6 benign, and 4 metastatic lesions. One patient received two allografts in separate procedures. Thirty-one percent of subjects were osteosarcoma patients with other cancers such as Ewing’s sarcoma, giant cell tumors, and aneurysmal bone cysts comprising the rest of the cases. Several modes of fixation were used to stabilize the allografts including standard plates, locking plates, and cementation. A single allograft was used in each of the procedures: 16 intercalary, 10 osteoarticular, 12 allograft-prosthetic composite, 2 morselized, and 1 alloarthrodesis. Among the 41 patients enrolled in the study, 6 were excluded from the outcome results: 4 patients were excluded because they had not yet reached the one year post op criteria while another 2 patients were excluded due to insufficient data. Among patients with successful graft incorporation (18), average time to union was 248.3 days. In total, 42 complications were encountered in 17 patients and consisted of: nonunion (18) which was defined as lack of radiographic incorporation at 12 months, infection (4), fracture (5), hardware malfunction (2), dislocation (1), DVT (1), metastasis (3), amputation (2), and recurrence (6). Many of the patients also received adjuvant therapy including chemotherapy (16), radiation therapy (3), or both (1). The results are summarized in Table 1 and Figure 1.
Discussion:

Allograft tissue transplantation is an effective procedure in reconstructing major skeletal defects following tumor resection in musculoskeletal oncology. Excellent functional results have been reported in many patients receiving large segment allografts. In this study, there was a 50.0% success rate, defined as those patients who showed complete graft incorporation without any major complications. This success rate is lower than that of a large retrospective study conducted by Mankin et al., which was reported to be approximately 75% [5]. This may be attributed to a number of variables such as significantly smaller sample size, the types of grafts used, the anatomic sites, and the stage of lesions and complexity seen in these cases.

As with any surgical procedure, there are several complications associated with bone allograft transplantation. In fact, nearly a third of patients receiving a bone allograft suffer from one or more complications, most notably infection, non-union, and fracture. The primary immediate concern for allograft transplantation is infection, with a post-operative infection risk ranging from 10-15% [3]. The highest risk of infection is during the first year post surgery [5]. This high infection rate can be attributed to factors related to nature of this operation such as wide resection for high grade tumors, frequent skin sloughs, and concomitant use of adjuvant radiation or chemotherapy, or both. There are several types of infection including bacterial surface contamination and viral or spore transfer. The infection rate in this study was 11.1%, which is similar to rates listed in literature. In a study by Mankin et al of 283 patients who received massive allografts, 11.7% of patients developed infections. Gram positive organisms were the most common cause of infection with 36% percent of these infection caused by Staphylococcus epidermis. The final outcomes of patients with infected allografts was poor compared those without infection [4].

Fracture is another major complication seen in approximately 10-15% allograft recipients [3]. The fracture rate in this study was approximately 13.9%, which is similar to rates reported in other studies. According to a retrospective study of 1046 allograft recipients conducted by Mankin et al., 17.7% of the allografts fractured at a mean of 3.2 years after transplantation [7]. There are several factors contributing to allograft fractures according to a retrospective study conducted by Cheng et al. There was a high incidence of fractures in those allografts that were

<table>
<thead>
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<th>Table I. Demographic Data</th>
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<tr>
<td><strong>N=</strong></td>
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<td><strong>Type of Graft</strong></td>
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<tr>
<td><strong>Gender</strong></td>
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<td><strong>Gender Distribution</strong></td>
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<tr>
<td><strong>Amputation</strong></td>
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<td><strong>Disarticulation</strong></td>
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Figure 1. Percent Complications

- Dislocation (3.8%)
- Amputation (4.7%)
- Nonunion (1.6%)
- Metastasis (5.9%)
- Infection (19.2%)
- Fracture (10.3%)
- Recurrence (10.3%)

46
fixed with methods requiring transcortical penetration. Furthermore, there was a correlation between anatomic site and fracture incidence, with the tibia at the greatest risk of fracture [10].

Perhaps the most significant complication in allograft recipients includes non-union of the allograft-host junction. Nonunion is defined as an osteosynthesis site that is not radiographically united at 12 months post-op[9]. There was a 50% non-union rate seen in this study. Nonunion rates reported in literature range considerably between 5-50%. For instance, a large retrospective study of 945 patients who received large allografts, conducted by Mankin et al. reported a 17.3% nonunion rate [6]. However, Donati et al. reported a 49.0% nonunion rate in another retrospective study of 101 patients who received large allografts [1].

There are numerous factors that affect nonunion of the allograft-host junction as demonstrated by Mankin et al [6]. One factor related to nonunion includes the type of graft transplanted. It was shown that the rate of nonunion was highest in patients who had an arthrodesis using allografts compared to patients who received osteoarticular grafts, intercalary grafts, or allograft prosthetic composites. Patients with concomitant infection and fractures also suffered a higher incidence of nonunion. Higher nonunion was also reported with subsequent revisional surgeries. Perhaps the factor that contributes most to allograft-host junction nonunion is adjuvant therapy, which includes chemotherapy and radiation. There was an 11.3% non-union rate among patients who did not receive adjuvant therapy, compared to 27% nonunion rate among patients who received chemotherapy. Among patients who received only radiation therapy, the non-union rate increased slightly to 18%. Patients who received both chemotherapy and radiation experienced a 19.7% nonunion rate.

This study demonstrates that allografts can be used to successfully reconstruct skeletal defects. Fifty-percent of the allograft recipients exhibited graft incorporation without any complications. Major complications of concern were nonunion, fracture, and infection. At one year post-operative, the nonunion rate was 50.0% which is considerably high, but comparable to some studies (49%). Several grafts went on to union after 1 year without surgical intervention, thereby lowering the nonunion rate to 36.1% when taking these cases into account. The fracture rate was 13.9% which is lower than rates reported in a similar study (17.7%). The infection rate was 11.1% which is comparable to rates reported in similar studies (11.7%). All data will be submitted to the MTF for inclusion in a comprehensive multi-institutional study. Results will serve as a control for the effectiveness of prospective biologic adjuvants and to improve the safety and efficacy of future allograft transplantation.
14. ALI HUSAIN (NJMS 2011)

FLORESCENT CELL-PENETRATING PEPTIDE UPTAKE BY TWO PROSTATE CANCER CELL LINES

Mentor: Beverly E. Barton, PhD (Department of Surgery)

Objective:
Prostate cancer is the second leading cause of cancer death in American men. During 2008, approximately 186,000 new cases of prostate cancer will be diagnosed in the United States. Prostate cancer accounted for approximately 27,000 deaths last year and more are predicted for 2008.

Signal transducers and activators of transcription (STATs) are key components of cytokine signal transduction pathways and cell signaling. STAT3 regulation is highly controlled and signaling is brief in benign cells. Constitutive activation of STAT3 is a feature of many malignancies, and results in expression of anti-apoptotic and cell cycling genes, angiogenic factors, and metastasis promoting factors. Ultimately, survival of the malignant cells depends upon continued expression of these genes. Therefore, inhibiting STAT3 may be useful in the treatment of prostate cancer.

Our lab has designed STAT3 inhibitors which inhibit its binding to the genome, however the plasma membrane of cells is extremely selective in what it permits to pass to the interior of the cell. Certain cationic peptides have a unique ability to penetrate the membrane and reach the nucleus. This particular class of peptides is known as nuclear localization signal (NLS) sequences. By adding therapeutic cargoes to NLS sequences, we may be able to treat prostate cancer in a novel way, alongside the contemporary modes of treatment.

The purpose of our experiments was to compare the uptake of four different synthetic peptide sequences, two of which contained NLS sequences on the C-termini, to determine which one might function best as a targeting peptide in prostate cancer cell lines.

Materials/Methods:
Cell Culture. All cell lines were grown as adherent monolayers in a humidified 5% CO2 atmosphere at 37°C in T75 flasks. DU-145 cells were the gift of Dr. James Turkson (University of South Florida). The DU-145 cells were grown in DMEM/Ham’s F-12 GlutaMax (Invitrogen) culture media supplemented with 10% newborn calf serum (HyClone) and HEPES buffer (Invitrogen). LNCaP cells were the kind gift of Dr. Fei Peng, University of Maryland Dental School. They were grown in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum, HEPES and GlutaMax (Invitrogen).

Peptides. Peptides were synthesized by the Molecular Resources Facility at the University of Medicine and Dentistry, New Jersey Medical School (Newark, NJ) on an Applied Biosystems synthesizer. The fluorescein isothiocyanate (FITC) labeled C-terminal resins were purchased from Bachem. Peptides were dissolved in sterile buffer; stock solutions were stored frozen until use. Peptides synthesized are given in the table below and are identified by their peptide synthesis numbers.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Synthesis #</th>
<th>Description</th>
<th>Sequence</th>
<th>Molecular Weight (Daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSMA substrate 1</td>
<td>350</td>
<td>EEE-FITC</td>
<td>776</td>
<td></td>
</tr>
<tr>
<td>PSMA substrate 2</td>
<td>351</td>
<td>EEEAA-FITC</td>
<td>919</td>
<td></td>
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<tr>
<td>PSMA substrate 3</td>
<td>352</td>
<td>EEEAAKKK-FITC</td>
<td>1309</td>
<td></td>
</tr>
<tr>
<td>PSMA substrate 4</td>
<td>353</td>
<td>EEEAAGRKRRKKRT-FITC</td>
<td>1930</td>
<td></td>
</tr>
</tbody>
</table>

Table 1- Fluorescent peptides used for uptake experiments.
The EEE sequence is a PSMA substrate that might be exploited for transport of the peptide into the cytoplasm of LNCaP cells. The AA dipeptide is a linker between functional peptides. Some cationic peptide sequences can transport proteins from the cytoplasm to the nucleus. These NLS sequences are rich in basic amino acids\(^4\). Two putative NLS sequences are included in peptides 352 and 353.

**Time Dependent Peptide Uptake.** 4 and 18 hour incubation

Cells from each cell line were seeded into 6-well plates and incubated at 37\(^\circ\) C for 4 and 18 hours. Fluorescently labeled peptides were added to the cells at varying concentrations. For each experiment, a control of cells that were not incubated with fluorescent peptide was also analyzed. Following incubation, the cells were harvested with trypsin, fixed and analyzed on the flow cytometer.

**Concentration-dependent peptide uptake.** We measured peptide 353 uptake in a one hour 37\(^\circ\) C incubation with concentrations of 0, 3, 30, 300, and 3000 nM. At the end of the incubation, cells were measured for cellular peptide uptake as described above.

**Flow cytometry.** All peptide uptake experiments were read and analyzed by Dr. Beverly Barton. Fluorescence was quantified using a FACScan flow cytometer (Becton Dickinson) and data was analyzed using CellQuest Pro software (Becton Dickinson) and an Apple Macintosh G4 dual coprocessor computer running OS X 10.3.9. Data are reported as geometric mean fluorescence intensities (GMFI).

**Imaging flow cytometry.** We also performed a 4 and 18 hour peptide uptake experiment with both cell lines. Sukhwinder Singh visualized and analyzed the cells using the AMNIS imaging flow cytometer. We used this apparatus in order to visualize the cells and determine localization of the peptide.

**Temperature dependent studies.** Fluorescently labeled peptides at a concentration of 300 nM were added to DU145 cells seeded in 6-well plates and incubated for 4 hours. For each experiment, a control of cells that were not incubated with fluorescent peptides was also analyzed at the same temperatures. The cellular peptide uptake was measured as described above.

**Results/Summary:**

1 hour concentration dependent peptide uptake.

We observed peptide 353 uptake in both cell lines with a concentration as low as 3 nM. We also observed increased peptide uptake as the concentration increased. An increasing GMFI corresponds to increased peptide uptake. The control samples, in which no peptide was added, had a GMFI value of 5 and 8 for DU145 and LNCaP cells respectively. At the maximum concentration of 3000 nm the samples had a GMFI value of 1757 and 1358 for DU145 and LNCaP cells respectively. These results are depicted in figure 1.

**Time Dependent Peptide Uptake.**

DU145 cells showed increased peptide uptake at both 4 hour and 18 hour incubations. For the 4 hour incubation, the control well GMFI value was 9. The GMFI values of peptides 350, 351, 352, 353, were 158, 153, 274, and 386 respectively. Peptides 352 and
353 showed significant increased uptake when compared with peptides 350 and 351.

For the DU145 cell 18 hour incubation, the control well GMFI value was 15. The GMFI values of peptides 350, 351, 352, 353, were 195, 192, 289, and 396 respectively. Peptides 352 and 353 showed significantly better uptake when compared with peptides 350 and 351.

The 18 hour incubation showed slightly increased peptide uptake in comparison to the 4 hour incubation. These results are depicted in figure 2.

LNCaP cells showed increased peptide uptake at both 4 hour and 18 hour incubations. For the 4 hour incubation, the control well GMFI value was 19.285. The GMFI values of peptides 350, 351, 352, 353, were 171, 131, 186, and 363 respectively. Peptide 353 showed significant increased uptake when compared with the other peptides.

For the LNCaP cell 18 hour incubation, the control well GMFI value was 26. The GMFI values of peptides 350, 351, 352, 353, were 435, 433, 533, and 1036 respectively. Peptide 353 showed significant increased uptake when compared with the other peptides.

Temperature dependent studies
DU145 cells varied in their peptide uptake with respect to temperature. As temperature increased, peptide uptake also increased. For example, peptide 353’s GMFI value was 153, 341 and 506 at temperatures of 4°C, 25°C or 37°C respectively. These results are depicted in figure 3.

Imaging flow cytometry. According to our imaging flow cytometry data, we found that all peptides showed cytoplasmic accumulation in both 4 and 18 hour incubations. Specific differences in peptide uptake with respect to each peptide were also observed. Figure 4 shows an 18 hour incubation of DU145 and LNCaP cells treated with peptide 351 and 353. The fluorescence intensity is much stronger with peptide 353 indicating increased uptake. Although there is significant cytoplasmic accumulation, there is not definite nuclear localization. Some images show diffuse staining indicating that there might be nuclear localization.
Conclusion:

Our results indicate that our synthetic peptides are transported into the cell readily. Peptide uptake is a concentration-, time-, and temperature- dependent process. Peptides 352 and 353 displayed more rapid and greater intracellular accumulation, compared to peptides 350 and 351.

In our concentration studies, a steady state or saturation point was not reached, even in the maximum concentration of 3000 nM. Peptide 353 was of significant interest and was used at this time and further concentration studies of each peptide will be performed.

Based on our flow cytometry data, all peptides show significant peptide uptake into the cytoplasm of the cells of both prostate cancer cell lines. According to our hypothesis we expected specific differences in the extent of peptide uptake. Indeed, peptides 352 and 353, each of which have an NLS sequence, displayed increased uptake.

DU145 cells varied in their peptide uptake with respect to temperature. As temperature decreased, peptide uptake also decreased. These results indicate that an energy requiring component, such as ATP, maybe involved in the mechanism of transport of these peptides.

According to our imaging flow cytometry data, we found that all peptides showed cytoplasmic accumulation in both 4 and 18 hour incubations. We did not expect peptides 350 and 351 to be transported to the nucleus. It is possible that 4 hours was not enough time for the nuclear transport of peptide 352 and 353. At 18 hours, all of the peptides again, showed cytoplasmic accumulation. However, some of the cells treated with peptides 352 and 353 did show diffuse nuclear staining which might indicate nuclear localization.

Our goals include designing efficient peptide delivery systems to transport therapeutic payloads to prostate cancer cells, and exploring the nuclear localization potential of these peptides with NLS sequences. Future studies will be performed in order to gain a better understanding of the mechanisms of uptake and will include use of inhibitors of nuclear export and endosomal accumulation.
15. IBRAHIM HUSSAIN (NJMS 2012)

TELOMERE DYSFUNCTION INDUCED SENESCENCE IN HUMAN BREAST CANCER PRECURSOR LESIONS

Mentors: Utz Herbig, PhD, (NJMS-UH Cancer Center), Anitha Suram, PhD, (NJMS-UH Cancer Center), Neena Mirani, MD, (Pathology), Meera Hameed, MD, (Pathology)

Objective:

Cellular senescence is an irreversible growth arrest that occurs in response to diverse stresses and signaling imbalances placing a cell at risk for malignant transformation. A critical role for this permanent growth arrest in preventing tumor progression was recently demonstrated by a number of laboratories who observed that certain benign human tumors and cancer precursor lesions are comprised of cells that had undergone cellular senescence. Together with other studies, these observations contributed to the now established fact that cellular senescence is a critical tumor suppressing mechanism that prevents malignant cancer progression. The signals that initiate this permanent growth arrest in human cancer precursor lesions however, remain poorly understood.

Studies in cell cultures revealed that cellular senescence is triggered by a number of stresses including dysfunction of telomeres, the physical ends of linear chromosomes. When telomeres become critically short, due to continuous cell proliferation and other stresses, they become recognized as double strand DNA breaks and initiate a signaling cascade that results in the senescence growth arrest. Cells that had undergone Telomere Dysfunction Induced Senescence (TDIS) are characterized by the presence of DNA damage foci that co-localize with telomeric DNA sequences. Using immunofluorescence microscopy we demonstrate that the majority of cells in early, pre-malignant human breast hyperplasia display dysfunctional telomeres and other markers of cellular senescence such as elevated levels of the heterochromatin protein MacroH2A. Senescence markers were severely reduced or absent in breast carcinomas, indicating that most cells within these advanced-stage human cancers have lost the ability to undergo TDIS. Our data suggest that TDIS is a critical tumor suppressing mechanism that limits the growth of cells in early breast-cancer lesions.

Methods:

Fifteen cases each of archival hyperplastic and invasive breast cancer tissue were obtained from the Dept. of Pathology at UMDNJ-University Hospital. 4 μm tissue sections were deparaffinized, hydrated through a graded ethanol series, and subjected to heat-induced antigen retrieval for forty minutes in sodium citrate buffer. The slides were stained using a telomere immunofluorescence in situ hybridization (FISH) assay. The antibodies used in my study were anti-53BP1, anti-MacroH2A, anti-p16, and anti-Ki67. The tissues were imaged using a Zeiss Axiovert 200 epi-fluorescence microscope equipped with ApoTome.

Summary:

The molecular changes that occur at different pathological stages during breast cancer development are poorly understood. By staining duct hyperplasias and invasive breast carcinomas for markers of cellular senescence we were able to deduce a number of key points that may explain why many breast cancer precursor lesions never progress to a malignant stage. First, we observed significantly more double-strand DNA breaks in duct hyperplasias compared to cells in invasive breast cancers (Fig.1). Second, MacroH2A, a senescence marker previously characterized in human cell cultures, identifies and labels senescent cells in vivo as...
co-staining with Ki67, a cell proliferation marker, is mutually exclusive in tissue (Fig. 2). Third, pre-malignant duct hyperplasias primarily were comprised of senescent cells, as the great majority of these cells displayed elevated levels of the heterochromatin- and senescence-marker MacroH2A (Figs. 2 & 3). In contrast, cells in malignant breast carcinomas contained few MacroH2A positive cells suggesting that the majority of cells in these cancers had bypassed cellular senescence. Fourth, all of the abnormal duct epithelial cells that displayed DNA damage foci also contained elevated levels of the heterochromatin protein MacroH2A, suggesting DNA damage as the causative factor for cellular senescence in these cells (Fig. 3). Finally and most importantly, we demonstrated that the double strand DNA breaks in hyperplastic cells co-localized with telomeres, while DNA damage in invasive breast cancer primarily was of non-telomeric origin (Fig. 4). This suggests that invasive breast cancer cells had stabilized their telomeres and evaded the tumor suppressing properties of telomere dysfunction induced senescence (TDIS). A final model for the progression of breast cancer is given, taking into account the newly discovered role of telomere dysfunction as a tumor suppressing mechanism in humans (Fig. 5).

Figure 1. Double strand DNA breaks in hyperplastic and invasive breast tissue. Tissues were immunostained with an antibody against 53BP1 (green) and counterstained with DAPI (blue). Representative images of pre-malignant human breast tissue (hyperplasia) and invasive breast cancer (invasive) are shown. Bar graph: quantification of 53BP1 positive breast epithelial cells in hyperplasia and invasive breast cancers. An average of 85 and 102 nuclei per tumor were analyzed for hyperplasia and invasive tissue, respectively. Images were acquired as z-stacks spaced 0.4 mm apart at 100x magnification and merged into a single layer for better visualization.

Figure 2. Cellular senescence in breast hyperplasia (top), but not invasive breast cancer tissue (bottom). Pre-malignant (hyperplasia, top) and invasive human breast cancers (bottom) were
immunostained with antibodies against MacroH2A (green) and Ki67 (red). Nuclear DNA was counterstained with DAPI (blue). MacroH2A is a heterochromatin protein that accumulates in senescent cells; Ki67 is a cellular marker for proliferation. Note that cells in breast hyperplasia are senescent (high MacroH2A, low Ki67), while the majority of cells in breast carcinomas were proliferating (low MacroH2A and high Ki67). Images were acquired at 63x magnification.

Figure 3. Double strand DNA breaks are positively correlated with cellular senescence in breast hyperplasia (top) but not in invasive breast cancer tissue (bottom). Pre-invasive and invasive breast cancer tissue was immunostained with antibodies against 53BP1 (red) and MacroH2A (green). Nuclear DNA was counterstained with DAPI (blue). Note that the great majority of cells with DNA damage foci also stain positive for MacroH2A in breast hyperplasia but not in breast carcinoma. Images were taken at 63x magnification.

Figure 4. DNA damage foci co-localize with telomere sequences in breast hyperplasia but not in invasive breast cancer tissue. 15 samples of each pre-malignant (hyperplasia) and malignant (invasive) human breast cancers were simultaneously immunostained using antibodies against 53BP1 (green) and a PNA complementary to telomeric DNA sequences (red). Nuclear DNA was counterstained with DAPI (blue). The great majority of cells in breast hyperplasia display discrete DNA damage foci that co-localize with telomeres. Although cells in invasive breast cancer tissue occasionally displayed DNA damage foci, telomere dysfunction induced DNA damage foci (TIF) were generally not observed. Bar graph: quantification of TIF (% of 53BP1
foci co-localizing with telomere foci) in hyperplasia and invasive breast tissues. An average of 40 cell nuclei per tumor was analyzed. Images were acquired as z-stacks separated by 4mm at 100x magnification.

Figure 5. Model of breast cancer progression. Ductal epithelial cells with an oncogenic mutation proliferate forming a lesion. Telomeres shorten with every cell division. If the senescence signaling pathways remain intact, cells will undergo telomere dysfunction induced senescence (TDIS) once telomeres become critically short. TDIS thereby limits progression of human breast cancer. Cells with defective senescence signaling pathways evade cellular senescence and progress to invasive carcinoma.

Conclusion:

Understanding the molecular mechanisms that control ductal epithelial cell growth will likely facilitate the development novel anti-cancer drugs and therapies to stop the advancement of malignant- and metastatic breast cancer. Our results demonstrate that the great majority of cells in pre-malignant human breast cancer lesions are senescent while those in invasive breast carcinoma are not. Cellular senescence was strongly correlated with telomere dysfunction suggesting that telomere dysfunction induced cellular senescence (TDIS) limits breast cancer progression. In addition, our data suggest that cells in invasive breast carcinomas have evaded TDIS, potentially by mechanisms that involve re-activation of telomerase. Finally, we have discovered a potential biomarker for tumor stage which could facilitate decisions on how to treat individual cancer patients.
16. TIFFANY JOW (STEVENS INSTITUTE OF TECHNOLOGY 2010)

CLONING AND INITIAL CHARACTERIZATION OF PROMOTERS OF THE GENES ENCODING MOUSE INTERFERON-λs AND HUMAN INTERFERON-λ RECEPTOR

Mentor: Sergei Kotenko, Ph.D. (Department of Biochemistry and Molecular Biology)

Objective:

Interferons serve as the body's innate immune response to viral infection. IFNs also demonstrate anti-tumor properties and are used in cancer therapy. While much more is known about Type I (INF-α, INF-β) and Type II (INF-γ) interferons, further characterization and investigation of Type III interferons (INF-λ) are necessary in order to fully understand the potential in healthcare of these antiviral proteins.

Background:

Fifty years after the breakthrough discovery of interferons, many questions still remain regarding the characterization of and potential in healthcare for these antiviral proteins. It is known that interferon production occurs as the body's innate response to viral infection and that these cytokines assist the immune system by inhibiting viral replication and by inducing resistance within host cells. In addition, interferons upregulate the production of other antiviral proteins as well as activate various immune cells such as natural killer cells and macrophages. Three types of interferons have been identified: Type I (INF-α and INF-β), Type II (INF-γ), and Type III (INF-λ). While INF-α and INF-β are secreted by all nucleated cell types in response to viral infection, the regulation of INF-λ production has not yet been extensively characterized. The human genome contains three IFN-λ genes: λ1, λ2, and λ3. In mice, there also exist three forms of IFN-λ: mλ1, mλ2, and mλ3. However, only mλ2 and mλ3 are expressed as the mIFN-λ1 gene contains a stop codon and does not code for a functional protein.

Important transcription factors involved in IFN-α/β production are IFN regulatory factors, IRF-3 and IRF-7, STAT proteins, as well as members of the NFKB and AP1 families. In order to characterize the regulation of INF-λ expression, it is important to demonstrate that these factors modulate the INF-λ transcription process. It has been demonstrated that the production of human IFN-λ1 is regulated similarly to INF-β, whereas human IFN-λ2 and IFN-λ3 are regulated similarly to INF-α. Because the murine genome only produces λ2 and λ3, it is important to identify which transcription factors stimulate the upregulation of murine IFN-λ2 and murine IFN-λ3 most effectively and whether these pathways more closely resemble those of INF-α or INF-β regulation. The promoter for murine IFN-λ3 has been cloned. It is important to continue with the cloning of the murine IFN-λ1 and murine IFN-λ2 promoters in order to test the different sensitivities of each to various transcription factors.

The IFN-λ proteins signal via a receptor complex comprised of two subunits, IL10R2 and IFNLR1. The IL10R2 subunit is expressed in the majority of cells. However, the production of the IFNLR1 subunit is tightly regulated and rarely produced except in plasmacytoid dendritic (pDC) and epithelial-like cells. Our objective is to clone the promoter region of the IFNLR1 gene into a luciferase-encoding reporter vector and perform successive tests measuring luciferase expression in order to determine which combination of promoter elements and transcription factors stimulates the IFNLR1 promoter most effectively.
Interferons are known to signal via the JAK-STAT pathway in order to upregulate expression of MHC class I antigens, various antiviral proteins, as well as transcriptional factors such as IRF-7 that enable cells to produce more interferons. The identification of STAT proteins in the cytoplasm via gel shift provides a way to detect interferon activity. The mIFIT-1 has been detected to be one of the antiviral genes that is strongly upregulated in response to either IFN-α/β or IFN-λs activity in the cell. Cloning the promoter of this gene will allow us to create reporter cells lines that respond to either IFN-α/β or IFN-λs by induction of luciferase expression, and to study the kinetics of mIFIT-1 gene expression in response to different IFNs.

Methods and Summary:

Amplification and Cloning of Promoter Regions:

The regions of interest were initially amplified using nested PCR (Polymerase Chain Reaction). This technique was used to amplify the IFNLR1-promoter, mIFIT-1, mIFN-λ1-promoter, and mIFN-λ2-promoter genes. After many trials, IFNLR1-promoter was finally successfully amplified using nested PCR and a gradient protocol (50°-65°C) for the annealing temperature on human PBMC cDNA library. The primers used for 1st round PCR were 12-23/12-21; the primers used for 2nd round PCR were 12-24/12-26. The optimal annealing temperature was 58°C.

Nested PCR was also used to amplify the mIFIT-1, mIFN-λ1-promoter, and mIFN-λ3-promoter regions on mouse C57/Bl6 genomic DNA. The primers used for the 1st round of PCR for mIFIT-1 were mIFIT-1/mIFIT-2; the primers used for 2nd round of PCR were mIFIT1-0/mIFIT1-2. The primers used in the 1st round of PCR for the muIFN-λ1 promoter were mifnl1pr1/mifnl1pr2-2 and for the 2nd round, mifnl1pr3/mifnl1pr2-2. The primers used in the 1st round of PCR for the mIFN-λ3-promoter gene were mifnl3pr1/mifnl3pr2 and for the 2nd round, mifnl2pr1/mifnl3pr2. The annealing temperature for these 3 nested PCRs was 52°C.

After amplification of the selected regions of interest was accomplished, the PCR products were purified by phenol chloroform extraction and digested with MluI/KpnI restriction enzymes at 37°C overnight. The digested products were run and DNA was extracted using Qiagen Gel Extraction Kit. The regions of interest were then ligated using T4 DNA Ligase at 16°C overnight to the pef2-luciferase vectors also digested with MluI/KpnI restriction enzymes. Ligated vectors
were transformed into DH/10B competent cells and plated on Ampicillin-Resistant LB plates, which were then incubated at 37°C overnight. Clones were picked up and grown in TB media at 37°C overnight. Mini-preps were performed and plasmid DNA was cut again with MluI/KpnI and verified on 1% agarose gels.

Transfection in COS cells:

Cos-7 cells were transiently transfected with a variety of plasmids (See Table 1) by DEAE-dextran method. After 36 hours, the cells were harvested, lysed (Triton–Gly–Gly Lysis Buffer), and assayed with a luciferin substrate in a luciferase assay buffer.

Table 1:

<table>
<thead>
<tr>
<th>Well</th>
<th>Luminescence Ct. 1</th>
<th>Luminescence Ct. 2</th>
<th>Average</th>
</tr>
</thead>
<tbody>
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</table>

Average of both Trials:

58
These preliminary results show that mIFN-λ3 is regulated similarly to INF-α and are thus IRF-3 and IRF-7 dependent. IRF-3 alone even shows a reduction in mIFN-λ3 upregulation, whereas IRF-3 and IRF-7 yield a stronger mIFN-λ3 expression.

Conclusion:

The promoter regions of mIFN-λ2, mIFN-λ1, and mIFIT-1 were cloned into luciferase vectors, as well as the promoter region of human IFNLR1. Preliminary results were also obtained that show mIFN-λ3 to be regulated similarly to INF-α as it is IRF-3 and IRF-7 dependent. Future directions for the project include performing tests using luciferase on the IFNLR1 promoter-driven luciferase expression vector in order to determine which combination of transcription factors is required to stimulate the hIFNLR1 promoter in epithelial cells. Additionally, we intend to create reporter cell lines using the mIFIT-1 promoter gene that respond to either IFN-α/β or IFN-λ3 by induction of luciferase expression and to study the kinetics of mIFIT-1 gene expression in response to different interferons. Lastly, we would like to further characterize whether mIFN-λ3 is regulated similarly to INF-α or INF-β, and study the regulation of the mIFN-λ2 gene expression.
17. VIDHI KAPOOR (NJMS 2011)

RELATION OF INTIMATE PARTNER VIOLENCE TO CERVICAL AND BREAST CANCER SCREENING

Mentors: Ping-Hsin Chen, PhD (Principal Investigator- Family Medicine), Sue Rovi, PhD (Family Medicine), Marielos Vega, BSN, RN, (Family Medicine) Mark S. Johnson, MD, MPH, (Family Medicine), Abbie Jacobs, MD, (Family Medicine)

Acknowledgment- Eugene Muchnik (NJMS 2011)

Objective:

The prevalence of domestic violence in an urban setting is about 14%. Although many studies of domestic violence are emerging, very few studies have addressed the effects of intimate partner violence (IPV) on cancer related health issues. However, one such study has established a strong relationship between IPV and cervical dysplasia. The purpose of our study is to examine the prevalence of cancer screening for victims and non-victims of IPV. We examined whether victims of IPV are up to date in their mammograms, Pap smears, and colonoscopies when compared with non-victims.

Methods:

A total of 421 patients at urban primary care settings in 3 different locations were part of the patient pool. Of these 421 patients, 70 were identified as victims of IPV during approximately a yearlong screening protocol that took place from 2004 to 2005. All patients screened were 18 and older. The analytical sample includes 399 patients of which 143 met the guideline criteria. Within the eligible patient pool, 25 patients (18%) were identified as IPV victims. Therefore all the patients included in this study were within the ages of 40 to 74.

The date of IPV screening was used as an index date for the chart reviewing period; a retrospective chart review was performed for every office visit beginning one year before the date of IPV screening to two years after the date of IPV screening using both computerized and paper charts. Specific abstraction forms were created which focused mainly on the patients' health care maintenance regarding mammograms, Pap smears, and colorectal cancer screenings. The guidelines used for an “Up to Date” screening are as follows: mammograms were considered up to date if the patient was at least 40 years of age and had a mammogram within two years leading up to the date of IPV screening. Patients were considered up to date with cervical cancer screening if patients were at least 40 years of age and had received a Pap smear within 3 years leading up to the date of IPV screening. Colorectal cancer screening was considered up to date if the patient was at least 50 years of age and had a colonoscopy/sigmoidoscopy within 10 years leading up to the IPV screening. Abstraction forms also focused on other information such as the type of insurance, smoking status, chronic diseases, height, weight, family histories of breast, cervical, and colorectal cancer.

Results:

Victims and non-victims were similar in race/ethnicity, educational attainment, and marital status. Overall, there were 84.8% of African Americans patients. Out of all the patients, 23.9% had completed college, and 33.2% were married.
In comparison, victims were more likely to have Medicaid/Medicare (56.3% non victims vs. 75.0% victims; p=.004), victims also tended to have lower income levels ($20,550 non victims vs. $14,100 victims; p=.004), and victims were also less likely to be employed (76.9% non victims vs. 63.6% victims; p=.022).

Screening rates of Pap smear, mammography, and colorectal cancer did not differ significantly between victims and non-victims (p>.05). Overall, screening rates were 49.0% for Pap smear, 49.7% for mammography, and 20.7% for colorectal cancer screening.

Conclusion:

Compared to the U.S. adult population, our study population has poorer cancer screening rates. According to the 2006 Behavioral Risk Factor Surveillance System (BRFSS) survey, screening rates of the U.S. adult population were 86% for Pap smear, 72% for mammography, and 55.6% for colorectal cancer screening. Based on these results, clinicians should make sure that the general female population (both victims and non-victims of IPV) are regularly screened for cancer. Also based on our results, it is apparent that victims of IPV tend to have lower income levels and are less likely to be employed resulting in a lower economic status, which in turn may affect their ability to maintain their healthcare. Although the final results show no significant difference between screening rates for victims and non victims, it is possible that since the overall cancer screening rates of our population (non victims and victims) are lower, the difference between their screening rates may not be apparent.
18. JILL KONOWICH, (NJMS 2011)

TARGETING ARID1B TO GLOBALLY SUPPRESS PROLIFERATION OF OSTEOSARCOMA CELLS

Mentor: Elizabeth Moran, PhD, (Orthopaedics)

Objective:

Appropriate regulation of gene expression requires the interplay of complexes, such as the SWI/SNF complex, that globally remodel chromatin structure through ATP hydrolysis coupled to nucleosome mobilization. Because the SWI/SNF complex is involved in regulating the accessibility of individual genes to sequence specific transcription factors, it is vital to proper cell cycle control. Since a loss of proliferation control can convert precursor cells to tumor cells, the SWI/SNF complex has become an important focus in anti-tumor targeting due to its ability to enact global changes in gene expression patterns and directly interact with tumor suppressors and oncogenes involved in transcriptional control, such as RB, BRCA1, c-Myc, and MLL. Initial difficulty with separating the pro-proliferative and anti-proliferative effects of SWI/SNF mediated-chromatin remodeling were overcome with the discovery that the SWI/SNF complex has two key dichotomies. Two protein subunits, ARID1A and ARID1B, are determinants of complexes with opposing roles with respect to cell cycle control. ARID1A is implicated in tumor suppression, while ARID1B has been deemed to be a pro-proliferative agent which allows promoter accessibility for multiple genes positively involved in cell growth. Preliminary studies, in which osteoblast precursors depleted of ARID1B appeared prone to senescence, have suggested that targeting ARID1B in osteosarcoma cells would significantly slow cell proliferation and would be advantageous because of its broad effect. Furthermore, ARID1B has been shown to promote maintenance of c-Myc expression and cell cycle specific gene expression of known retinoblastoma tumor suppressor gene product (pRb) targets. Therefore, if gene control equilibrium shifts away from ARID1A and toward ARID1B, then two major oncogenic events associated with osteosarcoma might be reversed: uninhibited activation of c-Myc and the effects of loss of pRb. Hence, targeting of the chromatin remodeling subunit ARID1B is hypothesized to have consequences similar to both restoring pRb and down-regulating c-Myc.

The objective of these studies is to show that depletion of the chromatin remodeling subunit ARID1B will reduce expression of multiple pro-proliferative genes in osteosarcoma cells. This will be accomplished by making stable knockdowns of ARID1A and ARID1B in Saos2 cells, an osteosarcoma cell line, using transfected plasmid vectors that deliver shRNA sequences that have been previously shown to work in osteoblasts. The level of ARID1B expression will be assayed in wild-type Saos2 cells using Western Blot analysis and will be later revisited to confirm if ARID1B has been successfully knocked down. Also, the level of c-Myc expression in wild-type Saos2 will be assayed and later compared to its level in proven ARID1B knockdown cells to determine if ARID1B depletion has had its hypothesized effect on c-Myc expression. The SWI/SNF chromatin remodeling complex has also been shown to directly associate with the c-Myc promoter through ARID1B, and c-Myc’s activation is ARID1B dependent. But, it has yet to be determined if this interaction occurs in osteosarcoma cells and whether it is linked with activation signals such as histone acetylation at the c-Myc promoter. By performing chromatin immunoprecipitation assays focusing on the c-Myc promoter region where ARID1B interacts with the E2F and STAT binding areas, we should be able to confirm the interaction of ARID1B with the c-Myc promoter region of the genome and then can further analyze the interaction’s
involvement with other chromatin remodeling agents such as histone acetyltransferase (HAT) or histone deacetyltransferase (HDAC).

**Methods:**

*Culture of Osteosarcoma cells*
Saos2 cells from ATCC were maintained in McCoy's 5A medium supplemented with 15% Fetal Bovine Serum and 1% Penicillin/Streptomycin.

*Transfection of Osteosarcoma cells to create stable ARID1A and ARID1B knockdown cell lines*
Saos2 cells were grown to 90% confluence on a 10 cm plate. 24 hours before transfection, fresh media with serum and antibiotics was added. Plasmids engineered to knockdown ARID1A and ARID1B via shRNA interference were delivered via Lipofectamine 2000. Protocol for Lipofectamine 2000 for sensitive cells lines was followed, and cells were incubated with Lipofectamine 2000 and plasmids for 5 hours. Afterwards, fresh medium with antibiotics and serum was added. On day 3, each plate was split 1:10 and McCoy's 5A media with 15% FBS, 1% Penicillin/Streptomycin, and 500 g/mL g418 was added. Media was changed every 3-4 days until colonies begin to grow.

*Western Blot Analysis*
Whole cell extracts were separated on a 7.5% SDS-PAGE gel and then transferred for 1 hour at 100 Volts to a polyvinylidiene difluoride membrane. A prestained protein marker was used as a molecular weight standard. The membrane was immunoblotted primarily with either mouse c-Myc or KMN (for ARID1B) and secondarily with anti-mouse HRP and then developed by chemiluminescence using a Western lighting kit (promega) to evaluate the levels of c-Myc and ARID1B expression in Saos2 cells.

*Primer design for Chromatin Immunoprecipitation assays*
Primers were designed for the human c-MYC promoter sequence to probe for the SWI/SNF subunit interaction on the c-Myc promoter (via ARID1B interaction).

**c-MYC set 1:** Fwd: TTA TAA TGC GAG GGT CTG GA  Rev: GGA TCT CCC TTC CCA GGA C
**c-MYC set 2:** Fwd: AGC AAA AGA AAA TGG TAG GC  Rev: GAA ACT TTG CCC ATA GCA GC

**Summary:**
Saos-2 cell transfections were not successful, so no stable cell lines have been successfully created yet. Even alterations to the protocol to minimize delivery toxicity did not prove successful as no colonies were seen after 5 weeks time. A new delivery system called NanoJuice will possibly be tried. The integrity of the plasmids is being checked via a maxi prep and restriction enzyme digestion, and a G418 concentration curve is currently being performed.

Western blots for both c-Myc and ARID1B were both inconclusive since there was not enough of a specific reaction. RNA has been isolated from Saos2 cells, and RT-PCR will be used to visualize expression levels of c-Myc at the RNA level.
Conclusion:

With further work on creating successful stable knockdown ARID1B cell lines, the lines will be characterized, permitting evaluation of the effect of ARID1B deficiency on expression of multiple pro-proliferative genes in osteosarcoma cells.

References:


19. YOOMIE LEE (TCNJ, 2011)

Fluorescent Resonance Energy Transfer (FRET)-Based Diagnostic Assay for Determining Drug Resistance in CML Patients

Mentor: Raymond Birge, PhD, (Biochemistry)

Introduction:

Chronic Myelogenous Leukemia (CML) is a multiphasic disease comprising 15-20% of all adult leukemia. The condition generally appears during the fourth and fifth decades of life and consists of a benign and easily treatable chronic phase, an ill-defined accelerated phase characterized by a rapid increase in blast cells, and a terminal blast phase that is refractory to treatment. The fusion tyrosine kinase Bcr-Abl, produced by the Philadelphia chromosome that is formed by a 9:22 translocation is thought to be the major driving force behind the disease and current treatments are aimed at inhibiting the kinase activity of this enzyme. Imatinib mesylate (Gleevec), which is currently first line therapy for CML, has shown remarkable success at controlling the chronic phase of this condition, however patients eventually do relapse into a treatment-refractory and terminal blast phase. Additionally, during the course of the chronic phase, drug resistance is a major issue and patients often need to be switched to different medications. It is our intention to use FRET technology to develop an assay which can be used to not only diagnose new cases of CML but will also be able to determine when a patient has become resistant to the current treatment, signifying that a different therapeutic agent should be employed by the physician. FRET (Fluorescent Resonance Energy Transfer) is based on the principle that, when two fluorophores of overlapping emission and excitation spectra are within close proximity, energy is transferred from the donor fluorophore to the acceptor fluorophore upon excitation of the donor fluorophore with light of a certain wavelength. In our case, the FRET probe that we will be using is called Picchu and is the CrkII protein flanked N-terminally by YFP (yellow mutant of green fluorescent protein) and C-terminally by CFP (cyan mutant of green fluorescent protein). CrkII is an adaptor protein that has been shown to interact with and become phosphorylated by Bcr-Abl. This binding causes and intramolecular interaction that brings the N-terminal and C-terminal ends of the CrkII protein together, a behavior that is mirrored by the Picchu probe, bringing the YFP and CFP fluorophores near each other. Once this close proximity is established, excitation of the CFP with light of wavelength 430 nm causes an energy transfer to YFP which emits at 530 nm. If the Picchu probe is not phosphorylated, excitation with 430 nm will cause CFP to emit at 475 nm. Hence, we should be able to gain information on the activity status of Bcr-Abl by analyzing the wavelength of light emitted by Picchu.

Objective:

We wish to develop a diagnostic fluorescent resonance energy transfer (FRET)-based assay to allow physicians to make early diagnoses and relapse determinations for Chronic Myelogenous leukemia (CML) -positive patients.

Methods:

Four western blots were performed. For each blot, 5x10^5 293T cells were transfected with 1 µg Picchu and 2 µg Bcr-Abl followed by either no treatment or treatment with 10 µM Gleevec. Forty eight hours later, the cells were lysed with 1% HNTG buffer and proteins were
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quantified with a Brønsted Lowry assay. A total of 15 μg protein was loaded onto a gel of 10% and 6% resolving and 4% stacking layers. The proteins were transferred onto a PVDF membrane which was then blocked using 5% skim milk or 5% BSA-Tween. The membrane was treated with primary antibody (Anti-Phospho-Abl 245, Anti-Abl, Anti-Phospho-CrkII and Anti-CrkII) overnight at 1:5000 dilution. After 3-4 washes with 1x TBS-Tween, the membrane was treated with secondary antibody overnight again at 1:5000 dilution. Afterwards, the membrane was treated with enhanced chemoluminescence (ECL) reagent and developed.

FRET assays were performed by first plating. 3x10⁵ 293T cells onto each well of a 6-well plate. 1μg of Bcr-Abl (WT and mutants Y253F, T315I and E225K) was transfected into cells. After twenty-four hours, cells were treated with Gleevec. Followed two hours later by transfection of Picchu. After twenty-four hours, cells were lysed, quantified and loaded onto a 96-well plate and, using a BIO-TEK Synergy HT plate reader, Picchu was excited at 433nm and emissions were read at 475 and 530nm.

Summary:

Four Western Blots (Figure 1), differentiated by type primary antibody used (Anti-phospho-Abl 245, Anti-Abl, Anti-phospho-CrkII and Anti-CrkII) provided the following information: tyrosine kinase Bcr-Abl, rather than chimeric protein Picchu, is responsible for phosphorylation of either Bcr-Abl itself or Picchu (Anti-phospho-abl 245 Ab blot). The similarities between the Anti-phospho-Abl 245 and Anti-phospho-CrkII ab blots show that Picchu phosphorylation indicates Bcr-Abl activity. Overall, the Western Blots show that it is legitimate to use Picchu as a marker of Bcr-Abl activity - a discovery that we exploited using FRET (Fluorescent Resonance Energy Transfer).

![Figure 1](image_url)
The FRET data (Figure 2) showed the effects of Gleevec, currently first line therapy for CML, on various mutants of Bcr-Abl, Y253F, T315I and E255K, using change in 530 nm emission. The 530 nm emission indicates that Picchu has been phosphorylated by Bcr-Abl, conferring onto Picchua phosphorylated closed configuration, which emits light at wavelength of 530 nm upon excitation at 430 nm. In comparison to WT Bcr-Abl, the Y253F mutant exhibits slight resistance to Gleevec treatment. The E255K mutant exhibits resistance to Gleevec treatment as evidenced by the way in which it exhibits no significant decrease in 530 nm emission. The T315I mutant is the most resistant form of Bcr-Abl, as evidenced by no decrease in the 530 nm emission.

**Conclusion:** We have developed and are currently standardizing a novel FRET-based assay that can reflect Bcr-Abl phosphorylation and activity. Our FRET probe Picchu is able to differentiate, through 530 nm emissions, between WT Bcr-Abl and mutant Bcr-Abl, such as Gleevec-resistant T315, in the presence of Gleevec. Further experimentation includes the standardization and optimization of this FRET-based assay, further development with Flow Cytometry, and testing this assay’s ability to recognize different Bcr-Abl positive cell types through use of fluorescent antibodies to cell surface markers.
Objective:

Glioblastoma Multiforme is the most common brain tumor and has poor prognosis. 40% of such tumors show amplification of the epidermal growth factor receptor (EGFR) gene, and they are often associated with increased expression of EGFR protein. It is thought that tumors arise as a consequence of genetically predisposing factors as well as a consequence of environmental stressors, such as hypoxic and hypoglycemic environments. Prior studies in our lab had shown that the combination of hypoxia and hypoglycemia induced EGFR expression in rodent neural stem cells. Therefore, we hypothesized that subjecting malignant glioma cells to hypoxia and hypoglycemia would induce expression of EGFR. Furthermore, the zinc finger transcription factor, egr-1 has been associated with cellular responses to hypoxic stress, is known to bind to the EGFR promoter and is essential for the increase in EGFR in neural stem cells. Thus, we further hypothesized that hypoxia and hypoglycemia would induce accumulation and nuclear translocation of egr-1, and that this would lead to increased EGFR expression in these cells.

Methods:

U-87 cells were grown to 70-80% confluency and subjected to four hours of hypoxia, hypoglycemia, both hypoxia and hypoglycemia, or control conditions. We produced hypoxia by placing cells in a 3 gas incubator with 2% O$_2$, 5% CO$_2$, and 93% N$_2$. We produced hypoglycemia by decreasing media glucose to 3 mM. Cells in control conditions were kept in the standard incubator with 21% O$_2$ and 5% CO$_2$ and media glucose of 17.5 mM. After subjecting cells to these test conditions, we harvested protein from the cells either immediately (for accumulation of egr-1) or after returning cells to control conditions for 20 hrs (for expression of EGFR).

Additionally, cytoplasmic and nuclear fractions were examined for egr-1, and a total membrane protein fraction was examined for EGFR protein levels. Proteins were separated by Western blot on 7% tris-acetate gels. Western blots were probed with antibodies against egr-1 (Santa Cruz biotechnology, sc-20689) or EGFR (Santa Cruz biotechnology, sc-03). Antibodies against Beta-tubulin (Santa Cruz biotechnology, sc-9104) and PCNA (Santa Cruz biotechnology, sc-56) were used as markers for cytosolic and nuclear proteins, respectively. Blots were developed with Western Lightning chemiluminescence reagent (perkinElmer) and visualized with UVP EpiChem3.

Results:

We found that neither hypoxia nor hypoglycemia changed the levels of egr-1 in total cell lysates from U87 in two separate experiments (fig 1). Hypoxia and hypoglycemia together slightly increased the levels of egr-1 in the cytoplasm of these cells. There was no egr-1 found in the nucleus of U87 cells regardless of exposure to hypoxia or hypoglycemia. To determine whether exposure to hypoxia and hypoglycemia would induce expression of EGFR, despite the absence of accumulation of egr-1 in these cells, we returned cells to control media for 20 hrs after
exposure to test conditions, and examined total membrane proteins. We found no difference in
EGFR protein levels between the control condition and the hypoxia and hypoglycemia condition.
To determine whether the absence of egr-1 accumulation holds also for non-tumorigenic
astrocytes, we subjected normal rat astrocytes to the same conditions. Western blot showed
no difference between egr-1 levels between control and hypoxic and hypoglycemic conditions.

Fig 1: egr-1 levels in total cell lysates of U87 cells

Conclusion:

Our results indicate that accumulation of egr-1 and expression of EGFR are not part of the
response of the general population U87 cells to hypoxia and hypoglycemia. However, one
possibility is that only a subpopulation of cells with stem cell like properties, known to exist in
some tumors, may exist within the U87 cells and those cells may increase EGFR expression
with exposure to hypoxic and hypoglycemic stress. However, our detection methods were not
sufficiently sensitive to measure changes in this subset.
DISTAL FEMUR DEFECTS RECONSTRUCTED WITH POLYMERMAETHACRYLATE AND INTERNAL FIXATION DEVICES: A BIOMECHANICAL STUDY

Mentor: Francis R. Patterson, M.D., (Orthopaedics)

Objective:

The distal femur is a common site for many benign bone tumors, such as giant cell tumor of bone (GCT). These locally aggressive tumors are treated with extended curettage, application of adjuvants such as phenol or argon, and eventually stabilized with polymethylmethacrylate (PMMA). Internal fixation with Steinmann pins, crossed screws, or locking condylar plates is typically employed in order to reduce the chances of post-operative fractures. Locking plates are now increasingly being used for peri-articular distal femur fractures; providing a rigid, toggle-free, fixed angle construct. The purpose of this study is to determine whether locking plate fixation would result in a stronger failure load and stiffness than either Steinmann pins or crossed screws after tumor resection at the lateral femoral condyle.

Materials and Methods:

Twelve pairs of matched femora from human cadavera obtained from the Musculoskeletal Transplant Foundation (Edison, NJ) were divided into 3 groups each containing 4 pairs of fresh-frozen femora. The average age of donors at the time of death was 38 years (range, 21-54 years of age). The femora were stripped of all soft tissues and radiographs were made to confirm osseus integrity. The fresh-frozen femora were thawed at room temperature. A high-speed burr (Midas Rex Institute, Fort Worth, TX) was then used to create a defect in the lateral condyle of all specimens, extending from the junction of the metaphyseal and diaphyseal areas down to the subchondral bone.

Three groups of 4 matched pairs of femora were organized for the following comparisons: 1) Steinmann pins vs crossed screws; 2) Steinmann pins vs locking plates; and 3) crossed screws vs locking plates. After all femora were reconstructed with either Steinmann pins, crossed screws, or locking plates, PMMA was prepared and mixed. The cement was molded into the defect, around the pins, screws, or locking plate and shaped to form the physiologic contour of the lateral femoral condyle. The cement was then given ample time to cure.

Each femur, set in a steel fixture at an angle that placed the transcondylar axis horizontally, was bolted into the load frame of the INSTRON machine (Instron, Norwood, MA). The loading nose was centered to apply a physiological load to both femoral condyles. Starting at 0 load and displacement the load was increased by 10 N/sec until the load reached 475 N. The load was then cycled in a sinusoidal pattern between 50 N and 900 N for 2000 cycles at 1 Hz. Each femur that survived was loaded at 1 mm/sec under displacement-controlled feedback until failure. For this trial, failure is defined as a sudden drop of 445 N from the maximum observed load.

For each femur, the load to failure (N) and the stiffness (N/mm) was calculated and recorded. In addition, the mode of failure was observed and recorded for each femur specimen. Within each group, a paired t test was used to compare differences between treatments or load to failure and stiffness.
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Results:

### Table 1: Data on Load to Failure and Stiffness

<table>
<thead>
<tr>
<th>Group</th>
<th>Type of Construct</th>
<th>Load to Failure (N)</th>
<th>P-value</th>
<th>Stiffness (N/mm)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PMMA + Steinmann Pins</td>
<td>14916 ± 2959</td>
<td>0.831</td>
<td>3274.3 ± 1220.265</td>
<td>0.334</td>
</tr>
<tr>
<td></td>
<td>PMMA + Crossed Screws</td>
<td>11651 ± 3074</td>
<td></td>
<td>2520.5 ± 486.6796</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>PMMA + Steinmann Pins</td>
<td>11728 ± 2724</td>
<td>0.013</td>
<td>2408.4 ± 650.0697</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>PMMA + LCP</td>
<td>24245 ± 8228†</td>
<td></td>
<td>4769.7 ± 1271.362</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PMMA + Crossed Screws</td>
<td>9880 ± 1130</td>
<td>0.004</td>
<td>2448.98 ± 1858.297</td>
<td>0.552</td>
</tr>
<tr>
<td></td>
<td>PMMA + LCP</td>
<td>22188 ± 3622</td>
<td></td>
<td>3192.1 ± 1406.521</td>
<td></td>
</tr>
</tbody>
</table>

*The values are given as a mean and the standard deviation
**All specimens were fresh frozen cadaver femora
† One of the locking plate constructs did not fail. Load to failure was considered 33506.62 N, the load at which the MTS machine failed.

Group 1 consisted of femora reconstructed with PMMA and Steinmann pins versus PMMA and crossed screws. The mean load to failure for femora reconstructed with PMMA and Steinmann pins was 14916 ± 2959 N compared to 11651 ± 3074 N for femora repaired with crossed screws augmenting PMMA (Table 1). There was no significant difference (p = 0.831) in load to failure between Steinmann pin and crossed screw constructs.

Group 2 compared femora repaired with PMMA and Steinmann pins against those fixed with PMMA and locking plates. Femora reconstructed with Steinmann pins augmenting PMMA failed at an average load of 11728 ± 2724 N compared with 24245 ± 8228 N for the femora reconstructed with locking plates and PMMA (Table 1). For all of the matched pairs, the femur samples reconstructed with locking plates maintained a more stable construct (p = 0.013) than femora repaired with Steinmann pins.

In group 3, comparing femora fixed with PMMA and crossed screws to those repaired with PMMA and locking plates, the average load to failure for femora reconstructed with crossed screws augmenting PMMA cement was 9880 ± 1130 N while the load to failure for femora repaired with locking plates and PMMA was 22188 ± 3622 N (Table 1). In all pairs of femora, the locking plate reconstructions were significantly stronger (p = 0.004) than their matched counterparts repaired with crossed screws augmenting cement.

Within each matched pair, the femora reconstructed with locking plates augmenting PMMA (p = 0.03) had significantly greater stiffness than the contra-lateral matched pair repaired with Steinmann pins and PMMA (Table 1). However, there was no significant difference in stiffness between locking plates versus crossed screws augmenting PMMA (p = 0.552). In addition, stiffness values for Group 1 femora, Steinmann pins versus crossed screws, were not significantly different (p = 0.334).

Table 2 displays the mode of failure for all femur samples. All 8 femora reconstructed with Steinmann pins and PMMA failed via a severe, intra-articular (intercondylar) fracture. 7 of the 8 femora repaired with crossed screws augmenting PMMA showed an expanded cortex resulting in bulging and impaction of the articular surface with minimal propagation down the shaft. One crossed screw and PMMA construct failed via an extra-articular fracture. Failure of 6 femora reconstructed with locking plates and PMMA resulted in an extra-articular spiral fracture either anterior or posterior to the locking plate screws. 2 of the 8 femora in the locking plate group did not show extra-articular fracture. One of these failed via a supra-condylar fracture while the other femur survived mechanical testing without fracture thereby reaching load capacity (~30,000 N) of the mechanical testing machine. In all femora that were fixated with LCP, the articular surface was noted to be intact without any signs of compromise or fracture.
Table 2: Mode of Failure ( # of Femora)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Intra-articular</th>
<th>Extra-articular</th>
<th>Expanded Cortex and Spiral Fracture</th>
<th>Supra-condylar?</th>
<th>No Fail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steinmann Pins</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Crossed Screws</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LCP</td>
<td>0</td>
<td>6**</td>
<td>0</td>
<td>1</td>
<td>1*</td>
</tr>
</tbody>
</table>

*Femur survived mechanical testing without fracture, thereby reaching load capacity of the mechanical testing machine (capacity ~ 30,000 N).
** The femora reconstructed with locking plates and PMMA failed with an extra-articular spiral fracture either anterior or posterior to the locking plate screws.

Conclusion:

Considering the extensive curettage required for treatment of giant cell tumors (GCT), durable reconstruction is needed to prevent post-operative fractures. Polymethylmethacrylate (PMMA) cementation is accepted as the foundation for noncontained GCT defect reconstruction for both the stability and adjunctive thermal necrotic effect it offers. The need for reinforcement in post-curettage defects has been debated in the literature and various methods of augmentation have been tested including stacked Steinmann pins and crossed screws. To our knowledge no studies have evaluated the biomechanical advantages offered by locking plate augmentation to PMMA for reconstruction of GCT defects.

A retrospective study conducted by Bini et al found that GCT patients whose lesions were fixed with threaded Steinmann pins and PMMA did not experience post-operative fractures. Randall et al. corroborated this conclusion in a biomechanical study of lateral tibial condyles showing that defects reinforced with PMMA and Steinmann pins displayed significantly greater load to failure than those repaired with PMMA alone. More recently however, Murray et al. found no significant biomechanical differences between femoral condyle defects repaired with PMMA and Steinmann pins or PMMA alone, further escalating the debate about the need for reinforcement in post-curettage defects.

In a biomechanical study using fresh-frozen femur samples, Toy et al. demonstrated that distal femur defects repaired with PMMA augmented by crossed screws resulted in a stronger reconstruction than PMMA alone or PMMA with Steinmann pins. In this study, large noncontained defects were created in the medial femoral condyles of 20 matched pairs of human femurs. The femora in various groups were tested to find stiffness and load to failure, with failure being defined as a sudden drop of 445 N. In all trials, the PMMA and crossed screws group represented a significant biomechanical advantage over PMMA alone and PMMA reinforced with Steinmann pins. As noted by the authors, the use of matched pairs of femora minimized variability and allowed the focus to remain on comparing the mechanical advantages of each construct. Acknowledging the power of this study, our design was modeled closely on the methods described by Toy et al. with the addition of PMMA reinforced with femoral locking plates.

Previously acknowledged as an effective mode of fracture fixation, the versatility of locking plates and their application in oncological treatment has been largely anecdotal. A recent study examined 25 patients receiving locking plates for oncological reconstruction. It was shown that 23 of 25 locking plates were intact after a mean follow-up of 18.2 months. These results are encouraging and show clinically, locking plates can provide a reliable and
Through biomechanical testing, our aim was to determine if this conclusion is applicable to distal femoral defects resultant from GCT tumor resection and curettage. Our study was modeled in hopes of elucidating the role locking plates could play in the treatment of this benign, locally aggressive bone tumor. To our knowledge the present study is the first biomechanical evaluation of distal femoral locking plates used for oncological reconstruction. This study compares PMMA augmented by a distal femoral locking plate with the previously examined crossed-screw and intramedullary Steinmann pin constructs. Fresh-frozen femurs were utilized to best represent in vivo bone quality, and cross-matched femur pairs allowed us to control for femur quality and size providing a more powerful comparison between the constructs. Defects of the lateral femoral condyle were created to mimic those caused by giant cell tumor excision and curettage. A load-to-failure biomechanical analysis of the lateral femoral condyle was then conducted comparing cross-matched femurs reconstructed with cement and augmented with intramedullary Steinmann pins, crossed-screws or condylar locking plate.

The significant differences in load-to-failure and stiffness between the different constructs validate our hypothesis that locking plate augmented PMMA is biomechanically superior to crossed-screw or Steinmann Pin augmentation. The locking plates failed at forces 43% and 53% greater than the Steinmann pins and crossed-screws, respectively. Furthermore the fracture pattern differed between the groups. The Steinmann pin reinforced femurs failed through a severe intra-articular fracture. All but one of the crossed-screw augmented femurs failed through bulging of the articular surface (expanded cortex) and spiral fracture. One crossed screw construct failed via an extra-articular fracture. For the femora reconstructed with plates, 6/8 failed via an extra-articular spiral fracture anterior or posterior to the locked screws. Of the other two locking plate reconstructions, one failed via supracondylar fracture while the other did not fail. As noted by Toy et al., failure via an extra-articular fracture is more desirable. These fractures often can be repaired with standard ORIF while fractures involving the articular surface are much more difficult to salvage often requiring allograft or endoprosthetic reconstruction.

Our explanation as to why the locking plate reconstruction showed a significant advantage over Steinmann pins or crossed screws is because of the additional stiffness the plates provide. As noted by Toy et al., the addition of screws crossing the midline in the condylar defect improves stability of the cement mantle thus transferring the load proximally. The multiple locking screws used in the locking plate head essentially act as crossed screws utilized in Toy’s study. As supported by the extra-articular type fractures seen in the locking plate reinforced femora, this allows the axial compression forces applied to the condyle to be transmitted proximally to the femur shaft that has better bone quality and is undisturbed by tumor and curettage. The superior fixation provided by multiple fixed-angle cortical screws also stabilizes femoral shaft to provide additional coronal stability.

In this in vitro study, reconstruction of post-curettage distal femora with locking plates and polymethylmethacrylate was biomechanically stronger in axial load testing than Steinmann pins or crossed screws augmenting PMMA. The locking plate constructs were able to withstand compression forces exceeding physiologic levels. This resilient construction may permit more rapid mobilization and prevent post-operative fracture. If the locking plate reconstruction failed, the ensuing fracture is likely to be extra-articular and would preserve the articular surface. Fractures of this type are easier to treat and rehabilitate than the intercondylar fractures associated with other modes of reconstruction.
CCAAT ENHANCER BINDING PROTEIN ALPHA IS A MOLECULAR TARGET OF 1,25DIHYDROXYVITAMIN D₃ IN BREAST CANCER CELLS

Objective:

Although a major function of the active form of vitamin D, 1,25dihydroxyvitamin D₃ (1,25(OH)₂D₃), is to maintain calcium homeostasis, 1,25(OH)₂D₃ has also been identified as a factor that negatively regulates the growth of a number of malignant cells in vitro and in vivo, including breast cancer cells. However, little is known about the molecular mechanisms and target genes mediating the antiproliferative effects of 1,25(OH)₂D₃.

1,25(OH)₂D₃ acts by binding to a high affinity intracellular receptor protein (the vitamin D receptor or VDR). 1,25(OH)₂D₃ bound to the VDR heterodimerizes with the retinoid X receptor (RXR) and along with coactivators and additional accessory nuclear proteins interacts with vitamin D response elements (VDREs) in the promoter of target genes and modulates their transcription (1).

Recently, the Christakos lab reported that CCAAT/enhancer binding proteins (C/EBPs) are 1,25(OH)₂D₃ target genes in kidney and in osteoblastic cells (2). The C/EBP family of transcription factors has been reported to be involved in the regulation of growth, differentiation, inflammation and the expression of cell type specific genes (3,4). The present study extends the initial observations in kidney and osteoblastic cells to cooperative effects between the vitamin D endocrine system and the C/EBP family of transcription factors in breast cancer cells.

In breast cancer cells expression of the transcription factor C/EBPα, a suggested tumor suppressor in breast cancer, is accompanied by cell cycle arrest and upregulation of the cell cycle regulator p21. Our hypothesis is that 1,25(OH)₂D₃ acts through the induction of C/EBPα to inhibit the growth of breast cancer cells. The goal of the present study is to examine C/EBPα as a 1,25(OH)₂D₃ target involved in the antiproliferative effects of 1,25(OH)₂D₃ in estrogen receptor positive and negative breast cancer cells. Through this we can begin to identify the mechanisms whereby C/EBPα may mediate the growth inhibitory effects of 1,25(OH)₂D₃.

Methods:

Cell Culture
Estrogen receptor positive (ER⁺ve) MCF-7 and estrogen receptor negative (ER⁻ve) MDA-MB-231 breast cancer cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotic mixture (penicillin, streptomycin, and neomycin). Cells were grown in a humidified incubator with atmosphere of 95% air-5% CO₂ at 37°C. For treatments, cells were grown to desired confluence and their medium was changed to DMEM supplemented with 2% charcoal-dextran-treated FBS. Treatments with vehicle or 1,25(OH)₂D₃ were done for the durations and with concentrations mentioned in the figure legends.

Cell transfections
In brief, cells were transfected using lipofectamine 2000 (Invitrogen) as per manufacturer’s protocol. After 6-16 hours of transfection the medium was changed to DMEM with 2% charcoal-
dextran-treated FBS. Transfected cells were treated with vehicle or 1,25(OH)₂D₃ as described in the figure legends. Cells were then harvested and cell viability was determined by trypan blue exclusion. Similarly transfected and treated cells were also used for preparation of nuclear extracts for Western blotting.

Nuclear extracts
For nuclear extract preparation from MCF-7 and MDA-MB-231 cells, cells were rinsed twice with PBS at 4°C, harvested by scraping, gently pelleted, washed, and lysed in hypotonic buffer containing 10 mM HEPES (pH 7.4), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium fluoride), protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 mg of pepstatin A per ml, 2 mg of aprotinin per ml, and 1% Triton X-100), and 1% Triton X-100. Nuclei were pelleted at 3,500 x g for 5 min, and cytoplasmic supernatants were separated. Nuclei were resuspended in hypertonic buffer containing 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, and the phosphatase and protease inhibitors indicated above. Soluble nuclear proteins were released by 60 min of incubation at 4°C, and insoluble material was separated by centrifugation at 12,000 x g for 5 min. The protein concentration of the supernatant was measured by Bradford’s method (5), and aliquots were stored at –80°C.

Summary of Results:
Earlier studies have demonstrated that C/EBPα and the vitamin D receptor (VDR) are significantly induced by 1,25(OH)₂D₃ at 8 and 24h (p<0.5 compared to 0 time) in ER⁺ve MCF-7 breast cancer cells (figure 1). The present study shows that 1,25(OH)₂D₃ dose dependently enhances the expression of C/EBPα in ER⁺ve MCF-7 cells (figure 2). Proliferation studies revealed that 10nM 1,25(OH)₂D₃ inhibits the proliferation of ER⁺ve MCF-7 cells over the period of four days (figure 3). ER⁺ve MDA-MB-231 cells, on the other hand, did not respond to 1,25(OH)₂D₃ over the same period (figure 4). Furthermore, 1,25(OH)₂D₃ failed to induce C/EBPα expression in ER⁺ve MDA-MB-231 cells (figure 5). However, proliferation of ER⁻ve MDA-MB-231 cells transfected with C/EBPα was significantly inhibited by 10nM 1,25(OH)₂D₃ (figure 6).

Conclusion:
We report for the first time that MDA-MB-231 breast cancer cells, which are estrogen receptor negative and not sensitive to 1,25(OH)₂D₃ treatment, can become sensitive to growth inhibition by 1,25(OH)₂D₃ in the presence of C/EBPα. Furthermore, these findings identify C/EBPα as a 1,25(OH)₂D₃ target in breast cancer cells and provide important evidence for C/EBPα as a candidate for breast cancer treatment.
Figure 1. C/EBPα is induced by 1,25(OH)₂D₃ in ER⁺ve MCF-7 breast cancer cells

Figure 2. 1,25(OH)₂D₃ dose dependently induces C/EBPα in ER⁺ve MCF-7 breast cancer cells

Figure 3. 1,25(OH)₂D₃ inhibits the proliferation of ER⁺ve MCF-7 breast cancer cells
Figure 4. 1,25(OH)$_2$D$_3$ does not affect proliferation of ER$^{-ve}$ MDA-MB-231 breast cancer cells

![Graph showing proliferation of cells](image)

Figure 5. 1,25(OH)$_2$D$_3$ does not induce expression of C/EBP$\alpha$ in ER$^{-ve}$ MDA-MB-231 breast cancer cells

![Western Blot showing expression of C/EBP$\alpha$ and $\alpha$-Tubulin](image)

Figure 6. 1,25(OH)$_2$D$_3$ inhibits the proliferation of ER$^{-ve}$ MDA-MB-231 breast cancer cells transfected with C/EBP$\alpha$

![Graph showing inhibition of proliferation](image)

REFERENCES:

23. HSIN JOU NG (TCNJ 2010)

EFFECTS OF ERBB-2/RAS SIGNALING NETWORK ON METASTATIC POTENTIALS OF PROSTATE CANCER CELL LINES

Mentor: Lizhao Wu, PhD, (Department of Cell Biology and Molecular Medicine)

Objective:
Prostate cancer cell growth is controlled by androgens which are required for their proliferation and continual survival. Therefore, in early stages of prostate cancer, patients respond to androgen deprivation therapy which effectively decreases the size of the tumor (1). However, in more advanced cases of prostate cancer, prostate cancer cells often mutate to an androgen independent phenotype that can persist after androgen ablation. It is at this stage when the tumor can begin to metastasize and consequently, become life-threatening (2).

Uncontrollable cell proliferation is the defining characteristic of cancer and therefore the signals that govern the cell cycle and cell growth have been studied for some time. It has been determined that mutations within the Ras/E2F pathway and ErbB-2 growth factor receptor are directly correlated with genetic alterations within the cell cycle that lead to increased cell growth (3). When overexpressed in the prostate, these genes activate the androgen receptor pathway and provide a method of survival when androgen is no longer available (4). ErbB-2 and Ras, which is downstream of ErbB-2, are believed to act through the androgen receptor signaling pathway, and are implicated in the development and persistence of prostate adenocarcinoma, as well as acting as key players in the progression towards androgen independent cells (5). If the overexpression of these two oncogenes can be proven to increase the metastatic potential of prostate cancer cells, measurable action could be taken in prevention, detection, and even treatment of the disease. There is evidence that suggests both Ras and ErbB-2 play major roles in transforming prostate cancer cells towards an increasingly malignant, androgen independent phenotype (6). This study addresses the effect of overexpression of ErbB-2 and Ras on metastatic potentials by studying migration and invasion properties of prostate cancer cells in vitro.

Wound healing and transwell assays were performed to assess cell motility in five different prostate cancer cell lines, DU-145, LnCap, Myc-Cap, PC3, and CWR22. Each cell line demonstrates different metastatic potentials. DU-145 and LnCap cells are human prostate cancer cells with low metastatic potentials, where PC3 and CWR22 are highly metastatic. Myc-Cap is a murine prostate cancer cell line derived from a non-metastatic prostate cancer model.

Methods:
1. Transfection/Infection
Retroviral pBabePuro-ErbB-2 or pBabePuro-Ras constructs were co-transfected with the packaging plasmids into amphotropic Phoenix cells. The retroviral supernatant produced was collected at 48-60-72 hours post transfection. The target cells were then infected with the retroviral supernatant and the successful transfectants were selected by culturing the cells in a medium containing puromycin.

A retroviral transfection was chosen to increase the flexibility of the experiment by permanently integrating ErbB-2/Ras into the genome. It also allows the injection of ErbB-2/Ras-overexpressing cancer cells into immuno-compromised mice to monitor metastasis ex vivo in the future.

2. Wound healing assay
The cells were cultured to confluence in p60 dishes. Three marker lines were drawn on the bottom of the plate as reference points and three “wounds” were scratched perpendicular to
the marker lines using a 200µl pipette tip. The cells were then rinsed with PBS, replaced with media, and pictures of the wounds were taken in intervals of 3 or 4 hours, beginning at 6 hours until the wound is closed.

3. Transwell assay

For a motility assay, the transwell inserts were coated with collagen at a concentration 3µg/ml, 50,000 cells were plated within the transwell inserts and cells were cultured for 19-24 hours. For an invasion assay, the cells were starved with serum-free medium for 24 hours and transwell inserts were coated as described above. In addition, 100µl of high concentration collagen (1.3mg/ml) was added on top of the transwell and allowed to solidify for 30-60 minutes. 50,000 cells were then plated within the transwell inserts and cultured for 48 hours. For both assays, at the end of culture, cells were stained with Diff-Quik Stain Set (Dade Behring) and quantified.

Results:

Ras- and ErbB-2-overexpressing PC3 cancer cells along with control cells transfected with an empty pBabe-puro vector were streaked as described in the wound healing assay. In both experiments, the control cells closed the wound significantly faster than either Ras- or Erb2-overexpressing cells (Fig. 1a & b). In the Ras and ErbB-2 experiments, at 20 and 18 hours respectively, the control cells had finished filling in the wound while the Ras/ErbB-2 cells had not finished the migration. However, the difference on closing the wound between the control cells and experimental cells is most likely due to their differential growth as during passage, we noticed that both ErbB-2- and Ras-overexpressing PC3 cells grew slower. Consistent with the notion, in a modified wound healing assay where cells were density arrested for 48 hours before subject to streaking we found no significant difference in closing the wound between the control cells and the experimental cells (Fig. 1c).

Figure 1. PC3 control and ErbB-2/Ras overexpressing cells migrate at similar rates due to their high metastatic potential. Measurements of the distance traveled by the cells in the wounding assay within a given time interval were recorded then averaged. The speed at each time point was calculated by the equation: ((average distance at point 0 – average distance at specific hour)/2)/the specific hour. The speeds were graphed and eventually combined to yield one final average per plate. When the cells were not under density arrest (a&b), the Ras- and ErbB-2-overexpressing cells migrated slower than the control cells. Because the rate of cell division could be a possible explanation for the faster closure of the wound streaks in the control cells, a density arrest wound assay was performed (c), yielding a similar rate of migration for both control cells and ErbB-2 overexpressing cells. (d). A Western Blot confirmed the overexpression of ErbB-2 in target PC3 cells. The SKBR3 cell line was used as a positive control as it is
known to express very high levels of ErbB-2. +: PC3 cells infected with pBabepuro-ErbB-2; -: PC3 cells infected with pBabepuro vector.

In parallel, we also infected Myc-Cap cancer cells with either control retroviruses or retroviruses expressing ErbB-2 or Ras. Both ErbB-2- and Ras-overexpressing cells closed the wound about the same time as the control cells, terminating the experiment at 36 hours (Fig. 2).

![Figure 2](image_url)

**Figure 2.** Myc-Cap control and ErbB-2-/Ras-overexpressing cells demonstrate a similar migration rate in a wound healing assay. Measurements of the distance were again, recorded to yield an average speed per plate at each time point. ErbB-2-overexpressing cells (a) and Ras-overexpressing cells had very similar migration speeds to the control cells.

To further understand the role of ErbB-2/Ras signaling pathway in cell migration and invasion, we used a transwell motility assay and a transwell invasion assay to measure metastatic potentials of ErbB-2 or Ras overexpressing cells. As shown in Fig. 3, although ErbB-2 overexpressing PC3 cells had similar motility as the control cells (Fig. 3a), they demonstrated much higher invasion potential, as evidenced by significantly higher number of cells invading the collagen layer (Fig. 3b). In addition, we also found that both ErbB-2 and Ras-overexpressing Myc-Cap cells had significantly higher motility than the control cells (Fig. 4).

![Figure 3](image_url)

**Figure 3.** PC3 ErbB-2-overexpressing cells have a similar migration potential but higher invasion potential than the control cells. ErbB-2 overexpressing PC3 cells and control cells were incubated in transwell motility and invasion assays as additional tests for metastasis potential. Cells were incubated in transwell chambers for 20 and 38 hours respectively and subsequently stained for cells that traveled to the bottom of the transwell membrane. Positively stained cells in the invasion assay were additionally required to invade through a 1.3 mg/ml collagen matrix.
Control                   ErbB-2 overexpression         Ras-overexpression          Control      ErbB-2     Ras

Figure 4. Myc-Cap ErbB-2/Ras-overexpressing cells have a higher migration potential than control cells. A transwell motility assay to assess metastatic potential of Myc-Cap cells showed that ErbB-2- and Ras-overexpressing Myc-Cap cells had a greater ability to migrate past the transwell membrane than our control cells, indicated by the greater number of cells collected on the bottom of the ErbB-2-/Ras-overexpression plates.

Conclusion:

Preliminary results show that Ras- and ErbB-2-overexpressing PC3 cells close wound streaks slower as compared to control cells. However, since we observed that the ErbB-2 or Ras-overexpressing PC3 cells divided at a slower rate than the control cells, and since ErbB-2 overexpressing PC3 cell closed wounds similarly as control cells under the density arrest condition, we suspected that the difference in closing the wounds between control cells and ErbB-2 or Ras-overexpressing cells is most likely due to their differential growth rates. Although ErbB-2 over-expressing PC3 cells exhibited similar motility in a transwell assay to control cells, they showed higher invasive potential (Fig. 3). Interestingly, for the Myc-Cap cell line, which is believed to have much lower metastatic potentials than the PC3 cell line, although there is no significant difference between control cells and ErbB-2 or Ras overexpressing cells in the wound healing assays (Fig. 2), the experimental cells exhibited much higher motility in a transwell assay than the control cells (Fig. 4). Taken together, our data are consistent with a positive role of the ErbB-2/Ras signaling pathway in mediating metastatic potentials of prostate cancer cells. Ongoing experiments utilizing other cell lines (DU-145, LnCap, and CWR22) with various metastatic potentials would further verify the role of ErbB-2 and Ras to increase the migration and invasion potential of the prostate cancer cells. Ideally, the over-expression cells can eventually be injected into immuno-compromised mice ex vivo to monitor the metastatic potential from the site of injection in hopes of creating a mouse model to monitor the various stages of prostate cancer.

References:

81
24.  MELISSA QUICK (COLLEGE OF HUMAN ECOLOGY, CORNELL UNIVERSITY, 2010)

ANALYSIS OF THE READABILITY OF ENGLISH LANGUAGE CANCER EDUCATION MATERIALS

Mentors: Stanley H. Weiss, MD; Daniel M. Rosenblum, PhD. Department of Preventive Medicine and Community Health, NJMS.

Background and Objectives:
Having any degree of illiteracy can affect one’s ability to complete job applications, to understand posted information, and can affect the comprehension of health information. According to the National Adult Literacy Survey conducted in 1992, nearly half of New Jersey adults have limited reading comprehension and have difficulty integrating information from “complex or lengthy texts.” Among these adults are many who have not attained a high school diploma or GED, as well as those born abroad whose native language is other than English (Jenkins, 1992).

The US Department of Health and Human Services (DHHS) defines the term “health literacy” as “the capacity to obtain, interpret, and understand basic health information and services and the competence to use such information and services to enhance health” (US DHHS, 2000). Pamphlets, flyers, and advertisements are all forms of written and printed material that are disseminated in order to raise public awareness of preventive measures. Cancer materials are an especially important resource in advertising the need for regular screening, as well as delineating the treatment options for those diagnosed as having cancer for patients and their families. While the average reading level in the U.S. is estimated to be at the 8th grade level, cancer education materials are typically written at about a 10th grade reading level (Cotugna, 2005)

In earlier phases of this project, we found that 92% of English materials were written at or above the 6th grade level (Pinto, 2005), which is quite far from the recommendation that essential materials be written at or below the 5th grade level (National Work Group on Literacy and Health, 1998). The average English reading level was 9.3 amongst 118 English materials in our analysis set of paired English and Spanish language documents. In a comparison of scores for 103 items obtained by 2 different scoring methods, we observed an average English reading level of 9.8 using the SMOG scale and 9.3 using the FRY readability scale (Luke, 2006); this systematic difference and direction has been noted by others.

Our current study sought to answer several questions. The first was whether there is variation in the reading grade levels between the materials designed for different types of cancer. Breast cancer, for instance, has enjoyed much media publicity, while other cancers may not be as well publicized. Second, we sought to assess whether revised pamphlets had improved readability. Third, we wanted to analyze how cancer materials prepared for general education, prevention and screening, and post-diagnosis patients compared in reading level. Lastly, we sought to determine how scores using the SMOG scale correlate with another widely used readability estimate, the FRY graph method.

Methods:
In 2005, New Jersey health departments were contacted via postal mail and email and asked to submit cancer education materials that they regularly distributed. The original publishers were then contacted so as to obtain a compendium of matching pamphlets (when only an English or a Spanish version had been provided) and to enhance the breadth of materials. 382 materials
were collected and scored, and came from 54 (47%) of the 116 health departments in New Jersey (plus the publishers). There were 118 with both English and Spanish versions. Additional literature has been steadily accrued over time for this project. In 2006, we scored 103 added pieces in English. For the current study, 117 more items were collected and analyzed, including some which were earlier or later versions of existing materials.

All accrued literature was coded as to cancer type. In order to utilize the FRY reading graph, an item must have a minimum of 100 words. There were four short items (of 35, 44, 86, and 90 words) that we therefore removed from analysis; all four were directed to a low grade level. All remaining items were scored using two separate scales known for their reliability (Friedman, 2006). The SMOG grading scale was developed by G. Harry McLaughlin in 1969 and integrates the number of polysyllabic words in a set number of sentences into a formula to derive the reading grade level. This scale is recommended by the National Cancer Institute for use in assessing the readability of cancer pamphlets (NCI, 1979). SMOG often results in slight inflation of the reading level due to its strict criteria requiring 100% comprehension. The FRY Readability Graph was created by Edward B. Fry in 1968, was extended in 1977, and uses a graph to assign a grade level based upon the average number of syllables and number of sentences in three 100-word passages. Both scales were applied to the remaining distinct 290 cancer materials written in English. We found 33 with 2 or more versions, 8 with 3 or more versions, and 3 with 4 versions.

Summary of Results:
These 290 unique pieces were collected from 37 different publishers, encompassed 13 types of cancer, and their most recent publication dates ranged from 1970 to 2008 (mean=1999). They have an overall SMOG readability score of 10.02 (SD = 2.15), ranging from grade 5 through 15. The mean FRY grade level was 9.18 (SD = 2.75) ranging from grade levels of 3 through 15. The mean SMOG score was almost a full grade level higher then FRY (p<0.001).

72 (24.8%) pieces focus on breast cancer, 36 (12.4%) on prostate cancer, 28 (9.7%) on colorectal cancer, 25 (8.6%) on lung cancer, 21 (7.2%) on cervical cancer, 18 (6.2%) on skin cancer, and 69 (23.8%) focused on general cancer information. The remaining 7.3% include pamphlets on blood (8), brain (1), liver (1), oral (2), testicular (6), and uterine cancer (3) (Figure 1). Only cervical cancer (mean SMOG=8.0, SD=2.2) significantly differed from the overall mean SMOG score, p<0.0001. For some other types in Figure 2 that tend to differ from the mean
SMOG score (such as brain and blood cancer), the small sample size may be limiting our statistical power to observe differences. In assessing the items with the FRY scale, both breast and cervical items differed significantly from the mean FRY at the $p<0.01$ level, while colorectal and general pamphlets differed from the mean at the $p<0.05$ level.

For the 33 pieces collected where we had one or more revised versions, the most recent versions were written a mean of 4.2 years later than the next most recent version (range 1-21 years, SD=4.7), yet the readability had not significantly improved (SMOG 9.97 vs. 10.27, $p=\text{ns}$).

205 materials focused on cancer prevention and/or the need for screening, 72 focused on treatment strategies and other therapy-related information. 13 were generally informative about cancer and did not belong in the other categories. We categorized each pamphlet by its primary focus (Table A). There were 11 pamphlets that included both treatment and screening/prevention information and could not be assigned to just one of the three categories; these were excluded from the analysis shown in Table 1. Reading levels based on SMOG were significantly, and almost 2 grade levels, higher for “treatment” compared to “screening/prevention”, which in turn was significantly, and 1.5 grade levels, higher than “general” materials. The reading levels observed with FRY showed a similar pattern. Given the intended audiences and level of complexity of the information to be conferred to the reader, these trends are not surprising.

SMOG reading levels also varied by the length of the type of item: small items (3 bookmarks, 12 cards, 7 door hangs; n=22) 8.8; one-page flyers (n=20) 9.3; pamphlets (n=137) 10.0; and booklets (n=111) 10.3.

**Table 1**

<table>
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<tr>
<th>Reading Level</th>
<th>N</th>
<th>General</th>
<th>Screening/Prevention</th>
<th>Treatment</th>
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<tr>
<td>N</td>
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<td>195</td>
<td>71</td>
<td>205</td>
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<tr>
<td>Mean SMOG score (SD)</td>
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<td>9.6 (2.0)</td>
<td>11.5 (1.9)</td>
<td></td>
</tr>
<tr>
<td>SMOG p-values</td>
<td>$\leftarrow - - - p &lt; 0.05 - - - \rightarrow$</td>
<td>$\leftarrow - - - p &lt; 0.001 - - - \rightarrow$</td>
<td>$\leftarrow - - - p &lt; 0.001 - - - \rightarrow$</td>
<td></td>
</tr>
<tr>
<td>Mean FRY score (SD)</td>
<td>7.4 (3.0)</td>
<td>8.8 (2.7)</td>
<td>10.4 (2.4)</td>
<td></td>
</tr>
<tr>
<td>FRY p-values</td>
<td>$\leftarrow - - - p = \text{ns} - - - \rightarrow$</td>
<td>$\leftarrow - - - p &lt; 0.001 - - - \rightarrow$</td>
<td>$\leftarrow - - - p &lt; 0.001 - - - \rightarrow$</td>
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</table>

**Conclusions:**
Cancer education materials are essential in order to properly inform the public of the need for healthy lifestyles that will help prevent cancer. Materials also serve to educate the public of different means of early detection so that malignancies can be caught early, when they are more amenable to treatment. After diagnosis, materials are utilized to educate patients and their families about treatment options, services available to them, and coping strategies.

We found that the SMOG tends to consistently score material at a higher level than FRY. We believe this is consistent with the fact that SMOG was developed based on 100% comprehension of material, while FRY is an approximation of grade level.

Despite general agreement for over two decades that materials are needed at lowered literacy levels, there remains a paucity of such material. We found almost no improvement in the SMOG
scores of items over the mean of 4.3 years elapsing between versions, and despite the fact that many versions had undergone one or more revisions relatively recently.

We found that cancer materials that focus on information for those who have already been diagnosed with cancer tend to have higher scores then those for the general population. We believe that those already diagnosed have more familiarity with cancer jargon and may therefore be able to understand complicated terms better. However, a score of 11.5 (SMOG), or 10.4 (FRY), is far above the recommended 6th grade reading level, as set by the National Cancer Institutes (NCI), for cancer literature. Most importantly, items that are designed with the general population in mind to increase knowledge of the need for preventive measures and adequate, regular screening are still set at too high a reading level for the average American (SMOG=9.3, FRY=8.7). Since the average American reading level is 8th grade, less then half of the U.S. adults can be expected to understand the average piece of general cancer literature.

If cancer education materials continue to be published and disseminated in such a way that they are not comprehensible by the majority of the population, they will continue to play only a limited role in fighting the war on cancer. Since low literacy is associated with poorer health status and more use of costlier health care services (National Work Group on Literacy and Health 1998), it is imperative that printed cancer materials be accurate, culturally relevant, and at a reading level that can be understood by all. We suggest that public health professionals, clinicians, health departments, agencies such as the NCI and The American Cancer Society, and publishers all work towards developing additional materials at appropriate levels to reach a broader portion of the population. Much recent work has further demonstrated that people may tend to have different learning styles, and therefore inclusion of graphics and/or multimedia may be more effective in achieving comprehension and reaching out to those with low reading literacy.

References:
25. **BOBBY REDDY (NJMS, 2011)**

**Tumor Suppressor Functions of RE-1 Silencing Transcription Factor in Breast Cancer Cells via TAC1 Regulation**

Mentor: Pranela Rameshwar, PhD (Medicine)

**Objective:**

Breast cancer remains the most prevalent cancer among women in the United States. The neuropeptide, substance P (SP), derived from the TAC1 gene has been shown to exhibit oncogenic properties in breast cancer. Also, TAC1 has been shown to mediate metastasis, including bone marrow. SP stimulates breast cancer cell (BCC) proliferation via autocrine mechanisms via the truncated neurokinin 1 (NK1) and NK2 receptors. The repressor element 1 (RE-1)-silencing transcription factor (REST) or neuron-restrictive silencer factor (NRSF) is a zinc finger transcription factor that suppresses neuronal genes in non-neuronal cells. In addition, REST has been reported to exhibit both oncogenic and tumor suppressor functions. The TAC1 gene has a functional REST binding site with the untranslated Exon 1. The objective of this study is to characterize the of REST in breast cancer development.

**Hypothesis:**

The overarching hypothesis states that REST mediates tumor suppressor functions in breast cancer cells. The specific hypothesis states that REST levels are indirectly proportional to aggressiveness of breast cancer cells and TAC1 induction.

**Materials and Methods:**

Breast Cancer Cells

In this study, we obtained and cultured the following BCC lines from American Type Culture Collection: MDA-MB-231 (highly aggressive), T47D (non-invasive), and MCF12A (non-tumorigenic). Primary breast tissues were acquired from Brookdale Hospital (Brooklyn, NY) and Cooperative Human Tissue Network. The studies were approved by the Institutional Review Board of the University of Medicine and Dentistry of New Jersey.

Loss of Function Studies

These studies were performed using REST siRNA to silence REST gene and protein expression. A mutant REST siRNA and untransfected cells were used as controls. Transfection efficiency was verified with RT-PCR and Western analysis. TAC1 levels in REST knockdown BCCs were determined using RT-PCR, and substance P (SP) production was calculated using ELISA. Functional analyses of REST knockdown was performed using growth curve analysis and migration assays.
Gain of Function Studies

These were performed through stable transfection of the pCMV6-XL4-REST vector to exogenously express REST in MDA-MB-231 cells. Untransfected cells and cells transfected with pCMV6-XL4 vector without the REST insert served as controls. The effect of ectopic expression of REST was determined using the methods described above in the loss of function studies.

Results:

![Graph A: REST mRNA Levels in BCCs](image1)

![Graph B: TAC1 Expression in REST knockdown BCCs](image2)

![Table 1: Control, REST, and REST-∆κB](table1)

![Fig. 1: A) REST mRNA levels in BCC lines. B) TAC1 levels in REST knockdown BCCs and controls. C) Substance P (SP) production in REST knockdown BCCs and controls.](figure1)

![Fig. 2: A) Growth curve of REST knockdown MDA-MB-231 cells and controls. B) Growth curve of REST knockdown T47D cells and controls. C) Migration assay of REST knockdown BCC lines and controls (not shown: MCF12A growth curve, which shows an increase in cell proliferation in REST knockdown BCCs compared to controls).](figure2)

![Fig. 3: A) Map of vector pCMV6-XL4-REST, which was used to exogenously express REST in BCCs. B) Growth curve of MDA-MB-231 after stable transfection with pCMV6-XL4-REST and controls. C) Migration assay of MDA-MB-231 after stable transfection with pCMV6-XL4-REST and controls.](figure3)
Summary:

In the studies, we found that REST knockdown in BCCs leads to increased TAC1 and substance P (SP) production. Also, loss of REST in non-invasive and non-tumorigenic BCCs led to increased cell proliferation and migration. We further demonstrated that ectopic expression of REST in highly aggressive BCCs leads to decreased TAC1 induction, SP production, decreased cell proliferation and migration, supporting tumor suppressor function. In addition, decreased REST mRNA and protein levels were observed in advanced stages of primary breast cancer tissues.

Conclusion:

Our data correlate decreased REST levels with increased tumorigenicity in both cell lines and primary malignant tissues, supporting the in vitro findings that suggest a tumor suppressor role for REST. These findings also support previous findings that report on an oncogenic role for TAC1. These studies have potentially significant clinical implications. REST may serve as a biomarker for carcinogenesis in the context of BC. If incorporated with existing diagnostic methods, it may lead to early detection. Also, since we showed decreased REST levels in more advanced stages of BC, REST may be indicative of prognosis. However, before the diagnostic and pharmacological value of REST can be further explored, the molecular mechanisms that lead to REST dysregulation, including its link to TAC1 needs to be characterized. Investigation of such pathways will lead to further knowledge of developmental as well as pathological process in neuroendocrine systems and cancer cell biology.

References

26. JAYANT REDDY (TCNJ 2010)

INVESTIGATION OF NBS1 TO DETERMINE NOVEL INTERACTING PARTNERS VIA A TWO-HYBRID ASSAY

Mentors: Masashi Inafuku, PhD, (Cell Biology and Molecular Medicine), Katsunori Sugimoto, MD, PhD, (Cell Biology and Molecular Medicine)

Objective:

DNA repair mechanisms are integral to cellular function and proliferation. DNA is often damaged due to factors such as chemicals, ionizing radiation, and normal cellular processes such as aerobic respiration. Improper repair of DNA leads to an accumulation of mutations and cancer. Therefore, further investigation and manipulation of DNA repair mechanisms may prove as an effective first line defense against cancer and other genetic diseases.

In this particular study we investigated the interactions of the protein Nbs1. Nbs1 is involved in double strand break repair and forms a complex with Mre11 and Rad50. This complex migrates to sites of double strand breaks and creates short oligonucleotide strands. ATM binds to the Nbs1 c-terminus and initiates a further cascade of DNA repair related processes such as recruitment of DNA repair proteins and halting of the cell cycle to allow for DNA repair.

Our primary goal was to investigate whether another protein was competing with ATM to bind with the Nbs1 c-terminus.

Methods:

To pursue this goal, we performed a two-hybrid assay. Nbs1 gene is joined to the binding domain sequence of the Gal4 transcription factor gene specific to the Gal promoter. The cDNA library is joined to the activating domain sequence of the Gal4 transcription factor gene. The Gal promoter is connected to the His3 coding sequence. When Nbs1 and a protein from the cDNA library bind, the activating domain increases transcription of the His gene. This “two-hybrid” interaction is monitored by cell proliferation on a media lacking histidine. cDNA from proliferating cells are sequenced with a primer specific to a tag in the cDNA library vector and the unknown protein X is identified.
Summary:

Western blot using anti-HA antibody was performed to confirm that PJ69-4A yeast were successfully transformed with the pGBDU-HA-Nbs1 plasmid.
Upon successful E.Coli transformation and extraction, the candidates were digested with Hind III restriction enzyme and run through agarose gel electrophoresis. All candidates except for those in lanes 8, 10, and 13 showed expected banding patterns when compared to a pACTII positive control. This confirmed that the plasmids were not contaminated and were viable for the two hybrid assay.

![Image of gel electrophoresis](image)

Five candidates (13, 17, 25, 30, and 37) successfully proliferated on a 30 mM 3AT SDAMW (-H,-U,-L) plate. This indicated a successful interaction between Nbs1 and the unknown protein coded for by the cDNA in each candidate.

![Image of plate with candidates](image)

The five positive candidates were sequenced with an HA primer for the HA tag located on each cDNA library. The following proteins were identified via a BLAST search of the sequences.

<table>
<thead>
<tr>
<th>Candidate:</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 <strong>GENE ID: 50628 GEMIN4</strong></td>
</tr>
<tr>
<td>17 <strong>GENE ID: 10456 HAX1</strong></td>
</tr>
<tr>
<td>25 <strong>GENE ID: 10456 HAX1</strong></td>
</tr>
<tr>
<td>30 <strong>GENE ID: 50628 GEMIN4</strong></td>
</tr>
<tr>
<td>37 <strong>GENE ID: 51061 TXNDC11</strong></td>
</tr>
</tbody>
</table>
Conclusion:

Three novel proteins were identified as novel interacting partners of Nbs1: Gemin4, Hax1, and Txndc11. We could not determine the significance of Gemin4 and Txndc11 in relation to Nbs1.

Hax1 has been identified as an anti-apoptotic protein. We propose that Hax1 competes with ATM for binding to Nbs1. ATM promotes a pro apoptotic pathway through p53. Hax1 may serve in an anti-apoptotic pathway that competes with ATM. Further work is necessary to determine the precise mechanism by which Nbs1 interacts with Hax1 as well as the competitive mechanism between Hax1 and ATM.

References:


27. ROOPA ROY (NJMS 2011)

TIMELY FOLLOW-UP OF ABNORMAL MAMMOGRAMS

Mentors: Kelly Kronhaus-Ussery, MD (Family Medicine, HUMC) and Steven Keller, PhD (Psychiatry and Family Medicine)

Background:

In the United States, breast cancer is the second most commonly diagnosed cancer, and the second leading cause of cancer death among women.\(^3\) Therefore, it is of utmost importance to ensure that detection and ultimate diagnosis of breast cancer is prompt and efficient. The use of mammograms has been shown to reduce mortality of breast cancer by 25-30% among women aged 50-74 years by advancing the date of diagnosis 3-4 years, when cancer is at an early and treatable stage.\(^4\) However, without successful follow-up, the potential benefit of programs focusing on increased use of screening mammography cannot accomplish their goal of reducing breast cancer-associated mortality. Subsequent follow-up may include additional diagnostic testing based on classification of abnormalities detected in mammograms using the BI-RADS (Breast Imaging Reporting and Database System) scale. A QA/QI study was conducted at an urban, multi-ethnic family practice center to assess the practices and efficiency of follow-up of abnormal mammograms.

Objectives:

♦ To examine the efficiency and timeliness of follow-up procedures among women with abnormal mammograms.
♦ To identify any trends of follow-up time of abnormal mammograms with the following variables among the patient population: BI-RADS classification, age, insurance, ethnicity, associated symptoms, family history and personal history.

Methods:

A retrospective chart review was conducted on 101 medical charts. Charts were randomly selected from a collection of all medical records available, and were excluded based upon the following exclusion criteria: Male, BI-RADS 1 and 2. Data collected included medical record number, date of birth, related symptoms, date of abnormal mammogram, BI-RADS classification of abnormal mammogram, radiologist’s recommendation on abnormal mammogram report, date of notifying patient of recommendation, follow-up procedure completed (Repeat Mammogram, Ultrasound, MRI, Biopsy), date of follow-up procedure, result of follow-up procedure, Insurance, Race/Ethnicity, relevant Personal History and Family History. The time to follow-up was defined as the time interval between the abnormal mammogram and 1st diagnostic test. Appropriate follow-up of abnormal test results is defined as 60 days unless the physician recommends a repeat mammogram in 4-6 months.\(^1,5\) Completion of follow-up procedures was also double-checked using Affinity software if not otherwise documented in the patient’s chart.

Data Analysis:

Standard descriptive statistics were used to analyze the patient population via SPSS software. Calculations were then made via Excel for determination of trends in data.
Summary of Results:

BI-RADS Classification vs. Follow-up time

69 (68.3%) patients with BI-RADS 0 (Additional Imaging required) had a mean age of 54 years. Their follow-up was either MRI, Biopsy, Ultrasound only, Mammogram and ultrasound, Diagnostic Mammogram with additional views, or Repeat Mammogram in 4-6 months. The median follow-up time interval after the abnormal mammogram was 33 days, under the recommended 60 days. Of these patients, 81% completed follow-up tests in under 60 days, 13% patients in over 60 days, and 6% with no documented follow-up in the chart or Affinity program. One patient with BI-RADS 0 had a recommended treatment of Repeat Mammogram in 6 months (180 days). Her follow-up time post-mammogram was 359 days; longer than the recommended follow-up time.

20 (19.8%) patients with BI-RADS 3 (Probably benign finding) had a mean age of 56.6 years. Of those who had to complete a diagnostic test in under 60 days (n=5), the median follow-up time was 73 days. Only 40% of these patients followed up in under 60 days. Of those patients (n=15) who had 6 months to repeat a mammogram, the median follow-up time was 239 days. Only 27% patients were able to follow-up on time.

11 (10.9%) patients had BI-RADS 4 (Suspicious Abnormality). The mean age of this group was 64.8 years. All patients were advised to undergo a biopsy in under 60 days. The median follow-up time was 31 days, and 64% patients were able to have a biopsy in under 60 days. There was 1 (0.9%) patient, aged 47 years, with BI-RADS of 5 (Highly Suggestive of Malignancy). This patient completed a biopsy in 7 days.

Age vs. Follow-up Time

Patients were aged from 33 years to 77 years, with a mean age of 55 years.

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Mean (median) Follow-up time (days)</th>
<th>% pts under 60 days</th>
<th>% pts over 60 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 50 years (n=30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diagnostic test in 60 days: (n=27)</td>
<td>45.3 (32)</td>
<td>81.48%</td>
<td>18.5%</td>
</tr>
<tr>
<td>50-59 years (n=36)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diagnostic test in 60 days: (n=25)</td>
<td>92.7 (33)</td>
<td>76%</td>
<td>24%</td>
</tr>
<tr>
<td>&gt; 60 years (n=35)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diagnostic test in 60 days: (n=29)</td>
<td>81.3 (36)</td>
<td>89.6%</td>
<td>10.3%</td>
</tr>
</tbody>
</table>

Insurance vs. Follow-up time

Over 55% of patients had Charity care. This group had one of the higher percentages of patients following up under 60 days (85.7%). Those with both Medicare and Medicaid, Private Insurance, and Self Pay also had most patients follow up in under 60 days, with percentages of 100%, 84.2%, and 100% respectively. The group with the lowest percentage of patients following up under 60 days had Medicaid (only 40% under 60 days), with the highest median follow-up time of 65 days. The remaining insurance groups had median follow-up times of less than 40 days. All insurance groups had less than 50% timely follow-up for patients advised to a repeat mammogram in 6 months.

Race/Ethnicity vs. Follow-up time

Of all patients in this study, 31.7% were White-Hispanic and Other Race each, 28% was White-Not Hispanic, 7.3% were Minority, and 1.2% were Black Hispanic. Of the 25 patients who had delayed follow-up, 7 (28%) were White-Hispanic, 7 (28%) were Other Race, and 4 (16%) were White-Not Hispanic. Of the 70 patients who followed-up on time, 19 (27%) were White-Hispanic, 18 (26%) were White-Not Hispanic, and 16 (23%) were Other Race. Of the 6 patients
with no follow-up documented, 3 (50%) were of Other Race and 1 (17%) were Minority. For tests recommended under 60 days, Minority population had the highest percentage (40%) of patients with untimely follow-up. White-Hispanic patients had the longest mean follow-up time (83.7 days).

**Personal and Family History vs. Follow-up Time**

14 (13.8%) patients had a relevant personal history such as breast cancer, benign breast history, or other form of cancer. 11 of these 14 (78.5%) patients followed up in a timely manner. Of the 25 patients who had untimely follow-up, only 1 of them had a personal history, specifically of breast cancer. All other 11 patients with documented personal history had a follow-up time of under 60 days, with the exception of 2 who had no follow-up indicated. Both patients had a history of breast cancer.

13 (52%) of the 25 patients who had untimely follow-up had family history that may increase risk for breast cancer. Of these patients, 23% had a 1st degree relative with breast cancer, and 30% of them had a 1st degree relative with a form of cancer. This is an overall higher percentage than the group of patients who followed up on time with a family history (20%). Of these patients, 28.5% of them had a 1st degree relative with breast cancer, and 42.8% of them had a 1st degree relative with some form of cancer.

**Associated Symptoms vs. Follow-up Time**

87% (20 of 23) of symptomatic patients followed up on time. 24.6% (17 of 69) of patients with timely follow-up had associated symptoms compared to the 16% (4 of 25) of patients with symptoms who followed up later than recommended by the physician. Of the symptomatic patients with timely follow-up, 59% (10) of them had a breast lump present, 29.4% (5) experienced breast pain, and 11.7% (2) had both breast pain and lump. Of the symptomatic patients who did not follow up on time, 75% (3) had a breast lump, and 25% (1) had both breast pain and breast lump.

**Discussion/Conclusion:**

Early breast cancer detection is only as useful as the subsequent follow-up after an abnormal mammogram finding. Over 30% of patients in this study with abnormal mammograms did not follow-up in an appropriate time frame. A major goal was to identify any trends in follow-up time with individual factors of patients. It is important for physicians to recognize which aspects of a patient may make them more likely to delay follow-up of abnormal test results so that proper counseling and support can prevent the risk of breast cancer to progress over time. Estimates for tumor doubling times range widely, with a median time of 260 days for mammographically detected tumors.1

Consistent with the literature, patients instructed to have repeat mammograms in 4-6 months have a higher proportion (75%) of untimely follow-up. This is a steady finding even when patients are further divided into different groups of BI-RADS, age, insurance, or ethnicity. These patients either have a BI-RADS 0 or BI-RADS 3. Patients with BI-RADS 4 and 5 have a much lower mean follow-up time than BI-RADS 3, the only group with a median follow-up time greater than the recommended 60 days (73 days). A potential reason for this finding may be that patients with BI-RADS 4 and 5 have a significantly higher risk of breast cancer (23-34% and 75-99% respectively2) than those with BI-RADS 0 or 3 (less than 2% chance of cancer2), and may therefore express more worry to consequences of delaying treatment. Also, physicians may be more proactive with BI-RADS 4 and 5 patients, as these women require biopsy for a more urgent scenario. Typically, they receive immediate appointments from triage nurses whereas BI-RADS 3 patients make a radiology appointment through their PCP. The average wait time for a mammogram is 4-6 weeks at HUMC, which may add an obstacle to patients' abilities to repeat mammograms in a timely fashion.
Patients aged 50-59 years old had the highest mean follow-up time of 93 days for a test recommended under 60 days. This is inconsistent with literature that shows women aged 65 and older to be more likely to delay additional follow-up\(^1\). In fact, in this study, women over 60 years had the highest percentage of timely follow-up (89.6%). This may be the case since women in this category have most likely retired from work and may have more time to arrange appointments for additional follow-up procedures. However, when median follow-up times were tabulated, there were no significant differences within the age groups. Future research is required to test this finding with a survey administered to patients in different age groups to see what their schedule permits and their viewpoints on having an abnormal mammogram result.

Insurance may play a role in ability to follow-up on time. This study revealed Medicaid patients to have the highest median follow-up time (65 days) as well as the lowest percentage (40%) of patients able to maintain follow-up in under 60 days. This is in contrast to patients covered by Charity Care, (85.7%), Private (84.2%), Self Pay (100%) and those with both Medicaid and Medicare (100%), all who were more able to follow-up on time. However, Medicaid patients had the shortest median follow-up time for patients who were recommended to repeat a mammogram in 180 days (199 days). This reveals that Medicaid patients may have difficulty with immediate follow-up, but are able to arrange follow-up more adequately after an extended time. Arranging transportation, financial burden, or disability may play a factor. Further studies are needed to uncover reasons why this group may have limitations on immediate follow-up.

There has been conflicting evidence of race/ethnicity playing a role in follow-up time. However, this data is consistent with the finding that Minority (Asian/Pacific Islander or American Indian) women have a slightly lower proportion (60%) of timely follow-up than White, African American, and Hispanic women (89.47%, 100%, and 77.27% respectively).\(^1\) Language and cultural beliefs may form a barrier to effective communication between the physician and patient that may cause this discovery.

Of the patients with relevant personal history, 78.5% followed up on time, 73% of whom had a benign breast history. These women may be accustomed to frequent testing and making/maintaining appointments, and may fear a benign condition developing into a more serious medical concern. Patients who had family history were surprisingly more likely to delay follow-up according to this data. This may be due to the increasing fear of finding out a negative result\(^1\), since these women are at a higher risk to hear life-altering news. Symptomatic women were more likely to follow-up on time (87%). This is a common finding in healthcare, as patients are less expected to ignore a tangible worry. Patients seem to want answers to these palpable concerns.

Since this study focused primarily on patient delay of follow-up, future studies can be conducted to determine potential factors in provider delay of communicating test results to patients in an efficient and timely fashion. With the knowledge from this study, physicians can help prevent specific groups of women delay important diagnostic tests that may be pivotal to potentially detecting and subsequently treating breast cancer.

References:

Objective:

The purpose of this QA/QI project was to determine and analyze rates of screening of cancers and other medical conditions at the Center for Family Health in Hoboken, NJ.

Preventive care is one of the most crucial parts in assuring superior patient care and quality of life. Family physicians have long found themselves at the forefront of preventative care in the US. However, studies show that preventive services are highly underused in the US, resulting in a preventable loss of lives, poor health, and inefficient use of healthcare dollars. The US Preventive Services Task Force provides guidelines for the screening of certain conditions. It provides grade definitions that determine whether screening is beneficial for patients based on the quantity and quality of evidence of the effectiveness of screening.

USPSTF GRADE DEFINITIONS:

<table>
<thead>
<tr>
<th>GRADE</th>
<th>RECOMMENDATION</th>
<th>RATIONALE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Strongly recommended</td>
<td>Good evidence was found that the service improves health outcomes and benefits substantially outweigh the harms.</td>
</tr>
<tr>
<td>B</td>
<td>Recommended</td>
<td>At least fair evidence was found that the service improves important health outcomes and concludes that the benefits outweigh the harms.</td>
</tr>
<tr>
<td>C</td>
<td>No recommendation</td>
<td>The service can improve health outcomes, but the balance of benefits and harms is too close to justify a general recommendation.</td>
</tr>
<tr>
<td>D</td>
<td>Not recommended</td>
<td>The service is ineffective or the harms outweigh the benefits.</td>
</tr>
<tr>
<td>I</td>
<td>Insufficient evidence to make a recommendation</td>
<td>Effective evidence is lacking, of poor quality, or conflicting, so the balance of benefits and harms cannot be determined.</td>
</tr>
</tbody>
</table>

Methods:

A retrospective chart review of 118 charts was conducted to assess screening rates for “A”, “B”, and select “I” conditions recommended for screening by US Preventative Task Force. Charts were excluded if the patients were not adults (under 18 years of age) or if the patients were seen less than three times at the Center for Family Health. Conditions looked at included immunizations for influenza, pneumococcal disease, and tetanus, diphtheria, and pertussis, screening for abdominal aortic aneurysm, chlamydia and gonorrhea, breast cancer, cervical cancer, colorectal cancer, depression, diabetes, drug use, glaucoma, cholesterol, HIV, obesity, osteoporosis, syphilis, and tuberculosis, and counseling for aspirin to prevent coronary arterial
The criteria used for determining whether a condition was adequately screening was defined by the USPSTF. According to USPSTF guidelines, breast cancer screening is indicated for women aged 40 and older. Mammograms should be performed every 1-2 years in such patients. Cervical cancer screening is indicated for women aged 21 or older, or within 3 years of onset of sexual activity, whichever comes first. Pap smears should be performed every 1-3 years, and should not be done on women who have had a hysterectomy for a benign condition, or on women older than 65 if they have a history of normal Pap smears. Colorectal cancer screening is indicated for all adults 50 and older. All indicated patients should have a colonoscopy every 10 years, flexible sigmoidoscopy or double contrast barium enema every 5 years, or fecal occult blood testing annually. Screening for every condition was marked as “indicated and done”, “indicated and not done”, or “not indicated” based on guidelines similar to the ones previously mentioned. Preventative care was considered “indicated and done” if one of the following three criteria was met: 1) the screen was documented as done at the Center for Family Health 2) the service was recorded as having been received elsewhere 3) if the service was offered and the patient declined.

Charts for patients who were indicated for breast, cervical, and colorectal cancer screening were then scrutinized to determine if there were any correlations among those who were screened and those who were not. Correlations among the patient’s age, gender, insurance carrier, and chief complaint were considered.

Summary:

At the Center for Family Health, the average combined screening rate for breast, cervical, and colorectal cancer was 82.5%. Individually, the screening rates were 91.7% for breast cancer, 82.8% for cervical cancer, and 72.7% for colon cancer (Figure 1). While the rates of screening for breast cancer and cervical cancer were acceptable, the rate of screening for colon cancer showed a need for improvement. A chart review was conducted for a sample population among those who were screened for any of the three cancers. While there were no correlations between patients who were screened for breast and cervical cancer, colon cancer screening rates showed a significant correlation between GI complaints and screening rates. Of the patients screened for colon cancer, 72.7% had a recurring GI chief complaint (Figure 2). Also, the percent of females that failed to be screened was significantly higher than the percent males that were not screened. An explanation for this was not determined, but may be due to a small sample size. The influence of age and insurance on screening was also analyzed but no significant correlation was found between them and screening rates for any of the cancer screenings.
Figure 1:

Screening And Immunization Rates

Colorectal Cancer
Breast Cancer
Cervical Cancer
Influenza
Pneumococcal
Tdap
Abdominal Aortic Aneurysm
Aspirin to Prevent CAD
Chlamydia, Gonorrhea
Dental Examination
Depression
Diabetes
Diet/Exercise
Drug Use
Eye Exam/Glaucoma
HIV
Obesity/BMI
Osteoporosis-DEXA
Osteoporosis- Ca/D
Problem Drinking
STI Counseling
Syphilis
Sun Exposure
PPD
Tobacco Use
Fasting Lipid Panel
Vitamins w/ Folic Acid

Percent Indicated and Performed

0 20 40 60 80 100

- Cancer Screening
- All Others

Figure 2:

Total Number of Patients 118

Patients indicated for Pap smear
58

Patients indicated for mammogram
48

Patients indicated for Colon Cancer Screening
70

Patients not screened
4 (8.3%)

Patients Screened
51 (72.9%)

Patients not screened
19 (27.1%)

Patients with GI related chief complaints
73.7%

Mean Age 65.3 yrs.

Percent Female: 68.4%

Initials: Primary Insurance Carrier: Medicaid/Charity Care 73.7%

Patients with GI related chief complaints
21.1%

Mean Age 67.6 yrs.

Patients not screened
10 (17.2%)

Patients Screened
48 (82.8%)
Conclusion:

Despite the need for improvement among cancer screenings, the rates of cancer screening was significantly higher than the screening rates of other conditions, which had an average rate of 46.2% (Figure 1). Possible reasons for the higher screening rates among cancers may be attributed to the clear screening guidelines for the cancers, thus encouraging physicians to routinely screen for these rather than other conditions which may have more obscure guidelines. Focus groups geared toward the conditions with lower screening rates can be conducted to determine the amount of physician knowledge on these guidelines. Some conditions may have had lower screening rates due to ineffective preventive care communication skills. Research shows that communication relevant to preventive services and practices is based on the physician’s skill in basic history taking and data collection, relationship building, facilitation skills, negotiation, and partnership skills. Physicians may be able to provide patient education on the conditions being screened for through effective communication. There may be a need for further physician education to improve communication skills. Previous studies also show that organizational changes such as encouraging separate preventive care visits are effective in improving adult cancer screening rates. Furthermore, it is possible that screening was more likely to be performed when it was indicated on a questionnaire that was included in the patient’s chart. Studies show that implementation of a preventive care flow sheet can improve the rates of screening significantly. After implementation of such a flow sheet at the Center for Family Health, a quality improvement project could be conducted to figure out whether a flow sheet should be used to improve screening rates.

References:

FIXATION IN MUSCULOSKELETAL ONCOLOGY PATIENTS: A RETROSPECTIVE ANALYSIS OF LOCKING VERSUS STANDARD PLATES

Mentors: Kevin C, Anthony D. Ugliarolo MD*, Kathleen S. Beebe MD*, Francis R. Patterson MD, Joseph Benevenia MD* (NJMS-Orthopaedic Surgery; Division of Musculoskeletal Oncology)
*Co-Mentors

Objective:

Over the past few decades, there have been many advances in fracture care in orthopedic surgery with the invention of intramedullary nailing, the external fixator and standard compression plating. These techniques of open reduction and internal fixation (ORIF) stress anatomic reduction & rigid internal fixation to achieve functional rehabilitation of the limb.

Following tumor resection, current preferred reconstructive procedures include placement of cadaveric allograft, autograft, or polymethylmethacrylate (PMMA). Plating techniques remain the mainstay for orthopedic oncology reconstructions which include fixation of pathologic fractures, allograft reconstructions, allograft-prosthetic composites and prophylactic fixation of impending fractures. [7] Standard Dynamic Compression plates (DCP), create an environment conducive to primary bone healing by providing absolute stability through reducing the gap by anatomic reduction and strain to less than 2%. By redirecting stress from the bone fracture to the plate, the bone is allowed to heal without significant strain. Conventional plates have the ability to resist axial, torsional, and bending loads. Although constructs are stable biomechanically, healing is often delayed because of the extent of soft-tissue dissections and bony devascularization. [1]

In recent years, locking compression plates (LCP) have become the method of choice for fixation. Locked plates rely on different mechanical principles to provide fracture fixation and in doing so provide for a different biologic environment for bone union. Whereas a standard compression plate achieves stability via friction created from a screw pressing it against bone, locking head screws mechanically couple the locking plate to the bone.[1] Reciprocal threads on the head of the screws and the holes of the locking plate allow the screw to be fixed directly into the plate. Because the screws do not toggle in the plate, these constructs function as multiple fixed angle devices providing additional stability and limiting screw pullout. Moreover, they conserve vascularity, thus allowing for a quicker union. Additionally, while compression plates must be contoured specifically, locking plates do not need to be.[1,2,5] However, it must be noted that locking plates can only be used to maintain fracture reduction, but not to obtain it. Therefore, the fracture must be reduced and the limb alignment, length, and rotation must be set before any of the locked screws are placed. Locking plate technology has shown to overcome many of the complications related to standard plate usage. The mechanisms determining locking plate fixation hint at their potential benefit to allograft fixation and oncological surgery. Locking plates with reduced screw toggle should theoretically lead to quicker healing by causing consistent, stable fixation. Such benefits have already been seen as Beucker, et al showed that locking plates used for allograft-host fixation were associated with improved union rates and fewer additional complications when compared to standard plates.[3]
This retrospective study was undertaken to evaluate the performance of locking plates in comparison to standard compression plates for fixation upon resection of skeletal tumors in patients of all age ranges.

Methods:

This study received institutional review board approval. Upon review of our orthopedic oncology database, patients were identified who had undergone resection of benign and malignant skeletal tumors with standard and locking plate reconstruction from December 1997 to April 2006. Each patient's clinic and hospital record was retrospectively reviewed. Those patients who had allograft reconstructions were noted. Demographic, oncological, surgical and follow-up data were collected. The demographic data included age and sex. Diagnosis, tumor location, type of lesion (benign versus malignant), presence of metastatic disease, and whether the patient received chemotherapy and/or radiation was reviewed and collected as part of the oncological record. The surgical date, operative time, blood loss, type of allograft, need for flap closure, length of hospital stay, and complications were also determined. Follow-up data included total follow-up time, presence of postoperative complications and the need for further operations. For many patients, follow-up extended beyond the radiographic union because of their underlying oncologic condition. Minimum follow-up for study inclusion was 4 months. Radiographs of each patient were reviewed from time of surgery to the latest follow-up to assess any complications such as non or malunion. The determination of radiographic union was made based on the presence of mature bridging bone at the junction site. Any patient who did not show healing by 12 months postoperatively or who required additional surgery was defined as having a nonunion. [1] Once all data were collected, the paired sample T-test was used to compare the 2 groups and to determine if there was a statistically significant difference in the union rates amongst them. A P<0.05 was considered to be statistically significant.

Summary:

Sixty six patients meeting the inclusion criteria were identified. There was homogeneity of the study group with regards to age, gender, lesion type, and adjuvant therapy. Surgical data, including type of allograft, estimated blood loss, operative time and need for flap closure were also similar for the two groups (Table 1).

In the standard compression plate group, 17 complications were encountered in 14 patients and consisted of: DVT (1), nonunion (8), hardware fracture (2), hardware loosening (1), infection (2), hematoma (1), death from metastasis (1), and amputations (1). 7 of the nonunion patients required second procedures. One required a (transforearm) amputation, four required replacement with another standard plate, two required removal of plate and allograft followed by an endoprosthetic replacement.

In the locking plate group, 13 total complications were encountered in 8 patients: nonunion (7), infection (1), Death from metastasis (1), DVT (1), hardware loosening (1), screw breakage (1), fracture of plate (1). 3 of the nonunion patients required second procedures, all of which required replacement of the locking plate.

There was no statistical significance between the two groups in terms of union (P< 0.25, 95% confidence interval). In all, nonunion rates were 38% and 32% (p = 0.245), complication rates were 58% and 41% (p = 0.076) and recurrence control rates were 74% and 90.625% (p= 0.164) for standard versus locking plates, respectively. Upon further subset analysis with regards to
plating, nonunion in single plate procedures was determined to be 8% and 34% for locking and standard plates, respectively (p = 0.008).

**Conclusion:**

Limb-salvage resection accompanied by adjuvant therapy has become the standard treatment for most patients with bone tumors. However, the quality of pathologic bone is quite compromised due to the underlying oncological condition, irradiation and chemotherapy. The decreased strength of the bone poses many challenges to healing and incorporation of grafts. Additionally, allografts also pose barriers to osteosynthesis as the allograft bone does not have a native blood supply. Fixation of the constructs includes both standard compression as well as locking plates. In recent years, locking plate technology has gained popularity due to its biomechanical and clinical advantages over standard compression plates. These advantages include unicortical screws, improved purchase and decreased vascular and tissue stripping.

To date, no study has compared the performance of locking to standard plates following tumor removal in musculoskeletal patients of all age ranges. Results indicate that there is no statistically significant difference in union rates at the allograft-host junction between the two groups. However, these data do suggest that the use of locking plates may provide a clinically significant benefit when compared to standard compression plates because of fewer revisions for plate and screw failure and the ability to obtain unicortical fixation in allograft segments.

Upon analysis of a subset group of procedures with single plate fixation, there was a substantially greater incidence of nonunion in single standard plate fixation as compared to single locking plate fixation. Certainly, more research is needed in this area. In subsequent studies, the efficacy of single plating in various types of allografts, namely intercalary, will be pursued. There are two main limitations to this study. The first is that of the shorter follow-up period for patients with locking plates. However, this was largely uncontrollable because of the fact that these implants have only been

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**TABLE 1. Comparisons of Locking and Standard Plate Cohorts**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Locking Plate</th>
<th>Standard Plate</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>28</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Procedures</td>
<td>32</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Path. Fx's</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>PMMA/Resection</td>
<td>10</td>
<td>4</td>
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</tr>
<tr>
<td>Allograft/Allograft</td>
<td>17</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Second procedures</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>14 M/15 F</td>
<td>21 M/15 F</td>
<td></td>
</tr>
<tr>
<td>Average Age(y)</td>
<td>35.8</td>
<td>31.8</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Surgical</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Length of Stay(days)</td>
<td>67.39</td>
<td>68.47</td>
<td>0.234</td>
</tr>
<tr>
<td>Flap closure</td>
<td>6</td>
<td>12</td>
<td>0.196</td>
</tr>
<tr>
<td>Operation Time(min)</td>
<td>303.1</td>
<td>336.16</td>
<td>0.44</td>
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<tr>
<td>Estimated Blood Loss(cm²)</td>
<td>533.47</td>
<td>641.57</td>
<td>0.522</td>
</tr>
<tr>
<td><strong>Allograft type</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IC</td>
<td>10</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>4</td>
<td>3</td>
<td></td>
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<tr>
<td>Scapular</td>
<td>0</td>
<td>1</td>
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</tr>
<tr>
<td>Femoral Head</td>
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<td>Humeral Head</td>
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<tr>
<td>Free Fibular</td>
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<tr>
<td>Hemiplakis</td>
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<tr>
<td>Allograft Crouch</td>
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<tr>
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<tr>
<td></td>
<td>Radiation</td>
<td>3</td>
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</tr>
<tr>
<td></td>
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<td><strong>Follow up</strong></td>
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<td></td>
</tr>
<tr>
<td>Mean Followup (mo.)</td>
<td>17.4</td>
<td>38.5</td>
<td></td>
</tr>
<tr>
<td>Nonunion</td>
<td>7</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Days to Union</td>
<td>165.21</td>
<td>196.1</td>
<td>0.389</td>
</tr>
<tr>
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<td>13</td>
<td>22</td>
<td></td>
</tr>
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<td>Procedures with Complications</td>
<td>8</td>
<td>12</td>
<td>0.076</td>
</tr>
<tr>
<td>Complication Rate</td>
<td>40.16%</td>
<td>57.9%</td>
<td></td>
</tr>
<tr>
<td><strong>Plating</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total No. of Plates</td>
<td>41</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Procedures with Single Plating</td>
<td>25</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Procedures with double or greater plating</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Single Plate Nonunion</td>
<td>2</td>
<td>11</td>
<td>0.003</td>
</tr>
</tbody>
</table>
recently used as compared to standard compression plates. The second limitation is that of the
varying anatomic locations for tumor resection and subsequent reconstruction. It must also be
noted that the same criteria were used to determine incorporation in both osteoarticular as well
as intercalary allografts. Intercalary allografts have two opportunities for nonunion while osteoarticular allografts display only one. However, it has also been noted in studies that with
regards to intercalary allografts, it is the diaphyseal junction that poses the challenge to union as
compared to the metaphyseal junction. [3, 8]

Certainly, a larger, multi-institutional retrospective study is needed to fully assess the long term
outcomes of locking plate fixation. However, the advantages of locking plates with respect to
ease of surgical technique and fixation into compromised bone suggest that they may be
attractive options in the field of musculoskeletal oncology.

References:

AN IMPROVED METHOD OF FUSION DISPLAY FOR CO-REGISTERED ONCOLOGIC IMAGES

Mentor: Lionel Zuckier, MD, Nuclear Medicine (Radiology)

Introduction:

Ease of perception, and the ability to identify lesions, has become an emerging topic in radiology (Kundel). With new methods available to display and manipulate the digital image, this is a relevant and important practical topic. The term “conspicuity” refers to ease of identifying a lesion, and is an important concept in radiologic image perception. One specific imaging technique, Positron emission tomography (PET), detects annihilation photons emitted by F-18, the radionuclide within fluorodeoxyglucose (FDG), a radiopharmaceutical, is injected into the patient in commonly performed oncologic PET examinations. PET renders an image of functional information, which in the case of FDG indicates cells showing high metabolic activity.

Recently, PET technology has been combined with computed tomography (CT), resulting in a dual modality imaging system capable of rendering both a CT and PET image. Combining the anatomical information from the CT and the functional from PET, produces “best of both worlds” in that functional information is precisely correlated with the exact anatomic location of the abnormality. Dual-modality PET-CT images are intrinsically co-registered and can be graphically combined to produce a single 2-dimensional “fusion image” (example, below).

There are no universally accepted standards for display of PET-CT images. While 2-D fused images are a useful method of correlating PET findings with anatomic location, some authors have displayed fused images without separate native CT and PET data.

Earlier work in the Department of Nuclear Medicine demonstrated that when comparing native FDG PET and CT with 2D fused images using a standard vendor workstation algorithm, 23% of lesions were missed, due to either partial or complete loss of conspicuity (Zuckier et al, 2007). This problem is conceptually applicable to all similar methods of combining image data.

Objective:

The goal of this work is to characterize a novel commercially-developed method of portraying fused PET-CT data with minimal loss of information, i.e. to clearly portray 2 dependent variables, CT density and FDG concentration, at each voxel location. We have worked with the vendor to optimize various strengths of the new fusion method that we are characterizing (Zuckier and Hu, 2008).
THE CLARIO FUSION TECHNIQUE:

Introduction
Our lab has worked with a commercial image software company, Clario Medical Inc., to validate a novel method of image fusion to PET-CT images. In this technique, a “base image” is composed using standard 2D fusion methods while the Z axis is used as a second dependent variable, to recode the FDG intensity as a 3-D rendered height map. It is anticipated that the additional information provided by the Z-axis is a means of conserving image information while concurrently displaying 2 dependent variables per pixel location.

Preprocessing
Co-registered PET and CT images are identified by examining the spatial location information in the DICOM headers. PET images are converted to Standard Uptake Values (SUV’s) and re-sampled to the resolution of CT using bilinear interpolation.

Surface creation
Pixels on each CT slice are elevated in the out-of-plane direction (the Z-direction) by their corresponding SUV’s to create a 3D surface. Standard computer graphics techniques such as smoothing, lighting, shading, and projection are applied to render the surface, example below (fig. 2a).

Views
Perspective projection is used in 3D View mode while orthographic projection is used in Quantitative View mode because it keeps the relative scale of objects. In Quantitative View mode the camera’s pitch angle is also fixed to 0 degree so the scene is always viewed parallel to the ground, which represents the plane of zero SUV. Horizontal marker lines are displayed as overlays on top of the surface. The lines are labeled by their altitude, i.e., the SUV’s example below (fig. 2b).

Methods:
Our experiments evaluated 2 aspects of the Clario display:
1) Ability to exploit Quantitative View mode for analysis of SUV (Quantitative Analysis).
2) Ability to portray fused images clearly (Conspicuity).
Quantitative analysis - Methods
1) 12 cases with at least 3 lesions in the liver were selected from an anonymized library of PET-CT images.
2) A subset of slices containing the liver was extracted from the DICOM image set.
3) An experienced radiologist (LSZ) was asked to quickly measure FDG uptake in the 3 hottest lesions of each of the 12 patients using standard (GE Xeleris and MIMVista) and new software (Clario) methods.
4) Time required for measurement in each patient was recorded and compared.

Quantitative analysis – Results
1) The average time for measuring the 3 liver lesions per case on Xeleris, MIMVista and Clario were 60.5, 62 and 66 seconds, respectively.
2) Plot of time required for task per case shows a virtually superimposable curve for each of the 3 image processing methods (fig. 3).

Conspicuity - Methods
1) 4 acquisitions of a Jaszczak phantom containing 6 artificial lesions of variable diameter were performed.
2) The 4 acquisitions had variable lesion intensity within a background intensity of 2.
3) Using Matlab, slices of the image containing the balls were replaced with “null slices” to create a “null” phantom. Each of the 6 spheres was then singly inserted into each of the 4 quadrants of the null phantom to form at total of 24 single-sphere images. This was repeated for all 4 intensities to create a test library of 96 images.
4) Newly created PET images were combined with a homogenous CT image to create fusion Clario images.
5) An experienced reader (LSZ), blinded to image composition, reviewed both the native AC images and the Clario fusion images and attempted to assign the ball to the correct quadrant.

Conspicuity - Results
1) Each ball/intensity combination appears 4 times, once in each quadrant. Error rates therefore range from 0 to 4. Errors have been graphed per combination of ball size and ball intensity (fig. 4).
2) For each method, conspicuity decreased with decreasing size (larger number) and intensity (larger numbers) of the ball.
3) Clario fusion performed in a similar and equivalent manner to reading the native AC images (14 errors out of 96 reads).
Conclusions:

1) We have successfully implemented and characterized a new method of image fusion that uses the Z-axis to code SUV intensity. Two features of this method are the ability to quantitate uptake using a graphical interface (Quantitative View mode) and preserved conspicuity of lesions.

2) With respect to quantitative analysis, the new Clario method performed similarly to the standard GE and MIM platforms with an improved user-friendly graphical interface. Future research will include analysis of more complicated natural or synthetic distributions of activity.

3) With respect to lesion conspicuity, the 3D image fusion had an identical error rate to the native PET images, demonstrating no loss of information when rendering an image in fusion mode. Future considerations for this aspect include fusion with a more complicated CT background, such as that of the lung and liver or even artificial images. As well, availability of additional test images will allow comparison of the new 3D method with the standard but limited 2D technique.

4) Ultimately, the 3D technique will be compared to the standard fusion technique on patient cases to see if it can be used in an efficient way and is able to reduce frequency of missed lesions.

References:

1) Hu, Patrick, Zuckier, Lionel. Z axis coding for PET-CT image fusion. 2008
2) Kundel H. History of research in medical image perception. J Am Coll Radiol 2006;402-8
3) Zuckier LS and Hu YP. Z-axis coding to portray FDG intensity on co-registered PET-CT slices. 55th Annual Society of Nuclear Medicine Annual Meeting, New Orleans, June 2008
4) Zuckier LS, Ghesani NV, Liu Y. Perspicuity of solitary oncologic lesions on fused 18F-FDG PET-CT images. Accepted for Poster presentation, 54th Annual Society of Nuclear Medicine Meeting, Washington DC, June 5, 2007
Cancer Summer Student Research Program - 2008

31. JASON TEICHMAN (NJMS 2011)

CURING MYCOPLASMA INFECTION ABROGRATES EXPRESSION OF BONE MORPHOGENETIC 2 PROTEIN IN TRANSFORMED BRONCHIAL EPITHELIAL CELLS

Mentor: Melissa B. Rogers, Ph.D. (Biochemistry & Molecular Biology)

Objective:

Lung cancer is the leading cause of cancer deaths among both men and women in the US. The ACS estimates that 161,840 people will die in this country from lung cancer in 2008, accounting for approximately 29% of all cancer deaths, more than colon, breast and prostate cancers combined. Only about 15% of lung cancer patients will survive 5 years. These statistics highlight the importance of developing new therapies for treating lung cancer.

We have previously identified a novel link between lung cancer, bone morphogenetic protein (BMP2), and mycoplasma infection. Lung carcinomas express significantly more BMP2 relative to normal tissues or benign lung tumors. BMP2 is a member of the transforming growth factor-b (TGF-b) superfamily that normally acts to regulate cell proliferation, apoptosis, differentiation, cell-fate determination, and morphogenesis. Aberrant BMP2 expression is associated with lung, breast, colon, prostate, and pancreatic cancer and melanomas. Recombinant BMP2 protein or overexpression of the BMP2 gene increased tumor growth of A549 lung cancer cells in nude mice, while anti-BMP2 antibodies or antagonists reduced tumor growth by 50%.

We have found that mycoplasma infections strongly induce BMP2 expression in many cell types, including BEAS-2B immortalized human bronchial epithelial cells. Mycoplasma also induced malignant transformation as demonstrated by growth in soft agar and tumor formation in nude mice. The incidence of mycoplasma infection was reported to be higher in lung carcinoma patients than in a healthy control group. People, particularly smokers, can harbor chronic mycoplasma pulmonary infections for years without detection. This suggests that their lung cells are likely to be subjected to elevated BMP2 signaling and is important because antibiotic treatment can eradicate mycoplasma infection. Indeed non-small cell lung carcinoma patient survival improved following clarithromycin treatment.

Hypothesis 1: Live mycoplasma infection is required for BMP2 expression in BEAS-2b cells.

Aim 1: Measure effect of curing mycoplasma in previously infected cells.

Hypothesis 2a: BMP2 overexpression stimulates the growth of non-transformed BEAS-2b cells.

Hypothesis 2b: BMP2 overexpression accelerates mycoplasma-mediated transformation

Aim 2: Compare growth rates and morphology of BMP2 overexpressing cells to normal cells in the presence or absence of mycoplasma.

This work can contribute to helping discover novel therapeutic approaches to lung cancer based on modulating BMP2 signaling and/or mycoplasma therapy.

Methods:

Cell Culture: BEAS-2B immortalized human bronchial epithelial cells were cultured in DMEM (Sigma) supplemented with 5% fetal bovine serum and 1% glutamine. BEAS-2B cells stably transfected with the pcDNA3.1 vector (Invitrogen) and cells transfected with the pcDNA3.1 vector containing the human BMP2 cDNA were obtained from Dr. Langenfeld’s laboratory at UMDNJ-Robert Wood Johnson Medical School. All cells were grown in 5% CO2 at 37°C.

Mycoplasma Infection: Uninfected BEAS-2B cells, including the transfected lines, were intentionally infected with mycoplasma as follows. Mycoplasma-free cells were seeded in
100 mm plates with 9 ml complete media and 1 ml conditioned media from mycoplasma-infected cells. After 24 hr, media was removed, cells were washed with PBS, and then cultured in fresh complete media. Infected and control uninfected cells were confirmed to be mycoplasma-positive or –negative, respectively, using the MycoAlert Mycoplasma Detection Kit (Cambrex) and by PCR of culture media using primers specific for mycoplasma rRNA (16S).

Eradication of Mycoplasma in Cell Cultures: BEAS-2B cells that had been previously mycoplasma-infected for 45 days and 6 months were grown with the antibiotic Plasmocin (25 μg/ml) (InvivoGen) for 41 and 33 days, respectively. Eradication of mycoplasma was confirmed as above.

RNA Isolation/RT-PCR: Total RNAs from mycoplasma-postive or –negative BEAS-2B cells were isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. 1 μg of RNA was reverse-transcribed using 100 units of SuperScript™ III RT (Invitrogen) and oligo dT according to the manufacturer's instructions. Five percent of the resulting cDNA was used for PCR to amplify BMP2 and Actin.

Cell Morphology Observation: Uninfected or mycoplasma-infected BEAS-2B cells were plated at various densities, and morphology was observed over time at 100x magnification by phase contrast. Images were captured using Spot Advanced Software (Diagnostic Instruments).

Summary:

Using both the enzymatic MycoAlert assay and PCR (Fig. 1) we confirmed the eradication of long-term mycoplasma infection in BEAS-2B cells following treatment with Plasmocin. In addition, we confirmed that our uninfected samples were mycoplasma-free, our long-term infected samples were mycoplasma-infected, and that conditioned media used initiate these studies carried mycoplasma.

PCR was performed on media taken from BEAS-2B cells in culture to detect mycoplasma. The 3-month mycoplasma infected samples were either untreated or treated with Plasmocin for 41 days following 45 days of continuous infection. The 6-month mycoplasma infected samples were similarly untreated or treated for 33 days. The results of the PCR and an enzymatic assay (MycoAlert, not shown) confirmed that the mycoplasma infection was not present in the Plasmocin-treated samples.

Figure 1. Plasmocin treatment cures mycoplasma infection

<table>
<thead>
<tr>
<th>Myco Duration</th>
<th>3 mo.</th>
<th>6 mo.</th>
<th>3 mo.</th>
<th>6mo.</th>
</tr>
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<tr>
<td>Myco Status</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Plasmocin</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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</table>

We showed by PCR (Fig. 2) that treatment of long-term mycoplasma-infected cells with Plasmocin abrogated the expression of BMP2. This data indicated that BMP2 expression ceased between 16 and 20 days of treatment in the 6-month-infected samples and before 24 days in the sample that began treatment after 45 days of continuous mycoplasma infection.
Figure 2. Plasmocin treatment of BEAS-2B cells abrogates BMP2 expression

<table>
<thead>
<tr>
<th>Myco</th>
<th>69 d</th>
<th>73 d</th>
<th>82 d</th>
<th>69 d</th>
<th>73d</th>
<th>94 d</th>
<th>6 mn</th>
<th>6 mn</th>
<th>6 mn</th>
<th>6 mn</th>
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<th>6 mo</th>
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</thead>
<tbody>
<tr>
<td>Plasmocin Dur.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>24 d</td>
<td>28d</td>
<td>43 d</td>
<td>-</td>
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<td>16d</td>
<td>20d</td>
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<tr>
<td>BMP2</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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</table>

Figure 3. BMP2 overexpression accelerates the loss of contact inhibition in mycoplasma-infected BEAS-2B cells.

Figure 4 shows that the growth characteristics and morphology of the BEAS-2B cells cured of mycoplasma infection remained transformed. These cells exhibited a rapid growth rate and a lack of contact inhibition which led to the formation of foci. The cured cells did not revert to their original non-transformed morphology.
Conclusions:

Based on the results of the enzymatic MycoAlert assay and PCR for mycoplasma rRNA, we can conclude that antibiotic treatment with Plasmocin effectively eradicates mycoplasma infection in BEAS-2B cells. Curing the mycoplasma infection appears to abrogate BMP2 expression, as shown in Fig. 2. Cells infected with mycoplasma for 6 months and treated with plasmocin for 16 days continued to show BMP2 expression, while those treated for 20 days show no BMP2 expression. This is reasonable because the manufacturer suggests that mycoplasma infections treated with Plasmocin take 2-3 weeks to cure. It is interesting that the transformed BEAS-2B cells treated with Plasmocin fail to revert to their original non-transformed morphology despite the lack of BMP2 expression (Fig. 4). This suggests that the changes that occur following mycoplasma infection and subsequent increased BMP2 signaling are not reversed simply by decreasing BMP2 expression.

Overexpression of BMP2 in uninfected BEAS-2B cells does not appear to be sufficient to generate transformation (Fig 3). This suggests that there are other changes associated with mycoplasma infection besides increased BMP2 expression that produce the transformed morphology and growth characteristics. However, increased BMP2 expression appears to accelerate the rate at which normal BEAS-2B cells transform (Fig 3). Cells overexpressing BMP2 showed increased foci formation and loss of contact inhibition and anchorage-dependent growth.

Literature Cited:

32. RYAN S. WEISS (THE UNIVERSITY OF VIRGINIA 2010)

THE BINDING OF FACONI ANEMIA PROTEINS TO CALMODULIN AND THE ROLE THIS MAY PLAY IN THE STABILITY OF THE STRUCTURAL PROTEIN NONERYTHROID α-SPECTRIN IN HUMAN CELLS.

Mentor: Muriel Lambert, PhD, (Pathology and Laboratory Medicine)

Objective:

Fanconi anemia (FA) is an autosomal recessive disease characterized by aplastic anemia, myelodysplasia, a markedly increased incidence of acute myelogeneous leukemia, progressive bone marrow failure and diverse congenital abnormalities\(^1\), \(^2\). FA cells are particularly hypersensitive to the effects of DNA interstrand cross-linking agents and are defective in the ability to repair damage caused by these agents\(^1\), \(^2\). Based on these findings, it has been proposed that the underlying mechanism for this disorder involves a DNA repair deficiency\(^1\), \(^2\). Our laboratory has identified a structural protein, nonerythroid α spectrin (αIISp), which is a major cytoskeletal protein contributing to membrane organization and integrity, in the nucleus of normal human cells\(^10\). It has been demonstrated that αIISp binds directly to DNA containing an interstrand cross-link and plays a role in repair of this type of damage\(^3\)-\(^6\). Studies in our laboratory have shown that cells from a number of FA complementation groups are deficient in αIISp\(^7\). We have developed a model (Fig. 1) in which we have hypothesized that αIISp localizes to sites of DNA interstrand cross-links and acts as a scaffold to aid in the recruitment of repair proteins to the site to damage\(^4\). In FA cells, there is reduced recruitment of repair proteins as a result of the decreased levels of αIISp, and thus, reduced levels of DNA repair in these cells\(^4\), \(^8\). Studies in our laboratory indicate that in FA cells reduced levels of αIISp are due to reduced stability of this protein rather than to reduced expression\(^7\). An important question is what factors are responsible for the reduced stability of αIISp in FA cells. We hypothesize that one or more of the FA proteins is involved in maintaining the stability of αIISp and that one
way they may do so is by binding to one of the proteins that is involved in the breakdown of αIIsp, such as calmodulin or calpain. One mechanism for the cleavage of αIIsp by calpain is thought to involve the stimulation of calpain activity by calmodulin. The binding of calmodulin to αIIsp (Fig. 2) enhances the susceptibility of spectrin to calpain cleavage. We hypothesize that the binding of one or more of the FA proteins to calmodulin may inhibit its ability to bind to αIIsp. This would prevent the stimulation of calpain activity, and as a result, lead to reduced αIIsp cleavage in the cell.

Methods:

An antibody against calmodulin (Santa Cruz Biotechnologies) was bound to protein-A-coated agarose beads (Sigma Aldrich Corp.) in binding buffer consisting of 25 mM Tris-HCl, pH 7.3, 150 mM NaCl, 1% Triton X-100 (Bachem Biochemicals) plus protease inhibitor mixture (Roche Molecular Biochemicals). Control beads were also prepared in which rabbit IgG was bound to the beads. The antibody beads were washed five times in binding buffer and blocked with 2% BSA overnight at 4°C with steady agitation. The blocked beads were subsequently washed and resuspended in binding buffer. The nuclear protein extracts were pre-cleared with protein A-bound agarose beads for 3 h at 4°C. The nuclear extract was then incubated with the antibody bound beads overnight at 4°C with steady agitation to allow antibody-antigen complex formation. The protein-A-agarose bead-bound immune complexes were then washed five times with IP wash buffer and resuspended in this buffer. The samples were centrifuged, the supernatant was removed, and loading dye was added to the pellet containing the beads. The samples were then placed in boiling water for five minutes, cooled on ice, and centrifuged.

The supernatant from the sample of each of the immunoprecipitations was subjected to SDS-PAGE using 8% or 10% polyacrylamide. Rabbit IgG was used as an internal loading control. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Bio-Rad) and immunoblotted. Antibodies against various FA proteins were used to determine whether any of the FA proteins had co-immunoprecipitated with calmodulin. Exposure times for all lanes in each immunoblot were the same. Immunoblots were developed using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology Inc.) and then exposed to X-ray film (Pierce). The primary antibodies used were: anti-α spectrin, anti-FANCA, anti-FANCC, and anti-FANCD2. When blots were reprobed, they were stripped using the Re-Blot Western Recycling Kit (Chemicon) and reblocked before reprobing with the secondary antibody.

Results:

In the present project, several of the FA proteins were examined for their ability to bind to calmodulin. Co-immunoprecipitation studies were carried out using nuclear extracts from normal human cells to examine this binding. The ability of the FA proteins to interact with calmodulin was detected by western blot analysis. The results showed that αIIsp bound to calmodulin, as expected (Fig. 3). Both FANCA (Fig. 4) and FANCC (Fig. 5) also bound to calmodulin; FANCD2 (Fig. 6) bound to a lesser extent. Based on these studies, it is possible to hypothesize that in normal human cells, FANCA and FANCC bind to calmodulin, thus, interfering with calmodulin’s ability to bind to αIIsp. This in turn could inhibit the ability of calmodulin to stimulate cleavage of αIIsp by calpain. Thus the ability of at least two of the FA proteins to bind to calmodulin and prevent the breakdown of αIIsp could lead to the maintenance of normal levels of αIIsp in the cell and normal levels of repair of DNA interstrand cross-links. In FA-A and FA-C cells, where there is a deficiency in FANCA and FANCC, respectively, this association between the FA proteins and calmodulin would be deficient, which could lead to cleavage of αIIsp. FA proteins,
therefore, by binding to calmodulin and maintaining the stability of αII-Sp in the cell, could be essential for any of the cellular processes in which αII-Sp plays a role, such as DNA repair, signal transduction, and cell growth and differentiation. A deficiency in these FA proteins, such as occurs in FA, could lead to reduced levels of αII-Sp and consequently reduced DNA repair which could play a role in the increased incidence of cancer observed in FA patients.

Figure 3. Non-erythroid α-spectrin in co-immunoprecipitated with calmodulin. Co-immunoprecipitations were conducted using an antibody to calmodulin and nuclear extracts from normal human cells. The IP reactions were examined by SDS-PAGE and western blot analysis. Immunoblotting was carried out using anti-αII-Sp. Western blot analysis showed that calmodulin bound to αII-Sp as expected. A positive control showed that αII-Sp was present in the nuclear lysate.

Figure 4. FANCA co-immunoprecipitated with calmodulin. Co-immunoprecipitations were conducted using an antibody to calmodulin and nuclear extracts from normal human cells. The IP reactions were examined by SDS-PAGE and western blot analysis. Immunoblotting was carried out using anti-FANCA (top panel). The bottom panel shows the IgG heavy chain which was used as a loading control. The control, which includes a co-immunoprecipitation using rabbit IgG, are seen in the left lane.

Figure 5. FANCC co-immunoprecipitated with calmodulin. Co-immunoprecipitations were conducted using an antibody to calmodulin and nuclear extracts from normal human cells. The IP reactions were examined by SDS-PAGE and western blot analysis. Immunoblotting was carried out using anti-FANCC (top panel). The bottom panel shows the IgG heavy chain which was used as a loading control. The control, which included co-immunoprecipitation using rabbit IgG, is seen in the left lane.

Figure 6. FANCD2 co-immunoprecipitated with calmodulin to a lesser degree. Co-immunoprecipitations were conducted using an antibody to calmodulin and nuclear extracts from normal human cells. The IP reactions were examined by SDS-PAGE and western blot analysis. Immunoblotting was carried out using anti-FANCD2 (top panel). The bottom panel shows the IgG heavy chain which was used as a loading control. The control, which included co-immunoprecipitation using rabbit IgG, is seen in the left lane.

Summary and Conclusions:

Studies have indicated that Ca^{2+}-activated binding of calmodulin to an unstructured insert in the 11th repeat unit of αII-Sp enhances the susceptibility of spectrin to calpain cleavage. This insert is the cleavage site for calpain, ubiquitous Ca^{2+} - dependent proteases, and various caspases. It has been shown that the binding of calmodulin to αII-Sp at this site affects the rate of proteolysis of the molecule by calpain by controlling the accessibility of calpain cleavage sites via conformational changes. Studies have shown that the calmodulin-αII-Sp complex actually accelerates the rate of calpain cleavage of αII-Sp by allowing calpain to access the cleavage site on the 11th repeat unit.

Based on our studies, it is possible to hypothesize that, in normal human cells, certain FA proteins aid in stabilizing αII-Sp by binding to calmodulin and interfering with calmodulin’s ability to bind to αII-Sp. This would decrease the accessibility of calpain cleavage sites on αII-Sp and in
turn, would inhibit the enzymatic breakdown of \( \alpha \)IISp that occurs when it is bound to calpain. Thus, the ability of at least two of the FA proteins, FANCA and FANCC, to stabilize \( \alpha \)IISp, which is involved in repair of DNA interstrand cross-links, would aid in maintaining normal levels of DNA repair in cells.

When there is a deficiency in these proteins, the resultant reduced levels of \( \alpha \)IISp would lead to a repair deficiency, particularly in repair of inter-strand crosslinks. Cells from Fanconi Anemia patients have been shown to be particularly sensitive to cross-linking agents and in these patients there is an increased incidence of cancer, which may be related to a failure in DNA repair. The reduced levels of \( \alpha \)IISp, and the resultant repair deficiency, could play an important role in this increased incidence of cancer observed in Fanconi Anemia patients.

References:

7. Lefferts JA, Lambert MW 2003. Fanconi anemia cell lines deficient in \( \alpha \)IIspectrin express normal levels of \( \alpha \)II spectrin mRNA. Biochem Biophys Res Comm, 307:510-515.
33. JANET WU (THE OHIO STATE UNIVERSITY 2011)

THE NOVEL SCAFFOLD PROTEIN, CCPG1, NEGATIVELY REGULATES RHO ISOFORM GTPASES AND MAY REGULATE CELL MOTILITY

Mentor: Ian Whitehead, PhD, (Microbiology and Molecular Genetics)

Objective:

Rho GTPases are involved in cell motility by regulating the actin cytoskeleton, which plays an important role in the formation of lamellipodia, contraction of cell bodies, and detachment of cells. The three isoforms of Rho proteins termed Rho, Rac, and Cdc42 are structurally similar but have different cellular functions. Rho isoform, consisting of RhoA, B and C participates in regulating the contraction of actin and myosin filaments as well as stress fibers (Wheeler and Ridley, 2004). Rac regulates actin in a different way by inducing the formation of surface protrusions (lamellipodia), and Cdc42 is key to the directional polarity of motile cells (Raftopoulou and Hall, 2004). Our lab recently identified a novel scaffolding regulator of Rho proteins termed cell cycle progression protein 1 (CCP) (Kostenko et al., 2006). Interestingly, we also found that Ccpg1 negatively regulates Rho isoform without affecting Cdc42 and possibly Rac. Since Rho proteins play a crucial role in cell motility, we hypothesized that Ccpg1 would inhibit cell motility. Through various experiments involving wound healing assays, time-lapse images, and motility assays with three different cell lines, HeLas (human cervical cancer cells), NIH/3T3s (mouse embryonic fibroblasts), and T47Ds (human breast cancer cells), we found that although Ccpg1 does not appear to affect cell motility of these three cell lines in wound healing assays, it significantly increases the motility of T47D cells in the transwell assays.

Methods:

Cell Culture and Reagents: HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, Penn/Strep, and Sodium Pyruvate at 37 °C in 5% CO₂. NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium and F-10 supplemented with Penn/Strep, and Sodium Pyruvate at 37 °C in 7% CO₂. T47D cells were grown in RPMI supplemented with 10% fetal bovine serum, Penn/Strep, and L-glutamine at 37 °C in 5% CO₂. Primary mouse IgG FLAG M2 monoclonal antibody was used for western and immunofluorescence detection of CCP. The α-actinin mouse monoclonal primary antibody was used as a loading control. Rabbit polyclonal antibody was used to stain for Ki67. HRP-conjugated secondary anti-mouse antibody was used for western detection whereas Alexafluor 488 (green) and 567 (red) secondary antibodies were used for immunofluorescence detection.

Transient Transfection: HeLa cells were transfected using FuGene according to the manufacturer’s description with 3 µg of plasmid (empty pAX vector or pAX containing CCP) when the cells were between 50-60% confluent. NIH 3T3 and T47D cells were transfected using Lipofectamine 2000 according to the manufacturer’s description with 3 µg of the indicated plasmids when the cells were between 50-70% confluent.

Wound Healing Assay: 24 hours after HeLa, NIH 3T3, and T47D cells were transfected with the appropriate plasmids, the media was changed and three scratches were made into the confluent monolayer of cells. The scratches were made perpendicular to a line drawn on the bottom of the plates using 20 µL pipette tips. An hour later, after the cells were allowed to
settle, pictures were taken of the wounds using the Zeiss microscope at 0, 6, and 24 hours after the cells settled.

Time Lapse Assay: HeLa cells transiently transfected with either vector or CCP were viewed at low confluence using the Zeiss microscope. Pictures were taken every 5 minutes and compiled together to make a time lapse movie of the cells’ movement.

Motility Assay: T47D cells were plated in triplicates into transwells 24 hours post transfection into low serum media. The transwells were inserted into collagen coated wells containing complete serum. Cells, kept in the incubator, were allowed to migrate towards a complete serum gradient for 24 hours after plating. Cells were then fixed and stained for quantification.

Immunofluorescence Staining: HeLa cells that were transfected with either vector or CCP were plated in triplicates at low density in complete media on poly-lysine treated cover slips in a 6-well dish. 24 hours after incubation, the cells were washed with PBS and then fixed with formaldehyde. After fixing, the cells were washed with PBS, permeabilized with a mixture of 0.1% Triton X-100 and 1% BSA in PBS. After an hour, the primary staining with the antibodies indicated was applied. An hour later, the secondary staining using the antibody indicated was done in the dark. DAPI at a concentration of 1:1,000 in PBS was applied to the cover slips to stain the nuclei. Fluorescence images of the cells were observed and noted using a Zeiss Axiovert 200M microscope fitted with an ApoTome Imaging System.

Lysate Extraction: Lysates were extracted after the experiments were performed. GST-FISH lysis buffer was used to lyse the cells and protease inhibitor cocktail set III was used to inhibit protein degradation. Cells were then scraped with a rubber policeman and centrifuged. The supernatant was then transferred to a new Eppendorf tube for storage or use in western blot.

Western Blot: Western blot analysis was performed to confirm protein expression. 50 µL of samples from lysates were loaded into each well. After transferring onto a membrane, the membrane was blocked with a 5% milk solution for an hour. The membrane was then rinsed with TBS/Tween and probed with a primary antibody at a concentration of 1:1,000. An hour later, the membrane was washed again with TBS/Tween and probed with a secondary antibody at a concentration of 1:10,000.
Results:

<table>
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<tr>
<th>A.</th>
<th>Effect of Cpgl on Wound Healing</th>
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<tr>
<td>Rate of Wound Closure (µm/hr)</td>
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<td>CCP</td>
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<table>
<thead>
<tr>
<th>B.</th>
<th>Effect of Cpgl on Wound Healing</th>
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<td>Rate of Wound Closure (µm/hr)</td>
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<tr>
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<table>
<thead>
<tr>
<th>C.</th>
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<td>Rate of Wound Closure (µm/hr)</td>
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<tr>
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</table>

Figure 1: The results from the wound healing assay in HeLa cells (A), NIH/3T3 cells (B), and T47D cells (C) were quantified and the average rate in µm/hr was graphed. Standard deviations and P values were calculated to show that the difference in the two averages is insignificant, and therefore, it can be concluded that CCP does not affect cell motility in wound healing assays in HeLa cells.

Figure 2: The results from the transwell motility assay in T47D cells were quantified and graphed and we can conclude that CCP induces cell motility.
Figure 3: Western blot analysis was performed to confirm the over-expression of Ccpg1 in the wound healing experiments in HeLas (A) and NIH/3T3s (B). α-actinin was used as a loading control.

Figure 4: Immunofluorescence images in HeLa cells using Ki67, an antibody which is a marker for cell proliferation show that there is no difference in the amount or intensity of CCP treated cells versus vector treated cells which shows that CCP does not affect cell proliferation in HeLa cells.

Conclusion:
After performing the assays, we have concluded that Ccpg1 does not affect motility in wound healing or time lapse assays in HeLa cells. There is no difference in the cell proliferation rate between HeLa cells containing Ccpg1 or vector. The activation of Ccpg1 does not affect motility in commonly used motile cell lines such as NIH 3T3 or T47D cells measured by wound healing assays. However, the activation of Ccpg1 enhances the motility of T47D cells in transwell motility assays.

Since Ccpg1 enhances motility in T47D cells, we plan to carry out additional transwell motility assays in the T47D cells to check if the results repeat, and we plan to perform motility assays in the HeLa and NIH 3T3 cell lines as well.

Literature Cited:
Introduction:

Loss-of-function mutations in tumor-suppressor genes have a well-established role in the cumulative events leading to cancer. Perhaps the most well-known tumor suppressor, p53, has been extensively studied as a target for cancer therapy [1]. Recently, the potential therapeutic value of interferon regulatory factor-1 (IRF-1) as another mediator of apoptosis has also been explored.

First discovered as an inducer of interferon pathways [2], IRF-1 has increasingly been attributed with tumor suppressor functions as well [3]. In immunohistochemical studies of primary breast cancer patient samples, IRF-1 expression occurs in normal breast epithelium but is lacking in invasive ductal carcinoma [4]. This is consistent with the putative role of IRF-1 as a tumor suppressor that is downregulated in malignant neoplastic tissue.

Previously, it has been unclear whether IRF-1 is differentially expressed within different grades of ductal carcinoma in situ (DCIS), a non-invasive form of breast cancer with a better prognosis. Our lab has explored IRF-1 expression in DCIS and has found that, unlike IRF-5—another member of the IRF family with tumor-suppressor activity—IRF-1 continues to be expressed in moderate stages of DCIS.

IRF-1 plays a role in both p53-dependent [5] and p53-independent [6] apoptotic pathways, and in the latter mediates the apoptosis signal induced by IFNγ [6, 7]. Ectopic expression of IRF-1 in breast carcinoma cells results in downregulation of the apoptosis inhibiting protein, survivin, independent of p53 [8]. IRF-1 overexpression in vitro upregulates apoptosis markers such as activated forms of caspase-3 and caspase-7 [9] and caspase-8 [9-11]. In mice, intratumoral adenovirus infection with IRF-1 in vivo induces apoptosis [9]. IRF-1 is also induced by epidermal growth factor [12] to suppress cell proliferation. Together, these data suggest that a method for targeted induction of IRF-1 apoptotic pathways in cells that have lost p53 function may be an effective treatment for breast cancer.

Interestingly, mice lacking IRF-1 were found to have decreased apoptosis in involuting mammary glands [13]. However, rates of apoptosis in control and IRF-1 null mice were no longer significantly different by 72 hours. This suggests that IRF-1 actually suppresses apoptosis under special conditions, although the full range of these conditions is still unknown.

IRF-1 has been indicated as a mediator in the apoptotic pathway induced by several breast cancer drugs, such as Mullerian inhibiting substance [14] and Faslodex [15-17], an antiestrogen treatment for estrogen-receptor-positive post-menopausal breast cancer patients. Thus, the pharmacological induction of IRF-1 may be useful as a method for targeted therapy in antiestrogen resistant breast cancers.

Due to the correlation our lab found between loss of IRF-1 function and disease progression from DCIS to invasive ductal carcinoma, we sought to construct a model for the inhibition of IRF-1 activity in normal mammary epithelial cells, in order to mimic breast tumors lacking IRF-1. Upon generation of this model, we would then conduct functional assays to study the role of IRF-1 in breast cancer tumorigenesis.
Methods:

**Cell culture and materials**
The starter quantity of dnIRF-1 construct contained the entire wildtype IRF-1 cDNA except for deletions of bp 647–1173 and the transcription-activating domain. The 3’ and 5’ untranslated regions and the DNA binding domain were unmodified. The MCF-12A non-cancerous breast epithelial cells were maintained at 37°C in 95% O₂/ 5% CO₂.

**Plasmid amplification**

Transformation of DH5α with pcDNA3/ dnIRF1 or empty pcDNA3 vector. 0.2 µL of pcDNA3/dnIRF1 or 0.2 µL of empty control pcDNA3 and 50 µL competent DH5α were placed on ice for 30 minutes, heat shocked for 1 minute at 42°C, then placed on ice for 2 minutes. 300 µL SOC medium was added. The transformation mixture was incubated at 37°C with shaking (200 rpm) for 1 h.

**Colony selection.** 350 µL of transformation solution was plated on 10-cm plates containing 0.75% agar with 100 µg/mL ampicillin. Plates were incubated overnight at 37°C. 2 colonies of each strain were selected for growth in 250 mL of LB medium and 100 µg/mL ampicillin overnight at 37°C with shaking (200 rpm).

**Plasmid purification.** Plasmid DNA was extracted using the Qiagen Plasmid Midi kit (Qiagen, Valencia, CA) following manufacturer’s instructions, and resuspended in sterile deionized H₂O. DNA concentrations were measured using the Nanodrop Spectrophotometer 1000 (Nanogen, San Diego, CA).

**Transient Transfection with dnIRF-1**
Cells were transfected with the dnIRF-1 or empty control plasmids using FuGENE 6 Transfection Reagent (Roche Diagnostics, Branchburg, NJ). For dnIRF-1 transfection, 3:1, 3:2, 6:1, and 6:2 of µL FuGENE to µg pcDNA3/dnIRF-1 were diluted with serum-free medium to 1:100. For empty plasmid transfection, a 3:1 ratio of FuGENE to pcDNA3 was used. Diluted transfection complex was added to MCF-12A cells grown to 80% confluency in 6-well plates. Cells were incubated for 24 hours at 37°C.

**Western blot analysis**
Confirmation of expression of dnIRF-1 in MCF-12A cells was performed using immunoblotting. After cell lysis and 10% SDS-PAGE, total protein was transferred to nitrocellulose membrane. Non-specific binding was blocked with 5% nonfat milk/ TBST for 1 h with shaking. Rabbit anti-IRF-1 antibody (1:1000; Cell Signaling, Danvers, MA) was added overnight at 4°C. After three washes with TBST, the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:2000, Cell Signaling, Danvers, MA) for 1 h at room temperature. After three washes with TBST, bands were visualized on HyBlot CL film (Denville Scientific, South Plainfield, NJ) after treatment with enhanced chemiluminescence detection reagents (GE Healthcare, Piscataway, NJ).

Results:

The dnIRF-1 construct encodes a deletion mutant of the IRF-1 protein lacking the transcription-activating domain and protein-binding domain. Thus, dnIRF-1 protein inhibits IRF-1 activity in apoptosis and transcription factor pathways by acting as a competitive inhibitor for endogenous wildtype IRF-1 protein. Since the anti-IRF-1 antibody binds to the N-terminus of IRF-1, which remains unaltered in dnIRF-1, a greater amount of IRF-1 total protein should be visualized in dnIRF-1 transfected cells.
Consistent with this theory, cells transfected with dnIRF-1 displayed greater detection by the IRF-1 antibody (Figure 1), suggesting that transfection was successful.

<table>
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**Figure 1.** Western blots of whole cell lysate from MCF-12A cells transfected with dnIRF-1.

**Conclusions:**

Inhibition of IRF-1 using dnIRF-1 transfection has been successful in T47D and MCF-7 breast tumor cells [16]. However, whether or not this technique could be applied to normal cells was uncertain due to the differences in immune response mechanisms between cancerous and non-cancerous cells. Continuation of this project in the lab will show that transfection of dnIRF-1 can be applied to normal cells to mimic a model for loss of IRF-1 function in normal breast epithelial cells contributing to tumorigenesis. Current data indicate that transfection appears to have worked best using 1 µg of IRF-1 and 3 µl of FuGENE, or the least amount of transfection material tested. This may be due to the fact that, at high concentrations, DNA and FuGENE can be toxic to cells.

We plan to use this model for inhibition of dnIRF-1 to explore how the loss of IRF-1 function in normal breast tissue contributes to tumorigenesis. In addition, continuing work to identify the pathways involving IRF-1 will benefit the development of more targeted therapies for breast cancer patients. While breast cancer therapy has been revolutionized by the use of the antiestrogen Tamoxifen since the 1980s, many tumors develop resistance to the drug linked to a loss of estrogen receptor (ER) signaling [17]. However, IRF-1 appears to mediate the apoptotic effects of an effective new steroidal antiestrogen, Faslodex, in ER+ tumors [17]. Uncovering a novel breast cancer drug that induces the IRF-1 apoptosis pathway in the absence of ER signaling may allow treatment of tumors that have developed antiestrogen resistance.

Furthermore, work in this field points toward a possible future diagnostic tool in identifying breast cancer. Immunohistochemical analysis of IRF-1 expression as a biomarker may be a cost-effective method of determining the presence of IDC in breast tissue. Evaluation of IRF-1 expression may complement sentinel node biopsy as a viable tool for diagnosing invasive breast cancer.
References:


