NEW JERSEY MEDICAL SCHOOL
OF UMDNJ

Funded by NCI Cancer Education Program Grant R25 CA019536-29

CANCER SUMMER STUDENT
RESEARCH ABSTRACTS

2009

Harvey L. Ozer, MD, Principal Investigator and Director
Gwendolyn M. Mahon, PhD, Co-Director
Lorie-Anne Phillips, M.S., Coordinator
FORWARD

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 40 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 18 medical students and 3 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.

The administration of the summer program is 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 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, and an 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/
FACULTY EXECUTIVE ADVISORY COMMITTEE

The faculty executive advisory committee is responsible for carefully reviewing and selecting the projects submitted by faculty, as well as reviewing 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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NJMS-UH Cancer Center and Department of Orthopedics

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Department of Pathology & Lab. Medicine

George Studzinski, MD, PhD (Chair)  
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CANCER EDUCATION PROGRAM FACULTY

While the Cancer Center building currently houses 13 laboratories, the broader Cancer Center Community (CCC) is composed of 121 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 2009 participating faculty and their respective departments are listed below.

Betsy Barnes, PhD  
NJMS-UH Cancer Center and Department of Biochemistry and Molecular Biology

Beverly Barton, PhD  
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Charles Cathcart, MD  
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Lionel Zuckier, MD  
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Lizhao Wu, PhD  
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Michael A. Lea, PhD  
Department of Biochemistry and Molecular Biology
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<th>Name</th>
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<tr>
<td>Nasrin Gheseani, MD</td>
<td>Department of Biochemistry and Molecular Biology</td>
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<td>Ping-Hsin Chen, PhD</td>
<td>Department of Family Medicine</td>
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<td>Pranela Rameshwar, PhD</td>
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<td>Richard Howells, PhD</td>
<td>Department of Biochemistry and Molecular Biology</td>
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<td>Stanley Weiss, MD</td>
<td>Department of Preventative Medicine and Community Health</td>
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<td>Sylvia Christakos, PhD</td>
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<td>Teresa Wood, PhD</td>
<td>NJMS-UH Cancer Center and Department of Neurology and Neurosciences</td>
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<tr>
<td>Utz Herbig, PhD</td>
<td>NJMS-UH Cancer Center and Department of Microbiology and Molecular Genetics</td>
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1. ALEXANDER ACEVEDO (NJMS 2012)

QUANTITATIVE CT (QCT) CAN SURVEY BONE DENSITY DURING PET-CT STUDIES OF ONCOLOGY PATIENTS – THE FIRST 100 STUDIES

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

Objective:
Oncology patients are at increased risk for developing osteoporosis as a result of their treatment. Several traditional chemotherapies have been documented to accelerate bone mineral density (BMD) loss in cancer patients. CMF (cyclophosphamide, methotrexate, fluorouracil), a common combination of chemotherapy used to treat breast cancer, induces premature menopause, resulting in decreased estrogen production. Aromatase inhibitors, such as anastrozole, prevent the conversion of androgens to estrogens, reducing BMD. An increase in fracture risk and mortality can result from this bone loss. However, cancer patients often have undiagnosed osteoporosis, with only 3%-32% of these patients receiving bone density testing.

Several guidelines have been developed for the screening and management of osteoporosis in high-risk oncology patients. The U.S. Surgeon General's office recommends BMD screening for postmenopausal women >65 years of age and those taking medications that are known to increase fracture risk. The Danish Bone Society also recommends bi-annual BMD testing in breast cancer patients with risk factors.

Bone mineral density values are typically measured as mass per unit cross-sectional area (g/cm²), expressed in terms of the number of standard deviations from the mean BMD of normal young adults. World Health Organization defines osteoporosis as T-score more than 2.5 SD below the mean. Dual-energy X-ray absorption (DXA) is the most commonly used technique for measuring BMD; however, its accuracy has been questioned for certain subsets of spinal testing. DXA scanning may falsely elevate BMD in patients with spinal degeneration, lumbar fractures, scoliosis, IVC filters and aortic calcifications. This results from the inability of DXA to discriminate between trabecular bone, cortical bone, and other densities present in the image.

Quantitative computed tomography (QCT) is an accepted method for determining BMD, which uses standard CT scanners and associated software. QCT’s main advantage over DXA examination lies in its ability to selectively measure the metabolically active trabecular bone in 3 dimensions. The computer software allows the operator to choose a 3-dimensional volume of interest (Figure 1), which can be chosen to avoid artifacts that may affect accuracy. This volumetric approach also avoids the underestimation of BMD in children, where variation in bone size affects the accuracy of DXA measurements. Despite its advantages, QCT is not widely used in clinical practice due to high cost of its hardware and the higher radiation dose relative to DXA. A patient may receive a dose as low as 90 mrad from a CT scan and only 0.1 mrad during a DXA examination.

The goal of this work is to describe a novel protocol for the use of QCT to measure BMD of high-risk oncology patients during routine PET-CT studies. The first 100 studies are presented here as proof of principle.
Methods:

115 studies (105 patients) scheduled for routine oncologic PET-CT using the radiopharmaceutical F-18 fluorodeoxyglucose (FDG) were entered into the study. In 10 patients, 2 studies were performed with an interval of 179 ± 39 days between them. The study was I.R.B. approved, and informed consent was obtained from all subjects.

Inclusion Criteria: Adult patients with malignancy in whom the L1-L3 vertebral bodies were visualized in their entirety without evidence of metastatic involvement or hardware were included in this study. Of the 115 studies, 4 were excluded due to non-malignancy, 3 due to boney metastases (2 lytic, 1 sclerotic), 7 due to technical image acquisition errors and 1 due to spinal surgery. 100 studies remained for analysis.

Quantitative Computed Tomography: A commercial software package (QCT PRO, Mindways Software, Austin TX) was used to capture BMD data from PET-CT studies. A phantom containing 5 cylindrical density calibration standards was placed beneath the patient during the scan (Figure 2). A gel “bolus bag” was placed between the patient and the phantom to reduce interface artifacts. Standard PET and spiral CT images were acquired on a GE Discovery LS scanner. A second set of CT slices were reconstructed for QCT (kV 140, mA 90, 2.5 mm thickness), a procedure that manipulates the raw CT data but does not expose the patient to additional radiation.

Image Processing: Acquired CT images were evaluated for the extent and severity of beam hardening on an ordinal rating scale of 1 to 3 (slight, moderate and severe). Vertebral bodies were manually reoriented in space and the trabecular regions defined on single CT slices through L1, L2 and L3. The QCT PRO software calculated the BMD for each vertebral body in units of mg/cm³. The T-score (standard deviation from the mean of young healthy adults) and Z-score (SD from the mean of an age and gender matched population) were derived from the normative UCSF database.

Statistical Analysis: Absolute values of BMD, T scores and Z scores were correlated with patient history, including history of chemotherapy. In the cohort of 10 patients with repeat studies, differences in BMD and Z score were correlated with history of interval chemotherapy. Beam hardening of CT images was correlated with clinical and technical factors. For normally distributed data, t-tests were performed to determine differences between groups. Non-normal distributions were evaluated using the Mann-Whitney Rank Sum Test. To assess relationships between variables, the Spearman Rank Order Correlation was employed. A p value equal to or less than 0.05 was considered significant.
Summary:
In the 100 studies performed, BMD of L1-3 vertebral bodies averaged 123.8 ± 40.3 mg/cm³ (Avg ± SD) (Table 1). The Z score, reflecting standard deviation from age and gender matched controls, averaged -0.45 ± 3.04.

BMD values for all patients resembled those of gender and age matched controls (Figures 2a and 2b). Average BMD, Z-score, and demographic variables were compared between groups of studies in patients who received chemotherapy and patients who were chemo-naïve. Patients who have received prior chemotherapy were noted to have slightly lower absolute BMD (119.1 mg/cc ± 36.3) than chemo-naïve patients (133.6 mg/cc ± 46.9), though the difference was not statistically significant (p value = 0.09) (Table 2). Z score values of post-chemotherapy patients (-0.76 ± 3.51) was also found to be slightly lower than chemo-naïve patients (0.20 ± 1.49), though this difference was also not statistically significant (p value = 0.14).

Subjective scoring of beam hardening in the acquired images was correlated with patient weight and arm positioning (Figure 3). Beam hardening correlated positively with patient weight (ρ = 0.500, p value < 0.05). A negative correlation was noted between beam hardening and positioning of the arms over the patient’s head (ρ = -0.709, p value < 0.05).
In the 10 patients with repeat studies, the average change in BMD (-3.3 ± 9.7) and Z-score (-0.10 ± 0.29) were calculated (fig. 3). Patients treated with chemotherapy in the interval between the 2 scans had larger decreases in BMD (-9.1 mg/cc ± 6.3) than those who had received chemo prior to both studies but not during the interval (-0.8 mg/cc ± 10.2) (p value 0.24). The decrease in Z-score in patients who received interval chemotherapy (-0.26 ± 0.21) was also greater than that in patients who did not undergo interval chemotherapy (-0.03 ± 0.30) (p value 0.26). So too, a greater % change in BMD was noted in patients treated with interval chemo (-8.37% ± 6.92) than those without interval treatment (-0.22% ± 7.01) (p value 0.12).

Conclusion:
All patients who were entered successfully completed the study without complication. No degradation was noted in the clinical CT images such that it impacted clinical PET-CT analysis. QCT methodology was readily applied to PET-CT patients, allowing absolute evaluation of BMD in this at-risk population, without an increase in radiation dose. In our preliminary 100 studies, below average Z scores were noted compared to age and gender-matched controls (average Z score = -0.45). Lower Z scores were slightly more pronounced in patients that received prior chemotherapy (-0.76 vs. +0.20) though not statistically significant. In our 10 repeat studies, greater decreases in BMD and Z scores were noted in patients receiving interval chemo (p value N.S.). These results agree with previously published literature, highlighting the need to routinely monitor bone density in oncology patients.

Beam hardening scores, which if severe can limit absolute measurements of BMD, increased in extent and severity with increasing body weight (ρ = 0.500, p value < 0.05). Additionally, beam hardening scores were lower in patients who were positioned with their arms above their head (ρ = -0.709, p value < 0.05). These findings indicate that image quality is optimal when the patient is positioned with their arms are over their head and body weight is not overly elevated.

The lack of statistical significance could be due to the small sample size and lack of statistical power. Consequently, a larger study group would be needed to demonstrate the effect of chemotherapy on BMD. The amount of beam hardening present raises concern over the potential degradation of analyzed images. However, the QCT PRO software automatically checks the quality of each slice in the image, removing slices that fail a quality control test and derives the calibration data from the remaining slices.

Although the practicality of QCT in a clinical setting has been demonstrated, a potential challenge to implementation may include the ability to bill for the concurrent procedure.

References:
2. KATHRYN CLEFFI (JOHNS HOPKINS UNIVERSITY, 2010)

RADON AWARENESS IN ESSEX COUNTY

Mentor: Stanley H. Weiss, MD (Preventive Medicine and Community Health)

Background:
Radon is an inert element and radioactive gas that is released from the breakdown of uranium in rocks and soil. It is invisible, odorless, and tasteless and can be found in higher concentrations in mountains regions. It can seep into buildings and homes through cracks in the foundation, the water supply, and openings around sump pumps, pipes, and drains. Radon further decays into radioactive particles that can persist for years. It is these particles that can become trapped in the lungs and cause damage to the lung tissue.

Exposure to radon is the second leading cause of lung cancer, linked to about 20,000 of the 160,000 deaths due to lung cancer in the United States each year. The higher the radon concentration one is exposed to, and the longer the exposure, the greater the risk of developing lung cancer. The risk from radon is even greater when exposed to tobacco smoke. The combined effect of tobacco smoke and radon on lung cancer risk is not completely understood, but they appear to be synergistic – they greatly enhance the risk as compared to the risk from either factor alone.

On January 13, 2005, U.S. Surgeon General Dr. Richard H. Carmona issued a national health advisory on radon. He stated, "Indoor radon is the second-leading cause of lung cancer in the United States and breathing it over prolonged periods can present a significant health risk to families all over the country. It's important to know that this threat is completely preventable. Radon can be detected with a simple test and fixed through well-established venting techniques."

As radon exposure is appropriately considered a major environmental problem, it is useful for public health educators to understand the level of radon knowledge in their community to determine the need for appropriate educational programs. A survey conducted in New York State sought to gain more information about the radon awareness, testing, and remediation knowledge of its residents (Wang et al. 2000). It found that 82% of respondents had heard of radon, but only 21% were “knowledgably aware” of radon. To be knowledgably aware, respondents had to have heard of radon, know it was a radioactive gas, and know it was unhealthy and causes lung cancer. Similar data to guide New Jersey efforts is not available.

Objectives:
- Create a questionnaire that included some of the same issues addressed by Wang et al. to assess the radon knowledge of residents in Essex County, New Jersey in order to help future public health efforts regarding radon awareness.
- Utilize this opportunity to educate the public about radon.

Methods:
We constructed an anonymous survey with several demographic questions and seven questions related to radon to measure the general knowledge of residents in Essex County about key radon related issues. All of the questions were multiple choice with three possible answers, with the option to skip questions given in the information sheet describing the survey. The surveys were given before any education on radon to assess current knowledge before intervention. It was deemed not feasible to systematically give a linked or unlinked post-test. A
protocol was submitted to the UMDNJ Newark Institutional Review Board, along with an information sheet (in lieu of a signed consent). It was approved two weeks before the end of the summer project. Data collection is expected to continue. Thus, this represents a preliminary analysis.

The surveys will be handed out to Essex County homeowners at various locations such as health fairs, hospital clinics or departments, and local health departments in conjunction with the Essex County Cancer Coalition (ECCC). The ECCC is taking part in two radon awareness programs sponsored by the New Jersey Department of Environmental Protection (NJDEP) to distribute free radon testing kits to homeowners. Our questionnaire is offered at the time participants receive free kits and is completed on a voluntary basis.

The NJDEP produced a map (Figure 1) showing the radon potential in each county (divided by town) as low, moderate, or high. All of Essex County was rated as having moderate radon potential, except for Newark, Irvington, East Orange, and Verona which had low potential. The moderate areas were chosen as the primary target population for the radon survey in Essex County.

Figure 1. Map of radon potential in Essex County, divided by town

The data were collected, entered into, and analyzed using Epi Info, which is freely available software from the Centers for Disease Control and Prevention.

Results:
A total of 65 surveys were completed on two occasions in Newark (in Essex County). One survey was omitted from analysis because 6 of the 7 questions were left unanswered. Of the 64 remaining respondents, 26 (40.6%) were Essex County residents and 22 (34.4%) were homeowners.
Table 1. Demographic characteristics of survey respondents and Radon Score

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<th>Total Number</th>
<th>Percent of Respondents</th>
<th>Radon Score % (Mean ± SD)</th>
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<tr>
<td>Respondents</td>
<td>64</td>
<td>100%</td>
<td>68.5% (4.8 ± 1.4)</td>
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<td>County of Residence:</td>
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<tr>
<td>Essex County, NJ</td>
<td>26</td>
<td>40.6%</td>
<td>65.7% (4.6 ± 1.6)</td>
</tr>
<tr>
<td>Other</td>
<td>36</td>
<td>56.3%</td>
<td>72.9% (5.1 ± 1.4)</td>
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<td>Missing</td>
<td>2</td>
<td>3.1%</td>
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<td>Homeowner:</td>
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<tr>
<td>Yes</td>
<td>22</td>
<td>34.4%</td>
<td>68.5% (4.8 ± 1.1)</td>
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<tr>
<td>No</td>
<td>40</td>
<td>62.5%</td>
<td>70.0% (4.9 ± 1.6)</td>
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<td>Missing</td>
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<td>3.1%</td>
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<td>Location of Distribution:</td>
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<tr>
<td>Farmers’ market</td>
<td>51</td>
<td>79.7%</td>
<td>71.4% (5.0 ± 1.3)</td>
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<td>Pediatric Clinic</td>
<td>13</td>
<td>20.3%</td>
<td>61.4% (4.3 ± 1.8)</td>
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Figure 2. Percent of All Respondents with Radon Questionnaire Scores 0-7

92.2% of people who completed the survey knew that radon was a radioactive gas (Question 1) and 93.8% knew that radon had no smell (Question 2). When asked if they should test their home for radon even if their neighbor had an acceptable radon level, 87.5% correctly chose “yes” (Question 3). Yet only 67.2% knew that radon is strongly associated with lung cancer (Question 4), and just 43.8% said that radon is more dangerous for smokers than for non-smokers (Question 5). 78.1% of respondents said that radon levels are highest in the basement (Question 6), but only 21.9% knew that better home insulation can (actually) increase the radon level inside the home (Question 7).

The mean overall correct score was 68.5% (4.8 ± 1.4, range 0 to 7). Figure 2 shows the distribution of radon scores for all respondents, which follows a unimodal distribution. On the first occasion surveys were distributed by a university hospital pediatric clinic (n=13); the average correct was 61.4% (4.3 ± 1.8). Nearly half of respondents (46.2%) at this location resided in Essex County. On the second occasion, respondents at a farmers’ market (n=51)
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held at New Jersey Medical School answered an average correct of 71.4% (mean 5.0, SD=1). Here, fewer were from Essex County (39.2% residents, 56.8% non-residents).

Respondents residing outside Essex County responded correctly to 72.9% (5.1/7 questions), while Essex County residents averaged 65.7% (4.6/7). Similarly, a higher percentage (72.2%) of non-Essex County residents than Essex County residents (61.5%) knew that radon is associated with lung cancer. Respondents who were homeowners and those who were non-homeowners had similar radon scores, answering 68.5% (4.8/7) and 70.0% (4.9/7) questions correctly.

In comparison with the survey conducted by Wang et al., we considered respondents to our survey who knew that radon was a radioactive gas and that it is strongly associated with lung cancer as “knowledgably aware.” Overall, 64.1% answered both questions correctly. 69.4% of non-residents were knowledgably aware of radon, while only 57.7% of Essex County residents were knowledgably aware.

Conclusion:
The survey was constructed to assess knowledge about seven key facts about radon. We distributed fact sheets about radon after the survey. Of 64 respondents to the radon awareness survey distributed in Essex County, 92.2% knew that radon was a radioactive gas, but only 67.2% knew that radon was strongly associated with lung cancer. Furthermore, people who completed the survey answered an average of only 68.5% (4.8/7) questions correctly. Essex County residents had a lower mean score on the survey (65.7%, 4.6/7) than non-Essex County residents (72.9%, 5.1/7) and also a lower percentage of respondents that knew radon is associated with lung cancer (61.5% vs. 72.2%). Similarly, only 57.7% of Essex County respondents were considered “knowledgably aware” of radon. Although this is much higher than the 21% of respondents that were knowledgably aware of radon in the survey conducted by Wang et al. in New York State, there is still much room for improvement among the residents in Essex County. These results from the survey suggest that more radon education is indeed needed in Essex County. Public health programs should focus on increasing general radon knowledge and stressing the risk of developing lung cancer due to radon exposure in the home. Furthermore, education should promote radon testing for homeowners and provide information regarding mitigation to reduce the level of radon in homes throughout Essex County. These preliminary results strongly support the decision of the ECCC to implement radon education and radon testing programs in the region. Other New Jersey county cancer coalitions may wish to embark on programs similar to the ECCC’s radon awareness program, especially since some funding for radon test kits from the NJDEP may still be available for those counties.

Acknowledgments:
Thank you to Chris Tuohy, MPH for all of his guidance and help in constructing and distributing this survey, and to both him and Daniel Rosenblum, PhD for their encouragement and involving me in many related ECCC activities.

References:
3. JILL K DEUTSCH (STEVENS INSTITUTE OF TECHNOLOGY 2010, NJMS 2013)

INHIBITION OF GROWTH AND INDUCTION OF DIFFERENTIATION BY APIGENIN, BAICALEIN, AND QUERCETIN IN COLON CANCER CELLS

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

Objective:
There is considerable data to support the concept that polyphenolic molecules that occur in fruits and vegetables may have beneficial effects as cancer chemopreventive agents. We have shown previously that these polyphenolic molecules can induce enzyme markers of differentiation either alone, or in combination with histone deacetylase (HDAC) inhibitors. These data raise the question whether the induction of enzyme markers for differentiation are due to the anti- or pro-oxidant properties of polyphenols. Part of our objective for this study was to compare the action of three flavonol molecules, apigenin, baicalein, and quercetin on four cell lines derived from human colon cells (Caco-2, SW1116, HT-29, and NCM460).

Since production of hydrogen peroxide has been reported when some polyphenolic compounds are incubated with serum-containing medium, we examined this phenomenon with the three polyphenols, and with hydrogen peroxide alone. Further studies were performed with baicalein because that molecule had resulted in the greatest production of hydrogen peroxide. A second objective was to test the significance of the hydrogen peroxide formation, so we added catalase in combination with baicalein.

In addition, we wanted to determine the potential of the three polyphenols in combination with butyrate, an HDAC inhibitor, to increase the activity of alkaline phosphatase in Caco-2 cells. Synergistic effects were seen for the increase in alkaline phosphatase activity in Caco-2 cells for baicalein and quercetin, but not with apigenin.

Methods:
Cells. Caco-2, HT-29, and SW1116 human colon cancer cells were incubated at 37 °C in RPMI-1640 medium with 5% fetal calf serum and 25 mM N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) buffer.

Enzyme assays. Caco-2, HT-29, SW1116, and NCM460 cells were incubated with 10 ml RPMI-1640 medium with 25 mM HEPES and 5% fetal calf serum. The medium was changed after 24 hours and the cells were incubated for 72 hours before harvesting. The cells were washed with phosphate-buffered saline and extracted with 0.5% NP40, 0.25 M NaCl, 5 mM EDTA, and 50 mM Tris (pH 8.0). The protein concentration of the extract was determined using the BCA Protein Assay Reagent from Pierce, Rockford, IL, USA. Enzymes were assayed at 37 °C. Alkaline phosphatase was assayed in a volume of 0.24 ml using para-nitrophenyl phosphate (4.5 mM) as the substrate and 6.8 mM 2-amino-2-methyl-1-propanol (pH 10.3) as the buffer. Incubations were for 30 minutes and were stopped by the addition of 2.0 ml 0.05 N NaOH. Formation of product was monitored by the change in absorbance at 410 nm. Aminopeptidase was assayed in a volume of 0.5 ml using 2 mM L-alanyl-4-nitroanilide as the substrate and 0.1 M Tris (pH 8.0) as the buffer. Incubations were stopped by the addition of 2.0 ml 1M acetate (pH 4.2). Production of the product, 4-nitroaniline, was monitored at 405 nm.

Measurement of hydrogen peroxide. FOX2 reagent was prepared by dissolving xylenol orange and ammonium ferrous sulfate in 250 mM H₂SO₄ to final concentrations of 1 and 2.5 mM, respectively. One volume of this concentrated reagent was added to 9 volumes of HPLC-grade methanol containing 4.4 mM BHT to make the working reagent which comprised 250 μM ammonium ferrous sulfate, 100 μM xylenol orange, 25 mM H₂SO₄, and 4 mM BHT in 90% (v/v)
methanol. The working reagent was calibrated against solutions of H₂O₂ of known concentration. A sample of culture medium (180 μl) was mixed with 20 μl methanol and incubated at 37 °C for 30 minutes. The FOX2 reagent (1.8 ml) was added, followed by vortexing and 30 minute incubation. Solutions were then centrifuged at 4000 rpm for 10 minutes at 4 °C and the absorbance at 560 nm was read against a methanol blank.

Statistical evaluation. Statistical significance of the results was determined by a two-tailed Student's t-test or by Dunnett's test for multiple comparisons using the Instat program (GraphPad Software, San Diego, CA, USA). A probability of less than 5% was considered significant.

Summary:
The structures of apigenin, baicalein, and quercetin are shown in Fig. 1. While all three structures are similar, apigenin and baicalein are trihydroxyflavones, whereas quercetin is a pentahydroxyflavone. It is most likely these differences in structure with respect to hydroxyl group number and placement that cause these three molecules to act differently when assayed for their ability to act as either anti- or pro-oxidants, as well as their ability to induce differentiation in colon cancer cells.

![Fig 1 Structures of flavonol molecules a Apigenin b Baicalein c Quercetin](image)

The hydrogen peroxide assay using the FOX2 reagent yielded results that indicate baicalein has the most potent pro-oxidant effects of these three molecules. Fig. 2 details the production of hydrogen peroxide over time in serum-containing medium. Production of hydrogen peroxide was greater with baicalein than with quercetin or apigenin. Fig. 3 indicates that at 100 and at 250 μM concentrations of hydrogen peroxide there was approximately a 40% increase in alkaline phosphatase activity but no increase in aminopeptidase activity in Caco-2 cells. These concentrations were higher than for the polyphenols and the magnitude of the effect was less. Furthermore, the effects of baicalein and catalase, either as single agents, or in combination, were measured on Caco-2 human colon cancer cells (Fig. 4). In this experiment, the increase in alkaline phosphatase and the decrease in protein synthesis in Caco-2 cells resulting from incubation with 50 μM baicalein was not blocked by catalase, suggesting that the breakdown of hydrogen peroxide did not prevent the action of baicalein.
Fig 2 Production of hydrogen peroxide analysis from apigenin, baicalein, and quercetin at varying concentrations in RPMI 1640 medium with 5% fetal calf serum measured using the FOX2 assay.

Fig 3 Enzyme assays for Caco-2 human colon cancer cells treated with 100, 250, and 500 μM hydrogen peroxide: a Alkaline Phosphatase, b Aminopeptidase, c Protein.

Fig 4 Enzyme assays for Caco-2 human colon cancer cells treated with catalase (100 units/mL), 50 μM baicalein, or catalase (100 units/mL) + 50 μM baicalein: a Alkaline Phosphatase, b Aminopeptidase, c Protein.

Finally, three experiments were performed with apigenin, baicalein, or quercetin and butyrate either as single agents, or in combination. The three polyphenols caused increases in alkaline phosphatase activity as single agents, but no increase or decrease in aminopeptidase activity in Caco-2 cells. There was no induction of differentiation in the other cell lines by the three polyphenols. Based on their effects on induction of differentiation alone, baicalein generally produced the strongest alkaline phosphatase response. These results are summarized in Table 1 at 25 μM concentrations for Caco-2 and SW1116 cells. Furthermore, in combination with 0.5 mM butyrate, a histone deacetylase (HDAC) inhibitor that usually increases alkaline phosphatase activity in Caco-2 colon cancer cells by about 300%, baicalein showed the greatest synergistic effect with butyrate, yielding a 900% increase in alkaline phosphatase activity compared to the controls.
Table 1 Results of enzyme assays for 25 μM apigenin, baicalein, and quercetin on Caco-2 and SW1116 cells

<table>
<thead>
<tr>
<th></th>
<th>Alkaline Phosphatase</th>
<th>Aminopeptidase</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.172 ± 0.029</td>
<td>0.674 ± 0.147</td>
<td>0.338 ± 0.113</td>
</tr>
<tr>
<td>25 μM Apigenin</td>
<td>0.296 ± 0.031</td>
<td>0.319 ± 0.076</td>
<td>0.22 ± 0.028</td>
</tr>
<tr>
<td>25 μM Baicalein</td>
<td>0.555 ± 0.105</td>
<td>0.442 ± 0.076</td>
<td>0.322 ± 0.038</td>
</tr>
<tr>
<td>25 μM Quercetin</td>
<td>0.305 ± 0.062</td>
<td>0.363 ± 0.073</td>
<td>0.49 ± 0.113</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SW1116</th>
<th>Alkaline Phosphatase</th>
<th>Aminopeptidase</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.878 ± 0.208</td>
<td>0.407 ± 0.072</td>
<td>0.16 ± 0.014</td>
</tr>
<tr>
<td>25 μM Apigenin</td>
<td>0.521 ± 0.047</td>
<td>0.339 ± 0.075</td>
<td>0.154 ± 0.007</td>
</tr>
<tr>
<td>25 μM Baicalein</td>
<td>0.759 ± 0.03</td>
<td>0.324 ± 0.045</td>
<td>0.17 ± 0.0342</td>
</tr>
<tr>
<td>25 μM Quercetin</td>
<td>0.735 ± 0.037</td>
<td>0.265 ± 0.051</td>
<td>0.187 ± 0.005</td>
</tr>
</tbody>
</table>

Table 2 Results for enzyme assays for apigenin, baicalein, or quercetin and butyrate either as single agents, or in combination

<table>
<thead>
<tr>
<th></th>
<th>Alkaline Phosphatase</th>
<th>Aminopeptidase</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.113 ± 0.006</td>
<td>0.554 ± 0.02</td>
<td>0.477 ± 0.043</td>
</tr>
<tr>
<td>0.5 mM Butyrate</td>
<td>0.47 ± 0.029</td>
<td>0.597 ± 0.004</td>
<td>0.527 ± 0.012</td>
</tr>
<tr>
<td>25 μM Apigenin</td>
<td>0.146 ± 0.013</td>
<td>0.441 ± 0.031</td>
<td>0.412 ± 0.022</td>
</tr>
<tr>
<td>0.5 mM Butyrate + 25 μM Apigenin</td>
<td>0.253 ± 0.018</td>
<td>0.437 ± 0.034</td>
<td>0.37 ± 0.036</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.214 ± 0.035</td>
<td>0.541 ± 0.039</td>
<td>0.507 ± 0.04</td>
</tr>
<tr>
<td>0.5 mM Butyrate</td>
<td>0.795 ± 0.044</td>
<td>0.613 ± 0.026</td>
<td>0.599 ± 0.027</td>
</tr>
<tr>
<td>25 μM Baicalein</td>
<td>0.476 ± 0.067</td>
<td>0.426 ± 0.062</td>
<td>0.435 ± 0.006</td>
</tr>
<tr>
<td>0.5 mM Butyrate + 25 μM Baicalein</td>
<td>1.929 ± 0.049</td>
<td>0.39 ± 0.045</td>
<td>0.428 ± 0.031</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>0.124 ± 0.013</td>
<td>0.34 ± 0.039</td>
<td>0.575 ± 0.016</td>
</tr>
<tr>
<td>0.5 mM Butyrate</td>
<td>0.95 ± 0.072</td>
<td>0.27 ± 0.018</td>
<td>0.508 ± 0.014</td>
</tr>
<tr>
<td>25 μM Quercetin</td>
<td>0.215 ± 0.037</td>
<td>0.323 ± 0.062</td>
<td>0.403 ± 0.009</td>
</tr>
<tr>
<td>0.5 mM Butyrate + 25 μM Quercetin</td>
<td>1.888 ± 0.236</td>
<td>0.288 ± 0.047</td>
<td>0.43 ± 0.056</td>
</tr>
</tbody>
</table>

Conclusion:
In Caco-2 cells, the effectiveness of the phenolic molecules as cancer chemopreventive agents was accompanied by increased activity of the differentiation marker alkaline phosphatase, but not of aminopeptidase. The Caco-2 human colon cancer cells were notably more sensitive to growth inhibitory and differentiating effects of apigenin, baicalein, and quercetin than the other cell lines that were examined (HT-29, SW1116, and NCM460).

Among apigenin, baicalein, and quercetin, baicalein yielded the most profound hydrogen peroxide production in serum-containing medium over time. Hydrogen peroxide only induces cell differentiation at concentrations significantly higher than those produced by apigenin, baicalein, and quercetin in vitro. Addition of catalase did not prevent the increase in alkaline phosphatase or the decrease in protein in Caco-2 cells after incubation with 50 μM baicalein. Apigenin, baicalein, and quercetin can have pro-oxidant effects, but our data suggested that this action was not the sole determinant of growth inhibitory or differentiating effects on colon cancer cells.

Baicalein, in combination with butyrate, has the greatest synergistic effect on colon cancer cell differentiation among the three polyphenols studied. Quercetin had a very similar effect, while apigenin decreased the induction of alkaline phosphatase resulting from incubation with the HDAC inhibitor, butyrate.
4. JING JING FENG (NJMS 2012)

EFFECTS OF 1,25-DIHYDROXYVITAMIN D$_3$ (1,25(OH)$_2$D$_3$) AND CDX2 ON LI-CADHERIN TRANSCRIPTION

Mentor: Sylvia Christakos, PhD (Biochemistry & Molecular Biology)

Objective:
Cdx2 and LI-cadherin expression have been linked to intestinal metaplasia. In addition, expression of Cdx2 and LI-cadherin are correlated in colorectal cancer. 1,25(OH)$_2$D$_3$ has been reported to inhibit the growth of a number of tumor cells including colon cancer cells. Epidemiological studies have documented an inverse correlation of 25(OH)D levels and vitamin D intake with cancer incidence and a recent clinical trial using 1,100 IU daily treatment with vitamin D showed a 77% reduction in cancers after the first year, including both breast and colon cancers (Lappe, JM et al. Am J. Nutrition 85: 1586-1591, 2007). However, the molecular mechanisms remain to be fully delineated. The goal of the present study was to determine the effects of 1,25(OH)$_2$D$_3$/vitamin D receptor (VDR) and Cdx2 on LI-Cadherin expression.

Methods:
Cell Culture
Cos-7 African Green Monkey kidney fibroblast cells and Caco-2 colon adenocarcinoma cells were cultured in DMEM supplemented with 10% FBS and 1% PSN. Cells were grown in 100x20mm polystyrene tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ) at 37°C in a humidified atmosphere of 95% air, 5% CO$_2$.

Cell transfections and luciferase assay
Cells were grown to 70% confluency and then were transfected with LI-cadherin promoter construct (-1000/+48) linked to the luciferase reporter gene, Cdx2 expression vector, and Vitamin D receptor expression vector using Lipofectamine$^\text{TM}$ 2000 (Invitrogen, Carlsbad, Ca). For studies using the VDR expression vector, cells were treated with 1,25(OH)$_2$D$_3$ (10$^{-7}$M and 10$^{-8}$M).

Summary:
In this study we found that Cdx2 induces the transcription of LI-cadherin. 1,25(OH)$_2$D$_3$ alone had no effect on basal promoter activity. However, 1,25(OH)$_2$D$_3$/VDR suppressed Cdx2 activation of LI-cadherin promoter activity. 1,25(OH)$_2$D$_3$ also decreased LI-cadherin and increased Claudin-2 protein expression in Caco-2 cells. We also looked at the effect of methyltransferase G9a on transcription of LI-cadherin. We observed G9a was able to enhance Cdx2 mediated induction of LI-cadherin promoter activity. 1,25(OH)$_2$D$_3$/VDR may suppress Cdx2 mediated activation by sequestration of Cdx2 by VDR or by binding of VDR to the Cdx2 site in the LI-cadherin promoter.

Conclusion:
These data suggest that 1,25(OH)$_2$D$_3$/VDR inhibits the Cdx2 expression of LI-cadherin at the transcriptional level. Further studies will be needed to determine the exact mechanisms involved. These findings represent the first studies related to modification by 1,25(OH)$_2$D$_3$ of Cdx2 induced LI-cadherin transcription. In light of the evidence linking LI-cadherin and Cdx2 to tumorigenesis, a further understanding of mechanisms involved in 1,25(OH)$_2$D$_3$ suppression will be of interest in the future.
Figure 1. Dose dependent increase of LI-cadherin transcription with Cdx2

![Graph showing dose dependent increase of LI-cadherin transcription with Cdx2.](image)

Figure 2. 1,25(OH)$_2$D$_3$ suppresses Cdx2 induced LI-Cadherin transcription

![Graph showing effect of 1,25(OH)$_2$D$_3$ on Cdx2 induced LI-Cadherin transcription.](image)

Figure 3. VDR dependent 1,25(OH)$_2$D$_3$ inhibition of Cdx2 induced LI-cadherin transcription

![Graph showing VDR dependent inhibition of Cdx2 induced LI-cadherin transcription.](image)
Figure 4. $1,25(\text{OH})_2\text{D}_3$ inhibits LI-cadherin protein expression in Caco-2 cells

Caco-2 Cells
Western Blot

<table>
<thead>
<tr>
<th></th>
<th>NT</th>
<th>$10^{-7}\text{M}$</th>
<th>$10^{-8}\text{M}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,25(\text{OH})_2\text{D}_3</td>
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</tr>
</tbody>
</table>

Figure 5. G9a methyltransferase enhances Cdx2 activation of LI-cadherin transcription

- Vehicle
- Cdx2
- Cdx2 + VDR + $1,25(\text{OH})_2\text{D}_3 (10^{-8}\text{M})$
Figure 6. Does $1,25(\text{OH})_2\text{D}_3$ affect the expression of other transjunctional or transmembrane proteins in the intestine?
5. YAN GAO (NJMS 2012)

TELOMERE DYSFUNCTION INDUCED CELLULAR SENESCENCE IN HUMAN COLON ADENOMAS

Mentor: Utz Herbig, PhD, (Microbiology & Molecular Genetics)

Objective:

Colon cancer is cancer of the large intestine, with 112,000 people diagnosed annually in the United States. Most cases of colon cancer begin as small, noncancerous (benign) clumps of cells called adenomatous polyps. Over time some of these polyps become carcinomas based on a staging system depending on the extent of local invasion, the degree of lymph node involvement and whether there is distant metastasis. While a critical tumor suppressing mechanism that prevents malignant colon cancer progression has not been identified, a number of laboratories have observed that certain benign human tumors and cancer precursor lesions are comprised of cells that have undergone cellular senescence. We hypothesize that cellular senescence, an irreversible growth arrest that occurs in response to stresses and signaling imbalances, is a critical tumor suppressing mechanism that prevents malignant colon cancer progression.

While a number of stresses can trigger cellular senescence, we have focused on dysfunctional telomeres. Because of continuous cellular proliferation or other stresses, telomeres can become critically shortened, leading to the activation of a DNA damage response that ultimately results in cellular senescence. DNA damage foci are seen with these shortened telomeres, showing that they have undergone Telomere Dysfunction Induced Senescence (TDIS). By using immunofluorescence microscopy, we observe that significantly more adenomatous polyp cells display dysfunctional telomeres and other markers of cellular senescence such as elevated levels of macroH2A. Colon carcinoma cells show a significant reduction in senescence markers and dysfunctional telomeres, suggesting that these cells have lost the ability to undergo TDIS. We suggest that TDIS is a critical tumor suppressing mechanism that prevents the growth of colon adenomatous polyp cells into carcinoma cells.

Methods:

We obtained adenoma and carcinoma tissue from the Department of Pathology at UMDNJ University Hospital and imaged them with a Zeiss Axiovert 200 epi-flourescence microscope equipped with ApoTome. 10 cases of adenoma and 12 cases of carcinoma colon cancer tissue were obtained as 4 μm tissue sections. Tissue samples were deparaffinized, hydrated through a graded ethanol series, and subjected to heat-induced antigen retrieval for forty five minutes in sodium citrate buffer. The slides were then stained using a telomere immune-fluorescence in situ hybridization (FISH) assay. Antibodies used were anti-53BP1 and anti-MacroH2A. Telomeres were detected using a Cy3-fluorescently labeled peptide nucleic acid (PNA) complementary to telomeric repeats.
Summary:

First we stained the tissues with antibodies against 53BP1 and a PNA complementary to telomeric DNA sequences. 53BP1 is a binding protein that participates in the cellular response to double stranded DNA breaks. Using a magnified view, I counted 53BP1 foci within each nuclei for 5 adenoma and 11 carcinoma tissue samples. Even though I started with 10 adenoma slides, 5 slides possessed too much background fluorescence and therefore could not be used. We can see that in the colon adenoma (below chart), there is significantly more 53BP1 foci than in the colon carcinoma. The bar graph shows the quantification of 53BP1 positive colon cells in adenoma and carcinoma tissue. An average of 478 and 312 nuclei per tumor were analyzed for adenoma and carcinoma tissue, respectively. The images were taken as z-stacks with 0.3 mm planes at 100x magnification and merged into a single layer.

Next, we quantified TIF+ and TIF- cells within the tissue. We establish TIF+ cells as a cell that has at least 50% co-localization between PNA foci and 53BP1 foci. Co-localization is defined as one 53BP1 focus overlapping with a PNA signal. Not only did we see more 53BP1 foci in adenoma cells, but we also saw significantly more co-localization between 52BP1 foci and PNA signals. The chart below shows that in adenoma cells, there is significant double stranded DNA damage to the telomeres. The bar graph shows the quantification of TIF+ cells in adenoma and carcinoma cells. An average of 56 cell nuclei per tumor were analyzed by taking z-stacks with 0.3 mm planes at 100x magnification. In previous experiments, it has been shown that there is significant co-localization between MacroH2A and 53BP1 as well. When we put this information
together, we can see that the evidence supports our theory of telomere dysfunction induced cellular senescence.

**Conclusion:**

Colon cancer takes the lives of 655,000 people each year worldwide. Although our treatments and preventative care have improved significantly, we still do not fully understand why some individuals merely develop benign polyps while others develop malignant colon cancer. However, this research has shown that much more cells in colon adenomas are senescent while carcinoma cells continue to proliferate. Within senescent adenoma cells, there was a significant amount of DNA damage co-localizing with telomeres, suggesting that telomere dysfunction induced cellular senescence (TDIS) is the mechanism for triggering senescence and ultimately preventing progression into malignancy.
6. LYDIA HAN (NJMS 2012)

OSSEOUS METASTASIS DETECTED ON WHOLE BODY PET/CT SCAN PERFORMED IN PATIENTS WITH HEAD AND NECK CANCER

Mentor: Nasrin Ghesani, MD, (Nuclear Medicine)

Objective:

Head and neck cancers comprise 3-5% of all cancers in the United States. One of the most common sites of distant metastasis in these cancers is the skeleton. Hematogenic dissemination is the most common mechanism of bone metastasis utilized by head and neck tumor cells. Bone is a favorable environment for growth of tumor cells, because bone matrix contains many factors (i.e. matrix metalloproteinases, TGF-β) that stimulate the proliferation of cancer cells.

The incidence of clinically detected distant metastasis in head and neck cancers ranges from 4% to 25%. Head and neck cancers are usually locally invasive, and they tend to metastasize to regional lymph nodes rather than spread hematogenously. Distant metastases usually occur during the late course of disease, with lung being the most common site of distant metastasis, followed by bone and liver. It is important to detect distant metastases of head and neck cancers as early as possible in order to stage and treat the disease properly.

Bone scintigraphy (BS) using technetium-99m-labeled diphosphonates is the most frequently used method of detecting bone metastases, because of its wide availability and low cost. A disadvantage of using BS is that it may identify many false-positive lesions, which could result from degenerative disease, fractures, and inflammatory changes. Furthermore, early bone metastases are frequently missed by BS, especially when most of the tumor cells are confined to the bone marrow. Therefore, BS may not be able to differentiate between malignant and benign lesions.

Positron emission tomography (PET) using $^{18}$F-fluorodeoxyglucose (FDG) has been used more frequently due to this technique’s ability to detect lesions with high glycolytic rates. Although FDG-PET is used widely in oncological practice, its ability to identify bone metastases of head and neck cancers is not clear. Compared to the conventional bone scan, FDG-PET has higher resolution and provides additional information regarding soft tissue disease, monitoring of treatment, and identifying response in skeletal metastases. Therefore, in recent years, FDG-PET has been commonly used for detection of recurrent neoplasm and/or metastasis, staging of cancer, and evaluation of therapeutic effect. The use of FDG-PET is based on the increased glucose metabolism of malignant cells. FDG, which is a glucose analog, is absorbed, phosphorylated, and trapped in the cytosol of these cells.

The purpose of this study was to evaluate the effectiveness with which FDG-PET detects osseous metastasis in head and neck cancer patients.

Methods:

A retrospective review of patient charts and medical records was conducted in order to identify all head and neck cancer patients who underwent $^{18}$F FDG PET/CT scan for work-up of malignancy from April 2003 to present. This group was further narrowed by identifying patients in whom PET/CT scans reported osseous metastasis. Laboratory data, including surgical pathology, reports of other radiographic studies, such as MRI and bone scan, and were used to confirm osseous metastasis.

This study was approved by the Institutional Review Board.
Summary:
970 patients with head and neck cancers from April 1, 2003 to July 27, 2009 were identified from the institutional cancer registry. Out of these 970 patients, 786 underwent $^{18}$F-FDG PET/CT scans. 49 (age range 29 - 93, 45 male, 4 female) out of 786 patients (6%) were reported to have osseous metastasis detected by PET scan. Bone metastasis was identified in 11 of these patients on initial staging PET/CT scan, while the remaining 38 patients in their surveillance scan. 22 patients were found to have a single osseous metastatic lesion while 27 had multiple lesions. 10 patients had metastatic disease limited to bones while 39 patients had metastasis to multiple other sites including lungs, liver, lymph nodes and adrenal glands.

Out of the 49 patients who showed bone metastasis, 6 of these patients were confirmed by biopsy, 2 by bone scan, 6 by follow-up CT, 1 by follow-up MRI, 10 by follow-up PET, and the remaining 24 by imaging features of concurrent CT scan. The primary cancers in these patients included 1 epiglottic, 7 laryngeal, 5 nasopharyngeal, 14 oral cavity, 3 oropharyngeal, 2 parotid gland, 2 sinonasal, 12 supraglottic, and 3 tonsillar.

Conclusion:
FDG-PET is being used extensively in oncological practice for the staging and management of cancers, as well as for therapeutic monitoring. There is growing evidence that FDG-PET is superior to conventional imaging work-ups in the evaluation of patients with head and neck malignancies, particularly because FDG-PET allows for whole-body surveillance which can detect distant metastasis. According to the data, it appears as though FDG-PET is an effective way of determining osseous metastasis, as well as other sites of metastasis, especially when it is used as a screening tool of distant metastasis in initial work or for surveillance in patients with head and neck cancer. Other modalities such as CT scan, MRI, or bone scan may be used for confirmation of the findings.

Clinical studies using a similar methodology will be conducted to examine the effectiveness of FDG-PET in determining:
1) Osseous metastasis in patients with other types of cancers, such as lung and breast
2) Metastasis to other parts of the body, such as liver and lung

Clinical follow-up studies will also be done to examine whether or not patients who had negative PET/CT scans, later developed bone metastases.
7. ERIC HOLDER (NJMS 2012)

IMPROVING BREAST AND PROSTATE CANCER SCREENING RATES IN UNIVERSITY HEIGHTS SUPER NEIGHBORHOOD

Mentor: Ping-Hsin Chen, PhD (Family Medicine)

Objective:

The objective of this project is to assess community members overall awareness of breast and prostate cancer screening practices. It is the goal of the research team through this Community Based Participatory Research (CBPR) to close the gap in breast and prostate cancer health disparities that are particularly prevalent within the University Heights Super Neighborhood. Nationally, Breast and Prostate cancers are the second leading cause of cancer deaths. As compared to the overall United States Population, the incidence, prevalence, and mortality rates from these cancers are higher for New Jersey and even more so within Essex County. Due to lack of education and awareness of the importance of cancer screening, screening rates are lower in minorities than in Whites. As such, minorities compared to Whites are more likely to be diagnosed in later stages and to die of breast and prostate cancer. We hypothesize that (1) breast and prostate cancer screening rates in underserved minorities were lower than the U.S. adult population and (2) various barriers lead to health disparities in breast and prostate cancer between minorities and Whites.

Methods:

This study utilizes the CBPR method in order to promote active participation of members of Newark’s Super Neighborhood Council as well as residents in all stages of the study. Specifically, the collegiate CBPR method was used, which focuses on community members and researchers working together as colleagues to format the research within the community. A community member paired with a UMDNJ medical student surveyed a total of 127 residents about their cancer screening beliefs and behaviors. The target community is the University Heights Super Neighborhood. In 2006, the City of Newark was divided into 21 Super Neighborhoods amongst Newark’s 5 wards. A super neighborhood by definition is a geographically designated area within the City of Newark, where residents and community organizations work together to identify, plan and set priorities to address the needs and concerns of their community. A needs assessment survey was formatted to determine the baseline knowledge, attitudes and screening behaviors of residents within the University Heights super neighborhood. The assessment was also formatted to determine the barriers to the promotion of breast and prostate cancer screening in the Super Neighborhood. The University Heights Super Neighborhood according to Census data has 3,700 households of which specific methods were used to collect data. These methods not only included recruiting participants directly from their households but also at community health fairs, soup kitchens and finally at the Family Medicine Practice at UMDNJ University Hospital, which is located within the University Heights Super Neighborhood.

The inclusion and likewise exclusion criteria were customized separately for men and women. Women aged 40 to 74 years of age who live in the Super Neighborhood were
considered eligible for the needs assessment. Women below 40 were excluded because breast cancer screening is not a recommended procedure. Women older than 74 were excluded because there is no universal recommended physician agreement on screening tests for this age group and furthermore there are no mammography trials that enroll women older than 74. Based upon the American Cancer Society recommendation for prostate cancer screening for African Americans, men aged 45 to 74 years were included.

Summary:

The results of the data collection provide insight into the overall health, attitudes and screening behaviors of participants. While 65% of male respondents stated that their health was very good or excellent, 44% of the male respondents BMI indicated that were either overweight or obese. Likewise, 63% of female respondents stated that their health was very good or excellent while 71.1% of female respondents BMI indicated that they were either overweight or obese.

In our efforts to obtain a whole view understanding of residents within this community to formulate a community specific intervention, educational achievement was assessed. Only 14% of female respondents and 20.6% of male respondents completed 4 years of college or more. In conjunction, income level was also studied. Half (50%) of male respondents and 41.8% of female respondents made less than $10,000 dollars a year. Demographically speaking, 91.1% of male respondents were African American and the remaining 8.9% were Hispanic. Likewise, 93.8% of female respondents were African American and the remaining 6.2% were Hispanic.

In the figures below, Figures 1 and 2 illustrate the percentage of men and women who have or have not been screened for prostate or breast cancer respectively. A total of 54.5% of men had not been screened for prostate cancer. Likewise 24.6% of women had not been screened or were unsure if they had been screened for breast cancer. Figure 3 illustrates the top 3 barriers to breast cancer screening indicated by female respondents out of a possible 14 barrier choices. The three major barriers specified by females are; lack of health insurance, lack of symptoms and lack of recommendation by their physicians to be screened. Figure 4 indicates top three barriers to prostate screening indicated by male respondents out of a possible 9 barrier choices. The three major barriers specified by males that have prevented timely and appropriate screening are; lack of awareness that they need the checkup, embarrassment associated with the procedure, and fear of the examination.
Q2B.1: A Prostate Specific Antigen test, also called a PSA test, is a blood test used to check men for prostate cancer. Have you ever had a PSA Test?

Figure 1.

Q2.1 A mammogram is an x-ray of each breast to look for breast cancer. Have you ever had a mammogram?

Figure 2.

Top 3 Barriers to Breast Cancer Screening for Women Surveyed

Figure 3.

Top 3 Barriers to Prostate Screening for Men Surveyed

Figure 4.
Conclusion:

As it stands the National Breast Cancer Screening rate is 72.1% according to the National Health Review Survey on cancer screening rates. In our study we found that the screening rates for female participants was 75.4%. For males the National Screening rate stands at approximately 61%, while the screening rates for male participants within University Heights Super Neighborhood was 45.5%. Women within the community have shown to have a slightly higher screening rate than the national average, while males have a significantly lower screening rate than the national average. It must be noted that although the screening rates are comparatively equal between the national average and the average obtained for women within this study, it has been determined in previous studies that the higher prevalence of breast cancer mortalities in African American women is due to a more aggressive and earlier onset form of the cancer. Thus when African American women do get breast cancer, it tends to be more advanced when diagnosed, have a higher likelihood of reoccurring and in the end lead to less favorable long term outcomes. This is very significant for the University Heights Super Neighborhood where 93.8% of female survey respondents were African American.

Our Second hypothesis was that various barriers lead to health disparities between minorities and Caucasians. It has been determined from the data, that there were overwhelming three major barriers affecting males and three major barriers affecting females from receiving cancer screening. With the screening rate being significantly lower for male respondents than the National average, these barriers may be much more of a hindrance for the males.

It is important to note a very contradictory and disturbing finding. As previously noted, the majority of female respondents noted that they believed they were in very good or excellent health, however the overwhelming majority of females (71.1%) were overweight or obese. Likewise most of the males believed that they were in very good or excellent health, while 44% were overweight or obese. With continued studies it may be elicited from this data that residents within this community may have a poor understanding of good health, which may translate to the untimely cancer screening rates and their belief or lack thereof for the need to be screened. This lack of understanding of proper health might also result from low educational achievement levels within the community or from lack of income needed to support proper health maintenance. With further studies, these parallels will be further inspected.

Presently African American women are more than 36% more likely to die of breast Cancer than Whites and African American males are 2.4 times more likely to die from prostate cancer. For breast and prostate cancer, the incidence and mortality rates for N.J are higher than in the U.S and Essex County ranks second highest for breast cancer deaths and first for prostate cancer within N.J. With the continuation of this study we hope to formulate a community specific approach to raise awareness, educate and enhance timely screening and reduce breast and prostate cancer related mortalities.

Literature Cited:
8. ZACHARY HOMA (NJMS 2012)

DETERMINATION OF INSULIN RECEPTOR AND IGF1R EXPRESSION IN BREAST CANCER CELLS

Mentors: Teresa L. Wood, PhD, Deborah A. Lazzarino, PhD (Neurology and Neurosciences)

Objective:
Insulin-like growth factor (IGF) and insulin signaling are highly implicated in the pathogenesis of many breast tumors. The two IGF ligands, IGF-I and IGF-II, bind to the IGF receptor (IGF1R) with high affinity whereas insulin at physiological concentrations binds to the insulin receptor (IR) with high affinity. Two isoforms of the IR are expressed based on alternative splicing of exon 11 (12 amino acids). IR-B is the more metabolic isoform and is associated with classic insulin signaling. IR-A, however, has a mitogenic function, and is stimulated by both insulin and IGF-II. Of particular interest is that IR-A is found at higher levels in some cancers including breast cancers, suggesting that it may be an important mediator of IGF-II and insulin signaling in tumors (1). In addition, insulin levels in conditions such as type 2 diabetes may be sufficient to cross-activate the IGF-IR. Thus, there is considerable interest in defining the expression levels of IGF1R and IR isoforms in tumors. RNA levels of IGF1R or of relative levels of IR-A vs. IR-B have previously been quantified, but a quantitative assay to determine relative levels of all three receptors has not been available. Our lab recently reported a highly specific RT-PCR assay to quantify the relative amounts of IR-A, IR-B and IGF1R on the same scale using mRNA isolated from rodent cells (2). However, the three receptors have never before been measured together on the same scale in human cell lines or specimens. The ability to do so would allow for associating different breast cancers with specific “molecular signatures” of IGF/insulin signaling receptors.

The purpose of this project was to develop a highly specific RT-PCR assay to quantify human IR-A, IR-B and IGF1R on the same scale using specially designed primers designed against the human receptor sequences and that address sequence homology between all three receptors. Data are presented showing validation of the primers for sensitivity, specificity and efficiency as well as initial validation of the assay for breast cancer cell lines.

Methods:
Primer Design
Due to the high degree of homology between the two IR isoforms as well as approximately 50% homology between IGF1R and IR mRNAs, primer design focused on those regions with low sequence homology. For the IR isoforms, sense primers were targeted to span the isoform specific exon/exon junction while the antisense-primer was common to both isoforms. For IGF1R, primers were designed to target regions with low homology to IR.

Sensitivity Assays
Primer pairs were tested for sensitivity with regard to their specified target. 10-fold serial dilutions, ranging in concentration from $10^7$ to $10^1$ copies of plasmid standards were used as the template for each primer pair. Each reaction contained final concentrations of 1X QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.25 µM of both sense and antisense primers and a particular concentration of plasmid standard, as mentioned above, and were performed in triplicate. Data from these experiments were used to generate amplification plots, which show the fluorescence from the SYBR Green plotted against the cycle number of the PCR protocol, as well as standard curves. Standard curves plot Ct, which is the cycle number at which the amplification passes an arbitrary threshold against the known amount of plasmid standard.

Competition Assays
Primer specificity was examined by testing their ability to amplify the intended target while in the presence of a homologous competitor. 10-fold serial dilutions of plasmid standards were repeated in the presence of $10^7$ of competing plasmid standards. Data from these experiments were used to generate amplification plots and standard curves.

**Efficiency Validation Experiments**

In order to accurately apply the $\Delta \Delta Ct$ method of relative quantification, it is necessary to ensure that the efficiency, which is a measure of how much PCR product increases during each cycle of all primer pairs, is equivalent for each primer pair including the primer pair for the endogenous control (3). Unlike previously described assays which use plasmid standards, in this assay we used cDNA from MDA-MB231, an aggressive epithelial breast cancer cell line. There are at least two methods used to calculate efficiency. One involved the following equation: $E = \left[10^{(-1/slope)}\right] - 1$, where slope is taken from the standard curve. $E = 1.0$, or 100% efficiency when the slope is approximately -3.33. Another method to verify efficiency is to plot $\Delta Ct$ (average Ct of IR or IGF1R primer pair – average Ct of $\beta - Actin$) against the log cDNA dilution. A linear regression curve is fitted to the data and the absolute value of the slope must be less than 0.1 for the efficiencies to be considered equivalent.

**Summary:**

**Sensitivity Assays**

Primer pairs for IR-A, IR-B and IGF1R were sensitive over 7 orders of magnitude, ranging from $10^7$ to $10^1$ copies of plasmid standards. The slope of the standard curve for IR-A is -3.44 (Figure 1) whereas the slopes for IR-B and IGF1R are -3.53 and -3.52 respectively (data not shown).

**Competition Assays**

IR-A primers were specific to their target in the presence of $10^5$ copies of IR-B plasmid standards, a homologous competitor. The slope of the standard curve for this assay over the range of $10^7$ to $10^1$ is -2.88, which, when calculated into efficiency equals 122% (Figure 2). Visual inspection of the curve shows that at lower concentrations of the plasmid standard, the
data shifts to lower values of Ct. However, the slope of the standard curve, when omitting the data point at $10^1$ is -3.32, an ideal slope.

![Figure 2](image)

**Figure 2**

Standard curves of the IR-A competition assay, plotting Ct vs. log concentration of the plasmid standards. Slope of the blue line is -2.88 and slope of the red line is -3.32.

Preliminary data using the competition assay show that as the IR-A sense primer concentration is titrated to lower concentrations, the slope of the standard curve shifts into a more ideal range. When the primer concentration is lowered from 0.25 µM to 0.05 µM, the slope becomes -3.04 and when reduced further to 0.025, the slope becomes -3.56 (data not shown). Data from competition assays for IR-B and IGF1R are unavailable at the present time.

**Efficiency Validation Experiments**

Primers for IR-A, IR-B, IGF1R and β-Actin were tested using 2-fold serial dilutions ranging from 200 ng to 12.5 ng of MDA-MB231 cDNA. The primer pairs of IR-A, IR-B and IGF1R were each tested for efficiency in relation to the endogenous control, β-Actin. When the IGF-IR sense primer concentration is reduced to 0.05 µM, IGF-IR and β-Actin have approximately equivalent efficiencies, as the slope of the linear regression curve is -0.0808, meeting the criterion that the absolute value be less than 0.1 (Figure 3). Preliminary data shows that the slope of the linear regression curve for IR-B and β-Actin is approximately -0.1, the threshold for validation. This requires further experimentation. The slope of the linear regression curve for IR-A and β-Actin is not close to the threshold although preliminary data suggest that titration of the IR-A sense primer would bring IR-A closer to an equivalent efficiency with β-Actin.
Figure 3
Efficiency plot of IGF1R and β-Actin, plotting Delta Ct vs. log concentration ng cDNA. The slope of the regression line is -0.0808

Conclusion:
The purpose of this project was to demonstrate the validation of specifically designed primer pairs for sensitivity, specificity and efficiency. When subjected to sensitivity assays, primer pairs for IR-A, IR-B and IGF1R were able to detect their target over a range of $10^7$ to $10^1$ copies of plasmid standards. With regard to competition assays, IR-A primers displayed overefficiency at low concentrations of IR-A standards, suggesting that they cross-reacted to IR-B standards when IR-B was $10^6$ more abundant than IR-A. It appears then, that the IR-A primer pairs are specific to IR-A standards in the presence of $10^5$ copies of a homologous competitor. However, titration of the IR-A sense primer appeared to minimize cross-reactivity. Thus, at an optimized level, the IR-A primers may exhibit even larger degrees of specificity. Although not tested, it is expected that the other primer pairs will have similar degrees of specificity and may also require primer concentration optimization. Efficiency validation experiments, which are critical for the accurate application of the $\Delta\Delta$Ct method of relative quantification showed that IGF1R primers, when titrated to appropriate levels had an equivalent efficiency to β-Actin when using MDA-MB231 cDNA as a template. The relative efficiencies of IR-A and IR-B with regard to β-Actin are not yet ideal, although primer optimization shows promise in making them equivalent.

In order for a RT-PCR assay to be meaningful, it is critical that the primers be thoroughly validated. For this particular assay, due to the significant homology among the two IR isoforms and IGF1R, it is imperative that the primers demonstrate not only sensitivity to their targets, but that they also exhibit specificity and not cross-react to other targets. Although it is not yet fully validated, this assay, which will measure IR-A, IR-B and IGF1R on the same scale, has the potential to be used as a powerful tool to be applied in both the laboratory and clinic in the classification of different cell lines and tumor specimens for insulin and IGF ligand sensitivity.

References:
3. Applied Biosystems User Bulletin 2 P/N 4303859
9. JAMES IMM (NJMS 2012)

EFFECT OF MAP3K COT1 ON DIFFERENTIATION OF VITAMIN D-RESISTANT HUMAN LEUKEMIA CELLS

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

Objective:
Investigate the effect of COT1, the protein kinase upstream in the Erk cascade (MAP3K), on vitamin D-induced differentiation in a vitamin-D resistant cell line of human leukemia.

Methods:
Experiments were conducted on HL60-40AF cells, a line of vitamin D-resistant cells derived from human myeloid leukemia HL60 cells. The cells were grown in RPMI 1640 medium with 10% bovine calf serum supplementation at 37°C. The cells were suspended into equal cell groups and treated with the appropriate factors of the triple combination and a pharmacological COT1 inhibitor. After 72 hours of incubation with CO₂ at 37°C, the cells were harvested and viability was determined with 0.5% trypan blue staining. The cells were washed in 1xPBS and centrifuged at 2000 rpm for 5 minutes at 4°C. The cells were then stained with anti-CD14 and anti-CD11b antibodies. CD14 and CD11b positive cells were detected with Cell Quest software and a Coulter flow cytometer. Slides for nonspecific esterase (NSE) activity were made and analyzed to observe monocyte-like differentiation.

Summary:
The physiological form of Vitamin D, 1,25-dihydroxyvitamin D₃ (1,25D), has been shown to induce differentiation in human leukemia cells. By adopting a more mature phenotype, the cancer cells gradually lose the ability to proliferate. However, in many cases of vitamin D-induced differentiation, a subset of the population remains resistant to the effects of vitamin D and retains the immature, neoplastic phenotype. A triple combination of 1,25D, an antioxidant carnosic acid, and a p38 kinase inhibitor has been shown to induce differentiation in these vitamin D-resistant populations.

Here we investigated the effect of mitogen-activated protein kinase kinase kinase 8 (MAP3K8) also known as COT1 on differentiation in vitamin D-resistant human leukemia cells and its effect when used in conjunction with the triple combination.

The experiment was set up with 11 different groups: an untreated group, an ethanol vehicle control, a DMSO vehicle control, a 1,25D (D) and carnosic acid (C) group, a 1,25D and p38 kinase inhibitor (S) group, a carnosic acid and p38 kinase inhibitor group, and a group with DCS, all 3 factors of the triple combination. Each of the latter 4 groups was accompanied by an identical group with the addition of COT1 inhibitor.
Figure 1. Expression of differentiation markers in 40AF cells after 72 hours. COT1 inhibition induced differentiation in resistant leukemia cells when combined with the effects of the DCS triple combination, especially in the expression of CD11b markers. COT1 inhibition increased the expression of CD11b markers in each of the other 3 groups as well but not to a statistically significant extent. The increase in expression of CD11b markers was consistently minimal in the DC + COT1 inhibitor group when compared to DC alone. Groups that contained the p38 kinase inhibitor consistently expressed greater levels of CD11b marker expression, especially the DS + COT1 inhibitor group. While the percentage of CD14-positive cells increased as well, the difference was not statistically significant in any of the groups.

Figure 2. Expression of CD11b markers over 72 hours. COT1 inhibition led to an increase in CD11b marker expression within 24 hours in parallel with the DCS without COT1 inhibitor group.
<table>
<thead>
<tr>
<th>Groups</th>
<th>Weak stain</th>
<th>Strong stain</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Untreated</td>
<td>2.3</td>
<td>0.6</td>
<td>2.9</td>
</tr>
<tr>
<td>2. EtOH control</td>
<td>7.0</td>
<td>0.3</td>
<td>7.3</td>
</tr>
<tr>
<td>3. DMSO control</td>
<td>2.3</td>
<td>0.2</td>
<td>2.5</td>
</tr>
<tr>
<td>4. DC</td>
<td>8.4</td>
<td>0.3</td>
<td>8.7</td>
</tr>
<tr>
<td>5. DC + COT1 inhibitor</td>
<td>4.7</td>
<td>4.3</td>
<td>9.0</td>
</tr>
<tr>
<td>6. DS</td>
<td>4.0</td>
<td>0.4</td>
<td>4.4</td>
</tr>
<tr>
<td>7. DS + COT1 inhibitor</td>
<td>3.1</td>
<td>0.7</td>
<td>3.8</td>
</tr>
<tr>
<td>8. CS</td>
<td>2.7</td>
<td>3.2</td>
<td>5.9</td>
</tr>
<tr>
<td>9. CS + COT1 inhibitor</td>
<td>8.2</td>
<td>2.2</td>
<td>10.4</td>
</tr>
<tr>
<td>10. DCS</td>
<td>7.9</td>
<td>1.4</td>
<td>9.3</td>
</tr>
<tr>
<td>11. DCS + COT1 inhibitor</td>
<td>8.5</td>
<td>3.1</td>
<td>11.6</td>
</tr>
</tbody>
</table>

Table 1. Percentage of NSE-positive cells after 72 hours. NSE staining for monocytic phenotype showed low positive cell counts, which together with the high CD11b to CD14 ratio suggested that differentiation induced by DCS and DCS + COT1 inhibitor may have produced granulocytic phenotype.

Figure 3. Viability of 40AF cells after 72 hours. Viability of the cells measured after 72 hours was not compromised by the addition of any of the experimental factors.
Conclusion:
Inhibition of COT1 activity increased the proportion of cells positive for the myeloid cell marker CD11b, suggesting that COT1 acts as a negative regulator of differentiation in vitamin D-resistant leukemia cells. The mechanism is likely to be suppression by COT1 of a still to be identified branch of a MAPK pathway required for differentiation; the inhibitor relieves this suppression. Groups containing a p38 kinase inhibitor expressed a higher percentage of CD11b-positive cells, especially the DS + COT1 inhibitor group. However, the greatest percentage of differentiated cells required the use of all four factors, DCS and COT1 inhibitor. Perhaps of clinical application, COT1 inhibitor can be used in conjunction with the DCS cocktail in the treatment of persons with myeloid leukemia resistant to differentiation therapy.

Future experiments should include replications of all experiments to determine reproducibility. Nitro Blue Tetrazolium (NBT) staining to determine the significance of the high CD11b to CD14 ratio should be carried out.

References:

Acknowledgements
Dr. George P. Studzinski, Xiangwen Chen-Deutsch, Dr. Xuening Wang, Jing Zhang, Urmia Soni, Ramya Takkellapati, Isaiah Scantlebury, UMDNJ – New Jersey Medical School. This research was supported by USPHS NIH grants R01 CA044722-19 and R01-CA117942-01 from the National Cancer Institute.
10. NEIL KAUSHAL (NJMS 2012)

EVALUATION OF A NONINVASIVE EXPANDABLE PROSTHESIS IN MUSCULOSKELETAL ONCOLOGY PATIENTS FOR THE UPPER AND LOWER EXTREMITY

Mentors: Neeraj Patel MBS, Anthony Uglialoro MD, Joseph Benevenia MD, Francis R Patterson MD, Kathleen S Beebe MD (Orthopaedic Surgery)

Introduction:
Typical treatment of malignant bone tumors in pediatric patients includes neoadjuvant chemotherapy followed by wide resection of the tumor. Unfortunately, resection often includes sacrifice of the epiphysis. Thus, continuation of normal contralateral limb growth can result in potentially significant limb length discrepancy. Recently, the implantation of an expandable prosthesis has replaced older methods as the primary surgery by which not only can an affected limb be reconstructed, but also by which limb length discrepancy can be equalized in skeletally immature patients.\textsuperscript{1,2}

The noninvasive expandable prosthesis is used for limb-salvage surgery following tumor resection in skeletally immature patients. It enables correction of limb length discrepancy in growing patients without the risks of open surgery. Despite the availability of the noninvasive endoprosthesis since the 1990s, only two published reports exist describing the functional outcomes of the implants in the lower extremity. Though this prosthesis is approved for the lower extremity, our experience has included compassionate use for the upper extremity, a practice that has not yet been reported.

The purpose of this study is to report our experience with the Repiphysis\textregistered (Wright Medical Technology; Arlington, TN) noninvasive expandable prosthesis for both the upper and lower extremity.

Materials and Methods:
We retrospectively reviewed twelve consecutive patients (Seven females, five males) between 2003 and 2008 who required implantation of the Repiphysis\textregistered noninvasive expandable prosthesis system. The diagnosis was osteosarcoma in 10 patients (6 of the femur, 2 each of proximal tibia and proximal humerus) and Ewing's sarcoma in 2 patients (1 of the femur, 1 of total humerus). Overall, the 12 patients underwent 15 procedures. Data was collected on demographics, pathology, complications, number of expansions, and total expansion length.

The Musculoskeletal Tumor Society (MSTS) functional score was administered for all patients for either the upper or lower extremity, depending on the site of pathology. This system consists of six criteria: pain, function of the extremity, emotional acceptance of any residual deficit, use of mechanical supports, and walking ability and gait (for the lower extremity). Each category is given a score from 0-5, leading to a maximum possible score of 30.

Results:
Thirteen prostheses were implanted in 12 patients. Mean age was 10 years (range; 7-16) with average follow-up of 31 months (range; 10-68). Seven patients underwent a total of 35 expansion procedures with an average of 4.7 lengthening procedures per patient (range; 1-8). There was an average of 1.03 cm of expansion (range; 0.5-2) at each visit. Mean total expansion was 4.89 cm (range; 1.9-9.45).
7 non-oncologic complications were noted; three of which required revision surgery and all patients with non-oncologic complications retained their limbs. One patient with osteosarcoma had a recurrence and underwent a hip disarticulation. One complication was noted in the upper extremity: elbow contracture with associated posterior radial head subluxation.

The mean MSTS score after physical rehabilitation was 25.3 (range, 23-27) and 23.4 (range; 13-30) for upper and lower limb implants, respectively.
Table 1.

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Gender</th>
<th>Age</th>
<th>Diagnosis/ Location</th>
<th>Date of Initial Surgery</th>
<th># of Expansions</th>
<th>Total Expansion (cm)</th>
<th>Follow-up (Months)</th>
<th>MSTS</th>
<th>Complications</th>
<th>Revision (Y/N)</th>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>7</td>
<td>Osteosarcoma/ Lt. distal femur</td>
<td>5/30/2006</td>
<td>0</td>
<td>N/A</td>
<td>10</td>
<td>21</td>
<td>D.O.D.- Local recurrence + Mets</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>11</td>
<td>Ewing sarcoma/ Lt. femur</td>
<td>4/27/2004</td>
<td>8</td>
<td>7.9</td>
<td>54</td>
<td>30</td>
<td>Contracture/ arthofibrosis; Septic loosening of femoral prosthetic component</td>
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<tr>
<td>3</td>
<td>M</td>
<td>12</td>
<td>Osteosarcoma/ Lt. prox. Tibia</td>
<td>3/19/2008</td>
<td>0</td>
<td>N/A</td>
<td>10</td>
<td>N/A</td>
<td>None</td>
<td>N</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>16</td>
<td>Ewing Sarcoma/ Lt. humerus</td>
<td>3/7/2006</td>
<td>5</td>
<td>5.5</td>
<td>34</td>
<td>26</td>
<td>Elbow Contracture</td>
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<tr>
<td>5</td>
<td>F</td>
<td>8</td>
<td>Osteosarcoma/ Lt. prox. Humerus</td>
<td>3/23/2004</td>
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<td>5.75</td>
<td>54.2</td>
<td>27</td>
<td>None</td>
<td>N</td>
</tr>
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<td>6</td>
<td>M</td>
<td>9</td>
<td>Osteosarcoma/ Rt. prox. Tibia</td>
<td>3/26/2003, 2/26/2008</td>
<td>2,0</td>
<td>1.75, N/A</td>
<td>60, 8</td>
<td>28 *</td>
<td>Failure of initial implant/ Reconstruction w/ new prosthesis</td>
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<td>26</td>
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<td>Aseptic loosening of tibial prosthetic component, Tibial fracture, SCFF, Mets, N..E.D</td>
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<td>9</td>
<td>M</td>
<td>9</td>
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<td>22</td>
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<tr>
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<td>M</td>
<td>11</td>
<td>Osteosarcoma/ Lt. femur</td>
<td>3/20/2008</td>
<td>0</td>
<td>N/A</td>
<td>12</td>
<td>24</td>
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<td>N/A</td>
<td>7</td>
<td>13</td>
<td>Hip Disarticulation Local recurrence + Mets- A.W.D.</td>
<td>N</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>6</td>
<td>Osteosarcoma/ Lt. prox. humerus</td>
<td>10/29/2008</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
<td>23</td>
<td>None</td>
<td>N</td>
</tr>
</tbody>
</table>

* MSTS score reflects functional analysis prior to the recent revision surgery that required a new prosthesis secondary to mechanical failure

*D.O.D= Dead of Disease; N.E.D= No Evidence of Disease; A.W.D= Alive with Disease
Conclusion:
Limb salvage procedures in skeletally immature patients have been a challenge for orthopedic oncologists. The advent of an expandable endoprosthesis has aided orthopedic oncologists in combating the problem of limb length inequality in skeletally immature patients. Earlier endoprosthesis designs have been succeeded by minimally invasive implants. The creation of a noninvasive expandable endoprosthesis originated with the Phenix endoprosthesis (Phenix Medical, Paris, France) and is now produced under the name Repiphysis® (manufactured by Wright Medical Technology). The goal of Repiphysis® is to allow for noninvasive expansions and to potentially limit risk of infection and morbidity.

Experience with the noninvasive expandable prosthesis has been increasing, but the body of literature surrounding these prostheses remains small. Past studies have solely focused on the endoprostheses in the distal femur and the proximal tibia; there is a lack of reporting on experiences with noninvasive endoprostheses in the upper limb. Our study reports our experience with twelve patients using the Repiphysis® in the distal femur, proximal tibia, and the proximal humerus. To date, there have been no studies on the use of the Repiphysis® in the upper limb. Our study aims to further the studies on the Repiphysis® use on the lower limb and to introduce our compassionate experience with the Repiphysis® on the upper limb.

We report our use of the Repiphysis® on the upper limb as an encouraging model for future studies. The upper extremity Repiphysis® prosthesis was placed in three patients (Table 1). No complications were encountered with any of the expansion procedures. The expansion procedures allowed for our patients to achieve satisfactory limb length equality.

The mean MSTS scores after physical rehabilitation were 25.3 and 23.4 for upper and lower limb, respectively. The upper limb MSTS scores were slightly higher, as was the range (23-30). The dimensions of the Repiphysis® prosthesis in the upper extremity are smaller in comparison to the custom make of the lower extremity. The upper extremity is not exposed to the same load and forces that is applied during simple daily activity, not to mention strenuous activity that may be encountered in running or other simple playful activity. The dimensions of the upper extremity prosthesis are made to endure the normal activity of the upper extremity. The MSTS scores appear to reflect this difference in usage and activity. The functional outcomes of our twelve Repiphysis® patients are very encouraging. Compared to prior studies on the Repiphysis®, the prevalence of complications and MSTS scores of our patients on the lower limb are encouraging.

The Repiphysis® noninvasive expandable prosthesis is functionally effective for both upper and lower limb implantation. This endoprosthesis is very useful for pediatric patients with bone malignancies and is associated with fewer complications at the time of lengthening than invasively expandable prostheses.

*Note – The authors do not have any potential or real conflicts of interest relating to this manuscript, nor do we have any financial or other significant relationship relating to the manufacture of Repiphysis/Phenix or its competitors.

References
11. VIVIAN KU (NJMS 2012)

DIFFERENCES IN T CELL POPULATIONS UPON WHOLE BODY γ-IRRADIATION IN WILD-TYPE AND IRF-5 KNOCK-OUT MICE IN VARIOUS IMMUNE SYSTEM ORGANS

Mentor: Betsy Barnes, PhD (Biochemistry and Molecular Biology)

Objective:

Interferon regulatory factor 5 (IRF5) belongs to a family of nine mammalian transcription factors which were identified in the late 1980s. Many IRF members play central roles in the differentiation of hematopoietic cells and in the regulation of gene expression related to innate and adaptive immunity. Several IRF family members have also been implicated in cell cycle regulation and apoptosis supporting their role in susceptibility to and progression of cancer (1). With the recent generation of irf5 knockout (KO) mice, previous data from our lab in human cells has been corroborated indicating that IRF5 plays a critical role in the cellular response to extracellular stress, i.e. pathogens, DNA damage (1). Indeed, previous studies have shown that the induction of proinflammatory cytokines, such as IL-12, TNF-α, and IL-6, is impaired in irf5 KO mice (2). Some of these cytokines are also important for immune cell differentiation. For example, IRF1 has been shown to regulate the expression of genes in developing thymocytes required for conversion and selection into CD8+ cytotoxic T lymphocytes (CTLs) (2). Also, the induction of the gene encoding p40 subunit of IL-12, the cytokine essential for Th1 type differentiation, is dependent on IRF1 (2). IRF1 KO mice have also been found to have reduced levels of IL-15, which impaired the development of natural killer T cells (3).

We hypothesize that loss of irf5 expression contributes to alterations in immune cell subpopulations that may contribute to a number of critical functions, such as antitumor immunity and DNA damage response. In order to address this hypothesis, we have examined and compared T cell (CD4+ and CD8+) populations in wild-type (WT) and irf5 KO mice. We have also exposed these mice to whole-body γ-irradiation (IR), which has been shown to not only cause DNA damage but also immunosuppressive and inflammatory effects in mammalian cells (4), and has also been used to induce thymic lymphomas in wt C57Bl/6 mice (5). We are particularly interested in these T cell populations since CD4+ cells play an important role in antitumor immune responses by priming tumor-specific CD8+ CTLs and activating their response to the specific tumor (6). Differences in immune cell subpopulations and induction of apoptosis were determined by flow cytometry.

Experimental Design

<table>
<thead>
<tr>
<th>Treated with IR (6.3Gy)</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>Wild Type</td>
</tr>
<tr>
<td>IRF-5 knockout</td>
<td>IRF-5 knockout</td>
</tr>
</tbody>
</table>

Method:

6-8 week old C57Bl/6 wt and irf5 KO mice were treated with 6.3 Gy of whole-body γ-irradiation. 17-19 hours later, the treated and untreated mice were sacrificed and the thymus, spleen, and peripheral blood were collected. Splenocytes, thymocytes, and peripheral blood
Cancer Summer Student Research Program - 2009

(PBL) were treated with 1X RBS Lysis buffer. After neutralizing and washing the cells in PBS, cells were resuspended in 1X Binding buffer for flow cytometry. Four color flow cytometry was used with the following extracellular markers: Annexin V- FITC for apoptosis, CD45-PE for lymphocytes, CD4-PerCP for helper T cells, and CD8-APC for cytotoxic T cells. The cells were fixed with 1% formaldehyde in PBS. Flow cytometry was performed on a FACSCalibur (BD Biosciences, San Jose, CA). Analysis of the data was done with FlowJo or CellQuest programs.

Summary:

Previous data from our lab has shown that there are differences in immune cell populations in WT versus KO mice. Moreover, recent data from our lab and others suggests that IRF5 has cell type-specific functions. As a result, we analyzed more specifically the differences in CD4+ and CD8+ T cell populations from different immune system organs. In the thymus, there was no significant difference in the numbers of CD8+ T cells between WT and KO mice; in the spleen, a slight increase in CD8+ T cells was observed in KO mice. More significant differences were observed in T cell population numbers from PBL, where CD8+ T cells were reduced by 32.32% in KO versus WT mice (Fig 1). For CD4+ T cells, there was no significant difference in thymus and spleen between WT and KO (Table 1). However, in the PBL, there was a 19.2% decrease in CD4+ T cells numbers in KO versus WT mice (Table 1).

Figure 1: FACS analysis of CD8 in untreated wild type and knock-out

<table>
<thead>
<tr>
<th>Spleen WT</th>
<th>Spleen KO</th>
</tr>
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<tr>
<td>11.56%</td>
<td>20.63%</td>
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<table>
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<tr>
<th>Thymus WT</th>
<th>Thymus KO</th>
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<td>83.98%</td>
<td>85.90%</td>
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<table>
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<th>PBL WT</th>
<th>PBL KO</th>
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<tr>
<td>40.11%</td>
<td>7.79%</td>
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Table 1: CD4 population in WT and KO

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<th>Wild Type</th>
<th>IRF 5 Knock-out</th>
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<tr>
<td>Thymus</td>
<td>85.92%</td>
<td>88.92%</td>
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<tr>
<td>Spleen</td>
<td>21.10%</td>
<td>21.22%</td>
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<tr>
<td>PBL</td>
<td>52.78%</td>
<td>38.58%</td>
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After observing the differences in CD8+ and CD4+ T cell populations between WT and KO mice, especially in PBL, we then treated mice from each group with IR to determine if loss of irf5 would affect the response of CD4+ and CD8+ populations to extracellular stress. Unlike the WT PBL, the CD8+ and CD4+ T cell numbers from the KO PBL increased after IR (Figure 2).
There was no effect from IR in the CD4+ T cells from WT PBL, but in the KO, there was an increase in CD4+ T cells after IR. For the spleen, WT and KO CD8+ T cells had a similar decrease after IR, and there was no significant effect from IR in the CD4+ T cells in either WT or KO mice (data not shown). Thymus CD8+ and CD4+ T cells had similar decreases seen after IR in both WT and KO (data not shown).

**Figure 2: CD8+ and CD4+ T cells from KO PBL increased after IR**

Figure 2: CD8+ and CD4+ T cell numbers were very different in the WT versus KO in PBL after IR treatment. The WT CD8+ T cells from PBL decreased by 28.5% after IR, whereas there was a 4.41% increase in KO. The WT CD4+ T cells from PBL had no change after IR, but there was a 30.47% increase in KO CD4+ T cells from PBL.

We then sought to determine why there was such a different response in CD8+ and CD4+ T cell populations from PBL in WT and KO mice after IR. One possible mechanism could be from a variation in the cellular response to DNA damage, such as induction of apoptosis. We used Annexin V as an apoptosis marker. In the CD8+ T cells from PBL we observed an increase in apoptosis after IR treatment in WT and KO mice, but there was less apoptosis occurring in the KO compared to WT (Table 2). Conversely, CD4+ T cells from KO mice PBL were slightly more sensitive to IR-induced apoptosis than WT mice. Neither of the observed differences were statistically significant given the number of mice utilized in each experiment. Similarly, no significant differences in apoptosis were observed in T cells from thymus of WT and KO mice (data not shown). However, CD8+ and CD4+ T cells from the KO spleen had a decreased amount of apoptosis when compared to WT (Table 2).

**Table 2: Decreased Amount of Apoptosis in CD8+ and CD4+ T cells from KO Spleen after IR**

<table>
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<tr>
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<th>Wild type after IR</th>
<th>Knock-out after IR</th>
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<tr>
<td>CD8+ PBL</td>
<td>18.6% increase</td>
<td>13.64 % increase</td>
</tr>
<tr>
<td>CD4+ PBL</td>
<td>22.62% increase</td>
<td>30.38% increase</td>
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<td>CD8+ Spleen</td>
<td>0.58% increase</td>
<td>2.89% decrease</td>
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<tr>
<td>CD4+ Spleen</td>
<td>5.94% increase</td>
<td>2.11% increase</td>
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**Conclusion:**

There were significant differences observed in CD8+ cytotoxic T cell and CD4+ helper T cell populations in WT and irf5 KO mice. In PBL, the loss of irf5 correlated with a decrease in
CD8+ and CD4+ T cells when compared to WT mice. These differences appear to be organ specific, suggesting that IRF5 may play a role in T cell development, trafficking and/or differentiation. After whole body IR treatment, CD8+ T cell numbers from PBL of WT mice were decreased, but the there was no affect of IR on the WT CD4+ T cell population. This suggests that the CD8+ T cells in the PBL of WT mice are more susceptible to gamma-irradiation and the double stranded DNA breaks it causes than the CD4+ T cells.

In irf5 KO mice, after whole body irradiation, there was actually an increase in the CD8+ and CD4+ T cells in the PBL when compared to WT. This suggests that loss of irf5 could desensitize these cell populations to IR-induced DNA damage. IRF5 has been shown to be involved in the DNA damage-induced apoptotic pathway in mouse embryonic fibroblasts but never before in T cells (1). To determine if alterations in apoptosis was one mechanism by which CD8+ and CD4+ T cell populations from KO mice were increasing in numbers after IR and DNA damage, we looked at the apoptosis levels in CD8+ and CD4+ T cells in PBL, spleen, and thymus. We found that there were no significant differences in apoptosis observed between CD4+ and CD8+ T cells from PBL of WT and KO mice, indicating that IR-induced apoptosis in these T cell populations from PBL may not be deficient. However, splenic CD8+ and CD4+ T cells from KO mice were less sensitive to IR-induced apoptosis than WT. This suggests that there may be organ specific roles for IRF5 in apoptosis. It is unclear at this point whether the increase in PBL T cell populations in KO versus WT mice after whole body IR is due to a loss in cellular apoptotic signaling based on the current data. It will be worthwhile for apoptosis in these cell populations to be measured by other techniques in the near future. However, we can not rule out an effect of IRF5 on pro-inflammatory cytokine expression that may also effect T cell populations after IR.

So, although loss of irf5 may contribute to alterations in T cell populations from different lymphoid organs, particularly after an extracellular stress like DNA damage, there are other mechanisms than the apoptotic pathway that irf5 is influencing to cause these alterations. Future research would include analysis using Flow cytometry or Western blot to look at IRF5 expression before and after IR in cell populations from WT and KO, as well as IR-induced apoptosis by other techniques, such as the TUNEL assay, comet assay, and Western blot of downstream mediators such as Parp cleavage. We also need to determine other IRF5-mediated mechanisms which may contribute to changes in T cell population numbers after IR, such as IRF5-mediated cytokine expression in response to IR. Cytokine expression will be measured by Q-PCR. More research into the mechanism by which irf5 is causing an alteration in T cell population is needed to fully appreciate the role that irf5 may play in the antitumor immune response and DNA damage.

References:
3. Ohteki, T., Yoshida, H., Matsuyama, T., Duncan, G.S., Mak, T.W., Ohashi, P.S. The transcription factor interferon regulatory factor 1 is important during the maturation of NK1+T cells, natural killer cells, and intestinal intraepithelial T cells. J. Exp. Med. 1998; 178: 967-972.
12. **JOEY LARATTA (NJMS 2012)

**ROLE OF MIR-15A/16 IN PROSTATE CANCER**

Mentors: Erica Salerno, MS, (Pathology), Frederick D. Coffman, PhD, (Pathology), Brian D. Brown, PhD, MSSM, New York, NY, Desiree Bonci, PhD, Istituto Superiore Sanita, Rome, Ruggero De Maria, PhD, Istituto Superiore Sanita, Rome, Elizabeth Raveche, PhD, (Pathology)

**Objective:**

Other than skin cancer, prostate cancer is the most frequent cancer in American men. The American Cancer Society estimates that 192,280 new cases of prostate cancer will be diagnosed this year in the United States.\(^1\) Although the advancements in prostate cancer diagnosis have ameliorated prognoses, some prostate cancer types are aggressive and have the ability to spread quickly. MicroRNA (miRNAs) are non-coding, single-stranded RNAs comprising 19-25 nucleotides that function in the regulation of eukaryotic gene expression. The primary miRNA transcript encoded by the host genome contains hairpin structures that undergo a series of cleavages to form a mature sequence. These single-stranded mature sequences are incorporated into an RNA-induced silencing complex (RISC) that then binds to the 3’ UTR of a target mRNA, resulting in post-transcriptional regulation of the gene. Depending on the degree of complementarity to the 3’ UTR of specific messenger RNAs, miRNAs associated with RISCs either target the message for degradation or block translation leading to a decrease in protein production.

There are over 200 miRNAs known to exist in the human genome, and each of these miRNAs can have over 400 targets.\(^2\) With the developments in microarray biotechnology, miRNA profiles have been identified for a variety of cancers. Some miRNAs overexpressed in cancers may act as oncogenes, while miRNAs with tumor suppressive potential may be underexpressed in cancers. Notably, the chromosomal region 13q14, containing the microRNA cluster **mir-15a/16-1**, which exhibits tumor suppressive qualities, is often deleted or altered in chronic lymphocytic leukemia and prostate cancer.\(^3\) This miRNA cluster has been shown to target the 3’ UTR of the mRNA for cell cycle stimulator **CCND1** (cyclin D1) and anti-apoptotic **BCL2**, leading to their decreased translation.\(^4\) We have previously found that the 13q14 region was point mutated in the New Zealand Black (NZB) murine model of chronic lymphocytic leukemia (CLL), leading to a reduction in mature miR-15a/16 expression.\(^5\)

In this study, we investigated the role of **mir-15a/16-1** in an aggressive human prostate cancer cell line (LNCaP). It has been shown that LNCaP also have a defect in interferon α and γ signaling due to epigenetic silencing of a key intracellular intermediate in the signaling cascade.\(^6\) We proposed that overexpressing miR-15a/16 may rescue the interferon pathway and stop cell cycle progression, functioning as a single therapy or enhance traditional chemotherapy allowing for effective therapy with a less toxic dose of the chemotherapeutic.

**Methods:**

**Cell lines:** LNCaP cells, courtesy of Beverly Barton at UMDNJ-NJMS, are derived from metastasis to the left supraclavicular lymph node in human prostate carcinoma. LNCaP were maintained in high glucose RPMI 1640 media + 10% FBS. 293T is an adherent, kidney epithelial human cell line. ARPE-19 is an adherent, retinal epithelial human cell line.

**RNA extraction and quantitative real-time PCR:** Total RNA was isolated according to Trizol (Invitrogen) methodology. PCR amplification of miR-15a and miR-16 was performed using TaqMan miRNA Reverse Transcription Kit and miRNA PCR assays, normalizing to 18S rRNA.
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The PCR reaction was run on the Applied Biosystems 7500 Real Time PCR System for 40 cycles. The relative quantification (RQ) values of LNCaP miRNA levels compared to normal ARPE-19 and 293T levels were determined using the standard $2^{-\Delta\Delta CT}$ method.

Lentiviral vector sensor assay: The mCherry sensor (courtesy of Brian Brown, Mt Sinai NY) contains antisense to miR-16 in 3'UTR, such that when mature miR-16 binds to the antisense with perfect complementarity, the target, mCherry, degrades. The TWEEN-RRmiRNA 15/16 (provided by Desiree Bonci and Ruggero De Maria of Istituto Superiore Sanita) is a double vector. A 724 bp fragment of the mir-15a/16-1 human genomic DNA (NCBI36: ch13:49519256:49523338 region) was cloned into a lentiviral vector under the control of the cytomegalovirus promoter and GFP under the control of the phosphoglycerate kinase promoter. LNCaP were transduced with either mCherry sensor alone or with TW-15a/16 and incubated at 37°C. Flow cytometric analysis for GFP and mCherry expression was performed at 9 days post-transduction. Five to ten thousand events/sample were acquired on LSR II and analyzed using FACSDiva Software Version 6.1.3 (BD Biosciences).

Microscopy: Images were obtained of LNCaP transduced with mCherry sensor after 5 day incubation using a Zeiss LSM 510 on Zeiss Axiovert 100M Base microscope with attached AxioCam. Images were analyzed on AxioVision (Release 4.7.1).

Cell cycle analysis: Both transduced (9d) and untransduced cells were stained with hypotonic propidium iodide (PI) and analyzed using ModFit LT V3.1 software (Verity Software House). Samples were acquired on FACSCalibur (Becton Dickinson).

Intracellular Flow Staining: LNCaP cells were fixed with paraformaldehyde (PFA) and permeabilized with 0.2% Triton X-100 in a phosphate buffered saline (PBS) solution. The anti-human Bcl-2 antibody was labeled with phycoerythrin (PE, Becton Dickinson) and the anti-human pStat-1 was labeled with pacific blue (Becton Dickinson). Unconjugated anti-human IRF-5 antibody was labeled with Alexa Fluor 680 using mouse IgG1 Zenon labeling kit (Invitrogen). LNCaP were incubated in antibody for 20 minutes at 4°C in the dark. Ten to twenty thousand events/sample were acquired on LSR II and analyzed using FACSDiva Software Version 6.1.3 (BD Biosciences).

Summary:

Real time PCR shows LNCaP to have a significant decrease in levels of miR-15a compared to non-prostate fibroblast lines 293T and ARPE-19 (Figure 1). We used a double lentiviral assay to generate a stable LNCaP cell line overexpressing miR-15a/16. First, LNCaP cells were transduced with a lentivirus containing a cherry sensor with the miR-16 antisense sequence in the 3' UTR, such that when mature miR-16 binds to the antisense with perfect complementarity, the target, mCherry, degrades. mCherry expression in the LNCaP transduced with mCherry sensor was observed via microscopic analysis (Figure 2). Next, LNCaP were double-transduced with both the cherry sensor and TWEEN-RRmiRNA 15/16 (TW15a/16), a lentivirus expressing GFP and miR-15a/16. If the TW15a/16 was successfully making miR-16 in the double-transduced LNCaP cells, the mature form of miR-15a/16 will bind to the antisense on the cherry sensor, thus decreasing mCherry expression. Flow cytometric analysis of mCherry expression in LNCaP 9 days after transduction with TW15a/16 compared the mean fluorescence intensity of mCherry protein in LNCaP with mCherry sensor alone and LNCaP with both sensor and TW-15a/16. mCherry protein levels decreased significantly following miR-15a/16 addition (Figure 3). Thus, the lentivirus delivered was successfully making the miRNAs, degrading the mCherry mRNA, resulting in a decrease in the mCherry signal. Hypotonic PI
staining was used to measure DNA content to investigate the cell cycle effects of exogenous miR-15a/16 restoration in LNCaP cell line. The change in the percent of cells in S and G2 phases in TW-15a/16 transduced LNCaP was compared to those transduced with mCherry sensor alone using hypotonic PI staining. Exogenous miR-15a/16 restoration in the TW-15a/16 LNCaP resulted in G2 arrest and decrease in S phase (Figure 4).

**Conclusion:**

LNCaP cells have decreased miR-15a levels. There is a reported loss of heterozygosity at 13q14.3 which would result in a hemizygous expression of the cluster. Surprisingly, LNCaP do not have decreased miR-16 possibly due to the fact that there are two genetic loci for miR-16: mir-16-2 on chromosome 1 and mir-16-1 on chromosome 13. The mature forms are indistinguishable. We suspect that the loci on chr 1 is excessively active, thus overcompensating its production of miR-16 due to the loss at chromosome 13q14.

LNCaP cells have a defect in interferon signaling due to epigenetic silencing of Jak1, a kinase responsible for the activation of Stat1 in the signaling cascade following interferon α and γ binding. Normally, Stat1 is phosphorylated to pStat1 by Jak1 leading to dimerization and subsequent transport to the nucleus to function as a transcription factor for antiviral, microbial, or tumor responses. miR-15a/16 target proteins that can affect epigenetic DNA modifications of the genome through methylation and deacetylation. We suspected that LNCaP overexpressing the mir-15a-16-1 locus may rescue the defect in interferon signaling. Flow cytometric analysis compared levels of fluorescently labeled antibodies against pStat1 and IRF-5 in the mCherry sensor LNCaP versus the TW-15a-16 transduced LNCaP, however, no significant difference was shown.

Exogenous miR-15a/16 addition resulted in a G2 arrest and a decrease in S phase. Cyclin D1, one of the targets of miR-15a/16, is responsible for the transition of G1 phase into S phase; however, we observed a G2 arrest. We plan to investigate what other targets of miR-15a/16 could be responsible for such an arrest and whether exogenous miR-15a/16 delivery can ameliorate traditional chemotherapy.

**Supplemental Figures**

**Figure 1**

![Figure 1](image1.png)

FIG-1: Real time PCR shows LNCaP to have a significant decrease in levels of miR-15a compared to non-prostate fibroblast lines 293T and ARPE.19

**Figure 2**
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FIG-2: Representative microscopic analysis of mCherry expression in LNCaP transduced with mCherry sensor. Left: Bright field, right: mCherry, phase contrast

FIG-3: Representative flow cytometric analysis of mCherry expression in LNCaP 9 days after transduction with TW15a/16. Mean fluorescence intensity of mCherry protein levels compared in LNCaP with mCherry sensor alone and LNCAP with both sensor and TW-15a/16. mCherry protein levels decreased significantly following miR-15a/16 addition.

FIG-4: Change in the amount of cells in S and G2 phases is shown as a mean percent change of TWEEN-RRmiRNA 15/16 transduced LNCaP compared to those transduced with mCherry sensor alone.

References:
13. EDWARD LEE (TCNJ 2012)

DEVELOPING A QUANTITATIVE MEASUREMENT OF RADIATION TREATMENT ADHERENCE

Mentors: Denise C Fyffe, Ph.D., (Kessler Research Foundation), Charles Cathcart, M.D., (Radiation Oncology)

Objective:
Patient adherence to prescribed radiation treatment is essential for survival and the relief of symptoms. Many factors play into the role of adherence to radiotherapy, and identifying these factors may lead to interventions that are geared to improving adherence (Cathcart et al., 1997). Conventionally, radiation oncologists determine adherence by dividing the number of fractions given by the number of days elapsed. It is the expectation that every patient will not miss any treatments. However, many reasons can cause a patient to miss treatments, including machine downtime, bad weather, admission to another hospital, or the inability to be transported for treatment.

Adherence has been measured quantitatively for treatments of different diseases, but there is still lack of a good standard of measurement (Partridge, 2002). In radiotherapy, criteria have been established in labeling a patient adherent or nonadherent to follow-up visits, but there is a lack of a standard quantitative measure of adherence during the treatment course itself (Moynihan, 2009).

The objective of this study is to derive and develop a way to quantitatively measure patient adherence to radiation therapy. There are some challenges to such a measurement that are unique to radiotherapy. Radiation prescriptions are usually split into fractions in which patients receive once treatment on consecutive weekdays. Radiation therapy is usually not given on the weekends, and this poses a challenge to determine the relative effect of the total number of elapsed days from the first treatment to the last treatment. This study compares two different calculations of patient adherence and examines the properties for each.

Methods:
An IRB-approved retrospective chart review of 479 consecutive patients treated by the Department of Radiation Oncology at a large, inner-city, public hospital from January 1, 2006 to March 8, 2007 was conducted for data collection. On each patient, two different calculations of adherence were calculated. Patients who were under the age of 18 at the beginning of treatment, who received less than five fractions of radiation treatment, and who received brachytherapy exclusively were excluded from this study.

Data collection:
The following data was abstracted: number of fractions prescribed, number of fractions given, number of elapsed days of treatment, and dates of the first and last treatment. Patient records were checked to see if they received treatment during weekends and departmental holidays. Data was obtained from patient charts and electronic medical record systems Multi-Access. The abstracted data was entered into a Microsoft Office Access database, and then exported to a Microsoft Excel database for further data cleaning.
Six theoretical patients were created to compare the measurements of adherence by two different formulas. They were used to see how well each formula measure different aspects of adherence, including missed treatments and days when the treatment center is closed.

**Measures of adherence:**

Two different calculations were used to measure adherence. The first formula has been routinely used in the Department of Radiation Oncology, and the formula has the label $r$. The formula is:

$$ r = \frac{F_c}{t} $$

where $F_c$ is the number of fractions completed and $t$ is the number of elapsed days. $t$ is measured by labeling the first day of treatment as day 0 and counting the number of days to the last day of treatment.

The second formula was designed to measure how well patients adhered to their schedules, regardless of the weekends and holidays that fell during their treatment course and to account for patients who do not finish their treatment. This formula has the label $\rho$ and is defined as

$$ \rho = \frac{F_c^2}{F_p \cdot t_{tx}} $$

where $F_c$ is the number of fractions completed, $F_p$ is the number of fractions prescribed, and $t_{tx}$ is the number of possible treatment days elapsed.

**Summary:**

Theoretical patients were created in order to compare the ability of the formulas to measure adherence. These patients, as discussed below, were developed based on the some problems seen during departmental use of $r$. Problems include: $r$ values greater than 1, no patient being able to receive a perfect $r$ of 1, and $r$ values skewing actual adherence to treatment.

Figure 1 shows a patient who received and was prescribed 5 fractions. By definition of $t$, the patient received 5 fractions in 4 elapsed days. Thus, $r = \frac{5}{4} = 1.25$, a value that does not make sense as it is greater than 1.

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Figure 1. Check marks indicate days when the patient received a fraction. By redefining the value $t$, this patient’s $r$ becomes 1.

This problem can be solved redefining $t$ by labeling the first date of treatment as day 1 and counting up to the last date of treatment. After that change, $r = \frac{4}{4} = 1$, which indicates a perfectly adherent patient.

The following discussion will discuss six patients that reveal the shortcomings of $r$. Figure 2 represents six patients who were prescribed 5 fractions of radiation:
**Patient 1:** starts on Monday and comes in for treatment every day the treatment center is open.

**Patient 2:** starts on Wednesday and comes in for treatment every day the treatment center is open.

**Patient 3:** starts on Wednesday and comes in for treatment every day the treatment center is open. However, the hospital is closed Monday for a holiday.

**Patient 4:** starts treatment on a Wednesday, but he skips treatment on Monday and finishes on Wednesday.

**Patient 5:** starts treatment on Wednesday and continues to come in until Friday. The patient does not finish treatment.

**Patient 6:** comes in for treatment on Monday and Tuesday, misses the next three days, and then comes in on Monday of the next week. The patient does not finish treatment.

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Figure 2. Check (✓) marks indicate days when the patient received a fraction. Shaded days represent days when the hospital is closed. X (x) marks represent days when patient did not come in on a day the hospital was open.

The $r$ column shows the shortfalls of $r$ in measuring adherence. Patients 1, 2 and 3 are identically adherent as they come in everyday possible, but they have different $r$ values. Patient 4 is less adherent than patient 3 as patient 4 misses a day when he could have received treatment, but they receive the same $r$ values. Patient 5 is nonadherent as he does not finish his treatment but receives a perfect $r$ value of 1. Patient 6 represents a very nonadherent patient who misses possible days of treatment and does not finish treatment.

The analysis of these six theoretical patients shows a need to develop a new formula that accounts for days when treatment is not possible and for incomplete treatments.

**Derivation and Discussion of $\rho$**

Since a patient cannot receive treatment on weekends and holidays, $t$ must be redefined again to eliminate weekends and holidays. This new definition will receive a new name $t_{Tx}$, to emphasize that $t_{Tx}$ is the number of elapsed possible treatment days. Our modified adherence ratio then is:

$$r' = \frac{F_c}{t_{Tx}}$$

The $r'$ column in Figure 3 shows that patients 1, 2, and 3 have received values of 1, as they are all perfectly adherent and shows that patient 4 received a value less than 1. However, $r'$ still fails to account for patients who do not finish their prescription as patient 5 still receives a value of 1.
A new formula, labeled $\rho$, has to contain some constant that ‘punishes’ or lowers a patient’s $r'$ for incomplete treatment. The new formula must take the form

$$\rho = k(r')$$

where $0 \leq k < 1$ when the patient does not finish the prescription and $k = 1$ when the patient does finish the prescription.

A very simple formula for a constant with these properties can be calculated by dividing the number of completed fractions by the number of prescribed fractions, that is:

$$k = \frac{F_c}{F_p}$$

where $F_c$ is the number of fractions completed, and $F_p$ is the number of fractions prescribed.

If the patient completes the prescription, then $F_c = F_p$ and $k = 1$. If not, then $F_c < F_p$ and $k < 1$.

Combining $r'$ and $k$, we define a new adherence ratio $\rho$:

$$\rho = \frac{F_c}{F_p} \cdot \frac{F_c}{t_{Tx}} = \frac{F_c^2}{F_p \cdot t_{Tx}}$$

$\rho$ seems very different from the formula for $r'$, but $\rho$ will reduce back to $r'$ for patients who finish their prescriptions. When patients who finish their prescriptions, $F_c = F_p$. When substituted into $\rho$

$$\rho = \frac{F_c}{F_p} \cdot \frac{F_c}{t_{days}} = \frac{F_p}{F_p} \cdot \frac{F_c}{t_{days}} = \frac{F_c}{t_{days}}$$

Figure 3 shows how $\rho$ is a better measure of adherence. Patients 1, 2, and 3 has received $\rho$ values of 1, as they are all perfectly adherent, and patient 4 received a value less than 1. Patient 5 finishes only 3 out of 5 fractions and thus has a lower $\rho$ value than patient 4. Patient 6 is the least adherent as he finishes 3 out of 5 fractions over a longer period of time than patient 5 and thus receives the lowest $\rho$ value.

**Conclusion:**
There is no accepted measure of adherence for radiation oncology patients in the literature. The conventional way of measuring a patient’s adherence has shortfalls as patients cannot come for treatments when the radiation treatment center is not open, such as weekends, holidays, and machine downtime.

Our method of calculating adherence $\rho$ appears to depict more accurately if a patient is adherent to the doctor’s recommended prescription. The redefined value $t_{Rx}$ eliminates the days when the patient could not possibly receive treatment, such as weekends and holidays. Also, $\rho$ punishes patients who do not complete therapy, unlike $r$. $\rho$ appears to be the better measurement for adherence in radiotherapy.

**References:**


14. Shawn Li (NJMS 2012)

EXPLORING TWO ALTERNATIVE SPLICE FORMS OF ARID1B

Mentor: Elizabeth Moran, PhD, (Orthopaedics)

Objective:

SWI/SNF is an ATPase powered chromatin remodeling complex that can slide or remove nucleosomes to allow for the expression of certain tissue specific genes or to repress cell proliferation. It has been an important subject of cancer research because its function is crucial for tumor suppression (Fig 1).

SWI/SNF has approximately ten subunits; of particular concern are the ATPase and ARID family of subunits. At any time, one of two ATPases powers SWI/SNF. These mutually exclusive ATPases are BRG1 and BRM. Similarly, one of two ARID family of DNA-binding proteins may associate with the complex. They are alternatively known as ARID1A and ARID1B.

Structurally, ARID1A and ARID1B are 60% identical. ARID1A, however, has distinct Glutamine(Q)-rich domains and a different pattern of LXXLL motifs. LXXLL motifs indicate a potential for association with various hormone nuclear receptors. The ARID region binds DNA without segment specificity and presumably stabilizes the interaction of the complex with chromatin. The ARID sequences are 84% identical (Fig 3).

Functionally, ARID1A and ARID1B have opposing roles in cell-cycle control. They are required for cell cycle arrest and progression respectively. Since cell cycle progression is directly related to cancer proliferation, ARID1B stands out as a protein of interest because it can potentially be a target of anti-tumor therapy (Fig 4).

Additionally, preliminary polymerase chain reaction (PCR) results indicate that ARID1B exists in two forms in Saos2 osteosarcoma cells, and both forms are expressed equally. Currently, no literature suggests a purpose for this alternative splicing. However, the unique exon is located just before the ARID domain, and can potentially influence its DNA binding activity. We intend...
to examine the expression pattern of ARID1B_v1 vs ARID1B_v2 by exploring whether these two forms are expressed in normal osteoblasts and whether they change during differentiation.

**Methods:**

We harvested RNA from various cell lines, synthesized their cDNA, and used appropriate primers to amplify the segment of interest (Fig 6).

- Human osteosarcoma: Saos2, OHS50, U2-OS
- Human lung fibroblast: WI-38
- Mouse calvarial pre-osteoblast: MC3T3-E1

Differentiation into mature osteoblasts was achieved using ascorbic acid and β-glycerol phosphate.

**Cell cultures.** Cells grown in 100 mm plates to confluency with corresponding growth medium.

- MC3T3- α-MEM + 10% FBS + 50 units/mL penicillin and 50 ug/mL streptomycin.
- OHS50, U2-OS, Saos2, WI-38 – McCoy’s + 10% FBS + 50 units/mL penicillin and 50 ug/mL streptomycin.

Differentiation induced at 90% confluency with 50 ug/ml ascorbic acid and 10 mM B-glycerol phosphate.

**RNA extraction.** TRizol Reagent according to manufacture’s protocol.

**cDNA synthesis.** SuperScript III First-Strand Synthesis SuperMix according to manufacture’s protocol.

**PCR.** Expand High Fidelity PCR System according to manufacture’s protocol.

**Conditions:** 94°C 2 min, 30x (94°C 1 min, 55°C 1 min, 72°C 2 min), 72°C 10 min, hold at 4°C. Run through 1.5% Agarose gel at 100 volts.

**Summary:**

While all three osteosarcoma cell lines expressed both forms of ARID1B, the normal osteoblastic MC3T3-E1 expressed only ARID1B_v2. Since osteosarcoma cells are partially differentiated, we speculate ARID1B_v1’s involvement in differentiation.

We induced MC3T3 cells for 4 days to simulate the primary differentiation state of osteosarcoma cells, but no change was seen. Saos2, OHS50 and U2-OS still expressed both forms while MC3T3-E1 only expressed ARID1B_v2. We induced MC3T3-E1 further for one and
two weeks to secondary (matrix formation) and tertiary (onset of mineralization) differentiation states respectively, but still saw no change.

**Conclusion:**

Unlike osteosarcomas, normal osteoblasts expressed only ARID1B_v2 and differentiation is not a direct stimulant to expressing both forms.

The difference in expression could possibly be species related. All osteosarcoma cells were human while MC3T3-E1 was murine-derived. Currently, we are growing human osteoblasts as a future direction for this project.
15. PHILIP LIM (NJMS 2012)

BREAST CANCER CELL DORMANCY INDUCED BY BONE MARROW STROMA

Mentor: Pranela Rameshwar, Ph.D (Medicine)

Objective:
Breast cancer is the most common form of cancer that affects women in the United States and is also the second leading cause of cancer death despite the fact that it is one of the more treatable forms of cancer. Breast cancer has been known to metastasize into other areas of the body including the lung, brain, liver, and bone marrow (BM). Furthermore, recent studies indicate that the recurrence of breast cancer is possible even after years of disease-free remission. We hypothesize that breast cancer cells (BCCs) that metastasize into the BM are capable of entering a state of dormancy through their interactions with BM stroma. Through these interactions, microRNA (miRNA) from BM stroma may enter BCCs through gap junctional intercellular communications (GJIC) and bind to specific mRNA targets. After binding to mRNA, the miRNA leads to the silencing of the target mRNA before it can be translated and results in a change in the protein expression profile when compared to normally mitotic BCCs. While in a state of dormancy, BCCs are less susceptible to chemotherapy and, more importantly, can remain “hidden” within the BM. This ability to evade detection is problematic because dormant BCCs may develop into new breast tumors years after a healthy prognosis. The study of the interaction between BCCs and stromal cells could lead to the identification of novel therapeutic strategies that target BCCs in a dormant state and prevent the recurrence of breast cancer.

Methods:
Cell Culture – T47D breast cancer cells were co-cultured with stromal cells in an equal mixture of each respective cell line’s media. When needed, T47D cells were isolated from co-culture using Invitrogen Dynabead separation.

Flow Cytometry - T47D/stroma co-cultures were stained with propidium iodide and co-labeled with FITC-anti-cytokeratin, and analyzed by a BD Biosciences FACSCalibur.

miRNA Array Analysis - miRNA from T47D, stroma, and T47D/stroma co-culture were isolated and analyzed using Applied Biosystems Taqman miRNA Array.

Western Analysis - Protein extracts were run on SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was incubated overnight with mouse polyclonal IgG cyclin D1 and CDK4 antibody (1:1000 dilution). Goat polyclonal to mouse IgG antibody (1:2000 dilution) was added and detected with Western Lighting Chemi-luminescence Reagent Plus.

Fluorescent Microscopy - T47D and stromal co-cultures were labeled with FITC-Cx-43, Texas-Red-Phalloidin, and DAPI observed with a fluorescent microscope for the presence of connexin-43, cytoskeletal actin filaments, and DNA, respectively.

Summary:
In order to determine if stromal cells had any effect on T47D, I did flow cytometry to see if there was a change in the cell cycle profile of T47D co-cultured with stroma. Flow cytometry indicated that the T47D in co-culture were transitioned into G1-phase arrest whereas T47D cultured alone remained mostly in S phase. This correlated with Western blot analysis showing the reduced expression of cell cycle proteins involved in early G1-phase progression, cyclin D1 and CDK4. Cyclin D1 and CDK4 are required in order to phosphorylate protein Rb and allow for cell cycle progression.
progression. Therefore, down-regulation of these proteins would cause cells to remain in early G1-phase. Using fluorescent microscopy, I then looked for the presence of connexin-43 (Cx-43), the protein involved in the formation of GJIC in T47D/stroma co-culture. In the absence of the GJIC inhibitor octanol, Cx-43 fluoresced between the borders of stroma and neighboring T47D, while co-cultures containing 0.3mM octanol in their media did not fluoresce for Cx-43. This confirmed the establishment of GJIC between stroma and T47D. Next, a miRNA array was performed to see if there was a change in the levels of miRNAs between T47D cultured alone and in co-culture. The array showed that there were 12 miRNAs upregulated (Range: 4 to 256 fold change) and 1 miRNA downregulated (-2 fold change). Figuring out which specific miRNA is responsible for the cell cycle profile change in the co-cultured T47D is the future direction of this project. I will use a pre-miR to over-express a specific miRNA and introduce it to T47D cultured alone to see if they can be transitioning into the dormancy. Additionally, I also plan to use an anti-miR in T47D co-cultures to see if there is inhibition of the miRNAs passing through GJIC and a return to cell cycling in the T47D cells.

**Conclusion:**
Gap juntional intercellular communications (GJIC) do exist between stromal cells and T47D, thus transitioning the breast cancer cells to G1 phase. This correlates with decreased expressions of cell cycle progression proteins: cyclin D1 and CDK4.
16. RICHARD MAY (NJMS 2012)

CELL INTRINSIC SYNERGY OF RB AND E2F8 IN THE CONTROL OF HEMATOPOIESIS AND ERYTHROPOIESIS

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

Objective:
The mx1-cre inducible knockout system has been shown to efficiently knockout DNA fragments flanked by LoxP sites in hematopoietic tissues after injecting polyIpolyC (pIpC) or IFN. Our lab has previously shown that mx1-cre mediated Rb and E2F8 double knockout mice lead to much more severe hematopoietic defects than in Rb (mild) or E2F8 (none) single knockout mice, including anemia, splenomegaly, expansion of myeloid and erythroid progenitors in the spleen and bone marrow (BM), myeloid hyperplasia in BM, and B cell suppression in BM. Despite high similarity and functional redundancy during embryonic development between E2F7 and E2F8, mx1-cre mediated deletion of Rb and E2F7 does not enhance the hematopoietic defects observed in Rb single knockout mice. These data suggest a novel and specific synergy of Rb and E2F8 (but not E2F7) in regulating hematopoiesis. However, it is unclear if this phenotype and synergy are cell intrinsic to hematopoietic stem cells (HSCs) since the mx1-cre deletion leaks into other tissues through non-hematopoietic stem cells. Our objectives are to determine if Rb and E2F8 are cell intrinsic and synergistic to hematopoiesis and erythropoiesis. This will be accomplished through the bone marrow cell (BMC) transplant system specifically targeting HSCs and the EpoR-cre mediated system specifically targeting erythroids.

Methods:
BMC Transplant
BMCs were collected from one month old donor mice. The recipient mice were placed in an irradiation chamber and exposed to a two round irradiation process at a rate of 7+6.5 Gy with a three hour interval. Immediately after irradiation the recipients were intravenously injected with BMCs from the donor mice via a tail vein. Five weeks later the recipient mice were administered seven doses of pIpC to induce LoxP recombination.

CBC and Flow Cytometry Analysis
Peripheral blood from each individual recipient was obtained from the retroorbital plexus and sent to Research Animal Diagnostic Laboratory (RADIL) of the University of Missouri for complete blood count analysis. Reticulocytes in the peripheral blood were stained using new methylene blue and counted under light microscopy. The spleen of each mouse was weighed. Single cell suspensions of bone marrow and spleen cells were prepared. BM, spleen cells, and peripheral blood cells were incubated with lineage specific markers and analyzed using FACSCalibur™ (Becton Dickinson) flow cytometry.

Colony Forming Assay
Single cell suspensions of BM and spleen cells were prepared. 1x10^5 spleen cells and 3x10^4 BM cells were plated in MethoCult® M3630 medium and incubated at 37 degrees Celsius for 14 days. CFU-GEMM, CFU-GM, and BFU-E colonies were then counted using light microscopy.

Statistical Analyses
Statistical analyses were performed using paired and unpaired Student’s t-test, with a P value ≤0.05 being considered significant. Results are depicted as mean +/- standard error of the mean (SEM) for n given samples.
Results:

BMC Transplant System

BMCs were collected from \( mx1-Cre^{+};Rb^{LoxP/LoxP}, E2F8^{LoxP/LoxP} \) and wild type donor mice and injected into lethally irradiated wild type recipient mice. In this system the microenvironment (osteoclasts, osteoblasts, and mesenchymal stem cells) remains wild type in the recipient mice. Only HSCs \( (mx1-Cre^{+};Rb^{LoxP/LoxP}, E2F8^{LoxP/LoxP}) \) in the recipient mice recombine after the transplant when the mice are injected with pIpC. Mice receiving Rb and E2F8 double knockout BM cells also showed severe anemia (Figure 1a).

![Figure 1. Deletion of Rb and E2F8 in only HSCs can cause severe anemia, splenomegaly, and increased erythroid progenitors in the spleen.](image)

In BM, the erythroid progenitor cells significantly increased in the double knockout (Figure 2a), the granulocytes slightly increased (Figure 2b), and the B cells significantly decreased (Figure 2c). In the spleen and BM, Rb, E2F7, and E2F8 triple knockout mice did not lead to drastic changes in the observed phenotypes compared to Rb and E2F8 double knockout mice, suggesting that the observed synergy results from Rb and E2F8, not Rb and E2F7. Subsequently, the defects from the Rb and E2F8 double knockout mice in this system recapitulated the defects observed in the mx1-cre system.

![Figure 2. Abnormal expansion of erythroid progenitors, slight expansion of granulocytes and suppression of B cells in the bone marrow of mice receiving Rb and E2F8 deficient BMCs.](image)

EpoR-cre Mediated System

The EpoR-cre system is using the erythropoietin receptor promoter to control the expression of cre, which is used to delete conditional alleles in the erythroid lineage. Recombination occurs after Epo expression, which begins at the BFU-E stage, which is the first committed cell stage
in erythropoiesis, maximized at the CFU-E and early proerythroblast stages, and decreases at the late proerythroblast stage\(^5\). In this system, Rb and E2F8 double knockout mice showed more severe erythropoietic effects than Rb and E2F8 single knockout mice. We found anemia and reticulocytosis in EpoR-cre mediated Rb and E2F8 double knockout mice (Figures 3a and 3b). Splenomegaly was observed in these mice (Figure 3c). There were also increased erythroid progenitors in the spleen (Figure 3d) and BM (Figure 3e). In these mice, there were no significant changes in granulocytes or B cells, as expected (data not shown). Rb, E2F7, and E2F8 triple knockout mice did not lead to drastic changes in the observed phenotypes. This suggests that there is synergy between Rb and E2F8 and not between Rb and E2F7 in the control of erythropoiesis.

**Figure 3.** Deletion of Rb and E2F8 only in erythroid lineage can cause anemia, reticulocytosis, splenomegaly, and increased erythroid progenitors in the spleen and BM. Hemoglobin levels (a), reticulocyte percentage (b), spleen weight (c), and early erythroblast percentage in spleen (d) and BM (e) in mice with the indicated genotypes.

There is a significant increase in BFU-E in the spleen (Figure 4a) and BM (Figure 4b) in Rb, E2F7, and E2F8 triple knockout mice and Rb single knockout mice, but no significant change in CFU-GEMM or CFU-GM, which is consistent with the expansion of erythroid progenitors. Further experiments will verify BFU-E increases in Rb and E2F8 double knockout mice. The defects from the Rb and E2F8 double knockout mice in this system recapitulated the erythropoietic defects observed in the mx1-cre system.

**Figure 4.** Rb, E2F7, and E2F8 triple knockout mice can lead to an increase in BFU-E colonies. CFU-GEMM, CFU-GM, and BFU-E colonies per 1x10^5 spleen cells (a) and 3.3x10^4 BM cells (b).

**Conclusion:**
Mx1-cre mediated Rb and E2F8 double knockout mice showed a more severe phenotype than predicted from Rb and E2F8 single knockout mice. This included severe anemia, splenomegaly, substantially increased erythroid progenitors in the spleen and BM, myeloid hyperplasia, and B cell suppression. The Rb and E2F8 double knockout mice in the BMC transplant system recapitulated these defects which led us to conclude that Rb and E2F8 are cell intrinsic and
synergistic to hematopoiesis. The Rb and E2F8 double knockout mice in the EpoR-cre mediated system recapitulated the erythroidic defects (anemia, splenomegaly, and increased erythroid progenitors), but it did not recapitulate myeloid hyperplasia in BM or B cell suppression in BM in the mx1-cre system which led us to conclude that Rb and E2F8 are also cell intrinsic and synergistic to erythropoiesis.

References:
17. VARUN NAKHATE (TCNJ 2010)

IDENTIFYING THE EFFECT OF MUTATED RFA 1 AND 2 GENES ON ddc2-S4 PHENOTYPE SUPPRESSION

Mentor: Katsunori Sugimoto, PhD, (Cell Biology and Molecular Medicine)

Objective:

DNA damage checkpoint mechanisms are integral in the study of controlled and uncontrolled cellular proliferation or inhibition. DNA damage can be the product of various detrimental pathogens or even regular bodily processes such as aerobic respiration.

In human cells ataxia-telangiectasia mutated (ATM) and ATM-Rad3-related (ATR), coordinate the cellular response to DNA damage. In *Saccharomyces cerevisiae* or budding yeast, the ATR kinase homologue Mec1 kinase, plays an integral role in DNA damage checkpoint activation along with ATRIP, whose homologue is Ddc2. Mec1 functions with Ddc2 in a complex that the two proteins form. They associate to the damaged DNA in the complex that they form. Mec1 phosphorylates substrates downstream to induce DNA damage checkpoint¹.

In this particular study *ddc2-S4*, a mutated form of Ddc2 was studied along with mutated subunits of Replication Protein A (RPA), a single stranded heterotrimeric DNA binding protein².

RPA is comprised of RFA 1, 2 and 3. RFA 1 and RFA 2 interact with the Ddc2-Mec1 protein complex after damaged DNA is identified.

It was hypothesized that only a single copy of *rfa1-1*, a mutation in RFA1 which is carried on the plasmid, is required to suppress the *ddc2-S4* phenotype in the presence of the wild type RFA 1 gene.

Some substitutions in RFA2 are expected to suppress the *ddc2-S4* mutation’s phenotype in a similar fashion as the *rfa1-1* mutation.

Methods:

*rfa1-1* Mutation Dominance

PCR was employed to generate mutate the RFA 1 gene and produce *rfa1-1* segments in the high copy plasmid YEplac 195. This was accomplished by the addition of MnCl₂ during PCR. The amplified product was then transformed with *ddc2-S4* containing colonies and plated out in Ura- media. Cells from the plate were later introduced in a high copy plasmid, YEplac 112, and low copy plasmids YCplac 22 and YCplac 33. The cells would undergo homologous recombination in-vivo and incorporate themselves in the above plasmids. Select cells were finally transferred to HU media to see if the *ddc2-S4* phenotype was suppressed.
RFA2 screening

RFA2 was mutagenized by random mutations. MnCl₂ was used again to introduce mutations in the PCR fragment. The mutated rfa2 fragment was introduced in the high copy plasmid YEplac 195 by homologous recombination in vivo. Cells harboring the rfa2 gene were grown in Ura- media and select colonies were then introduced in Hydroxyurea (HU) media that inhibits DNA replication. After these colonies grew in the HU media, they were then transferred to Ura- and FOA media. All transformants at this stage have a Ura gene because the gene is carried by the YEp 195 plasmid, and therefore should grow in Ura- media. Cells from both these plates were then introduced to HU Media and Ura- media to investigate cell proliferation in the presence and absence of a plasmid.
Summary:

*rfa1-1 Mutation Dominance*

A mutation would be considered dominant negative if a single copy of the mutation is enough to suppress cell proliferation in the presence of one copy of its wild type counterpart. Yeast cells have a recombination efficiency of 50%.

*ddc2-S4* is sensitive to HU media. Therefore suppression of the *ddc2-S4* phenotype would result in cell proliferation in the media. Our data shows that there was almost complete suppression of the *ddc2-S4* phenotype in the low copy plasmids. This was indicated by four of five colonies selected showing cell proliferation. The fifth colony showed some cell growth but was indicative of incomplete suppression of the phenotype.

It can therefore be concluded that the mutation is dominant as it masks the effect of the wild type gene and suppresses cell proliferation in low copy plasmids.

*Figure 2: Experimental approach used to conduct RFA2 screenings*
RFA2 Screenings

Among the two screenings carried out to isolate RFA2 mutations, the first screening had considerably less numbers of colonies growing in the Ura- plates. It was believed that the sparse amounts of transformants in this screening may have been due to the usage of a smaller amount of mutated rfa2 gene during the transformation. Therefore, during the second screening, the amount of rfa2 used increased 8-fold. The second screening of rfa2 yielded about 1000 transformants as expected in five plates. Twenty colonies were acquired from the plates and were then transferred to HU media.

Later these colonies were transferred to Ura- media and FOA because the one of the goals of the experiment was to deduce whether the rfa2 mutation lies on the plasmid or not. FOA media is known to drop the plasmid out. Colonies were acquired from FOA media and introduced in HU media in the final step. It was predicted that these colonies would demonstrate complete suppression as it was hypothesized that the rfa2 mutation lies on the plasmid. Therefore, cell proliferation would be impossible due to the loss of plasmid in FOA media.

Contrary to our hypothesis, there were equal amounts of growth in colonies acquired from both Ura- media and FOA media suggesting that the loss of plasmid plays no role in suppression of the mutation. In order to further study the phenomenon, it is recommended that another screening be carried out to understand the role of the plasmid. If similar results are again acquired, it may be an appropriate measure to revise the hypothesis.

Conclusion

rfa1-1 Dominance

Almost complete suppression of ddc2-S4 was observed in low copy plasmids, masking the effect of the wild-type RFA 1 gene. Therefore, the rfa1-1 mutation is dominant negative. Failure to see complete inhibition in all cases could be attributed to contamination or one colony reverting to wild-type phenotype.

RFA2 Screenings

It was hypothesized that some substitutions in RFA2 were expected to suppress the ddc2-S4 in a similar fashion that rfa1-1 did. However, the screenings did not support the hypothesis as the two screenings conducted both indicated that the colonies didn’t carry the rfa2 mutation on the plasmid. It is recommended that further screenings be done that will support the hypothesis or that the hypothesis be changed if similar results are observed again.

Works Cited

18. SHEENAL PATEL (NJMS 2012)

EFFECT OF HYPMETHYLATION ON STAT3 ACTIVATION IN PROSTATE CANCER

Mentor: Beverly E. Barton, PhD (Surgery)

Introduction:
In 2009, prostate cancer is once again expected to be the most diagnosed cancer (excluding skin cancers) and the second greatest cause of cancer death in men. It is expected that there will be about 192,000 new cases and about 27,000 deaths due to prostate cancer in men this year (1).

Signal transducers and activators of transcription (STATs) are proteins involved in growth factor and cytokine signal transduction pathways. They mediate gene expression through their effects on transcription and are activated by phosphorylation (2). When phosphorylated, STATs will ultimately migrate to the nucleus and affect gene transcription (3). One particular STAT, STAT3, has been shown by our lab and many others to be involved in the malignant phenotype of prostate cancer among many other types of cancer. Specifically, STAT3 has been shown to be expressed constitutively in malignant cancer cells and is key to their survival (2). Therefore, therapies that may affect the expression or phosphorylation state of STAT3 may be important therapies in the treatment of prostate cancer.

5-aza-2’-deoxycytidine (5-aza-CdR), a DNA methyltransferase inhibitor, has been shown to decrease levels of P-STAT3 in some cancers, including multiple myeloma and large cell lymphoma (3, 4). However, depending upon the cancer type, 5-aza-CdR may or may not induce apoptosis. Moreover, the effect of 5-aza-CdR on prostate cancer with respect to P-STAT3 has not been evaluated. In this study, we hypothesized that treating a prostate cancer cell line with 5-aza-CdR decreased STAT3 phosphorylation and thus activation, thereby leading to apoptosis.

Methods:
Cells and reagents: DU-145 cells were maintained in DMEM/Ham’s F12 (Invitrogen) culture medium plus 10% newborn calf serum (Hyclone) as described (2). 5-aza-CdR was obtained from Alexis Biochemicals. It was dissolved in DMSO, which was used as the vehicle control in some samples.

Cell viability and proliferation studies: DU-145 cells were seeded in 12 well plates at 100,000 cells/well, and treated with 5-aza-CdR or vehicle for 24, 48, 72, and 96 hours. Harvested cells were analyzed for cell viability using fluorescein diacetate (FDA) staining and a Neubauer hemocytometer with a Zeiss Universal RIII fluorescence microscope. The following equations were used to determine % viability and % inhibition of proliferation:

\[
\text{% viability} = \frac{\# \text{ FDA stained cells}}{\# \text{ total cells}} \times 100
\]

\[
\text{% inhibition of proliferation} = \frac{\# \text{ total cells at each concentration of 5-aza-CdR}}{\# \text{ total cells in the vehicle control}} \times 100
\]

Determination of STAT3 and P-STAT3 by western blot: DU-145 cells were seeded at 200,000 cells/well in 6 well plates and treated with 5-aza-CdR or vehicle for 48 hours, 72 hours, and 96 hours. Lysates were prepared using standard procedures. STAT3 was detected by western blot analysis using anti-STAT3 antibody (Santa Cruz Biotechnology) after transferring
proteins from gels to PVDF membranes (Millipore) using a Hoefer transfer apparatus. PVDF membranes were blocked with 5% non-fat dry milk prior to probing with antibody. Blots were read on a Typhoon imager (Molecular Devices). Immunoprecipitation was done for determination of P-STAT3 using Protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Pre-cleared beads were rotated with cell lysates in the cold overnight. Washed beads were then heated with sample buffer and loaded onto precast gels. Western blots were performed as described, using anti-P-STAT3 antibody (Santa Cruz Biotechnology).

Results:
**Effect on DU-145 viability and proliferation:** Treatment of DU-145 cells for 24, 48, 72, or 96 hr with 10, 30, and 100 μM 5-aza-CdR or vehicle had no significant effect on viability. These results are shown in Figure 1. However, as little as 10 μM 5-aza-CdR over 48 hr inhibited proliferation by about 50%. Increased inhibition of proliferation was not observed over 96 hr or by increasing the concentration of 5-aza-CdR to 100 μM. These results are shown in Figure 2.

**Expression of STAT3 in DU-145 cells:** Treatment of DU-145 cells with up to 100 μM 5-aza-CdR for 48, 72, or 96 hr did not affect the expression of STAT3 as shown by western blot analyses in Figures 3a and 3b.
Expression of P-STAT3 in DU-145 cells: Treatment of DU-145 cells with up to 100 \( \mu \text{M} \) 5-aza-CdR or vehicle followed by immunoprecipitation and western blot analysis did not show expression of P-STAT3 in either untreated and treated samples. These results are shown in Figures 4a and 4b.

Conclusions:
Previous studies showed that 5-aza-CdR, a DNA methyltransferase inhibitor, plays a role in reactivating expression of genes that may have been epigenetically silenced by hypermethylation in tumor cells (6). Through its hypomethylating effects, 5-aza-CdR affects regulatory pathways for STATs and other intracellular signaling pathways in hepatoma cells, colon cancer cells, lymphoma cells, and myeloma cells (3, 4, 6, 7). In DU-145 prostate cancer cells, studies have shown that treated cells with 5-aza-CdR at concentrations up to 100 \( \mu \text{M} \) exhibited <7.5\% cytotoxicity over 5 days. Additionally, DU-145 cells treated with 8.8 \( \mu \text{M} \) 5-aza-CdR exhibited 40-50\% inhibition of proliferation over 5 days (5). Therefore, 5-aza-CdR inhibited proliferation without inducing apoptosis in DU-145 cells.

We observed that DU-145 cells treated with 5-aza-CdR for 96 hr exhibited no significant effect on viability at concentrations up to 100 \( \mu \text{M} \) (Figure 1). However, as little as 10 \( \mu \text{M} \) 5-aza-CdR decreased the proliferation of DU-145 cells by ~50\% (Figure 2). Similar decreases in proliferation were seen when cells were treated with 30 and 100 \( \mu \text{M} \) 5-aza-CdR. As reported previously, DU-145 cells remained viable but their growth was inhibited after treatment with 5-aza-CdR (5). Because the viability of DU-145 cells treated with 5-aza-CdR remained fairly constant, we concluded that apoptosis was not induced.

Expression of STAT3 in DU-145 cells treated with up to 100 \( \mu \text{M} \) 5-aza-CdR was not changed when the drug was present in the cells for up to 96 hr (Figure 3). These data match what was observed in other studies with large cell lymphoma and myeloma cells (3, 4).

Expression of P-STAT3 in DU-145 cells treated with up to 100 \( \mu \text{M} \) 5-aza-CdR or vehicle was not observed (Figure 4). Although the amount of cell lysate used in this experiment was within limits of volume suggested by the manufacturer, our results indicate that a greater amount is required.
to visualize P-STAT3. P-STAT3 could not be visualized even after approximately doubling the amount of cell lysate used initially in both untreated and treated samples. Further experiments will be conducted using immunoprecipitation and western blot analysis to visualize P-STAT3.

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I would especially like to thank Adetola Shodeinde for her assistance with western blot and immunoprecipitations. I would also like to thank H. Dan Lewis and Kalyani Ginjupalli for their assistance with general laboratory techniques, especially cell culture.

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19. ERICA PIMENTA (NJMS 2016)

CHARACTERIZATION OF THE INHIBITORY EFFECTS OF NALTRINDOLE ON MULTIPLE MYELOMA CELL PROLIFERATION

Mentor: Richard D. Howells, PhD, (Biochemistry & Molecular Biology)

Objective:
The goal of this study was to characterize the naltrindole binding site in human U266 multiple myeloma cells and to investigate the anti-proliferative effect of naltrindole on this cell line. Multiple myeloma (MM) is characterized by the uncontrolled growth of plasma cells originating from post-germinal center B cells in the bone marrow (1). Only 1% of unchecked plasma cell growth actually progresses to myeloma; the others remain asymptomatic. These cells typically produce huge amounts of immunoglobulin (IgA, IgG) or immunoglobulin light chains, kappa or lambda (2). MM cells activate osteoclasts and suppress osteoblasts; these cells work together to maintain a healthy, normal bone density. However, when osteoclasts are activated relative to osteoblasts, more bone is broken down leading to bone lesions, one of the physical manifestations of the disease. Other symptoms include anemia, infections and extreme renal dysfunction. This disease is not seen in children, and typically effects individuals at a median age of 70 years. MM makes up 10% of all hematological and 1% of total cancers (2). An exceptionally poor prognosis accompanies the diagnosis of MM. The median survival is only 3 years with the current treatment options available. The disease is difficult to treat for several reasons. Constant genetic change renders MM cells resistant or adaptable to most chemotherapeutic agents and MM cells are protected by the bone marrow microenvironment (2). Interleukin-6 (IL-6) is an abundant growth factor present within the bone marrow that contributes to an anti-apoptotic atmosphere, along with insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF), that promotes angiogenesis (1). Current treatments include the use of thalidomide and lenalidomide that induce DNA damage, and bortezomib, a proteasome inhibitor that activates caspase 5 and decreases MM cell proliferation. Combinatorial therapy including bortezomib may be more effective after more is known about targets for the disease. Bone marrow transplants have also been utilized but still 95% of treated MM patients relapse (2).

Opioid agonists, such as morphine, are potent analgesic drugs and are commonly used to decrease the bone pain that usually accompanies MM. Opioid antagonists, however, are used to treat opioid drug overdoses, and are also prescribed to decrease relapse in alcoholic patients under treatment. Naltrindole is a delta opioid receptor antagonist that our laboratory has recently shown to inhibit the proliferation of human MM cells. This effect, however, is not mediated by the well-studied mu, delta or kappa opioid receptors, and appears involve a novel naltrindole binding site. Two important questions are investigated here: What are the properties of the naltrindole binding site and how does naltrindole affect cell proliferation in MM cells?

Methods:
Cell culture. U266 cells were maintained in RPMI media supplemented with 10% Cosmic Calf Serum and 1% Penicillin Streptomycin. Cell dishes were split or utilized at confluence. Each method/experiment described below was performed at least twice.

Binding Assays. [3H]-Naltrindole binding assays were performed on whole cells in 100 μl of phosphate-buffered saline (PBS) at room temperature for 30 min. This time has been found sufficient to reach equilibrium. Nonspecific binding is determined in the presence of 200 μM unlabeled naltrindole. Assays were terminated by filtration through Whatman GF/B filters and the filters are washed twice with 4 ml PBS. Filters were soaked in EcoScint H liquid scintillation fluid (National Diagnostics, Somerville, NJ) before determination of filter-bound radioactivity.
using a Beckman LS 1701 scintillation counter (Beckman Coulter Inc., Fullerton, CA). Receptor binding data was analyzed by nonlinear regression using Prism 3.0 (GraphPad Software, San Diego, CA). Protein concentrations were determined with the DC protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard.

Detergent Extraction of MM Cells. To determine if the naltrindole binding site was viable after cell membranes were solubilized, U266 cells were resuspended in PBS containing 0.5% dodecyl maltoside or digitonin solution for 15 min on ice. Detergent extracts were then spun at 40,000 and 13,000 rpm, respectively, for 30 min. The supernatant was recovered and binding assays were conducted as described above, except that proteins were precipitated using 0.5 ml of 7% PEG and 0.1% γ-globulin prior to filtration and washing.

Inhibiting Protein Synthesis. U266 cells were incubated for varying lengths of time (from 30 min to 9 h) in 5 µM cycloheximide in culture media. Cells were then centrifuged and re-suspended in PBS. Naltrindole binding assays were performed as described above.

Reactive Oxygen Species (ROS) Assay. U266 cells were pre-incubated in 50 µM naltrindole overnight. The Cell Technology Fluorescent Thiol Detection Kit protocol was followed using H₂O₂ (100 µM) as a positive control. The 96-well plate was read at 485 nm excitation and 535 nm emission.

A second ROS assay was also used. The procedure followed was as described in the Molecular Probes Reactive Oxygen Species Detective Reagents manual. The U266 cells were cultured in RPMI/phenol red-free media overnight. The cells were incubated with 10 µM chloromethyl-H2DCFDA dye for 1 h and allowed a 15 minute recovery time following resuspension in phenol red-free medium before reading the plate at 485 nm excitation and 535 nm emission.

Histone Deacetylase Inhibition. U266 cells were incubated overnight in 5 mM valproic acid (VPA). Cells were then centrifuged, rinsed in 1x PBS and resuspended in PBS prior to conducting naltrindole binding assays.

Summary:

The purpose of this study was two-fold: to further characterize the naltrindole binding site in MM cells and to examine the effect of naltrindole on MM cell proliferation. By using cycloheximide to inhibit protein synthesis, we were able to analyze the turnover time of the naltrindole binding activity. Nonlinear regression analysis of the decay curve indicated that the binding site activity had a half-life of 4.55 h.

Our laboratory has previously demonstrated that in addition to its high affinity for the delta opioid receptor, naltrindole also binds to the serotonin 5-HT₁B receptor. To observe if other 5-HT₁B receptor ligands may bind to the site we looked at competitive inhibition of naltrindole binding with serotonin hydrochloride (5-HT), 5-carboxamidotyramine (5-CT), trifluoperazine dimaleate (TFP), and dihydroergotamine tartrate (DE). Consistently, 5-HT and 5-CT did not interfere with naltrindole binding at all but DE and TFP both did. Naltrindole has an IC₅₀ of 18 µM while that of DE was 39 µM. Another characteristic displayed by the naltrindole binding site was that it must remain intact within the lipid bilayer to functionally bind naltrindole. Little to no binding activity was recovered in detergent extracts. In addition, binding activity was maximal when binding assays were performed at room temperature rather than 37 °C. An interesting characteristic of the naltrindole binding site was that its level was greatly affected by HDAC inhibition. Previous studies using valproic acid (VPA) to block HDACs within the cell yielded a 2.5 fold increase in naltrindole binding activity following overnight treatment. Saturation curve analysis revealed that more than twice as many naltrindole binding sites were present after HDAC inhibition, with no change in affinity of naltrindole, however, these results were not consistently observed and require further study.

To explore possible mechanisms responsible for the anti-proliferative effect of naltrindole on MM cells, a reactive oxygen species (ROS) assay was preformed. The assay measured...
“free” or reduced glutathione (GSH) within cells. GSH is one of the ways the cell quenches dangerous ROS that may cause it damage. Presumably, the smaller the amount of reduced GSH within the cell, the larger the amount of ROS. With the addition of naltrindole, a 50% reduction in free GSH was observed. Also, naltrindole seems to act additively with the positive control hydrogen peroxide to increase the amount of ROS even more so. This may be one of the mechanisms through which naltrindole exhibits its anti-proliferative effect in U266 cells.

**Conclusion:**

Multiple myeloma is a devastating disease that is characterized by the uncontrolled growth of plasma cells within the bone marrow. Patients diagnosed with MM have a typically poor prognosis and usually succumb to the disease. With only a 3 year median survival and 95% relapse rate even after the best current therapy, myeloma is one of the most difficult cancers to treat. Opioids have been used for pain management for many different diseases, including myeloma. Interestingly an opioid antagonist, naltrindole, has been found to decrease cell proliferation in MM cell lines. Naltrindole does not exert this effect through an opioid-receptor mediated pathway. Therefore, the novel naltrindole binding site must be characterized and the mechanisms through which its anti-proliferative activity occurs must be explored in order to elucidate new targets and treatments for MM.

In terms of characterizing the binding site, room temperature has emerged as the best condition for conducting binding assays. The half-life of binding activity was determined to be 4.55 hours. HDAC inhibition has been shown to significantly increase naltrindole binding due to an increase in the amount of naltrindole binding sites.

While naltrindole may act on several different cellular pathways to inhibit U266 cell proliferation, generation of ROS was observed to be one of them. Incubating U266 cells with naltrindole decreased GSH levels by approximately 50%.

Initial *in vivo* studies conducted in our laboratory have indicated that naltrindole significantly decreases the rate of MM tumor growth in a SCID mouse xenograft model. In the future, additional *in vivo* studies will be conducted to find an optimal dose, treatment regimen and chemotherapeutic combination for effective tumor inhibition. The binding site will continue to be characterized while other anti-proliferative mechanisms are explored. It has been previously demonstrated that, in rodents, blockade of the delta opioid receptor by naltrindole attenuates the development of morphine tolerance while not affecting its analgesic activity. Thus naltrindole may be prove to be a powerful weapon for treating MM, by decreasing tolerance to morphine for pain management, and, at the same time, as an anti-proliferative agent for MM cells.

**References:**

20. CHRISTOPHER STADLER (NJMS 2012)

THE FEMORAL NECK-LESSER TROCHANTERIC AXIS AS A LANDMARK FOR TUMOR ENDOPROSTHESIS PLACEMENT

Mentors: Francis R. Patterson, MD, Joseph Benevenia, MD, Kathleen S. Beebe, MD, Howard Goodman, MD (Orthopaedic Surgery; Division of Musculoskeletal Oncology)

Objective:
Following osteosarcoma of the distal femur and subsequent resection of the affected portion of the bone, a distal endoprosthesis is often used to replace the removed section. The goal of distal femoral prosthesis implantation is to reconstruct the original femoral neck anteversion angle, which is generally accepted to be 15 to 20 degrees with respect to the intercondylar axis. Correct prosthetic placement is challenging due to the tumor’s destruction of bony landmarks and the absence of proximal femoral anatomy exposure during surgery.

The purpose of this experiment is to use the lesser trochanteric axis as a landmark to be used in distal femoral replacement. It is hypothesized that pre-operative measurement of this angle, combined with visualizing the lesser trochanter with intra-operative fluoroscopy would allow for accurate rotational placement of the prosthesis in accordance with the patient’s individual anatomical axes. In the end, normal, or near-normal, limb function would be restored.

Methods:
A) Measurements of the femoral neck axis and the lesser trochanteric axis were taken of 50 patients from axial femoral CT scans that are on electronic record in a picture archiving and communication system (PACS). The angle tool on PACS was used to determine version angles. With the horizontal plane at zero degrees, the femoral neck and lesser trochanter angles were additive values. Average values and variability of these data were computed.

B) Sixteen cadaveric femur specimens were CT scanned and actual femoral neck and lesser trochanteric angles were measured. This represents the pre-operative measurement of a patient’s original femoral neck version angle.

C) The distal portion of the femurs were then removed, and the previously measured lesser trochanteric axis was translated to the bone by rotating the specimen under “real-time” fluoroscopy until the lesser trochanter is at maximal view on the monitor. A distal femoral endoprosthesis was then positioned and anteverted from this alignment in accordance with the original angle of retroversion of the lesser trochanter. Finally, the post-implantation lesser trochanter angle was measured with respect to intercondylar axis using a goniometer. This value was then subtracted from the femoral neck to lesser trochanter angle to give the angle of femoral neck anteversion. This measurement was statistically compared to the measurement of the pre-implantation lesser trochanter angle.

Summary:
The measured angles from the fifty patients on the PACS system revealed a considerable amount of variability in both femoral neck anteversion and lesser trochanter retroversion. The average value for femoral neck anteversion was 13.2 (standard deviation of 6.5). Values ranged from 2.7 degrees to 27.3 degrees. The values for retroversion of the lesser trochanter showed an average angle of 22.0 degrees below the horizontal (standard deviation of 8.2). The values ranged from 2.5 to 40.9 degrees.

The cadaver bones showed less variability in the femoral neck angles than did the PACS CTs with an average of 6.4 degrees (standard deviation of 5.0). The range was from -2.7 to 14.8 degrees. The lesser trochanter numbers showed more variability than the PACS CTs with an average of 28.9 (standard deviation of 9.1). The range shrunk to 10.7 to 39.6 degrees.
The recreated femoral neck angles were found by subtracting the recreated lesser trochanter angle from the total value of the femoral neck to lesser trochanter axis.

There were small differences between the angles measured on the “pre-op” CT and the angles that were recreated using fluoroscopy and the goniometer. The differences between the femoral neck angles measured on the CT scan and those recreated with this technique ranged from zero to 7.7 degrees with an average difference of 2.08 degrees.

**Conclusion:**
In view of the purpose of recreating an individual’s original anatomical axes after a distal femur resection, the real-time fluoroscopy proved to be a reliable method. Considering the high variability between femoral neck and lesser trochanter version angles among the CTs stored on the PACS system, it would seem a necessary step to perform a pre-operative CT scan in order to determine these angles on each surgical candidate. These pre-operative measurements would serve as faithful markers for the insertion of the distal endoprosthesis. In the case of this experiment, the lesser trochanter was a readily apparent and reliable landmark (when maximized under fluoroscopy) for recreating the angles that were measured pre-operatively. Despite variability between patients’ angles, the technique of rotating the lesser trochanter into maximal view, treating this as the horizontal, and then using the lesser trochanter retroversion value as the angle for intercondylar anteversion (from the lesser trochanter) is clearly a reproducible method. An average difference of 2.08 degrees between actual, “pre-op” angles and the fluoroscopically recreated angles is negligible. A formal study was not performed which tested this technique in an actual intra-operative setting.

**References:**
21. BENJAMIN TAYLOR (NJMS 2012)

MAPPING OF XPB AND p210 BCR/ABL INTERACTION

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

Objective:
Patients with chronic myelogenous leukemia (CML) typically carry a balanced reciprocal translocation between chromosomes 9 and 22, resulting in the production of an in-frame fusion protein known as p210 BCR/ABL. Expression of p210 BCR/ABL in myeloid cells is associated with a variety of transformed cellular phenotypes including changes in nucleotide excision repair (NER). Consistent with this, previous studies have demonstrated that p210 BCR/ABL interacts with xeroderma pigmentosum group B (XPB), a protein necessary for both transcription and NER. The lab has previously mapped the docking site for XPB within the RhoGEF domain of BCR. It has also been shown that disrupting the interaction between p210 BCR/ABL and XPB by removing the binding site from BCR, mice lived significantly longer as shown by using a bone marrow transplantation model. Therefore, the aim of this project was to better understand the p210 BCR/ABL and XPB interaction by mapping potential binding sites of BCR to XPB sequences.

Methods:
The full length XPB that was used had never been fully sequenced. In order for our experimental results to be valid, several internal primers were created to ensure there were no mutations in the full length XPB. Then, using site-directed mutagenesis, complementary sets of primers were designed at residues 305 and 629 on full length XPB, which introduced double base pair substitutions to create stop codons.

The truncated variants of XPB were created using pcr-based site directed mutagenesis, and then were digested with the restriction enzyme dpn to remove all bacterially methylated DNA. Both pcr reaction mixtures were run on a gel and then extracted and purified. Next, the 1-305 and 1-629 fragments were transformed with DH5α bacterial cells and plated on LB agar overnight. After picking and growing the colonies overnight, they were then mini-prepped to extract the DNA. The 1-305 and 1-629 fragments were verified initially by cutting them with the restriction enzyme SmaI and running them on a gel. Then the fragments were sent for sequencing using several internal primers to ensure there were no additional mutations. After verifying the DNA sequence, more DNA was needed to run the yeast 2-hybrid analysis, so after another round of transforming, plating, and mini-prepping, the DNA was cleaned using a DNA cleanup kit.

To prepare for the yeast 2-hybrid experiment, the yeast reporter strain, PJ694A, which included the empty vector, pGBT9, was re-plated on YPD media and also media lacking tryptophan. Then, the same re-plating was done with the yeast reporter strain and the vector, which included full length BCR. When the yeast 2-hybrid experiment took place, there were 8 plates. The empty vector pGBT9 was used for the first four plates. The full length BCR in the pGBT9 vector was used in the last four plates. The first of four conditions included the vector, pGAD, with full length XPB. The second condition included pGAD with the 1-629 fragment. The third condition included pGAD with the 1-305 fragment. And the fourth condition was just the pGAD vector alone. These plates were all lacking leucine and tryptophan. After 2-4 days of incubation in 30°C, 4 colonies were removed off each of the 8 plates. Each colony was then put into a well dish, stamped, and plated. Plates lacking leucine and tryptophan (Leu/Trp) were examined for
growth on histidine deficient plates (Leu/Trp/His). Interactions between proteins were demonstrated by the ability to activate the his3 reporter gene.

**Summary:**
The full length XPB and the two mutagenic clones were all validated by sequencing before being tested for their ability to interact with full length BCR. (Figure 1)

The yeast 2-hybrid analysis revealed in the first four conditions that there was no binding as indicated by lack of growth on the Leu/Trp/His lacking media. This was expected as there was no full length BCR present for XPB to bind. In the last four conditions, there was binding with XPB, which was expected since there was no alteration to the full length DNA between BCR and XPB. The 1-629 fragment showed binding, however, there was no binding with the 1-305 fragment. These results are novel and indicate there is most likely a binding site between residues 305 and 629. (Figure 2)

**Conclusion:**
It was found that the 1-305 fragment did not bind to full length BCR, however, the 1-629 fragment did demonstrate binding. These results suggest that at least one binding site for BCR on XPB is between residues 305 and 629, and this binding may be important for both disease progression and lineage determination in CML.

Future direction would include constructing two fragments: between residues 305-629, and from 629-782. Then after a yeast 2-hybrid analysis narrowing down which fragment contains the binding domain for BCR, the binding site could be further narrowed down using site directed mutagenesis.
References: