NEW JERSEY MEDICAL SCHOOL
Cancer Education Program
ABSTRACTS
2010
NEW JERSEY MEDICAL SCHOOL
OF UMDNJ

Funded by NCI Cancer Education Program Grant R25 CA019536

CANCER SUMMER STUDENT
RESEARCH ABSTRACTS

2010

Harvey L. Ozer, MD, Principal Investigator
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 (competitive renewal in 2010). This program, which has been continuously funded by the NCI for 41 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 21 medical students and one undergraduate 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, 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 New Jersey Medical School website under the NJMS on iTunes tab at the following URL:

http://njms.umdnj.edu/
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.

Raymond Birge, PhD  
Department of Biochemistry & Molecular Biology

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NJMS-UH Cancer Center and NJMS Office of Research

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NJMS-UH Cancer Center and Department of Microbiology & Mol. Genetics

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CANCER EDUCATION PROGRAM FACULTY

While the Cancer Center building currently houses 14 laboratories, the broader Cancer Center Community (CCC) is composed of 92 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 2010 participating faculty and their respective departments are listed below.

Beverly Barton, PhD  Department of Surgery
Kathleen Beebe, MD  Department of Orthopedics
Joseph Benevenia, MD  Department of Orthopedics
Raymond Birge, PhD  Department of Biochemistry and Molecular Biology
John Capo, MD  Department of Orthopedics
Susan Feldman, PhD  Department of Radiology
Christopher Fritton, PhD  NJMS-UH Cancer Center and Department of Orthopedics
Roger Howell, PhD  NJMS-UH Cancer Center and Department of Radiology
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Ana Natale-Pereira, MD,PhD  Department of Medicine
Nicholas Ponzio, PhD/ Vincent Tsiagbe, PhD  Department of Pathology and Laboratory Medicine
Pranela Rameshwar, PhD  Department of Medicine
Elizabeth Raveche, PhD  Department of Pathology and Laboratory Medicine
Melissa Rogers, PhD  Department of Biochemistry and Molecular Biology
George Studzinski, MD, PhD  Department of Pathology & Laboratory Medicine
Katsunori Sugimoto, PhD  Department of Cell Biology and Molecular Medicine
Robert Wieder, MD, PhD  NJMS-UH Cancer Center and Department of Medicine
Teresa Wood, PhD  NJMS-UH Cancer Center and Department of Neurology and Neurosciences
Lizhao Wu, PhD  NJMS-UH Cancer Center and Cell Biology and Molecular Medicine
Lionel Zuckier, MD  Department of Radiology
NJMS-UH Cancer Center
2010 Cancer Summer Student Research Program

Abdel-Kareem Beidas
Natale-Pereira Lab

Daniel Rubinstein
Sugimoto Lab

Deepa Charla
Levison Lab

Frank Wang
Wieder Lab

Eliza Lamin
Ponzio Lab

Mihir Shah
Birge Lab

Dena Abdelshahed
Rogers Lab

Jeffery Moore
Benevenia Lab

Heather Hokaian
Howells Lab

Jeffery Kim
Feldman Lab

Jerel Chacko
Lee Lab

Jeffery Suell
Zucker Lab

Na'im Ali
Howell Lab

Jill Deutsch
Wu Lab

Kevin Chou
Beebe Lab

Meneka Dave
Rameshwar Lab

Quasim Husain
Capo Lab

Matthew Moralle
Fritton Lab

Paul Therattil
Barton Lab

Prasanthi Yelavarthy
Wood Lab

Ramya Takkellapati
Studzinski Lab

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1. DENA ABDELSHAHED (NJMS 2013)

BMP2 KNOCKDOWN SLOWS LUNG CANCER CELL GROWTH

Mentor: Melissa Rogers, PhD (Biochemistry)

Objective:
Lung cancer (both small cell and non-small cell) is the leading cause of cancer death for both men and women [American Cancer Society 2010]. More people die of lung cancer than of colon, breast and prostate cancers combined [American Cancer Society 2010]. Although smoking accounts for about 80% of global lung cancer deaths in men and 50% of the deaths in women, the pathogenic mechanisms leading to lung cancer are mostly unknown and there are currently no effective treatments for lung cancer [Jemal et al., 2010].

Bone morphogenetic proteins, part of the transforming growth factor-β family, are necessary for maintenance and control of cell proliferation and apoptosis. In lung, BMP2 synthesis is significantly increased in all types of lung carcinomas compared to normal tissues or benign lung tumors. High BMP2 expression in non-small cell lung carcinomas predicted poor patient survival [Beer et al., 2002]. Elevated BMP2 levels promote malignant lung cancer cell behaviors in vitro and in vivo by increasing cell proliferation and migration and stimulating angiogenesis [Feeley et al., 2006; Langenfeld et al., 2003]. Additionally, experimentally reduced BMP2 levels functionally inhibit lung tumor formation and metastasis in mice [Feeley et al., 2006]. Reducing BMP2 levels is a novel approach to treating lung cancer.

BMP2 RNA and protein levels can be modulated by shRNA. ShRNA is RNA that forms a tight hairpin loop. When cleaved intracellularly into siRNA, it binds to RNA-induced silencing complex (RISC). The RISC then binds to and cleaves mRNA, thus silencing it. We utilized two cell lines, namely A549 human lung adenocarcinoma cells and transformed Beas-2B human bronchial epithelial cells (Beas-2B were oncogenically transformed by Mycoplasma infection to become Beas² cells) [Jiang et al., 2008]. The following hypothesis was tested: If BMP2 promotes cell growth, then shRNA knockdown of BMP2 will slow growth.

Methods:

ShRNA Lentiviral Transduction: Cells were grown in Dulbecco’s Modified Eagle’s Medium, supplemented with 5% Fetal Bovine Serum and 2mM Glutamine. We transduced the cells as follows: remove media and add media+polybrene (a cationic polymer that neutralizes charge interactions between the pseudoviral capsid and the cell membrane); infect cells by adding thawed shRNA lentiviral particles to culture. We used two types of shRNA: a control, scrambled sequence that does not block BMP2 and a BMP2 directed shRNA. Cells infected with control shRNA were called C1, C2, and C3; cells infected with BMP2 shRNA were called B1, B2, and B3. Media+puromycin was added to the cells; the puromycin selects cells that have been infected by the lentivirus. After selection, we plated C1, C2, C3, B1, B2, and B3 on P100 plates at 5x10⁵. This was Day 0. Plates were kept in incubator at 37 degrees Celsius and 5% CO₂ injected constantly.

Growth Curve: In order to measure cell growth, A549 cells were counted from May 15, 2010 to May 21, 2010 and Beas² cells were counted from June 22, 2010 to June 28, 2010. Cells were plated to 5x10⁵ on Day 0 and kept in the incubator. The procedure on each day of the growth curve was as follows: Take pictures of cell plates for comparison; remove media and retain for ELISA study; wash cells with 10 ml PBS; suspend cells in 3 ml Trypsin; add 10 ml fresh media and move cell suspension to 15 ml conical tube; centrifuge suspension to get cell pellet; remove media and trypsin; resuspend cells in 10 ml fresh media; add 20 ul to hemacytometer and count cells; remove media and add 1 ml Trizol to 10 ml cell solution for RNA extraction.
**RT-PCR:** In order to measure RNA levels in our cells, we followed the Trizol manufacturer protocol to extract total RNA as follows: Centrifuge cells+Trizol for 20 minutes, add 200ul chloroform; cap tube securely and shake vigorously by hand for 15 seconds; centrifuge cells at 14x1000 rcf for 15 minutes to separate into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase; transfer aqueous phase to fresh tube and add 500 ul isopropyl alcohol; centrifuge at 14x1000 rcf for 10 minutes after which the RNA precipitate will form a gel-like pellet on side and bottom of tube; remove supernatant and wash with 70% ethanol, allow RNA to air dry then dissolve in RN-ase free water; quantify RNA at Nanodrop machine and prepare 100ng/ul RNA solutions. To denature the RNA, we added 1 ul primer and 1ul 10 mM dNTPs, incubated at 65 degrees Celsius for 5 minutes, then left on ice for 5 minutes. To create cDNA, we added 4 ul 5x first strand buffer, 1 ul 0.1 M DTT, 1 ul Millipore H20, and 1 ul Supercrypt II RT to the RNA. To amplify the cDNA, we did PCR, adding 5 ul 10x thermo buffer, 1 ul each of forward and reverse primer, 1 ul 10 mM dNTPs, 0.5 ul TAQ DNA polymerase, and 39.5 ul H20 to 1 ul of cDNA and subjected to 27 cycles in the thermocycler. We then ran the samples through agarose and polyacrylamide gels and quantified the bands using GeneSnap technology. We manually quantified each band while using the software’s automatic background correction.

**ELISA:** To measure protein levels, we concentrated media from the cells using 10,000 K 10ml columns from Amicon. Media was collected from culture plates and centrifuged in the columns at 5000 rpm for 40 minutes. We collected about 200ul for each 8 ml of media concentrated and conducted Elisa studies as follows: in a 96 well microplate coated with mouse monoclonal antibody against BMP2, add 100ul Assay diluent and 50 ul sample to each well; incubate on shaker for 2 hours; aspirate and wash 4 times; add 200 ul conjugate to each well and incubate on shaker for 2 hours; aspirate and wash 4 times; add 200 ul substrate solution to each well; incubate in dark for 30 minutes; add 50 ul stop solution to each well; Measure absorbance at 450 and 562 nm.

**Mycoalert Assay:** To assess mycoplasma levels in our cultures, we performed a MycoAlert assay as follows: collect 100 ul cell-free media; add 100 ul MycoAlert reagent, incubate for 5 minutes; take luminometer reading (this is A); add 100 ul MycoAlert substrate, incubate for 10 minutes; take luminometer reading (this is B). If B/A is much greater than 1, the sample is mycoplasma infected; if B/A is less than 1, the sample is uninfected.

**Summary/Results:**

A549 cells were counted on Days 1, 2, 3, 6, and 7 of 1 week. Beas were counted on days 1, 2, 3, 4, and 5 of 1 week. An obvious difference in growth was observed in the A549 cells (Figure 1). Control cells grew much more than BMP2 cells. Although the same trend was observed in Beas cells, the difference was not as significant (Figure 1). Also, there was a change in cell morphology in the A549 cells by Day 7.

RT-PCR studies showed control A549 cells produced almost three-fold more RNA than BMP2 shRNA A549 cells. Control Beas cells also made more BMP2 RNA, but the difference was not as significant (Figure 2). Our studies of protein levels showed five-fold greater BMP2 protein in control A549 than BMP2 A549. However, in Beas cells, no significant difference was observed in BMP2 protein levels between control and BMP2 cells (Figure 3). This could be due to the fact that the Beas cells produce significantly more BMP2 relative to A549 cells.

Lastly, because mycoplasma infection also increase BMP 2 concentration, we wanted to rule out any off target effects of the lentiviral shRNA on mycoplasma mRNA [Jiang et al., 2008]. We did a Mycoalert assay to measure mycoplasma in the cells. We tested uninfected cells and cells treated with control and BMP2 shRNA. Our results showed that the uninfected Beas-2B cells were not Myco-infected but the control and BMP2 cells showed a high level of infection as we expected (Figure 4). This means that the lentiviral shRNA was not acting on the mycoplasma’s mRNA.
Conclusion:

ShRNA silencing of BMP2 effectively slowed growth of A549 cells. Silencing of BMP2 was not as efficient in Beas\textsuperscript{t} cells, but a subtle difference in growth was seen. Some future directions of research include soft agar experiments to assess anchorage independent growth and malignancy of BMP2 knockdown cells, injection of cells into nude mice to assess effect of BMP2 levels on in vivo tumorigenesis, and optimization of BMP2 knockdown in Beas\textsuperscript{t} cells.

The greatest clinical implication of this work is, given the stimulatory effect of BMP2 on lung cancer cell growth, reducing infection-associated BMP2 synthesis may slow the progression of this disease. Our research provides insight into this possible treatment for lung cancer.

References:


Figures:

Fig. 1: Knocking Down BMP2 Expression Reduces Transformed Lung Cell Growth. The reduced inhibitory effect on the BEAS\textsuperscript{t} cells likely reflects the reduced knock down efficiency demonstrated in Fig. 2 and 3.
Fig. 2: A. A representative RT-PCR using primers specific to BMP2 and the constitutively expressed cyclophilin RNA. B. The average BMP2 RNA level from several RT-PCRs ± SEM (n=2 to 3).

Fig. 3: The average BMP2 protein levels ± SEM (n=2 to 3) from media conditioned for the number of days in parentheses.

Fig 4: BMP2 Knockdown did not alter Mycoplasma levels in the infected cells.
2. **NAIM ALI (NJMS 2013)**

**EVALUATION OF COMBINED MODALITY RADIOIMMUNOTHERAPY FOR TREATMENT OF MAMMARY ADENOCARCINOMA USING IN VIVO BIOLUMINESCENCE IMAGING**

Mentors: Roger Howell, PhD, John Akudugu, PhD, Lionel Zuckier, MD

**Objective:**
While chemotherapy provides an appropriate means of initially eradicating large numbers of cancer cells, there is a need for a second-line therapy option that is capable of targeting potentially metastatic cancer cells that survive chemotherapy. This study examines the ability of radioimmunotherapy to fill that need. Radioimmunotherapy agents such as Iodine-131 labeled anti-EpCAM target cancer cells for irradiation by using an antibody that is known to recognize cancer cells. A combined treatment of both chemotherapy and radioimmunotherapy should, in theory, treat both chemo-sensitive and drug-resistant cancer cells. As such, the goals of this study are to evaluate such combined treatment on a cellular level through flow cytometry and secondly to determine its macroscopic effect on breast tumors through IVIS imaging.

This past summer, the primary work done was in preparation for this project. This preparation encompassed three broad objectives: developing a protocol for radiolabeling and purifying of the antibody to be used for radioimmunotherapy, establishing appropriate imaging of the fluorescent drugs and luminescent cells to be used, and learning proper mouse injection techniques.

**Methods:**

Pierce iodination beads (Thermo Scientific, Rockford, IL) were used to iodinate 50 ug of anti-EpCAM 9C4 with 500 uCi of Iodine-131 at room temperature. A time study was performed in order to determine the optimal reaction time. 1 uL aliquots of the reaction mixture were removed at 8.5, 10, 12.5, 15 and 18.5 minutes of incubation. These aliquots were then plated onto a glass-backed silica gel thin-layer chromatography (TLC) plate (Whatman, Piscatway, NJ). Iodination and plating were done in a hooded glove box in the NJMS Radiation Safety office (MSB A679) to safeguard against potential contamination from volatilized radioactive iodine. Following thin layer chromatography, autoradiography was performed on the plate for an exposure time of 30 minutes. The resultant radiograph was analyzed for presence of free iodine along with labeled product.

High performance liquid chromatography was attempted as a means of purifying the reaction product. A sequence was developed in the Chemstation software (Agilent, Santa Clara, CA) used with the Hewlett Packard Series II 1090 Liquid Chromatograph. The solvent system used was: solvent A- 0.1% trifluoroacetic acid (TFA) in 2% acetonitrile in water; solvent B- 0.085% TFA in acetonitrile. A gradient of 2 to 45% B over 30 minutes was used for the mobile phase. The sequence developed consists of three methods: Startup, Purify, and Shutdown. Startup establishes the initial required solvent system from baseline. Purify injects the product and creates the appropriate gradient. Shutdown brings the solvent system back to baseline.

A Strata C-18U column (Phenomenex, Torrance, CA) was first used for filtration. The column was first preconditioned with 3 mL of methanol followed by 3 mL of PBS. The labeled product was then added to the column. PBS was added in 3 mL increments until all the fractions could be collected. Effluent was collected ten drops at a time for a total of 50 fractions. The radioactivity of the fractions was then analyzed using a dose calibrator.

Following a subsequent labeling, a PD-10 column (Amersham Bioscience, Uppsala, Sweden) was used for filtration. The column was preconditioned with 25 mL of PBS. Product
was added and PBS was added in 1 mL increments. 20 total fractions, 1 mL per fraction, were collected. As before the radioactivity of the fractions was analyzed using a dose calibrator.

A metastatic, human breast cancer cell line that has been transfected with the luciferase gene (MDA-MB-231-luc-D3H2LN) is being used for this study. Cells were cultured in 20 wells of a 96-well plate overnight in medium. 10 of the wells were treated with luciferin for 15 minutes prior to imaging. The remaining 10 wells were treated with a control of PBS. The cells were also treated with serial dilutions of the drugs to be used: paclitaxel labeled with Oregon green 488 (flutax), daunomycin, and doxorubicin. After a two-hour treatment, the cells were washed with PBS. Empty wells without any cells were also plated with serial dilutions of flutax, daunomycin, and doxorubicin. The plate was imaged for both luminescence (for luciferase activity from the cells) and fluorescence (for the fluorescent chemotherapeutics) using the IVIS 200 (Caliper Life Sciences, Hopkinton, MA). Optimal excitation and emission wavelength windows were determined by imaging with all possible fluorescence options.

With the assistance of Ms. Tracy Davis and Dr. Elmer David, tail vein injection (for injecting radioimmunotherapy) technique was learned. Mice were first sedated with a standard xylazine/ketamine cocktail delivered intraperitoneally and placed in a restraint specifically designed for tail vein injections. 27G, ½” needles on 1 mL syringes filled with 0.1 mL normal saline were used for the practice injection. An observable white “flash” in the vein was used to determine success. Success rate was tracked over time.

Orthotopic mammary fat pad injection (for injecting cancer cells) was learned with help from Dr. Deborah Lazzarino. Six 6-month old female Swiss Webster mice were euthanized via CO₂ inhalation. Limbs were secured using pins. Toothed forceps were used to lift the nipple. A 25G, 5/8” needle was used to inject 50 uL of 50% Trypan blue/50% glycerol into the mammary fat pad. Trypan blue was used to track the success of the injection and glycerol was used to mimic of the viscosity of the Matrigel to be used with cells. Bilateral inferiormost thoracic (3rd) mammary fat pads and bilateral abdominal (4th) mammary fat pads were injected for each animal, giving a total of 24 practice injections. Mice were dissected taking care not to disturb the peritoneum, and location of the injected material was tracked. 25 female NIH III homozygous (NIH-Lyst/+ Fxi 1+/- Btk xid nu) nude beige tumor-bearing mice 4-6 weeks old are being used for this study. 2*10⁶ cells in 50 uL of 50% medium / 50% Matrigel was injected into the 4th right mammary fat pad of each mouse using the technique practiced. 5 of the mice were injected with luciferin and imaged using IVIS 16 days following injection.

Summary:

Optimal iodination reaction time was determined to be less than 12 minutes as a second TLC band perhaps indicating cleaving off of the fluorochrome on the antibody used was noticed in the aliquots corresponding to reaction times greater than 12 minutes.

HPLC results were inconclusive as the intensity vs. time graph showed no peak corresponding to the passage of the antibody through the column as was expected.

A discoloration of the C-18U column was noticed following filtration. Measurement of the column with the dose calibrator also indicated a high level of radioactivity in the column. It is suspected that either the iodinated fluorochrome that has been cleaved off or the labeled antibody remains stuck in the column.

PD-10 filtration showed peaks of radioactivity around fraction 4-6 and 9-11 corresponding to labeled antibody and free iodine respectively, as was expected.
When the cells were imaged for fluorescence, the only light seen on the resulting image was from those treated with luciferin. It is likely that the light emitted through the metabolism of luciferin is of a wide spectrum and is orders of magnitude greater in intensity than the fluorescence associated with the drugs. Therefore, the luminescence could be masking the fluorescence making it impossible to image both simultaneously.

In the practice of tail vein injections, the technique was modified over practice sessions. These modifications included: changing the grip of the syringe closer to the needle, bending the tail to avoid the left thumb, and grasping the tip of the tail between the left ring and pink fingers. Success rate was improved from 10-20% to 80-90% over the various practice sessions.

Throughout all 24 practice orthotopic mammary fat pad injections attempted, the peritoneum was avoided. Injections that missed the mammary fat pad remained localized subcutaneously in the region immediately surrounding the gland. IVIS imaging of animals to be used in the study confirmed the presence of a tumor in all five of the mice imaged.

Conclusion:
The Iodogen method with Pierce iodination reagent has been used to label the antibody, however the exact procedure of labeling in order to optimize the labeling is yet to be determined. A PD-10 column will be used in order to purify the resultant reaction product.

A protocol for appropriate imaging has been determined. It was found that the more intense, broad spectrum luminescence masks any fluorescence from the drugs. Therefore, in order to image both fluorescence and luminescence in mice, each must be done separately. This can be accomplished by first imaging mice for fluorescence followed by an injection of luciferin and imaging for luminescence. While this may create issues with the ability to fuse these images as mice will need to be moved for injection of the luciferin, it will allow for a gross appreciation of the drug delivery or lack thereof.

The establishment of proper techniques for tail vein and orthotopic mammary fat pad injections allows for a greater degree of reproducibility between animals. Varying grip positions and identifying a means of visualizing a successful tail vein injection ensures that all animals will receive the same radioactive dose. For orthotopic mammary fat pad injections, the majority of the fat pad was missed in some of the practice injections. However, the injections did remain in the subcutaneous space without violating the peritoneum. IVIS imaging of 5 of the mice injected with cells shows the presence of a tumor near the 4\textsuperscript{th} right nipple in each of the mice imaged.
3. ABDEL-KAREEM BEIDAS (NJMS 2013)

THE IMPACT OF PATIENT NAVIGATION ON CANCER PERCEPTION IN THE LATINO COMMUNITY

Mentors: Ana Natale-Pereira, MD, MPH (Medicine) & Olympia Cepeda-Cotto, MPH (School of Public Health)

Objective:

The CMS-CPTD project is a randomized control trial designed to show the effectiveness of a community based patient navigation program in improving cancer screening, patient satisfaction, and informed decision-making. A total of 1271 participants have been enrolled. Of those, near 500 are active intervention participants, receiving facilitation of services through patient navigation (PN). The control group participants receive educational materials on a quarterly basis, but no navigation.

The objective of this cross sectional ancillary project is to assess more in detail, the effectiveness of navigation from the intervention participant’s perspective, with respect to satisfaction with the service provided, self awareness, and behavioral influence towards cancer.

Methods:

All CMS-CPTD project participants complete a baseline survey upon enrollment. Intervention participants complete an annual survey. Data collected includes their use of cancer screening procedures for cervical, breast, prostate and colorectal cancers, and perception about their health status, their cancer knowledge, and their utilization of services. Using the database of intervention participants of the CMS-CPTD project, a simple random sample of 100 intervention group participants was selected to complete a patient satisfaction telephone mode survey. Data were analyzed to identify the correlation between the PN program and the effect on the patients’ cancer knowledge and perception, as well as their willingness to advocate on their behalf as well as their family members. Statistical analysis was conducted using JMP 8 computer software. In addition, an observation was made between the baseline and annual survey responses of the intervention participants in the parent project, looking for changes in knowledge of, and attitudes and beliefs about cancer.

Summary:

In the CMS-CPTD study, it was possible to gauge the patients’ knowledge and perceptions of cancer through the surveys that were given upon entry into the program, yearly, and upon exiting the study. One of the requirements of the study is not to report any of the findings until all final reports are submitted to Congress. Hence, no results shall be presented before that point.

As part of an ancillary cross-sectional study of 100 randomly selected patients from the intervention group, we were able to show that at the very least, the program introduced the participants to the concept of Patient Navigation. When asked if they had known what a Patient Navigator was before participating in the program, 92 of 100 responded no. Those who answered no were asked a follow-up question, “Would you describe a Patient Navigator…” (Question 7), 70 were able to correctly answer “Contact between health provider and patient.” Lastly, when asked, “Has this program inspired you to speak to your family and friend about obtaining cancer screenings?” (Question 25), 92 of the 100 participants responded positively. This points to the effect the Patient Navigation program has had on the patients, as well as the relationship that the patient navigators were able to form with the patients.
Cancer Summer Student Research Program - 2010

### Did you know what a patient navigator was before you enrolled in the program?

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<tbody>
<tr>
<td>Yes</td>
<td>8</td>
</tr>
<tr>
<td>No</td>
<td>92</td>
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### Would you describe a Patient Navigator as a…

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<th>Description</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>I don’t know</td>
<td>8</td>
</tr>
<tr>
<td>Lawyer</td>
<td>5</td>
</tr>
<tr>
<td>Contact between health provider</td>
<td>70</td>
</tr>
<tr>
<td>Scientist</td>
<td>2</td>
</tr>
<tr>
<td>Referee</td>
<td>3</td>
</tr>
<tr>
<td>Doctor</td>
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**Conclusion:**

The results of the NJMS CMS-CPTD project show that the materials provided to by the program and the interaction with the Patient Navigators increased the cancer knowledge—as well as the perceptions of cancer—of the participants in the intervention group, thereby positively affecting their informed decision-making capacities.

There was a demonstrable increase in the cancer awareness of the patients after being enrolled for two years compared to their baseline at the time of enrollment.

The high level of patient satisfaction is a reflection of the relationship that the Patient Navigators were able to foster and build over the course of the study as well the trust they were
able to garner from the patients. Although much remains to be analyzed, the patient navigator model to facilitate utilization of services in healthcare may prove once more to be an effective way to reduce disparities that exist for cancer screening, diagnosis, and treatment among minority and underserved populations.
4. JEREL CHACKO (NJMS 2013)

PHENFORMIN INHIBITS TUMOR GROWTH AND INDUCES DIFFERENTIATION POSSIBLY VIA AN AMPK-DEPENDENT PATHWAY IN COLON CANCER CELLS

Mentor: Michael A. Lea, Ph.D. (Biochemistry & Molecular Biology)

Objective:

AMPK is a ubiquitous multisubunit serine-threonine protein kinase that forms heterotrimers composed of a catalytic subunit (α1 or α2) and two regulatory subunits (β and γ) that stimulate glycolysis via both 6-phosphofructo-2-kinase and gene upregulation in response to energy depletion (Hardie 2007). Essentially, it is a fuel gauge that balances growth against survival.

Activation of AMPK has been shown to reduce plasma glucose, a potential treatment for type 2 diabetes. Metformin, a common antidiabetic drug, has been shown to be an AMPK activator. Epidemiological studies have shown that metformin reduces the risk of cancer. Research has supported this finding, showing that activation of AMPK by metformin reduces tumor growth in p53- cells in vivo (Algire et al. 2010, Buzzai et al. 2007). Phenformin, a related compound, was used to treat diabetes but due to its potency, has a higher tendency to cause fatal acidosis. However, used concentratedly and briefly, it may be a powerful anti-tumor drug. In order for phenformin to be a viable option, its concurrent lactic acidosis must be reversed.

This study aims to research the effects of phenformin and if its acidosis can be reversed by 2-deoxyglucose, a glycolysis inhibitor, or compound C, an AMPK inhibitor. Not only will this research help find a new treatment for cancer, but its implications may help uncover more about the AMPK-based link between type 2 diabetes and p53- cancer.

Methods:

Cell lines Caco-2, HT29, SW1116, and NCM460 were used in this study. The cells were plated in 5mL RPMI 1640 with 25 mM HEPES and 5% FCS for 24 hours. Next, the cells were incubated for 72 hours in 10 mL medium with the presence or absence of either (a) 0.1 mM phenformin and/or 1 mM 2-deoxyglucose, (b) 0.1 mM phenformin and/or 10 µM compound C, or (c) 0.5 mM butyrate and/or 0.1 mM A 769662. The medium was collected and absorbance was measured at 560 nm as well as tested for pH levels. Treated cells were washed with 10mL of 1x PBS and transferred to centrifuge tubes and centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded by inversion and the cell pellets were mixed with 150 µL NP40 lysis buffer and left on ice for 30 minutes. The supernatant was collected after centrifuging at 3000 rpm for 10 minutes.

Alkaline phosphatase assay

100 µL of 100mM p-nitrophenylphosphate and 100 µL of 1.5M alkaline buffer solution was mixed with 50 µL extract and incubated at 37°C for 60 minutes. The reaction was stopped with 2 mL 0.05M NaOH and absorbance was measured at 410 nm.

Protein levels assay

10 or 20 µL extracts were brought to 100 µL with distilled H₂O and mixed with 2 mL of freshly-mixed BCA protein assay reagent and incubated at 37°C for 30 minutes. The solution’s absorbance was measured at 562 nm.

Summary:

The first graph and table show an average of the medium absorbances and pHs. The remaining graphs are results of a typical experiment.
Effects of Compounds on Medium 0D at 560 nm

Figure 1. Medium 0D values of Caco-2, SW1116, HT29, and NCM460 cells after a 72 hour incubation. The data are given as the mean for 4 determinations (control and phenformin) or 2 determinations.

<table>
<thead>
<tr>
<th></th>
<th>Caco-2</th>
<th>SW1116</th>
<th>HT29</th>
<th>NCM460</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>7.56 ± 0.05</td>
<td>7.57 ± 0.01</td>
<td>7.33 ± 0.03</td>
<td>7.47 ± 0.03</td>
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<tr>
<td>phenformin</td>
<td>7.44 ± 0.15</td>
<td>7.31 ± 0.06</td>
<td>7.17 ± 0.03</td>
<td>7.24 ± 0.09</td>
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<tr>
<td>2-deoxyglucose</td>
<td>7.67 ± 0.07</td>
<td>7.70 ± 0.01</td>
<td>7.41 ± 0.01</td>
<td>7.60 ± 0.01</td>
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<tr>
<td>compound C</td>
<td>7.77 ± 0.00</td>
<td>7.71 ± 0.05</td>
<td>7.46 ± 0.05</td>
<td>7.55 ± 0.01</td>
</tr>
<tr>
<td>phenformin + 2-deoxyglucose</td>
<td>7.68 ± 0.10</td>
<td>7.68 ± 0.04</td>
<td>7.38 ± 0.11</td>
<td>7.55 ± 0.05</td>
</tr>
<tr>
<td>phenformin + compound C</td>
<td>7.67 ± 0.05</td>
<td>7.43 ± 0.04</td>
<td>7.24 ± 0.02</td>
<td>7.37 ± 0.06</td>
</tr>
</tbody>
</table>

Table 1. Medium pH values of Caco-2, SW1116, HT29, and NCM460 cells after a 72 hour incubation. The data are given as the mean ± S.D. for 4 determinations (control and phenformin) or 2 determinations.

Figure 2. (A, B) 1.17x10^6 Caco-2 cells treated with phenformin and/or 2-deoxyglucose. (C, D) 1.50x10^6 Caco-2 cells treated with phenformin and/or compound C. The data are given as the mean ± S.D. for 3 determinations. Data were evaluated by ANOVA and Dunnett’s test. Bold values indicate P < 0.05.
Figure 3. (A, B) 1.50x10^6 SW1116 cells treated with phenformin and/or 2-deoxyglucose. (C, D) 1.50x10^6 SW1116 cells treated with phenformin and/or compound C. The data are given as the mean ± S.D. for 3 determinations. Data were evaluated by ANOVA and Dunnett’s test. Bold values indicate P < 0.05.

Figure 4. (A, B) 1.41x10^6 HT29 cells treated with phenformin and/or 2-deoxyglucose. (C, D) 1.50x10^6 HT29 cells treated with phenformin and/or compound C. The data are given as the mean ± S.D. for 3 determinations. Data were evaluated by ANOVA and Dunnett’s test. Bold values indicate P < 0.05.

Figure 5. (A, B) 1.20x10^6 NCM460 cells treated with phenformin and/or 2-deoxyglucose. (C, D) 1.50x10^6 NCM460 cells treated with phenformin and/or compound C. The data are given as the mean ± S.D. for 3 determinations. Data were evaluated by ANOVA and Dunnett’s test. Bold values indicate P < 0.05.
Alkaline phosphatase activity increases indicate differentiation in colon cancer cells. This was observed after treatment with AMPK activators, with the exception of A 769662 (Figs. 2A, 2C, 3A, 3C, 5A, 5C, 6A). Protein level decreases indicate lessened cell growth. This was observed after treatment with both AMPK activators and inhibitors (Figs 2B, 2D, 3B, 3D, 4B, 4D, 5B, 5D, 6B). A 769662 opposed the action of butyrate, although both have been shown to activate AMPK (Figs 6A and 6B). 2-deoxyglucose and AMPK have opposing physiological actions, but functioned similarly in cancer cells. Figures 2, 3, 4, and 5 show synergy between phenformin and 2-deoxyglucose. Phenformin and compound C additively inhibited growth but in terms of differentiation, they functioned opposingly. Individually 2-deoxyglucose and compound C increased pH, but 2-deoxyglucose was more effective at reversing phenformin’s acidosis.

Conclusion:

Phenformin works synergistically with 2-deoxyglucose and compound C to inhibit growth. However, phenformin works additively only with 2-deoxyglucose (and compound C in Caco-2) to induce differentiation. Caco-2 is known for spontaneous differentiation in long term culture. This increased tendency for differentiation was enhanced by compound C. HT29 did not have definitive differentiation results but that may be due to their inherently low levels of alkaline phosphatase. The opposing behavior of butyrate and A 769662 may be explained by the presence of 2 different AMPKs. Most studies only measure total AMPK without distinguishing the β and γ forms even though AMPK can be activated via different mechanisms (Hawley 2010). Ultimately, it can be concluded that phenformin is an effective anti-tumor drug, a finding made more relevant by the fact that 2-deoxyglucose, more so than compound C, is able to reverse phenformin-related acidosis.

Warburg claimed that cancer was a result of altered metabolism. His early prediction of increased glycolysis remains true, yet it is still debated if it is the cause or result of cancer (Garber 2004). Although his theory isn’t supported by many, it can be agreed that cancer can be conceptually simplified to uncontrolled growth. In that same manner, I propose that type 2 diabetes be considered uncontrolled survival. The faulty mechanism behind p53- cancer and type 2 diabetes (explaining their related treatments), may in fact be AMPK.

References:
5. DEEPA CHERLA (NJMS 2013)

PDGF-RESPONSIVE GLIAL PROGENITORS IN THE MOUSE SUBVENTRICULAR ZONE
Deepa Cherla,

Mentor: Lisamarie Moore, and Steven W. Levison, PhD (Neurology & Neurosciences)

Objectives:
The specific goals of these studies were to: 1. Compare the propagation of neural precursors as neurospheres or spheroids from the mouse subventricular zone (SVZ) and neocortex. 2. Compare sphere/sphereoid formation with EGF and FGF2; PDGFaa; and PDGFbb. and 3. Evaluate differentiation potential by staining cells for O4 (oligodendrocytes, or OLs) and GFAP (astrocytes).

Methods:
Cells were isolated from the newborn murine SVZ and neocortex. 1.5 x 10⁵ cells/mL were then placed in cell culture conditions that propagated mouse neurospheres: DMEM:F12-supplemented with B27 and EGF (10 ng/ml) and FGF-2 (20 ng/ml); or spheroids: PDGFaa (10 ng/ml); or PDGFbb (10 ng/ml) under 20% O₂ and 2% O₂. Spheres/spheroids were cultured for 5 to 7 days. Whole spheres/spheroids were plated onto chamber slides and differentiate in 2% O₂ for 5 days. They were then fixed, and stained using antibodies against cell surface and cytoplasmic markers to phenotype the cells. Stains included O4 (for Oligodendrocytes), GFAP (for astrocytes), vimentin, and nestin (for precursors).

Summary:
Recent studies in the Levison lab have established that glial progenitors exist in the rat SVZ and cortex (Bain et al., 2010). However, it has not yet been established whether these progenitors exist in the mouse SVZ nor has their growth factor responsiveness been evaluated.

Mouse neonatal SVZ cells were cultured in the described conditions, with subsequent quantification of spheres/spheroids generated in each condition. Data in the following table represent the number of spheres/field of view at 4X.

<table>
<thead>
<tr>
<th></th>
<th>O2 level</th>
<th>EGF+FGF-2</th>
<th>PDGFaa</th>
<th>PDGFbb</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVZ</td>
<td>2%</td>
<td>34.31 +/- 8.01</td>
<td>37.94 +/- 11.52</td>
<td>34.31 +/- 24.74</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>43 +/- 9.97</td>
<td>32 +/- 5.72</td>
<td>26 +/- 4.97</td>
</tr>
<tr>
<td>CX</td>
<td>2%</td>
<td>1.375 +/- .29</td>
<td>1.125 +/- .58</td>
<td>1.125 +/- .82</td>
</tr>
</tbody>
</table>

Growth factor-responsive SVZ cells formed spheres/spheroids when cultured in the EGF/FGF-2, PDGFaa, or PDGFbb. However, very few spheres/spheroids formed from the mouse neocortex. In general, there was no significant difference in sphere formation in any of the conditions except for EGF/FGF-2 generated more spheres at 20% O₂ vs 2% O₂ (much to our surprise). There was a trend for spheres grown in EGF and FGF-2 to produce more spheres. PDGFaa and PDGFbb favored the formation of O4+ cells (Oligodendrocytes) but also supported the production of GFAP+ cells (astrocytes) over O4+ cells (although to a lesser degree). This was partially expected since PDGFaa has been shown to promote the proliferation of OL lineage cells (Pringle et al., 1992). EGF and FGF-2 favored the formation of GFAP+ cells over O4+ cells, although some O4+ cells were observed.

Conclusion:
These data demonstrate that in addition to there being EGF/FGF-2 responsive cells in the SVZ that there also exist PDGFR responsive precursors in the mouse SVZ. Furthermore,
these precursors are rare in the neocortex. Our results also show that growth in PDGFs promotes the production of oligodendrocytes versus astrocytes. These observations provide insights into the origins of glioblastoma multiforme, a malignant and dangerous brain cancer, which is known to contain PDGFbb-responsive cells and which contains oligodendrocytic and astrocytic cells (Assanah et al., 2009).

References:


6. KEVIN CHOU (NJMS 2013)

THE EVALUATION OF THE INTERCALARY PROSTHETIC DEVICE IN MUSCOLOSKELETAL ONCOLOGY: A RETROSPECTIVE ANALYSIS

Mentors: Kathleen Beebe, MD, John C. Neilson, MD (Orthopedics)

Objective:

Diaphyseal are an uncommon site for bone tumors, but must be dealt with accordingly. After proper resection, a recurring problem for doctors is selecting the most appropriate method for obtaining the defect. The two most common methods at the time of this writing are intercalary allografts with hardware and intercalary endoprosthetics. Though endoprosthetics are more commonly used for their strength and stability, they have problems with modularity of their components and proper rotation of the prosthesis. Merete (Berlin, Germany) has released an intercalary diaphyseal segmental fixation (IDSF) device known as the OsteoBridge© that uses separate components to better fit the resected segment and obtain premorbid length. Before becoming FDA approved in the United States, another study by Sakellariou, et al was performed which retrospectively looked at 6 patients implanted with the IDSF. The hypothesis of this study is that the IDSF device could provide early pain-free functionality after tumor resection, and provide a marketable alternative to current intercalary endoprosthetics.

Methods:

In this IRB-approved retrospective study, the patients surgically implanted with the IDSF endoprosthesis between July 2008 and January 2009 were identified by database review and implant records. The inclusion criteria required patients to have a primary or metastatic malignant tumor necessitating diaphyseal resection reconstruction. Surgeries to revise previously stabilized pathological fractures were also included. Exclusion criteria eliminated patients with nonresectable tumors, had a follow-up of fewer than 12 months, and were under 18 years of age. Demographic data including age, sex, and race were noted along with tumor-related and endoprosthetic data.

To determine short-term outcome of the patients, the Musculoskeletal Tumor Society (MSTS) scoring system was used to quantitatively evaluate the outcome of the endoprosthesis surgery. It provides a 1-5 scale evaluation for the categories of pain, activity functioning, emotional acceptance, support use, dexterity or ambulation, and range of motion or lifting. The scores are then calculated as a percentage, with a higher percentage indicating greater return to premorbid functions. Other outcome measures included qualitative analysis of patients' survival, overall pain post-operationally, limb function, additional surgeries for complications, and integrity of the prosthetic at latest follow-up. All post-operative complications were properly documented, and placement of the IDSF was confirmed by radiologists at our institution.

Summary:

This retrospective study had 4 patients implanted with the OsteoBridge IDSF who were analyzed for survival, limb function, prosthesis integrity, and pain during follow-up. Of the 4 patients, 2 were male and 2 were female with a mean age 69 years (range, 60-75 years). Mean follow-up time was 15.5 months (range, 13-20 months). Of the 4 patients, the operative lesions included a myxoid malignant fibrous histiocytoma (MFH) of the left femur, a high grade pleomorphic sarcoma of the left tibia, a metastatic lesion from renal cell carcinoma to the right humerus, and a metastatic lesion from multiple myeloma to the right humerus (Table 1). All patients had good functioning until their last follow-ups. The average MSTS score for all patients was 26 points out of 30 (range, 22-30), indicating an 86.7% return to premorbid function.
Patient information is as follows: Patient 1 was a 69 year old female who presented with a 1 year history of a mass in her left anterior thigh that enlarged and became painful two months prior to diagnosis. Biopsy determined this to be a high grade malignant fibrous histiocytoma myxoid type. Surgical resection with negative margins and reconstruction with the IDSF device were performed. At 13 months follow-up, the patient had complaints of mild leg weakness and mild spasm. She had an MSTS score of 26, due to weakness that required occasional analgesics and requiring a knee-brace 1 year follow-up.

Patient 2 was a 74 year old female who presented with a 5 month history of intermittent leg pain. Radiographic images revealed a suspicious neoplasm on the lateral midshaft of the tibia. Biopsy revealed the mass to be a pleomorphic high-grade sarcoma. Patient received chemotherapy in addition to deep resection and reconstruction with an intercalary endoprosthesis. She required flap coverage to correct a wound breakdown, and the patient returned to the operating room for a cross leg tissue transfer secondary to the non-healing wound. At 20 month follow-up after weight-bearing therapy, the wound was clean, dry, and intact with no complaints. Patient 2 had an MSTS score of 22, due to chronic use of a cane for mobility, and an ankle-foot orthosis.

Patient 3 was a 68 year old male status post right nephrectomy secondary to metastatic renal cell carcinoma who presented with pathological fracture of the right humerus. Initial imaging studies displayed no indications of pathological disease, metastatic progression, or cortical destruction, confirmed with a biopsy from the clavicle. However, 1 year later after complaints of pain, radiographs and CT revealed increased size and lucency with destruction of the cortex at the proximal diaphysis of the right humerus, which indicated a possible risk of repeat fractures in those areas. Surgery was performed, and at approximately 14 month follow-up, patient was free of pain in the right arm but had global weakness secondary to chemotherapy. There was also mild tenderness in the left clavicle. Patient 3 had an MSTS score of 30.

Patient 4 is a 58 year old male with history of multiple myeloma who complained of 3 months right arm pain. Approximately 2 months later, he underwent radiofrequency ablation and intramedullary nailing. At 21 months follow-up, x-rays revealed a fractured interlocking screw requiring intercalary replacement. Revision surgery was done 1 month later using the IDSF. At 6 months post-op, the patient had full arm function. At 15 months follow-up, patient reported mild discomfort on forward flexion, but bone bridging was seen around the prostheses. Patient 4 had an MSTS score of 25 due to rotator cuff insufficiency and weakness in flexion that did not interfere with his daily activities.

During surgery, the average spacer length required to match the resected segments in patients had a mean length of 10.25 cm (range, 4-13 cm). The mean proximal stem length was 12 cm (range, 9-15 cm), and the mean distal stem length was 13.3 cm (range 9-20 cm) (Table 2).

Conclusion:
The goal of the musculoskeletal oncologist is first and foremost tumor removal and, when possible, to salvage the limb in order to return the patient’s premorbid function. Because survival rates are similar and patients are still susceptible to recurrence following amputation, limb-salvage is gaining popularity as a first-line attempt for treatment of bone tumors, though the challenge has now becoming selecting a technique to maximize function and minimize failure. In this study, follow up data from the 4 patients implanted with the IDSF device illustrates the effectiveness and relative safety of this new system.

In the study conducted by Sakellariou et al, 6 patients implanted with the Merete system were followed to assess functional and oncologic outcome. The post-operative MSTS evaluations displayed an average of 87.2% return of premorbid function in femur, tibia, or humerus. Of the six patients, five were free of local recurrences and did not need revision.
surgery. This study lends support to their promising results, as our 4 patients displayed an average MSTS score of 86.7%. In both studies, stems were cemented into the bone.

Studies using other endoprosthetic intercalary devices have demonstrated less successful outcomes. The majority of literature on this topic reports average MSTS scores between 60% to low 80%. Furthermore, these patients suffered notable complications such as aseptic loosening at an average follow-up of 14-20 months, infections at 10 months follow-up, and requirement of cane usage. This data marks a promising future for the OsteoBridge© system over other available intercalary devices; however, there is a paucity of literature examining long-term post-operative results. Severe mechanical complications have been reported with use of other intercalary endoprostheses with device failure reported at the 5 and 10 year follow-up mark. Given that the OsteoBridge has only been used clinically in the United States for 2 years, there have been no studies to determine the 5 or 10 year survival rate of the prosthetic nor reports of any resultant changes in patient MSTS scores.

Without long-term follow-up, it is not fully certain if the OsteoBridge© system provides an advantage over other intercalary endoprostheses. In addition, the small sample size in both this study and the Sakellariou study limits the ability to extrapolate this data to an expanded population. Further studies with larger patient populations and long-term follow-up are needed to confirm the promising short-term results. Future studies may also wish to examine ways to incorporate chemotherapy medications within the hollow canal comprising the modular shaft of the endoprosthesis.

Combined with the initial follow-up data however, this new IDSF endoprosthetic has great promise in the future of orthopedic oncology due to its ease of implantation, stability, and modularity for all bone sizes. With all factors considered, this device should be considered in the musculoskeletal oncologist armamentarium when addressing diaphyseal bone loss using an endoprosthetic.

References:


<table>
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<tr>
<th>Patient</th>
<th>Pathology</th>
<th>Location</th>
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<tbody>
<tr>
<td>1</td>
<td>Myxoid MFH</td>
<td>Left Femur</td>
</tr>
<tr>
<td>2</td>
<td>High Grade Pleomorphic Sarcoma</td>
<td>Left Tibia</td>
</tr>
<tr>
<td>3</td>
<td>Metastatic Renal Cell Carcinoma</td>
<td>Right Humerus</td>
</tr>
<tr>
<td>4</td>
<td>Multiple Myeloma</td>
<td>Right Humerus</td>
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Table 2 – Patient Post-Operational Data

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<th>Proximal Stem (mm)</th>
<th>Distal Stem (mm)</th>
<th>Follow-up (months)</th>
<th>MSTS</th>
<th>Compression screws?</th>
<th>Additional Surgery?</th>
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<td>13</td>
<td>150 mm</td>
<td>130</td>
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<td>N</td>
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<td>2</td>
<td>13</td>
<td>90</td>
<td>150</td>
<td>20</td>
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<td>110 mm</td>
<td>15</td>
<td>25</td>
<td>N</td>
<td>N</td>
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</table>
MENEKA DAVE (NJMS 2014)

OCT4 EXPRESSING BREAST CANCER CELLS: IMPLICATIONS FOR DORMANCY

Mentor: Pranela Rameshwar, PhD, (Medicine)

Objective:
Cancer is one of the most prevalent diseases in the world, reported by the National Cancer Institute to cause more than half a million deaths every year (CA Cancer J Clin, 2010). This particular study tested a hypothesis that has implications for mechanisms involved in cancer dormancy; in particular, immune protection within the bone marrow. It is thought that a small sub-population of stem-like cells within the heterogeneous tumor population is responsible for tumor propagation. This population of cells is believed to resist current therapy and can result in tumor relapse years after remission.

The transcription factor/stem cell related gene, Octamer-4 (Oct4) is associated with a primitive phenotype of breast cancer cells with stem-like properties. We hypothesized that these Oct4(+) cells, in the presence of mesenchymal stem cells (MSCs), induce regulatory T-cells (Tregs) whereas the Oct4(-) cancer cells induce T-helper 17 (Th17).

Methods:
Human subjects: The use of human peripheral blood mononuclear cells (PBMCs) and bone marrow aspirates were approved by the Institutional Review Board of the UMDNJ-Newark Campus.

Cells: MSCs were cultured from human bone marrow aspirates; Peripheral blood was obtained from the same donor for the isolation of PBMCs by Ficoll-Hypaque density gradient centrifugation (see diagram on right). Human breast cancer cell lines MDA-MB-231, and T47D were purchased from the American Type Collection and maintained according to manufacturers' instructions.

Tregs and Th17: PBMCs were co-cultured with MSCs and/or breast cancer cells. After 48 h the cultures were stimulated with PMA (20 ng/mL) and ionomycin (1 μg/mL) for 16 h. At the final 4 h of cultures, cells were treated with Brefeldin. The non-adherent cells were collected and subjected to 3-color labeling with APC-anti-CD4, FITC-anti-IL17 and PE-anti-FOXP3. Samples were analyzed using the FACScalibur flow cytometer.

In vivo Studies: Oct4(+) and Oct4(-) breast cancer cells (10^6) were injected with BD Biocoat™ Matrigel™ Matrix subcutaneously in female athymic nude BALB/c mice (age 3-4 weeks). Tumor size was monitored over 3 weeks. Parallel groups were studied for their response to chemotherapy by intraperitoneal injection with carboplatin (50mg/kg). Tumor sizes were recorded every 2 days.

Immunofluorescence for gap junctions: T47D breast adenocarcinoma cells were seeded at 2x10^5 cells per well into 6-well plates. Bone marrow stromal cells were seeded at 2x10^4 cells per well. 1-octanol was added at concentrations of 0mM, 0.3mM, and 1mM for gap junction inhibition. After 3 days of co-culture, cells were fixed and permeabilized then stained with rabbit anti-Cx43 overnight at 4°C. Cells were incubated in goat anti-rabbit IgG-FITC for 2 hours at room temperature, and finally in Texas Red-X phalloidin (F-actin staining) and DAPI (nuclear staining). For detecting gap junctional intracellular communication (GJIC), BCCs were stained with CFDA and co-cultured with MSCs for 2 days. Samples were viewed using an inverted fluorescent microscope.
Summary:

After co-culturing PBMCs with Oct4(+), Oct4(-) and heterogeneous BCCs, both in the presence and absence of MSCs, percentages of Tregs and Th17 cells were quantified using flow cytometry. In co-cultures with Oct4(-) breast cancer cells and MSCs, the percentage of Th17 significantly increased in comparison to cultures without MSCs, while Tregs only increased slightly in the presence of MSCs. Parallel experiments with Oct4(+) breast cancer cells showed almost a 5 fold increase in induction of Tregs with the addition of MSCs, with Th17 remaining relatively constant in both cases.

Positive immunofluorescent staining for Cx43, one of the proteins involved in the formation of gap junctional intracellular communication (GJIC), in co-cultures of stroma and heterogeneous breast cancer cells confirmed the establishment of gap junctions between the two cell populations. In order to test for GJIC, heterogeneous breast cancer cells were dyed with CFDA and then cultured with MSCs. After 2 days, cells were viewed using a fluorescent microscope and MSCs were checked for dye transfer which would show GJIC between the breast cancer cells and MSCs. It was found that dye transfer did indeed occur, however at a very low frequency. In a similar experiment conducted with Oct4(+) cells, the number of MSCs positive for dye transfer was higher, indicating higher incidence of GJIC between Oct4(+) cells and MSCs, compared to Oct4(-) breast cancer cells.

Further characterization of the Oct4 (+) breast cancer cells was done by conducting proliferation assays. As expected of these primitive cells, it was found that the Oct4(+) cells had a significantly higher doubling time (~60 hrs) compared to both heterogeneous breast cancer cells (~20 hrs) and Oct4(-) cells (~19 hrs).

Preliminary in vivo data shows that in mice injected with Oct4(+) cells and treated with chemotherapy, tumor volume initially decreases but levels off around 0.07 cm$^3$. This may indicate that cells within these tumors are resistant to chemotherapy. This is in contrast to tumors in mice injected with heterogeneous breast cancer cells which had a >95% response to treatment with carboplatin.

Conclusion:

In the presence of MSCs, Oct4(+) cells induce T$_{reg}$, whereas Oct4(-) cells induce Th17, confirming the dual role MSCs play in immunoregulation amidst the tumor microenvironment. Oct4(+)-cancer cells appear to form GJIC with MSCs and stroma at higher efficiency compared to Oct4(-) breast cancer cells which could explain the induction of differing immune responses in co-cultures of these cell populations. In general, Oct4(+) cells show longer doubling times than Oct4(-) cells and this could be one factor explaining resistance of these cells to chemotherapy, confirmed by in vivo studies. Additionally, in vivo studies have shown Oct4(+) cells to have a higher tumorigenic potential as compared to Oct4(-) and heterogeneous breast cancer cells.
8. JILL K DEUTSCH (NJMS 2013)

DISSECTING THE ROLE OF ERBB2/RAS SIGNALING NETWORK ON METASTATIC POTENTIALS OF PROSTATE CANCER CELL LINES

Mentor: Lizhao Wu, PhD (Microbiology and Molecular Genetics)

Objective:
The ErbB2/Ras-Rb/E2F signaling network has been implicated in several fundamental biological processes that are directly related to cancer development including cell growth, cell death, terminal differentiation, cell migration, and cell invasion. Essentially all human malignancies analyzed to date carry genetic alterations in this signaling network, either by Rb mutations, by the inactivation of negative cell cycle regulators such as cyclin-dependent kinase inhibitors, or by the activation of positive acting components such as ErbB2, Ras, and Myc. In addition, metastatic tumors or those at advanced stages tend to have increased levels of ErbB2 or Ras, suggesting that these two oncogenes may also be involved in cancer metastasis.

In American men, prostate cancer represents the highest estimated number of new cancer cases and the second leading cause of cancer deaths. Unfortunately no effective therapy is currently available for patients with advanced disease or metastasis primarily due to our limited understanding of specific molecules and signaling pathways involved in the progression of this cancer to an androgen independent state and metastasis. These are the two most frequent events that lead to hormone-ablation therapy failure and eventual patient death. Therefore, understanding the molecular changes that are responsible for these two critical events is the key to an effective cancer therapy. To determine whether the ErbB2/Ras signaling network plays a role in prostate cancer metastasis, we first used a retroviral system to over-express the ErbB2 or Ras oncogene in well-characterized human prostate cancer cell lines derived from secondary metastases. These cell lines range in motility and invasiveness, allowing us to determine whether over-expression of either oncogene in these lines increased their metastatic potential. Metastatic potential was measured by motility using a scratch-induced wound migration assay and a Transwell motility assay.

Methods:

Cells. Phoenix amphotrophic cells were stably co-transfected with pBabePuro-ErbB2 or pBabePuro-Ras retroviral constructs with packaging plasmids. The retroviral supernatant was collected at 48 and 72 hours post transfection, and PC3, DU145, and LNCaP cells were infected with the retroviral supernatant at the two time points. Selection of transfectants was performed by culturing cells in medium containing puromycin at a concentration of 2 mg/ml for five days. PC3 and LNCaP human prostate cancer cells were then maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS); DU145 human prostate cancer cells were maintained in DMEM medium with 10% FBS, glutamax, essential amino acids, and sodium pyruvate.

Wound Healing Assay. Cells were cultured to confluence in p60 dishes and starved for 24 hours with the corresponding media listed above containing 0.1% FBS. Three wounds were created with a 200 μL pipette tip vertically across a horizontal marked reference line. The cells were rinsed with PBS twice and subsequently at each time point, and starvation media was replaced. Pictures were taken at intervals of every 2, 4, or 8 hours.

Transwell Motility Assay. Becton Dickinson wells were placed into a 12 well tissue culture dish and coated with 3% collagen. Cells were plated into the Transwell chambers in 0.5% starvation media and placed in a 12 well tissue culture dish with 10% FBS growth media. Cells were fixed and stained at 18 or 54 hours.
Statistical evaluation. Statistical significance of the results was determined by a two-tailed t-test using Microsoft Excel. A probability of less than 5% (p<0.05) was considered significant.

Summary:

Our lab has previously shown that it is possible to produce a transgenic mouse model of prostate cancer in which the Myc oncogene is overexpressed to produce a non-metastatic prostate carcinoma. It was our goal with this study to determine, in cultured human cell lines, if the ErbB2/Ras signaling pathway plays a role in proliferation, motility, and invasion of these prostate cancer cells. To assess the motility of these cell lines and the various cell conditions, we performed a wound healing assay and a Transwell migration assay.

The three cell lines that were used are well characterized in literature and some of their features are shown in the following table.

Table 1. Derivation, androgen dependence, metastatic potential, and PSA expression of three well-defined human cell lines, PC3, DU145, and LNCaP.

<table>
<thead>
<tr>
<th></th>
<th>PC3</th>
<th>DU145</th>
<th>LNCaP</th>
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<tbody>
<tr>
<td>Derivation</td>
<td>Advanced Bone Metastasis</td>
<td>Brain Metastasis</td>
<td>Left Supraclavicular Lymph Node Metastasis</td>
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<td>Androgen Dependence</td>
<td>Androgen Independent</td>
<td>Androgen Independent</td>
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<tr>
<td>PSA Expression</td>
<td>Expresses PSA</td>
<td>Does Not Express PSA</td>
<td>Expresses PSA</td>
</tr>
</tbody>
</table>

In the wound healing assay, it was observed that the Ras-overexpressing cells closed the wound faster than either controls or ErbB2-overexpressing cells for the cell lines with the highest and lowest metastatic potentials; the control cells were slowest to fill in the wound. DU145 cells moved opposite to this pattern.

Figure 1. Wound healing data for three human prostate cancer cell lines: PC3, DU145, and LNCaP. Ras- and ErbB2-overexpressing cells, in addition to control cells were plated at 100% confluence and starved for 24 hours when wounds were made as described for the wound healing assay. Pictures were taken every 2, 4, or 8 hours to monitor cell migration. Time 0 is presented here along with T6 for PC3 and T24 for DU145 and LNCaP cell lines. In PC3 and LNCaP, Ras-transfected cells filled in the wound most rapidly, while controls were slowest. DU145 cells moved opposite to this pattern.
The western blot analysis of these three cell lines to ensure that each infected cell did actually overexpress the particular oncogene indicated that both DU145 and LNCaP cell lines had a low endogenous level of ErbB2 expressed. This can be one possible explanation for why the ErbB2 and control cells moved similarly in these two cell lines. It has to be noted that in all three cell lines ErbB2- and Ras-overexpressing cells grow slower compared to control cells. To minimize the effect of the intrinsic differences on growth rates of control cells and ErbB2- or Ras-overexpressing cells, we starved cells for 24 hours before injuring. This allowed us to measure the migration rate of the cells in this study, with minimal contribution from differential growth.

The Transwell motility assay was also used to assess the migration property of these cells. In PC3 and LNCaP, control cells moved through the membrane most rapidly, while ErbB2-transfected cells were slowest. In DU145 control cells moved slowest, while Ras-transfected cells moved the most rapidly.

One possible explanation for the abundance of control cells in this experiment is that the pore size for the wells that were used was 8 μm. ErbB2- and Ras-overexpressing cells are enlarged, making it more difficult to pass through the membrane in 18 hours. Furthermore, especially the LNCaP cells required longer time to migrate through the membrane, increasing the time where cells were exposed to the media containing 10% FBS. Based on our observations control cells proliferate faster than Ras- or ErbB2-overexpressing cells, indicating that higher cell counts seen in control cells resulted not only from the motility, but also from their higher cell growth rate. Taken together, this may indicate that the LNCaP cell line may not be suitable for the Transwell motility and invasion assays, and that the wound healing assay may be the best approach to assess the motility of these cells in vitro.

Conclusion:
Ras-overexpressing cells tend to migrate into a wounded area faster than ErbB2 or control cells (for both the PC3 and LNCaP cell lines). In both cell lines, the control cells migrated into the wounded space slower than Ras and ErbB2, possibly indicating the efficacy of these oncogenes in the motility of prostate cancer cells. It should be taken into consideration that control cells grow faster than Ras- and ErbB2-overexpressing cells, which may eliminate the difference in motilities observed in this group. Cell motility through a microporous membrane...
indicated that control cells were the most motile and ErbB2-transfected cells were the least motile in the PC3 and LNCaP prostate cancer cell lines. In the moderately metastatic line, DU145, controls were the least motile, while Ras-transfected cells were the most motile.

Future plans for this study include an invasion Transwell assay which mirrors the motility assay described, but where a Matrigel layer mimicking an extracellular matrix, is formed within the Transwell. The next step is to perform an in vivo study in immuno-compromised mice where ErbB2- and Ras-overexpressing cells from each of the prostate cancer cell lines would be injected to monitor their metastatic potentials. This data indicates that Ras- and ErbB2-overexpression in prostate cancer cell lines may facilitate the process of prostate cancer metastasis.

References:

MATTIE HANLON (NJMS 2013)

MOLECULAR ANALYSIS OF MALIGNANT B-1 CELL CLONES

Mentor: Elizabeth Raveche, PhD, (Pathology)

Objective:

Identify the presence of NZB malignant B-1 cell clones by analysis of the highly variable CDR3 region at the IgH loci.

Explore a possible connection between previously defined depressed levels of the microRNA miR15a/16 and elevated levels of BSAP, a member of the PAX5 gene family of transcription factors, in malignant CLL and NZB B-cells.

Methods:

DNA from malignant NZB spleen cells, an in-vitro NZB-derived malignant B-cell line, LNC, and a wild-type control, C57, was amplified via PCR, gel electrophoresis, and gel extraction. Primers to be used in PCR which would amplify the IgH CDR3 sequence of interest (~140-170 base pairs) were chosen. Capillary electrophoresis and fragment analysis of DNA PCR products were performed in order to determine clonality on the basis of fragment size. Delivery of naked antisense oligonucleotide specific for BSAP mRNA into a culture of malignant LNC cells, along with a negative control scrambled oligonucleotide, was carried out to determine its effects on miR15a/16 levels and cell-cycle arrest. Cell cycle arrest was analyzed via flow cytometry. RNA was isolated and quantified through reverse transcription and Real Time PCR.

Summary:

Fragment analysis confirmed the presence of dominant clones in the NZB CDR3 region, which were of similar fragment size to the CDR3 region of an in-vitro NZB-derived malignant B-cell line, LNC, and dissimilar to the wild-type control profiles. (see "IgH Region DNA PCR Fragment Analysis" below)
Since the \textit{mir}15a/16 gene is located in an intronic region of the DLEU2 gene, we investigated the effect of decreased transcriptional regulation of DLEU2 on miR15a/16 microRNA levels.
Since BSAP is a transcription factor thought to be a negative regulator of DLEU2, decreased BSAP via antisense should result in increased miR15a/16 levels which would lead to decreased proliferation of malignant B-1 cells. miR15a/16 levels increased in a dose-dependent manner following antisense BSAP treatment. (see “miR-15a/16 expression in LNC B-cells” below)

**miR-15a/16 expression in LNC B-cells**

Addition of BSAP antisense oligonucleotides caused significant elevation of miR-15a/16 levels in a dose-dependant fashion.

In addition, cell cycle arrest was proportional to increased miR15a/16 levels. (see “Effect of Antisense BSAP on LNC Cell Line” below)

**Effect of Antisense BSAP on LNC Cell Line**

Flow cytometry illustrates the dose-dependant effects of BSAP Antisense oligonucleotide on the LNC cell cycle causing a marked increase in the percentage of cells arrested in G2.

**Conclusion:**

The observed fragment size similarities between the dominant clones in the NZB CRDR3 region and the LNC CDR3 region may indicate the well-conserved nature of a unique clonal rearrangement. In the future, the CDR3 region of NZB B-cell DNA will be sequenced with the goal of further characterizing the molecular abnormalities which contribute to clonal selection.

We propose that by binding to the DLEU2 promoter, BSAP acts as a negative regulator of mir15a/16, thereby promoting B-cell proliferation and clonal development. Early results indicate targeting BSAP may be a potential therapy for CLL.
10. HEATHER HOLAHAN (NJMS 2013)

NALTRINDOLE AND RELATED OPIOIDS INHIBIT HUMAN MULTIPLE MYELOMA CELL PROLIFERATION

Mentor: Richard D. Howells, PhD, Department of Biochemistry and Molecular Biology

Objective:

Multiple Myeloma (MM) affects approximately 20,000 people in the United States each year with an average age at diagnosis of 61 years old in women and 62 years old in men (Raab et al, 2009). The risk of being diagnosed with MM across a lifetime is .66% for men and .55% for women (Anderson et al, 2007).

The malignancy involves the uncontrolled growth of plasma cells in the bone marrow which produces lytic bone lesions and monoclonal proteins such as IgA and IgG (Brown, 2005). The bone marrow environment plays a pivotal role in the viability of the cancer cells by supplying them with chemokines, cytokines, and relevant growth factors (Markovina et al, 2010). MM cells adhere to extracellular proteins in the bone marrow, and are bathed in fluid full of cytokines, IL-6, VEGF, IGF1, TNF super family, and IL-10. Bone lesions and destruction occur from increased osteoclast production and function after the release of Osteoclast Stimulatory Factor (OSF) by the myeloma and bone marrow cells (Roodman, 1995; Choi et al, 2000).

While treatment options have lengthened survival time, MM still remains a terminal disease. Initial response by patients to treatments such as chemotherapy, radiotherapy, bisphosphonates, and stem cell transplantation, is met with later relapse and treatment refractor. Treatment refractor has been attributed to the factors contributed by the bone marrow environment like fibronectin, insulin growth factor (IGF-1), stromal derived factor 1 alpha (SDF-1), tumor necrosis factor alpha (TNF-α), and B cell activating factor family (BAFF) (Markovina et al 2010). Present treatments such as Velcade have aimed at blocking the interaction between MM cells and the bone marrow. Velcade reversibly inhibits the catalytic site of the 20S proteosome and has shown to be effective in patients after relapse (Park et al 2008).

Naltrindole (Nti) is a delta opioid antagonist which has shown some immunosuppressant and anti-proliferative characteristics in mixed lymphocyte reactions, renal transplants, small cell lung cancer and MM cells (Kieffer et al 2001; Yulong et al 2004). In small cell lung cancer, Nti appears to block the Akt/PKB pathway, which is involved in glucose uptake, protein synthesis, and the inhibition of cell death (Chen et al, 2004). Interestingly, Nti binding does not appear to involve the mu, kappa, or delta opioid receptor as when all three are knocked out in mice, Nti is still able to express its immunosuppressive properties (Gaveriaux-Ruff et al, 2001). Our lab has determined that Nti binds optimally at room temperature, and that the cell must be intact in order for Nti to bind. Apart from these characteristics, the exact binding site is still unknown. Nti also attenuates opioid tolerance without affecting the pain relief properties of opiates such as morphine at the mu receptor (Abdelhamid et al, 1991). Naltrindole has great therapeutic potential for MM not only due to its anti-proliferative effects, but also due to its prevention of opiate tolerance.

Curcumin is the effective ingredient in turmeric, derived from the plant Curcuma longa, and has been used in Ayurveda and Far Eastern medicine to treat inflammatory diseases for centuries (Shishodia et al 2006). Presently, curcumin has been found to have anti-proliferative activity in MM cells by increasing apoptosis, by suppression of the NF-κB pathway, by inhibition of STAT3 phosphorylation, and IL-6 signaling (Park et al 2008). When curcumin was used in
combination with Velcade, MM cell lines showed elevated apoptosis than when either treatment was used alone (Park et al 2008).

The aims of this study were to determine the effect of curcumin on cell viability and to check whether it inhibits naltrindole binding to U266 MM cells. This investigation is important because present treatments for MM can have cytotoxic effects. It has been shown, that when using curcumin, which has much less cytotoxic effects, in combination with Velcade, a lower concentration of Velcade was required to obtain the same effect. Therefore, curcumin and naltrindole could also be used together for the treatment of MM however it is necessary to investigate their binding properties.

Methods:
U266 cells were plated and maintained in RPMI 1640 media, 10 % FCS and 1 % Penicillin/Streptomycin at 37° C.

In order to determine naltrindole’s binding in the presence of curcumin, a binding assay was used. U266 cells were harvested, spun, and re-suspended in 2mL PBS. 10 nM of \(^3\)H-Nti was added to duplicate test tubes containing .05 %, .2 %, .5 %, 1 % and 2 % DMSO and duplicate test tubes containing 5 µM, 20 µM, 50 µM, and 200 µM of Curcumin. 500 µM Nti was added to duplicate tubes to assess non-specific binding. Samples were incubated for 30 minutes at room temperature, filtered under reduced pressure through Whatman GF/B filters and specific binding was determined by liquid scintillation counting.

A glutathione assay was employed in order to detect the presence of reactive oxygen species (ROS). Glutathione is an antioxidant which protects cells from the cytotoxic effects of ROS. U266 cells were incubated with varying concentrations of Nti and Curcumin overnight. 20 mM of Monochlorobimane was added to each condition and washed at 1 hour. Monochlorobimane forms a fluorescent complex with reduced glutathione, hence the less the amount of reduced glutathione in the cell, the more ROS produced. Fluorescence was assayed with excitation at 380 nm and emission at 460 nm. The procedure was modeled after the Molecular Probes Invitrogen Protocol.

In order to assess cell viability, U266 cells were treated with 10 M Nti, 10 M curcumin, and 10 M Velcade both alone, and in combination. Cells were incubated for 24 and 48 hours, and 1mL aliquots were assessed viability using a Vi-Cell analyzer.

Summary:
The aims of this study were to determine whether curcumin, a compound shown to have anti-proliferative effects in Multiple Myeloma cells, has an effect on the ability of naltrindole to bind. Secondly, we sought to determine how curcumin affects proliferation of MM cells and its effect on cell viability.

Curcumin appears to inhibit naltrindole binding in a dose-dependent manner with an IC\(_{50}\) of 65 µM. This suggests that curcumin and naltrindole share a common binding site.

Curcumin also appears to produce ROS in cells after incubation overnight. As documented in the literature, one of curcumin’s anti-proliferation mechanism involves the production of ROS while naltrindole doesn’t seem to use this mechanism.

Curcumin as well as Velcade, a proteosome inhibitor used in the treatment of Multiple Myeloma, both seem to decrease cell viability over 24 and 48 hours while naltrindole appears to stabilize it. Proliferation assays conducted by our lab have shown that naltrindole decreases cell proliferation in a dose dependent manner, however it doesn’t have a significant effect on cell viability.
Conclusion:

Multiple Myeloma is presently a fatal disease with a median survival of 3 to 4 years. Our lab has identified naltrindole, a delta opioid antagonist, as a compound that decreases the proliferation of Human Multiple Myeloma U266 cells. Interestingly, curcumin, the main ingredient in the Curcuma longa plant, has also been shown to decrease the proliferation of human Multiple Myeloma cells. We have shown that curcumin decreases the binding of naltrindole to MM cells. Further studies such as proliferation assays and in vivo experiments, using both naltrindole and curcumin need to be performed in order to determine if these drugs can be used in combination for MM treatment.
11. JEFFREY KIM (NJMS 2013)

THE USE OF BLOOD OXYGEN LEVEL-DEPENDENT FUNCTIONAL MRI SIGNAL AS A DIAGNOSTIC TOOL TO DISTINGUISH DIFFERENT TYPES OF BRAIN TUMORS

Mentor: Susan C. Feldman, PhD (Radiology)

Objective:

Blood Oxygen Level-Dependent Functional MR imaging (BOLD fMRI) is a non-invasive technique that detects functional cerebral-hemodynamic changes as a signal that is dependent on the concentrations of oxyhemoglobin and deoxyhemoglobin. BOLD fMRI has been traditionally used to map functionally related areas of the brain. In a preliminary investigation of brain tumors, we showed that BOLD fMRI is a useful diagnostic tool to identify the extent of tumor infiltration and to distinguish brain tumor vs. non-tumor areas (*Feldman et al., 2009; AJNR*). In this study, we hypothesized that BOLD fMRI signals could also be used to differentiate between different types of brain tumors, specifically gliomas and meningiomas, two of the most prevalent brain tumor pathologies. This approach to quantifying imaging may be of use as a supplement to traditional imaging techniques, allowing neuro-oncologists and neurosurgeons to visualize lesions that are difficult to be seen in their entirety, and therefore, to better tailor the course of treatment for individual patients.

Methods:

Subjects and Tasks

In this retrospective study, ten neurosurgery patients were scanned with BOLD fMRI (as well as normal MRI, diffusion, and perfusions scans) before surgery. There were five cases of gliomas and five cases of meningiomas. Informed consent was obtained from all subjects at the time of scanning. All patients performed a bilateral, thumb-to-digits opposition motor task during the BOLD fMRI scan. This motor paradigm lasted 3 minutes, alternating between 20-second “on / finger tapping” and “off / resting” periods.

fMRI Acquisition and Processing

The BOLD fMRI images were acquired on a 1.5T GE EchoSpeed Horizon scanner. Patients were given a normal MRI scan (T1-weighted, gadolium-enhanced images) for anatomical overlay, followed by the BOLD scan. Raw data was pre-processed using the neuroimaging software SPM (Statistical Parametric Mapping) 99 (Wellcome Dept. of Imaging Neuroscience, London, UK). Functional analysis of BOLD fMRI signals were processed using Analysis of Functional NeuroImages (AFNI) software (developed by RW Cox in 1996; [afni.nimh.nih.gov](http://afni.nimh.nih.gov)). The AFNI program analyzes the BOLD fMRI signal from a single, “seed” point of interest, also known as a voxel of interest (VOI). By adjusting to any particular correlation value, \( R \) \((-1 \leq R \leq 1)\), the AFNI program highlights any other voxels that have a similar BOLD fMRI signal characteristics. The \( R \) value for each VOI was selected so to either maximize the amount of tumor area highlighted, and any normal brain excluded (Tumor \( R \) value) or so that the maximum non-tumor area was highlighted with any tumor excluded (Normal \( R \) value) – see Table 1 and Figure 1.

*Figure 1. Anatomical and fMRI Overlay. Patient 9, Meningioma. Tumor \( R = .99 \)*

Top row shows standard MRI scan images (T1-weighted, gadolium-enhanced images) that were used to identify tumor area. Bottom row shows functional BOLD fMRI with tumor area highlighted for a VOI with a given correlation (in this case Tumor \( R = .99 \)). \( R \) values for each
VOI were selected so that either the maximum amount of tumor area was highlighted with any normal brain excluded (Tumor $R$ value) or so that the maximum non-tumor area was highlighted with any tumor excluded (Normal $R$ value). Note from L to R: Coronal, Sagittal, 3 Axial images.

**Data Analysis**

For each patient, 6 VOIs within the tumor areas were sampled according to the following protocol: 2 points located at the center of the tumor; 2 points between the center and the periphery of the tumor; 2 points at the periphery of tumor. For normal tissue areas, we sampled 3-5 VOIs depending on the mass of tumor: at least one point contralateral to the center of the tumor and at least one point adjacent to periphery of the tumor area - see Figure 1. BOLD signals from tumor VOIs were compared to each other within a tumor area, from tumor to non-tumor area within a single subject, and from glioma to meningioma using paired Student’s T Tests.

**Figure 2. Sampling of VOIs in Tumor and Non-Tumor Areas**

Left: Axial Flair MRI Image. Right: Functional BOLD fMRI showing highlighted tumor area. Numbers within ovals represent VOIs within tumor area; numbers outside oval represent non-tumor areas. 6 points were selected for analysis from tumor area and 3-5 points were selected for analysis from normal, non-tumor areas.

**Summary of Results: Tumor vs Non Tumor**

As expected, the analysis of the BOLD signal confirmed the results of our previous studies – that BOLD signals can be easily and quickly used to identify the extent of tumor infiltration and
to distinguish between tumor vs non-tumor areas within patients with either gliomas or meningiomas – as shown by P values comparing the BOLD signal from tumor and non-tumor areas (Table 1). Moreover, for both meningiomas and gliomas, VOIs in tumor areas under extremely high $R$ values highlighted the whole tumor mass while excluding any normal brain tissue - see Figure 1.

Table 1. Statistical Results: Tumor vs. Non-Tumor

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor</th>
<th>Tumor $R$ Value</th>
<th>Normal $R$ Value</th>
<th>Tumor Mean &amp; (SD)</th>
<th>Non-Tumor Mean &amp; (SD)</th>
<th>P value comparing Tumor to Non-Tumor</th>
<th>“Tumor Mean” minus “Non-Tumor Mean”</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glioma</td>
<td>0.99</td>
<td>0.95</td>
<td>2494 (414.8)</td>
<td>1997 (40.78)</td>
<td>&lt;.0001</td>
<td>497.1</td>
</tr>
<tr>
<td>2</td>
<td>Glioma</td>
<td>0.99</td>
<td>0.98</td>
<td>5614 (109)</td>
<td>(4527) (762)</td>
<td>&lt;.0001</td>
<td>1087</td>
</tr>
<tr>
<td>3</td>
<td>Glioma</td>
<td>0.99</td>
<td>0.97</td>
<td>4484 (121.7)</td>
<td>3690 (84.85)</td>
<td>&lt;.0001</td>
<td>794.0</td>
</tr>
<tr>
<td>4</td>
<td>Glioma</td>
<td>0.99</td>
<td>0.96</td>
<td>4956 (515)</td>
<td>3815 (86.7)</td>
<td>&lt;.0001</td>
<td>1141</td>
</tr>
<tr>
<td>5</td>
<td>Glioma</td>
<td>0.99</td>
<td>0.95</td>
<td>3930 (432)</td>
<td>3810 (82.24)</td>
<td>&lt;.0001</td>
<td>120.56</td>
</tr>
<tr>
<td>6</td>
<td>Meningioma</td>
<td>0.99</td>
<td>0.94</td>
<td>6232 (533)</td>
<td>4142 (471)</td>
<td>&lt;.0001</td>
<td>2090</td>
</tr>
<tr>
<td>7</td>
<td>Meningioma</td>
<td>0.99</td>
<td>0.95</td>
<td>3062 (175)</td>
<td>2334 (87)</td>
<td>&lt;.0001</td>
<td>728</td>
</tr>
<tr>
<td>8</td>
<td>Meningioma</td>
<td>0.99</td>
<td>0.98</td>
<td>2628 (16)</td>
<td>2241 (73)</td>
<td>&lt;.0001</td>
<td>387</td>
</tr>
<tr>
<td>9</td>
<td>Meningioma</td>
<td>0.99</td>
<td>0.99</td>
<td>4830 (176)</td>
<td>4155 (140)</td>
<td>&lt;.0001</td>
<td>676</td>
</tr>
<tr>
<td>10</td>
<td>Meningioma</td>
<td>0.99</td>
<td>0.97</td>
<td>5614 (109)</td>
<td>3975 (112)</td>
<td>&lt;.0001</td>
<td>1639</td>
</tr>
</tbody>
</table>
Summary of Results: Glioma vs. Meningioma

When we analyzed whether the BOLD fMRI signal itself could differentiate meningiomas from gliomas, there was no statistical difference between the two tumor populations. It is possible that this result was due to the limited population studied. See Table 2.

### Table 2. Glioma vs. Meningioma: Statistical Results

<table>
<thead>
<tr>
<th></th>
<th>Glioma Mean &amp; (SD)</th>
<th>Meningioma Mean &amp; (SD)</th>
<th>P value comparing Glioma Mean vs. Meningioma Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Mean &amp; (SD)</td>
<td>4296 (1182)</td>
<td>4473 (1575)</td>
<td>0.89</td>
</tr>
<tr>
<td>Mean of (“Tumor Mean” minus “Non-Tumor Mean”) &amp; (SD)</td>
<td>727 (426)</td>
<td>1104 (724)</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Conclusion:

This study is a continuation of previous work in our lab that is designed to identify novel techniques in brain tumor imaging using the BOLD fMRI signal. The present study confirmed our past results in that the tumor BOLD signal itself can be used to quickly define the extent of brain tumor vs. normal tissue. In regards to our hypothesis, that the BOLD fMRI signal can be used to distinguish between glioma and meningioma, our results are inconclusive based on the limited size of our patient cohort. Further studies should include a larger patient cohort, including a diversity in the grades of tumors analyzed.
12. **ELIZA LAMIN (NJMS 2013)**

**USING IN VIVO CELL TRACKING TECHNOLOGY TO UNDERSTAND HOW T CELLS PROTECT AGAINST TUMOR DEVELOPMENT AND GROWTH**

Mentors: Yu Li, PhD (Oral Biology), Vincent Tsiagbe, PhD (Oral Biology), Nicholas M. Ponzio, PhD (Pathology & Laboratory Medicine)

**Objective:**

Although T cells are found in many different tissues of murine and human neoplasms, the reason they migrate to these sites of cancer growth is not fully known. Previous studies have shown that the presence of tumor responsive T helper-1 (Th1) cells at sites of tumor burden can inhibit tumor development and growth in an experimental lymphoma model in SJL mice. To investigate the migration pattern of these cells, tumor specific Th1 cell lines were developed in vitro by stimulation of naïve T cells with irradiated lymphoma cells in the presence of the Th1-polarizing cytokine, Interleukin (IL) 12. These tumor specific Th1 cells were then expanded and maintained in vitro in medium supplemented with the T cell growth cytokine, IL-2.

Using a limiting dilution cloning technique, several cloned lines were derived from one parental Th1 cell line. Cells from one of these cloned Th1 cell lines were transfected with a vector (pGL4.51[ luc2/CMV/neo]) that contained a luciferase gene. Since the bioluminescence of the luciferase-transfected cells can be measured using a sensitive imaging system, the in vivo migration and localization of these tumor specific Th1 cells can be tracked in living animals over a period of time. Therefore, in future studies it will be possible to inject these cells into SJL mice, and locate them in vivo. Since SJL mice spontaneously develop B cell lymphomas at approximately 13 months of age, it will be possible to demonstrate the migration patterns of the injected cells before, during, and after tumors begin to develop. It will also be possible to determine the role of the injected Th1 cells in mediating immune responses in the host that inhibit the development of primary lymphomas.

There are many immunotherapy techniques that are currently being investigated to treat human cancers. T cells that can produce a cytotoxic response in vitro are especially beneficial (1). Th helper 1 cells have been shown to produce a cytotoxic effect on host neoplasms when the Th1 cells have been grown with IL-12 in primary cultures (2). Th helper 1 cells mature in the thymus and then travel throughout the body combating infection and non-self antigens. In SJL mice the spontaneous tumors that develop are called Reticulum Cell Sarcomas (RCS) and they stimulate proliferation of Th1 cells in culture and in vivo (5). A monoclonal population of Th1 cells is needed in order to proceed with transfection. Once this is done, using a limiting dilution technique, it is possible to transfect the population. The method used was a luciferase assay system. The light emitted from the firefly luciferase was measured, the luciferin-luciferase reaction is very strong and the method of choice in the field of molecular biology (7). It has a sensitive detection and is better than chemiluminescence and fluorescence for comparison. In order to view a transfected colony of cells an imaging system is necessary. The IVIS imaging system provides a visual of the luminescence of the cells, using a CCD camera.
Methods:

**Cell lines**

The cell lines were derived from lymph node cells in SJL/J mice. The cells were cultured in complete media with irradiated SJL lymphoma (γ-RCS) to expand tumor-responsive T cells, and with Interleukin 12 to drive differentiation toward Th1 and with IL-2 to promote Th1 cell growth. They were stimulated with irradiated SJL lymphoma (γ-RCS) biweekly to maintain specificity, and given irradiated spleen cells as feeder cells every four weeks. Parental Th1 cell lines were cloned, and one clone (AML-1.5.1) was selected for luciferase transfection.

**Flow Cytometry**

Immunofluorescence assay was done initially to test for expression of surface markers CD4 (all Th cells), Tim-3 (Th1 cells) and T1ST2 (Th2 cells). *(Fig. 1)* A separate experiment was done to test for intracellular markers; IL-4 (Th2 cells) and IFN-γ (Th1 cells). *(Fig. 2)*

**ELISA**

IL-2 production was tested with an Enzyme-Linked ImmunoSorbent Assay (ELISA), which detects the presence of the IL-2 protein using a sandwich technique. A capture antibody was bound to the plate, and then nonspecific binding sites are blocked. Test samples were added, and the anti-IL-2 capture antibody binds to IL-2 in the sample. Finally, the enzyme-linked secondary anti-IL-2 antibodies were added; substrate was added which was converted by the enzyme to a color signal, and absorbance is measured. Cloned AML-1.5.1 Th1 cells were cultured with γ-RCS to stimulate cytokine production, and supernatant culture fluid was collected 24 hours later. ELISA was also done to test for production of IFN-γ.

**CTL Assay**

A $^{51}$Cr release assay was done to determine levels of cytotoxic activity. A spleen was taken from an SJL mouse and a cell suspension was made at 20x10^6 cells/mL. RCS tumor cell suspension was prepared from an SJL mouse, irradiated at 7500R, and co-cultured with SJL spleen cells at different ratios with or without additional stimulation. In the first culture, there was no stimulus, the second had 60 µL of IL-12 @ 1µg/mL, the third through sixth had AML1.5.1 cells (Th1 Clone) @ 20x10^6 in ratios of 1:1, 2:1, 4:1, and 10:1. These effector cells were incubated for four days. Then, target cells (NJ117) were labeled with $^{51}$Cr. Isotope release from target cells incubated alone and cells incubated with 4% Nonidet P-40 were measured for minimum and maximum release, respectively.

**Transfection**

The transfection of cloned cells was done in serum-free media with four different concentrations of DNA. Viable transfected cells were purified after 24 hours, and G418 antibiotic selection was initiated 48 hours post transfection. They were grown continuously with the antibody as well as IL-2 and IL-12 to promote growth. The transfected cells contained the gene that made them resistant to the antibiotic, thereby forcing the non-transfected cells to die.

**Imaging**

In order to view the transfected cells and measure their luminescence an IVIS machine was used. The machine contained a CCD camera that was initialized and then provided an image of the cells showing the degree of their luminescence. The cells that were positive
controls and transfected Th1 clones showed a moderate to bright signal, but the untransfected cells did not appear at all.

**Summary:**
FACS analysis showed that 95% of the AML-1.5.1 clone were positive for the Th1 marker, Tim3 (Fig. 1; lower right quadrant). The intracellular FACS (Fig. 2) showed that these cloned cells were also positive for the Th1 marker IFN-γ. 2.

*Fig 1. FACS analysis of Cell Line AML1.5.1 for Th1 Markers-Extracellular*

![FACS analysis of Cell Line AML1.5.1 for Th1 Markers-Extracellular](image1)

*Fig 2. FACS analysis of Cell Line AML1.5.1 for Th1 Markers-Intracellular*

![FACS analysis of Cell Line AML1.5.1 for Th1 Markers-Intracellular](image2)

The ELISA for IFN-γ (Fig. 3) shows that the amount of IFN-γ produced by the clones increased with time after stimulation by γ-RCS. The supernatants were collected 16 hours, 24 hours, and 48 hours after stimulation with irradiated tumor.

*Fig 3. IFN-γ ELISA of AML 1.5.1 Supernatants*

![IFN-γ ELISA of AML 1.5.1 Supernatants](image3)

**Conclusion:**
Cloned tumor responsive cell lines were produced by in vitro stimulation of naïve T cells with B lymphoma cells. FACS analysis revealed that the cloned T cell line, AML-1.5.1, was positive for CD4 and Tim3 cell surface markers expressed by Th1 cells. These cells were also positive, by intracellular stain, for IFN-γ. Tumor-activated clones produced IFN-γ, and individual
clones grew at varying rates and produced different amounts of cytokine. Tumor-reactive Th1 cells were transfected with luciferase, for in vivo tracking of tumor killing. Over time it will be possible to track the Th1 cells in vivo to help determine the role that these tumor responsive cells play in mediating inhibition of tumor development and growth. The results from these future experiments will help to develop new strategies using Th1 cell immunotherapy for cancer.
13. JEFFREY MOORE (NJMS)

THE EFFECT OF SUPPLEMENTAL BONE GRAFTING IN BENIGN AGGRESSIVE EPI-METAPHYSEAL BONE TUMORS

Mentors: Joseph Benevenia, MD, Kathleen Beebe, MD, John C. Nielsen, MD (Orthopaedics)

Objective:
Curettage and adjuvant treatment in epi-metaphyseal, benign-aggressive tumors can destroy the subchondral surface causing early articular fracture and eventual osteoarthritis. Filling defects with Polymethyl-methacrylate (PMMA) cement may accelerate this via thermal damage and un-cushioned contact stress. To lessen these effects, reconstruction with PMMA and supplemental subchondral bone allograft has been suggested. The purpose of this study is to determine whether subchondral grafting, with or without PMMA, is comparable to treatment with PMMA alone in minimizing the rate of articular fracture and degenerative changes, while maintaining similarly low recurrence rates.

Materials and Methods:
39 patients with a histologically confirmed benign aggressive tumor of the epi-metaphyseal region were treated from 1994-2008. The inclusion criteria were a histologically confirmed giant cell tumor or chondroblastoma treated with manual curettage and mechanical adjuvant high speed burr, defects were required to be filled with PMMA, bone allograft, or PMMA and subchondral bone allograft, and some form of adjuvant treatment. Sex and age demographic data was recorded. Tumor characteristics collected included the type of defect, location of lesion, primary treatment, adjuvant treatment, prophylactic fixation utilized, days of follow up, and pre-operative conditions such as severe subchondral bone thinning, arthritis, recurrence, and articular fractures. After data collection, patients were separated into two groups for statistical analysis. In group A, patients were treated with cement alone. In group B, patients were treated with allograft or subchondral allograft with cement. A subgroup of B was created to evaluate recurrence rates with the hybrid technique. The same operative technique was performed for all patients. A cortical window the size of the lesion was made so that the entire cavity was visible, and the tumor was thoroughly manually excavated with a hand held curette. After removal of the bulk of the tumor, a hand held curette was used to identify the intact bone edge. The cavity was enlarged with a high-speed burr. Adjuvant treatment was administered, ranging from argon laser coagulation to liquid nitrogen cryosurgery. Patients who were placed in group A had their lesions filled with cement alone. Patients whose lesions had been filled with bone allograft alone or allograft supplemental to cement, were placed in group B. If deemed necessary by the primary surgeon, prophylactic fixation devices were implanted, such as locking plates, Steinmann pins, and various others.

Results:

Table 1: Post-Operative Outcomes

<table>
<thead>
<tr>
<th></th>
<th>Group A- Cement Only</th>
<th>Group B- Graft +/- Cement</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractures</td>
<td>5</td>
<td>0</td>
<td>0.010</td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>5</td>
<td>2</td>
<td>0.227</td>
</tr>
</tbody>
</table>
Patients presented with a total of 17 post-operative complications upon follow-up (Table 1). Group A presented with 13 complications (65%), while group B presented with only 4 (21%). Outcome measures were articular fractures, osteoarthritic changes, and tumor recurrences, all post-operatively. The results of each outcome measure were compared between groups A and B. The first outcome measured was post-operative articular fracture rate. In regards to articular fractures, 5 (25%) patients presented with a fracture upon follow up. All 5 fractures occurred in group A, which is statistically significant ($p = .010$). Further subgroup analysis was done on group A to determine if any variables were significantly correlated with post-operative fractures. The presence of extensive pre-operative complications such as a early pathologic fracture, marked arthritis, or tumor recurrence, was the only variable which correlate significantly with the patient developing a post-operative fracture ($p = .035$).

The next outcome measure was post-operative degenerative arthritic change. 7 total patients (17.95%) presented with osteoarthritis upon follow up; 5 (25%) patients from group A and 2 (10.5%) patients from group B. This difference between the two groups, although promising, did not prove to be statistically significant ($p = .227$) (Table 1). Subgroup analysis was done on group A and B respectively, to determine in any variables correlated with significance to the development of post-operative arthritis. In group A, the location of the tumor significantly correlated with the patient developing osteoarthritis ($p = .044$). Of the four patients treated for lesions in the distal tibia, 3 of them experienced degenerative changes. Only 1 of the 6 patients with lesions in their proximal tibia presented with arthritic change and only 1 out of 9 patients with lesions in their distal femur showed evidence of osteoarthritis (Table 2). In group B, the type of adjuvant treatment utilized by the surgeon correlated significantly with the development of arthritic changes. Specifically, 2 out of the 3 patients treated with cryosurgery showed degenerative changes upon follow-up. None of the other patients in group B developed arthritic degeneration, regardless of the type of adjuvant treatment utilized.

The last outcome measured was tumor recurrence. 5 patients (12.82%) presented with a recurrence. In group A, 3 patients (15%) showed recurrence during follow-up, while 2 patients in group B experienced recurrence (10.52%). This difference was not statistically significant ($p = .674$) (Table 1). Subgroup analysis of both group A and B respectively, yielded no statistically significant correlation between treatment variables and recurrence rates.

### Table 2: Patient Data (subgroup analysis done from this data)

<table>
<thead>
<tr>
<th>Group</th>
<th>#</th>
<th>Age (range)</th>
<th>Sex</th>
<th>Location</th>
<th>Adjuvant Treatment</th>
<th>Fixation Device</th>
<th>Follow-up (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cement</td>
<td>20</td>
<td>34.2 y (18-53)</td>
<td>11 Males</td>
<td>1 prox. humerus 9 distal femur 6 prox. tibia 4 distal tibia</td>
<td>3 Argon 13 Argon/Peroxide 3 Cryosurgery 1 Phenol</td>
<td>6 None 14 Devices</td>
<td>564 (12-2808)</td>
</tr>
<tr>
<td>Graft +/ cement</td>
<td>19</td>
<td>29.5 y (12-66)</td>
<td>9 Males</td>
<td>6 distal femur 12 prox. tibia 1 distal tibia</td>
<td>5 Argon 4 Argon/Peroxide 1 Peroxide 3 Cryosurgery 6 Phenol</td>
<td>6 None 13 Devices</td>
<td>1027 (0-3758)</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Conclusion:

When treating benign tumors of bone in the epiphyseal region such as giant cell tumors and chondroblastomas, surgeons attempt to remove the tumor, stabilize the area, minimize recurrence, and limit secondary degenerative damages. Curettage followed by filling has become generally accepted as the preferred treatment. Unfortunately, tumor and treatment can weaken subchondral bone and directly damage cartilage, ultimately leading to post-operative articular fractures and osteoarthritis.

The type of filler used in treatment plays a role in both maintaining the structural integrity of the joint, as well as the influencing the likelihood that a patient will develop long-term degenerative changes. Structurally, PMMA is used by surgeons because it is nearly as rigid and strong as native bone and it sets almost immediately post-operative. Its exothermic nature helps kill residual tumor cells, limiting recurrence and re-operation. These characteristics make PMMA an excellent choice to fill curettage bone cavity. However, these same desired traits, rigidity and exothermic nature, as well as other drawbacks of PMMA have been shown suggested to expedite some of the long-term degenerative changes seen in patients treated with cement, specifically articular fractures and osteoarthritis.

Autograft and allograft are other types of defect filler. They provide an attractive choice for surgeons as they show complete incorporation and restoration and of new subchondral bone after 6 months. However, there are many disadvantages associated with their use. Autograft involves increased operative time, blood loss, and risk of infection. Both types of grafts have been shown to only provide slight increases in subchondral strength then when the defect is left unfilled. This means there is a high probability of collapse and fracture of the subchondral bone, and surrounding articular cartilage, especially in the first six months after treatment, before complete incorporation of the bone graft. The lack of stability the bone graft construct can cause additional wear during everyday movements, increasing the risk of degenerative changes.

To strike a balance between the benefits and drawbacks of graft alone or cement alone, many surgeons have adopted a hybrid filling technique consisting of a bone graft liner followed by complete filling with PMMA. They hypothesized that bone graft will provide a cushion between bone and cement, limiting contact wear. The results of our study support this hypothesis as all five fractures seen in follow-up were found in Group A, patients treated with PMMA alone. There were no articular fractures in those patients treated with bone graft +/- cement; lending support to the idea that the hybrid reconstruction provides cushioning, limits contact stress, retains construct strength, and shields tissue from thermal damage, resulting in a decrease in degenerative changes.

In addition to articular fracture, degenerative joint disease remains a long-term concern following subchondral reconstruction. Although able to extend tumor margin, the thermal heat from PMMA can damage cartilage and limit the healing potential of local tissue. This exothermic reaction may result in increased risk of developing osteoarthritis after treatment. Mjoberg et al. concluded that a post-operative radiolucent zone at the intersection of bone and cement is a direct result of PMMA thermal damage. All patients in that study developed the radiolucent zone, a precursor to osteoarthritis, but none showed any symptoms of arthritis at latest follow-up. In turn, few studies have been able to prove the direct link between cement usage and osteoarthritis. Despite this, surgeons are wary of PMMA’s potential role in long-term degeneration, suggesting that the ill-effects may develop many years post-operatively; beyond the scope of most available studies.

Unlike PMMA, bone graft does not have the potential to cause thermal damage provides more cushion. Despite these beneficial factors, Slazay et al. showed that bone graft provides poor structural integrity to treated bone. The weaker infrastructure at the joint causes an increase in movements and bone contact which actually leads to more degenerative changes than seen with PMMA.

The hybrid method seeks to retain the benefits of each filler material while minimizing their drawbacks. Studies hypothesize that in using the hybrid method for treatment, the bone graft liner will shield healthy tissue from the potential thermal damage of PMMA, while still retaining the construct strength of the cement. We compared bone graft +/- cement to defects treated with cement alone; however, the results of our study do not conclusively link PMMA to an increased chance for developing osteoarthritis. In group A, cement only, 5 patients developed osteoarthritis. In group B, graft +/- cement, only 2 patients experienced arthritic...
changes, of which only one was symptomatic. Although group A presented with more cases of osteoarthritis and did group B, the difference was in not statistically significant. Our results demonstrate the use of bone graft shows potential, although not statistically significant, for decreasing risk of arthritic change.

Finally, post treatment tumor recurrence represents a calculated risk that is minimized through careful confirmation of clear margins. It has been shown that thermal nature of PMMA extends tumor margin; reducing tumor recurrence⁵. Also, PMMA is far easier to remove and replace than bone graft when a re-operation is necessary. Bone graft is not associated with an exothermic process; therefore there is no evidence to suggest graft will prevent recurrence⁶. Our study was inconclusive in regards to differences in tumor recurrence. Of the 6 total recurrences in our patient population, 3 occurred in Group A while 3 occurred in Group B. Our results seem to support that hypothesis that the hybrid method maintains low recurrence rates, similar to cement only.

To our knowledge our study represents the first reported in the literature comparing articular fracture, degenerative change, and tumor recurrence in patients treated with PMMA alone versus those treated with bone graft +/- cement filler. The results of our study indicate that graft +/- cement shows great promise in preventing articular fractures when compared to using cement filler alone. Osteoarthritis occurred less frequently in the graft +/- cement group. Although not statistically significant, this result is likely due to the bone graft lining providing a protective effect against the thermal and mechanical stress induced by cement. There was no difference in recurrence rate between the two groups. At minimum, the use of hybrid method, as well as bone graft alone, represents no additional harm to the patient and may help prevent post-operative fracture. In light of these results we recommend the use of cement with a bone graft lining for repair of subchondral bone defects after tumor resection.

References:

Objective:
To investigate the stimulation of new cancellous bone formation by an anabolic agent, parathyroid hormone (PTH), in a mouse model for post-menopause with reduced circulating estrogen and insulin-like growth factor-1.

Low bone mineral density (BMD) and increased fracture risk are associated with several cancers and the therapies used for treatment.\(^1\) The reduction in BMD can be as severe as that due to postmenopausal bone loss in women.\(^1\) Low BMD is also associated with lower insulin-like growth factor 1 (IGF-1) in patients with acute lymphoblastic leukemia (ALL),\(^2\) the most common pediatric malignancy.\(^3\)

IGF-1 is a hormone important for normal growth and also has anabolic effects on bone in adults.\(^4\) Circulating IGF-1 is produced primarily by the liver. Peripheral tissues also produce IGF-1 in a paracrine/autocrine fashion in response to a variety of anabolic factors including growth hormone (GH) and parathyroid hormone (PTH). For example, the IGF-1/GH axis regulates longitudinal growth of bone by stimulating the proliferation and function of osteoblasts and chondrocytes.\(^2\)

Parathyroid hormone (PTH), prescribed in an injectable synthetic form under the name Teriparatide (Forteo), is an FDA-approved anabolic agent for treatment of osteoporosis. While PTH reduces fracture risk for some patient populations, PTH’s mechanisms are not completely understood. Additionally, the effects of the PTH therapy are quite variable in both post-menopausal women and animal models of post-menopausal osteoporosis.

A previous study by our group revealed that a serum balance in mice that favors GH over IGF-1 diminished the bone loss that occurs after ablation of ovarian function by ovariectomy (OVX).\(^4\) In this study we hypothesized that the stimulation of new bone formation after OVX would be positively affected by PTH treatment, despite reduced levels of circulating IGF-1.

Methods:
The liver IGF-1 deficient (LID) mouse (n=30, 12 weeks old) generated using the Cre-loxP system was utilized.\(^5\) LID mice exhibit 75% lower circulating IGF-1 levels.\(^4\) Ovariectomy (OVX) surgery was performed on 12 week old mice followed by 4 weeks of daily PTH (50 ng/g body weight) intraperitoneal (i.p.) injection. Basal controls were 12 weeks old. The mice were administered double calcein labels by i.p. (10 mg/kg), the first at 8 days and the second at 2 days prior to euthanasia. Bones were dissected immediately after euthanasia, fixed in 10% neutral phosphate-buffered formalin, and embedded in poly-methyl methacrylate plastic. All mouse protocols were approved by the Institutional Animal Care and Use Committee (MSSM).

Embedded bones were sectioned transversely with a diamond-coated wafering saw and polished using silicon carbide abrasive papers with successively smaller grit size to a mirror finish. Bone formation was quantified by measuring the two calcein labels on the lumbar
vertebral sections. The polished sections were mounted on glass slides and multiple fields were captured under a confocal microscope (Zeiss LSM 510) using epi-fluorescence mode with wavelength of 488nm (argon). Then, the bone sections were polished to remove approximately 50 µm of material. This was done to allow analysis of three consecutive sections. The captured image fields were stitched together to create complete cross sections (Figures 1 and 2).

Analysis of bone formation was performed using Image J and Adobe Photoshop 7.0 software. The two formation labels and the trabecular bone surface (BS) were traced on a Wacom graphics tablet and assigned different colors using the drawing tool in Photoshop (Figure 3). Figures 4 and 5 illustrate where sections were imaged. The length of each label was then measured using NIH Image J (Bone J plug-in) and bone formation was quantified as a function of bone surface length. Tracking and analysis was performed by two independent observers who were blinded to the treatment groups. Statistical differences among groups were assessed by a Student’s t-Test with p < 0.05 considered statistically significant.

Equations used to assess osteoblast activity were:6

- \( sL = \) single labeled surface, \( dL = \) double labeled surface
- \( MS = \) mineralizing surface = \( (dL+sL)/2 \)
- \( MAR = \) mineral apposition rate = distance between double labels / 6 days
- \( BFR = \) bone formation rate = \( MAR \times MS/BS \)

Summary of Results:

Bone formation rate (BFR) was significantly higher in LID mice treated with PTH after OVX compared to baseline control (Figure 6a). This new bone resulted in a larger bone surface (BS) in these mice (Figure 6b).

From left to right, Figures 1-3: Fig. 1 depicts a light microscopy image of a cross-section of a vertebra in the lumbar spine of a mouse. Fig. 2 shows a confocal microscopy image of a cross-section of a mouse lumbar spine with the calcein labels. Fig. 3 illustrates the tracing of the calcein labels where yellow is the single-labeled (sL) surface, red is the double-labeled (dL) surface, and blue is the outline of the trabecular bone surface (BS).
From left to right, Figure 4 (left, ventral view) and Figure 5 (right, cross-section): Micro-computed tomography images of the mouse lumbar spine. The green line is the rostral to caudal axis. The red lines denote the 3 transverse planes analyzed; each are 50 µm apart.
Conclusion:

Our results suggest that LID mice, when treated with PTH form new cancellous bone following OVX. Greater bone surface in LID mice may be the result of increased mineral apposition rate (MAR) due to PTH treatment. Since MAR measures osteoblast activity, it can be hypothesized that with a reduction in IGF-1, PTH has a more profound effect on osteoblasts to lay down more new bone. Additionally, 3D structural analysis via micro-computed tomography (Figures 4 and 5) confirmed an increase in bone surface area.

LID mice with OVX surgery represent a model for post-menopausal women. Low IGF-1 is also associated with low bone mineral density in patients with acute lymphoblastic leukemia. Therefore, our results with anabolic PTH in mice with IGF-1 deficiency may have implications for age-related osteoporosis and cancer therapy related bone loss. The strength of vertebrae might be increased and fracture risk decreased with PTH in IGF-1 deficient cancer patients and post-menopausal women.

References:

2. Inas T.H. et al., Bone Mineral Density in Young Adult Survivors of Acute Lymphoblastic

Acknowledgements:

We thank David Lagunoff, MD and Markus Meyenhofer from the New Jersey Medical School for their assistance with the confocal microscope and polishing, respectively. Additionally we thank: Shoshana Yakar, Ph.D., Wilson Mejia PhD, Hui Sun PhD, and Yinjgie Wu PhD from the Mount Sinai School of Medicine (MSSM).
Objective:
Previously, amputation has served as the standard treatment for sarcomas involving the distal leg, ankle, and foot. Today, due to the advances in flap coverage procedures, a variety of limb-sparing procedures are utilized. Despite these new advances, coverage of soft tissue defects in the distal leg, ankle, and foot regions has posed great difficulty for viable reconstruction. These difficulties are due in large part to the lack of available local tissue as well as to the sensitivity of the vasculature and decreased mobility of the skin.\(^1\) Soft tissue defects at can be caused by traumatic injury, tumor ablation, or infections such as osteomyelitis.\(^2\) A variety of reconstructive options exist such as skin grafts, free flaps, local flaps, and cross leg flaps; however, due to unique limitations at the distal leg, many of these techniques are difficult and even contraindicated.\(^1,2,3,4\)

The report by Masquelet et al.\(^3\) provided the groundwork for skin island flaps supplied by vasculature along superficial nerves. It has lead to the introduction of lower leg fasciocutaneous flaps such as the sural nuerocutaneous flap. While there are other fasciocutaneous flaps that may also be able to provide great coverage, the reverse sural artery flap proves to be the most effective due to its greater reliability and ease of surgery.\(^4\)

Within the literature there are no reports specifically documenting the use of the reverse sural artery flap after musculoskeletal oncologic resections. This study will evaluate patients treated with reverse sural artery flaps following soft-tissue sarcoma resections and validate its efficacy as the primary coverage option for defects at the distal leg.

Methods:
A prospective, IRB approved protocol was followed for six patients over the course of ten months at one institution. Adult patients (>18 years old) with primary soft tissue sarcomas of the distal leg, ankle, and foot that were confirmed by biopsy were included in the study. Patients who required a free flap during preoperative planning or if the patient’s tumor involved bone were not included in the study. In addition, patients with peripheral vasculature disease were excluded from the study. Each patient underwent a resection for a soft tissue sarcoma and the defects were reconstructed using the reverse sural flap. Demographic data collected included age and sex as well as co-morbidities such as breast cancer, diabetes mellitus, hypertension, and hypercholerestemia. Oncologic details were collected for the type of tumor, resection size, use of radiation and/or chemotherapy, and recurrence. Flap viability, healing time, donor site morbidity, functional outcomes, range of motion, and need for further surgery were identified post operatively (Table I).

Functional outcomes were evaluated based on the revised Musculoskeletal Tumor Society scale (MSTS). The MSTS scaling system for the lower extremity examines general factors such as pain, function, and emotional acceptance as well as specific factors to the lower extremity including the use of support and walking ability. Each item is given a value from 0 to 5 points, with 5 points indicating the highest function. The values are added for each subcategory and the percentage of the maximum score (30 points) was calculated. The functional scores were grouped into four categories as described by Aksnes et al.\(^5\) 1) poor ( < 25%); 2) fair (25% to 49%); 3) good (50% to 75%) and 4) excellent (> 75%) of the maximum score.
Surgical Procedure

Surgical resection with wide margins was performed by one of two surgeons in the division of musculoskeletal oncology in one hospital. All patients were prepared for a free flap reconstructive procedure if required. Once in the OR, the patient was given preoperative IV antibiotics (Ancef), placed in the lateral decubitus position, and was steriley prepped and draped. The tumor was excised along with a cuff of normal tissue. Intraoperative margins were taken to ensure the elimination of the tumor. Hemostasis was obtained and the area was irrigated. After frozen section analysis confirmed negative margins, reconstruction using a reverse sural rotational fasciocutaneous flap was done under the direction of the division of hand and microvascular surgery.

The reverse sural rotational flap, a fasciocutaneous flap based off of the superficial sural artery, was outlined in the distal third of the leg. The center of the flap was placed at the midline of the posterior surface of the leg with the pivot point of the pedicle located 5 cm from the lateral malleolus. A tourniquet was placed prior to harvesting the flap in the thigh at 350 mm Hg. Posterolateral incisions toward the gastronemius allowed for elevation of the soft-tissue flap. The sural artery vein and nerve were identified, ligated, and included with the deep fascia pedicle, which was elevated from the gastronemius and reflected anteriorly. The fasciocutaneous flap was rotated around the lateral side of the ankle to provide effective coverage of the defect and was sutured into place using 3-0 nylon. A split-thickness skin graft was used to cover the donor defect in the posterior calf. The patient was given VAC treatment, sterile dressings, and a splint in neutral dosiflexion before discharge.

Summary:
Six patients qualified to enter the study. Among the six patients there were five females and one male with an average age of 60 years (range 27-78). Co-morbidities included breast cancer (n=2), diabetes mellitus (n=2), hypercholerestemia (n=2), and hypertension (n=1). The defects resulted from the surgical resection of malignant fibrous histiocytomas (MFH) (n=3), chondrosarcomas (n=1), fibrosarcomas (n=1), and undifferentiated high grade sarcomas (n=1). The average size of the defect after resection was 94 cm^2 (range 50-143). The largest flap harvested was 10 cm x 13 cm, and the smallest flap was 10 cm x 5 cm.

All flaps were eventually viable yielding 0% flap failure rate, and 83% of flaps (5/6 patients) healed normally without complications in an average of 4.7 months (range 1-8). The single complication occurred in the patient with the largest defect who presented with partial necrosis of the distal aspect of the flap one month after surgery. This was treated with irrigation, debridement, and VAC treatment on 8 separate occasions after the original surgery. Eventually, in order to gain complete closure of the wound, split thickness skin grafting was used. There was no donor-site morbidity. Radiation therapy was utilized in patients with high-grade tumors and was delayed if there was prolonged healing time. No patients had recurrent tumors at the time of follow up. Average revised MSTS score was 80% (24/30) (range 15-29). Average Range of Motion (ROM) for dorsi flexion was 0 and plantar flexion was 17 (range 10-25). Average follow up was 8.8 months (range 4-14). (Please see Table 1 for a complete list of results).
## Table 1: Results

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>F/U (mo)</th>
<th>Anatomic Site</th>
<th>Pathologic Diagnosis</th>
<th>Tumor Dimension (cm)</th>
<th>Defect Size (cm²)</th>
<th>Co-morbidities</th>
<th>MSTS Score</th>
<th>Ankle ROM</th>
<th>Healing (mo)</th>
<th>Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>78</td>
<td>4.5</td>
<td>Lateral lower leg</td>
<td>Non-onc excised MFH</td>
<td>5</td>
<td>80</td>
<td>Breast cancer</td>
<td>21</td>
<td>Dorsi 0, Plantar 20</td>
<td>2</td>
<td>Pulmonary Embolism</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>14</td>
<td>Anterior ankle</td>
<td>Fungating Giant cell rich MFH</td>
<td>5</td>
<td>50</td>
<td>Multiple sclerosis, 1/2 PPD smoker</td>
<td>29</td>
<td>Dorsi 5, Plantar 25</td>
<td>5</td>
<td>Delay in radiation</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
<td>10</td>
<td>Anterior medial leg</td>
<td>Recurrent myxoid MFH</td>
<td>5.5</td>
<td>110</td>
<td>Diabetes, hypertension, high cholesterol</td>
<td>15*</td>
<td>Pre-op equinus contracture</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>67</td>
<td>9</td>
<td>Anterior pretibial</td>
<td>Non-onc excised extraskeletal myxoid chondrosarcoma</td>
<td>3.2</td>
<td>63</td>
<td>Diabetes, high cholesterol</td>
<td>29</td>
<td>Dorsi 0, Plantar 10</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>54</td>
<td>8</td>
<td>Anterior ankle</td>
<td>Fungating Myxofibrosarcoma</td>
<td>7</td>
<td>130</td>
<td>Breast cancer</td>
<td>27</td>
<td>Dorsi 0, Plantar 10</td>
<td>7</td>
<td>Delay in radiation</td>
</tr>
<tr>
<td>6</td>
<td>62</td>
<td>7</td>
<td>Anterior distal pretibial</td>
<td>Undifferentiated high grade sarcoma</td>
<td>6</td>
<td>130</td>
<td>Diabetes, high cholesterol, mitral valve prolapse</td>
<td>25</td>
<td>Dorsi 0, Plantar 20</td>
<td>5</td>
<td>Partial Flap Necrosis</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>60</td>
<td></td>
<td></td>
<td>5.3</td>
<td>94</td>
<td></td>
<td>24**</td>
<td>Dorsi 0, Plantar 17 (5 Patients)</td>
<td>4.66</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** * Wheelchair bound pre-op; **Mean = 26 without wheel chair patient*

Defects after oncologic resection involving the distal leg, ankle, or foot are challenging to adequately cover due to the lack of local tissue, sensitivity of the vasculature, and limited rotational flap movement. There are a variety of reconstructive techniques that can be used to cover these defects. A model flap should have an easy dissection, constant vascularity, preserve major arteries or sensory nerves, and have insignificant morbidity.

The reverse sural flap is indeed a model flap due to its simplicity of execution, reliability, and the straightforwardness of postoperative care. There are many advantages to this particular type of flap. Practically, the surgery is significantly less complex when compared to a free tissue transfer and does not require a costly microvascular team. Surgically, the use of this procedure allows good mobility and rotation of the flap, preservation of a major artery, and acceptable donor-site morbidity.

Clinically, the sural flap provides an ideal tissue match with similar thickness to the area of the defect. The disadvantages of the flap include the aesthetically unappealing skin graft at the donor site and the sensory loss from the sural nerve, which eventually becomes less noticeable over time. Contraindications for the reverse sural artery flap include venous insufficiency due to the retrograde flow of blood, and may be the main cause of sural flap necrosis.

The primary outcome measure of the study, flap viability, involved the adequate coverage of the defect without further need for reoperation due to flap failure. Out of the six patients in our series, five patients had normal healing and only one flap had surgical complications. The flap failure rate of our study was 0% and is similar to those reported in the literature. Al-Qattal et al. reported a flap failure rate between 5%-36% for 13 studies using the reverse sural artery flap, with an average of approximately 15.6%. Complications leading to flap necrosis are likely due to problems with the vasculature and perfusion. Baumeister et al. described venous insufficiency, arteriosclerosis and diabetes as risk factors for flap failure by necrosis. The one patient with partial flap necrosis in this study was co-morbid with diabetes and high cholesterol, which suggests the role those problems have upon flap failure. Other risk factors for flap necrosis include the size and position of the flap.

Since the study dealt with soft-tissues sarcomas, tumor recurrence must be evaluated. Although the follow up period was relatively short, there was no tumor recurrence. Adjuvant radiation was also administered in some patients in order to decrease the chance of local recurrence. Patients were considered for radiation if the tumor extended into the deep fascia, was larger than 5cm, or was high grade. In some instances delayed radiation was administered.
with the intent to improve patient tolerance to treatment, specifically to prevent wound breakdown. This study included two patients who received delays in radiation. While neither patients exhibited recurrence, no effective conclusions can be drawn at this time.

This study also examined functional outcomes and range of motion to identify the efficacy of the reverse sural artery flap and its effects on patients and their quality of life. MSTS scoring system was developed to evaluate the functional results and overall effectiveness of various surgical techniques after tumor removal.\textsuperscript{10} It takes into account general factors such as pain, function, and emotional acceptance as well as the use of supports and gait for the lower extremity.\textsuperscript{10} There are variety of factors that negatively impact postoperative MSTS including large tumor size, complications during surgery, bone resection, and old age.\textsuperscript{11} On average our patients treated with the reverse sural artery flap exhibited good MSTS scores (80\%) demonstrating the effectiveness of limb-salvage procedures on an individual’s overall health.

Conclusion:

The reverse sural artery flap is a reliable procedure for repair of soft-tissue defects in the distal region of the leg, malleolus, and dorsum of the foot. It affords several advantages such as providing a reliable blood supply, the maintenance of major arteries, ease and short surgical time, and low donor-site morbidity. This is the first report of the use of the reverse sural fasciocutaneous flap in musculoskeletal oncology following sarcoma resection. With limitations of size and excursion, this flap is a viable first line alternative for coverage that provides an ideal tissue match after tumor resection.

References:

Objective:

DNA damage checkpoint and repair mechanisms provide the cell with a method of preventing uncontrolled proliferation. Mutations in the genes controlling these pathways substantially increase the risk of cancer development. In humans, Mre11, hRad50, and Nbs1 from a heterotrimer (the MRN complex) which recognizes double stranded DNA breaks, and then phosphorylates downstream targets in order to activate checkpoint and repair pathways. In budding yeast (*Saccharomyces cerevisiae*), this function is served by the MRX complex, consisting of Mre11, Rad50, and Xrs2. Mec1 and Tel1 are two specific targets of MRX implicated in parallel pathways regulating the phosphorylation of downstream targets to activate the checkpoint response (Figure 1A). Mec1 knockout (Mec1Δ) yeast cells are highly sensitive to DNA damage, and are unable to proliferate in the presence of DNA damaging agents (Figure 1B).

Two studies were undertaken; one to investigate the suppression of the Mec1Δ phenotype by randomly mutagenized Rad50, and another to investigate the suppression of the Mec1Δ phenotype by randomly mutagenized Mre11.

Figure 1. A. The DNA damage checkpoint pathway in budding yeast consists of two parallel pathways dependent on activation of Mec1 and/or Tel1 by the MRX complex. Activation of both pathways is functionally required for DNA damage repair and eventual continued proliferation under normal conditions. B. The DNA damage checkpoint pathway in Mec1Δ yeast with WT MRX complex. Normal activation of only the Tel1 mediated pathway is insufficient for damage repair and results in non-proliferation
Methods:
Mutations were generated by PCR amplification of Rad50 or Mre11 in the presence of MnCl$_2$ using ChoiceTaq, a non-proofreading DNA polymerase. The fragments were then co-transformed into S. cerevisiae cells along with the low copy shuttle vector YCpLac33. The mutagenized fragments and plasmid vector underwent homologous recombination \textit{in vivo} to produce an expressible copy of the gene of interest. The cells were then plated on SDC + tryptophan (Ura$^-$) plates to select for transformants. The surviving transformants were transferred to yeast peptone dextrose + hydroxyurea (YPD+HU), a DNA damaging medium, to screen for Mec1$\Delta$ phenotypic suppression. Surviving colonies were transferred from the YPD+HU plate to a plate containing 5-Fluoroorotic Acid Monohydrate (FOA) in order to select for cells that lost the plasmid. Colonies from the FOA plate were cultured on both YPD+HU and Ura- media to test the dependency of Mec1$\Delta$ phenotypic suppression on plasmid presence (Figure 2). The genomic DNA was extracted from candidate colonies and transformed into the \textit{Escherichia coli} (\textit{E. coli}) strain JA226 by electroporation. The \textit{E. coli} were allowed to proliferate to amplify the candidate plasmids. The plasmids were then recovered from the \textit{E. coli} cells and retransformed into S. cerevisiae cells, which were subsequently retested on YPD+HU plates to confirm the previous results.

![Figure 2. Plating scheme used for screenings](image)

Summary:
Two candidate Rad50 mutations were found that successfully rescued the Mec1$\Delta$ phenotype. Budding yeast cells transformed with the candidate DNA demonstrated plasmid dependent proliferation in the presence of HU which was confirmed upon retesting. Additionally, one candidate Mre11 mutation was found which demonstrated similar Mec1$\Delta$ phenotypic suppression (Figure 3).
Conclusion:

The suppressor mutations were able to successfully activate the DNA damage checkpoint response in the absence of Mec1. These mutations can be considered dominant because the presence of a single RAD50 or MRE11 allele was sufficient to induce the checkpoint response in cells carrying the endogenous wild-type RAD50 or MRE11 gene at its own locus. It has previously been demonstrated that over expression of Tel1 can partially compensate for the loss of the Mec1 pathway in Mec1Δ budding yeast.5 We hypothesize that the candidate MRX mutations are able to suppress the Mec1Δ phenotype by hyper-activation of the Tel1 dependent DNA damage checkpoint pathway. The induction would then be able to compensate for the loss of the Mec1 dependent DNA damage checkpoint pathway and successfully induce the delay in mitosis associated with DNA damage checkpoint activation. This would in turn allow the cellular DNA damage repair machinery to repair the detected damage and the cell to continue to proliferate (Figure 4). Future investigations could focus on testing this hypothesis by analyzing the phosphorylation of downstream targets in the Tel1 dependent DNA damage checkpoint pathway by western blot analysis. Furthermore, a microarray analysis could be used to investigate which other genes are involved in suppression.

![Figure 3. Rad50 and Mre11 screening candidates proliferate in the presence of HU. P represents a positive control with WT Mec1, and N a negative control with the Mec1 deletion. V represents Mec1Δ transformed with empty vector. Candidate 1 failed to proliferate in the presence of HU, and therefore does not harbor a suppressor mutation. Candidates 2 and 3 have suppressing Rad50 mutations, and candidate 4 contains a suppressing Mre11 mutation. All three proliferated in the presence of HU in triplicate.](image)

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Figure 4. Proposed mechanism of Mec1Δ phenotypic suppression by Rad50 and Mre11 screening candidates. DNA damage results in the hyper-activation of the Tel1 pathway by the MRX complex, resulting in a sufficient checkpoint response to enable damage repair and continued proliferation.

References:

NON-CANONICAL SIGNALING PATHWAY FROM THE CRK ONCOGENE

Mentor: Raymond B. Birge PhD (Biochemistry and Molecular Biology)

Objective:

The EGFR family is comprised of HER1 (EGFR), HER2, HER3 and HER4. These are cell membrane receptors that have intrinsic tyrosine kinase activity which further creates a cascade of cellular signals when a ligand binds to its extracellular domain. In human tumors, these receptors have been identified as key players in the behavior such as increased proliferation, migration, angiogenesis and decreased cell death [1]. One of the signaling pathways initiated by EGFR involves tyrosine phosphorylation of Crk. This Crk family includes v-Crk, (an oncogenic fusion protein derived from avian CT10 retrovirus) [2,3] Crk I, Crk II, CrkL and comprise a class of regulatory proteins, called adaptors, which are known to assemble signaling complexes via the SH2 (Src Homology - 2) and N-terminal SH3 (Src Homology - 3) domains. The Crk SH2 domain binds to specific pTyr-X-X-Pro [4], whereas the Crk SH3 domain binds to specific Pro-X-X-Pro-X-Lys,Arg [5]. The CrkII adaptor protein also has a carboxy-terminal SH3 (SH3C) domain, which has not been well studied or understood thus far. Recent studies in our laboratory have identified Y251 in the atypical SH3C in hCrk II to be a phosphorylation site for the Abl Kinase. However, the function of this phosphorylation event has not been characterized yet. In this study, we have found for the first time that Y251 is phosphorylated after Epidermal growth factor (EGF)-induced stimulation of breast and ovarian cancer cells. This data suggest that tyrosine phosphorylation of Crk might play a regulatory role in cancer.

Methods:

**Western blotting:** Western blotting was performed following SDS-PAGE and transfer to Nitocellulose (BioRad) or polyvinylidene difluoroide (PVDF) membranes (Millipore). Membranes were blocked in 5% nonfat dry milk solution made up in TBS and were subsequently incubated with appropriate primary antisera at 4 C overnight. The membranes were then washed three times for 10 min each in TBS solution containing 0.1% Tween-20. Subsequently, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (Jackson Immunological Laboratories) in blocking buffer for 45 minutes at room temperature. Blots were developed with an enhanced chemiluminescence kit (Perkin-Elmer).
**Generation of phospho-specific antiCrkY251 Antibodies:** The phospho-specific antibodies; pY251 7415/5194E and pY251 7416/5194E were generated by coupling a phosphopeptide to a carrier protein KLH. Rabbits were immunized with this phosphopeptide attached to the carrier protein and the serum was collected. The serum was passed through an affinity column which captures these phosphospecific antibodies via the same phosphopeptide with a different carrier protein that lines the affinity column. These phosphospecific antibodies are finally eluted by a pH shock.

**Cell culture and Growth factor stimulation:** HEK 293T cells, T47D cells, MDA-MB-231 cells and MDA-MB-468 cells were maintained in Dulbecco's modified Eagle's medium [DMEM(Cellgro); 4.5g of glucose/L with 1-glutamine] supplemented with 10% fetal calf serum (Sigma). Lysates of these cells were made after culturing them in order to probe with the phosphospecific antibodies. The MDA-MB-231 cells were starved overnight and stimulated with 100 ng of each growth factors such as EGF, FGF, PDGF and IGF2. The A431 cells were starved overnight and then stimulated with 100 ng of EGF.

**Summary:**

**A.**

![Image of Western blot showing pY251 7415/5194E antibody binding to wild type hCrkII but not the Y251F mutant in presence of hAbl.]

**B.**

![Image of Western blot showing pY251 7416/5194E antibody binding to wild type hCrkII and the Y251F mutant in presence of hAbl.]

Fig 4: We tested two batches of antibodies; pY251 7415/5194E and pY251 7416/5194E. A) pY251 7415/5194E antibody was seen to be specific to phospho-hCrkII as it bound only to wild type hCrkII and not the Y251F mutant in presence of hAbl.

B) pY251 7416/5194E antibody was seen to bind to wild type hCrkII and the Y251F mutant in presence of hAbl.

We concluded that pY251 7415/5194E specifically detects phosphorylated hCrkII where as pY251 7416/5194E did not.
Fig 5: In unstimulated breast cancer cell lines T47D, MDA-MB-231, MDA-MB-468 Y251 is not detectably phosphorylated. MCF-10A is a non-transformed human breast epithelial cell lines. Anti-actin served as a loading control. hCrkII co-expressed with hAbl served as a positive control and Y251F mutant served as a negative control.

Fig 6: EGF induces phosphorylation of hCrkII at Y251 in A431 cells.

Fig 5: Upon EGF stimulation of A431 cells for 1, 5 and 30 minutes hCrkII was seen to be phosphorylated at Y251 above baseline phosphorylation seen without EGF stimulation.

Fig 6: In unstimulated breast cancer lines hCrkII was not detected to be phosphorylated. pY251 7415/5194E antibody was used to probe various breast cancer cell lines for presence of phosphorylation at Y251 in hCrk II. Positive control showed that the antibody was only bound to hCrk II in presence of hAbl and not Y251F mutant in presence of hAbl.

Fig 7: Western blot showing presence or absence of phospho Y251 in response to growth factor stimulation of MDA-MB-231 cells. EGF and IGF2 stimulation induced phosphorylation of hCrkII at Y251 in 30 minutes. Anti-actin served as a loading control.

Fig 7: pY251 7415/5194E antibody was then used to probe MDA-MB-231 cells upon stimulation by various growth factors for 30 minutes. EGF and IGF2 stimulation induced phosphorylation of hCrkII at Y251 in MDA-MB-231 cell line.
Conclusions:
Studies from our lab previously identified Y251 on hCrk II as a novel phosphorylation site. Generation of phospho-specific antibodies to phospho-CrK Y251 enabled us to examine phosphorylation in-vivo. Our results suggest that Y251 is phosphorylated upon EGF stimulation in A431 cells and also upon EGF and IGF2 stimulation in the MDA-MB-231 human breast cancer cell line.
Previously hCrkII has been known to be involved in assembling signaling complexes but the role of the SH3C was not clear. However, our study shows that hCrkII is phosphorylated at Y251 in the SH3C and this suggests the existence of novel modes of signal tranduction by an adaptor protein.

References:
18. JEFFREY SUELL (NJMS 2013)

ANALYSIS OF INCIDENTAL FINDINGS IN SERIAL FDG PET/CT STUDIES OF ONCOLOGY PATIENTS

Mentor: Lionel Zuckier, MD (Nuclear Medicine)

Objective:

18F-fluorodeoxyglucose positron emission tomography (18F-FDG-PET) has become a common procedure in the field of Oncological Radiology[1, 2]. 18F-FDG-PET is a molecular imaging modality used to assess metabolic activity in potentially cancerous sites. Due to its poor ability to distinguish anatomical landmarks, the PET scan is commonly paired with a co-registered CT scan (PET/CT). This hybrid is valuable as a means of identifying potentially malignant growths, however the 18F-FDG tracer is not tumor specific [3]. As a result, the patient may show variable uptake in regions such as the brain, heart, liver, and spleen that do not necessarily indicate cancer. When well defined and relatively intense, areas of uptake may resemble focal disease, and these have been termed “incidental findings”. The frequency of incidental finding has been well-documented in the population [4], but to our knowledge no study has been done that examines the variation in uptake of these regions over time in individual patients. The objective of our research is to review FDG-PET/CT findings in patients with multiple scans to determine if there were any common patterns in the incidental 18F-FDG uptake and to see if we can define a range of normal uptake. The research also aimed to look at if the patient’s past uptake values might serve as a better guide for predicting future findings than comparison to population controls alone.

Method:

The Picture Archiving and Communication System (PACS) database at UMDNJ-UH was reviewed to cull patients who had received ≥ 5 PET/CT scans between 2003 and June 2010. The studies were then segregated by sex and persistence of demonstrable disease. Twenty-two male post chemotherapy patients who had successful therapy without recurrence of their primary tumor were then identified in whom at least three normal ‘surveillance’ scans were obtained following their last chemotherapy treatment. The PET/CT studies were examined using a workstation running MIMVista software to measure the distribution of the radiopharmaceutical. SUVs were tabulated for several organs known to demonstrate variable or relatively constant uptake: testes, liver, spleen, gastroesophageal junction, vertebral marrow, thyroid, heart, brainstem and psoas muscle (Table 1). After preliminary assessment, evaluation was limited to the initial 5 regions listed above and the psoas.

When measuring the SUV_mean, partial volume effects were minimized by maintaining a 9mm margin between the Region of Interest (ROI) and the organ border, in order to prevent any signal from adjacent tissues from ‘bleeding’ in and distorting the true value of that particular tissue/organ (“partial volume effect”). The size of the ROI was set in order to maximize the area included while maintaining an adequate margin of intervening tissue.

<table>
<thead>
<tr>
<th>Region</th>
<th>ROI (mm)</th>
<th>SUVmax</th>
<th>SUV mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>10</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Liver</td>
<td>25</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Marrow (L1-L4)</td>
<td>5</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Thyroid</td>
<td>5</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Brainstem</td>
<td>5</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Testes (L/R)</td>
<td>10</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>15</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>GE Junction</td>
<td>10</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Muscle (L/R Psoas)</td>
<td>10</td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>
Table 1: ROI on PET scans for SUV measurements.

Summary:

The Standardized Uptake Value (SUV) of any area within a PET scan is the measure of the relative intensity of the injected radioactive \(^{18}\)F-FDG distribution taking into account the patient’s weight, amount of radiopharmaceutical injected and time elapsed between injection and scan (to account for radioactive decay). Several variables are known to introduce variation into the calculation of SUV [5]. These include technical factors such as system resolution and the image reconstruction filter as well as unaccounted physiologic variable such as the percent of body fat. Some variables such as serum glucose, uptake time and muscular activity[6, 7] can be monitored and controlled by staff, but others such as partially infiltrated injections are not easily avoided [8].

The purpose of reducing SUV variation is to create a dependable prognostic index with which abnormal organs might be more readily and accurately defined. Better delineation of normal and abnormal will potentially decrease both false positives and negative cases. Several studies have discussed the value of an internal measure as a standard for making SUV measurements more consistent and accurate; however the same studies differ on what organ makes the best internal standard. Use of the patient’s liver[9], basal ganglia [8]muscle [10] or blood pool of the thoracic aorta [11] have all proven to mitigate variation inherent to uncorrected SUV.

The general trend noted throughout earlier studies was that effective normalization was based upon finding a standard that best mimics the area of interest [8]. Because these variations are not related to a disease process, the ideal normalization would utilize an area that remains stable across scans and which is relatively inert physiologically. Such a region would create the best possible standard for eliciting any common uptake trends in post-chemotherapy patients.

Three regions that were measured throughout the 22 patients were examined as possible candidates for use in normalizing the SUV: muscle, liver and spleen. While muscle theoretically matches the requirement for an ideal, internal standard, we found that in the case of testicular uptake, using the psoas muscle for normalization (Testicular SUV\(_\text{max}\) / Psoas SUV\(_\text{mean}\)) actually increased the coefficient of variation versus the uncorrected testicular SUV\(_\text{max}\). We hypothesized that the limitation with using muscle as a standard was that the average value of muscle SUV was low; it has been suggested that regions with SUV\(_\text{mean}\) < 1 are particularly susceptible to small value changes that would cause large relative variations [12].

Although prior studies had utilized the liver as their standard [9], we found that, the spleen was superior to the liver. In a comparison of liver and spleen, each was used to normalize muscle SUVs. The spleen normalized muscular SUV\(_\text{mean}\) showed a reduced overall variation 24% more than the liver. To ensure validity, splenic and hepatic normalizations were also performed on the testes, GE junction, liver and lumbar vertebral marrow. Utilization of the spleen for normalization proved to be a statistically significant improvement over using uncorrected SUV. Spleen normalized SUV showed a decrease in average coefficient or variation by 10.7% (P<0.05) across all other target organs (Table 2). Conversely, SUV normalizations done by the liver for the same tissues had an increase in the coefficient of variation by 5.1% (P<0.05). Once the spleen had been proven optimal as a normalization measure, it was used to normalize all other SUV measures over time. Incidental findings in the marrow of the lumbar vertebrae, testicles, GE junction, and liver were then examined.

<table>
<thead>
<tr>
<th>ROI</th>
<th>Coefficient of Variation (normalized – SUV target/SUV spleen)</th>
<th>Coefficient of Variation (uncorrected – SUV)</th>
<th>Corrected/ Uncorrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscles</td>
<td>0.266</td>
<td>0.296</td>
<td>0.898648649</td>
</tr>
<tr>
<td>Testes</td>
<td>0.158</td>
<td>0.171</td>
<td>0.923976608</td>
</tr>
<tr>
<td>GE</td>
<td>0.246</td>
<td>0.256</td>
<td>0.9609375</td>
</tr>
</tbody>
</table>
Table 2: Spleen normalized SUV measurements for target areas in the body that are common for incidental findings. The coefficient of variation (standard deviation / mean) shows the range of variation for the sample. On average normalizing areas by using the spleen as the internal standard reduced the interscan variation by 10.8%.

The data acquired from MIMVista of the PET scans were analyzed in two ways:
(1) According to the ordinal number of scans following the scan post-final therapy. For example, the first scan following the last round of chemotherapy was numbered ‘0’ for all patients. Each patient’s next scan was categorized as scan ‘1’; any scans that preceded chemotherapy were accordingly given a negative number. Patients had scans that ranged from -3 to +4. The purpose of arranging them in this manner was to elicit any general trend in SUV that would correlate with post-chemotherapeutic recovery and group patients to improve statistical power.
(2) According to the interval since the scan post-final therapy. For example, the scan done immediately following the last treatment of chemo was marked as T = 0. All other scans were categorized by the number of months preceding or following the time zero scan. Patients had scans that ranged from -29 months to +60 months. The purpose of organizing the scans in this manner was to better elucidate chronological changes related to post-chemotherapeutic recovery.

A review of the PET/CT studies by both of the methods mentioned above could not derive any significant trends for any of the tissues examined. No correlation with normalized SUV or change in normalized SUV was noted. An example of one such investigation is shown below (Figure 1). The large distribution of incidental SUVs prohibited any statistically significant patterns from being elicited.

![Figure 1](image1.png)

**Figure 1:** Absolute spleen normalized testicular SUVmax. (A) Depicts the mean and 1 std. dev. of testicular SUV normalized to splenic SUV as a function of the number of scans since the scan following the last episode of chemotherapy (B) A time based categorization of the same data with T=0 as the scan immediately following the last episode of chemotherapy. There was a high degree of variation for both groupings of the data (n=22). Due to the variation no statistically significant trend could be extracted.
A visual inspection of a scatter plot that charted each of the individual studies graphed in figure 1 lacked definable trends. The organ normalized scatter plots revealed that while the group was random, there was a far greater degree of consistency from scan to scan within an individual. A statistical analysis of an individual’s deviation (Table 3) was done for the normalized SUV measurements quantitatively confirming that an individual’s past findings were a significantly better predictor of their own future than the population mean across all times or each specific time.

Table 3: Comparison of coefficients of variation (CoV) for different organs based on single patient values vs. the whole group’s value. The individuals’ averages were significantly smaller than group averages and time based averages. Utilization of the individual’s distribution significantly decreased the variation of normalized SUV.

The value of knowing a patient’s history has always been essential in the practice of quality patient care. Incidental organ uptake was found to be more predictable when based on the patient’s prior history. In 18 out of 22 patients, (82%), the coefficient of variation for that patient’s data was smaller than that of the entire group (averaging 33%, p<0.001). Utilizing patient history in medicine is obviously standard practice, but interestingly the value in doing so was only significant when the entire sequence was considered. Variation from individual scan to the next was so noisy that using only the most recent scan as a reference point for these incidental findings was no better than using the entire population as a reference.

We also compared an individual’s serial scans to those of all patients at single time points in their post – chemotherapy course. For example patient A’s scan 12 months after his last chemotherapy treatment was weighed against the normalized SUV variation of his entire history compared to the averaged, normalized SUV of patients B,C and D for their 12 month scans. In such a case, the individual’s history taken in its entirety was a more stable and reliable indicator of future scans. In 18 of the 22 patients (82%) the sequence of the individual’s past values showed a decreased coefficient of variation (averaging 30% p<0.001) than that of all patients at the same time-point in their post-chemotherapy progression. Our study suggests that ‘incidental findings’ are not completely random, for if they were, we would not expect there to be any significant variation between the individual and the group or the individual and others at the same time point.

**Conclusion:**
In our data sample we have demonstrated that the spleen was the best choice for an internal standard as it was proven to reduce overall variation in our patients better than other organs such as the liver or muscle. There is significant value in examining a patient’s prior PET history in order to establish a personalized normal by which variation can be limited to reduce false positives and allow actual anomalies to be more readily indentified.

EFFECTS OF INHIBITION OF COT1 ACTIVITY ON VITAMIN D-INDUCED DIFFERENTIATION OF HUMAN LEUKEMIA CELLS

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

Introduction:
The physiological form of Vitamin D, 1,25-dihydroxyvitamin D3 (1,25D3), has been shown to induce differentiation in human leukemia cells. Differentiation in leukemia cells results in a more mature phenotype and gradual loss of the ability to proliferate. These experiments investigated the effect of a specific pharmacological inhibitor of mitogen-activated protein kinase kinase kinase 8 (MAP3K8), also known as COT1, on vitamin D-induced differentiation in three human leukemia cell lines.

The COT1 gene, also known as Tpl2 (tumor progression locus 2), has been associated with regulating the activity of MAP kinase pathways, including ERK, JNK, and p38.

The specific pharmacologic inhibitor used in these experiments is 4-(3-Chloro-4-fluorophenylamino)-6-(pyridin-3-yl-methylamino)-3-cyano-[1,7]-naphthyridine (C21H14ClFN6). This compound inhibits kinase activity of COT1.

Objective:
To determine the effect of inhibition of COT1 activity on Vitamin D-induced differentiation

Hypothesis:
COT1 inhibitor enhances differentiation only in the initial stages of vitamin D-induced differentiation.

Materials and Methods:
Two cell lines were used in these experiments:
- HL60 – derived from a patient with promyeloblastic leukemia (M2 on FAB classification)
  - HL60 ATCC and its subline HL60 G
- U937 – derived from a patient with human histiocytic lymphoma (M5 on FAB classification)

Cells were cultured in IMDM medium supplemented with 15% BCS (HL60 ATCC) and RPMI 1640 medium supplemented with 10% BCS (HL60 G, U937). Experiments were seeded at 3x10^5 cells/ml and were incubated at 37°C in the presence of 5% CO2 for 72, 96, and 120 hours.
Groups:
1. Untreated
2. 1 nM 1,25D (72h)
3. 1 nM 1,25D (72h) + COT1 inhibitor (73h, starting 1 hr before 1,25D)
4. 1 nM 1,25D (72h) + COT1 inhibitor (last 48h)
5. 1 nM 1,25D (72h) + COT1 inhibitor (last 24h)
6. 1 nM 1,25D (96h)
7. 1 nM 1,25D (96h) + COT1 inhibitor (last 72h)
8. 1 nM 1,25D (120h)
9. 1 nM 1,25D (120h) + COT1 inhibitor (last 72h)

To test this hypothesis, COT1 inhibitor was added at several time points after exposure of the cells to 1,25D. Differentiation was measured at intervals by determining surface differentiation markers and cell cycle arrest using a Coulter flow cytometer.

Results

![CD11b Expression in HL60 ATCC Cells](image)

Figure 4: CD11b expression in HL60 ATCC cells. A significant (p < 0.01) increase was observed only between 1 nM 1,25D (72h) and 1 nM 1,25D (72h) + COT1 inh (73h), i.e. when COT1 inhibitor was added at the same time as 1,25D.
Figure 5: CD14 expression in HL60 ATCC cells. A significant increase was observed when COT1 inhibitor was added in combination with 1,25D for 72h compared to 1,25D alone (p = 0.013).

Figure 6: HL60 ATCC cell cycle analysis. Data show that addition of COT1 inhibitor after 48h of incubation with 1,25D results in a significant increase in percentage of cells in G1 phase compared to addition of COT1 inhibitor 1 hr prior to addition of 1,25D.
In HL60 ATCC cells, adding COT1 inhibitor with 1 nM 1,25D at 0 hours and incubating for 72 hours showed a significant increase in both CD11b and CD14 expression compared to the control group of 1 nM 1,25D alone.

Adding COT1 inhibitor after 48 hours of incubation with 1 nM 1,25D did not increase the differentiation as determined by CD11b and CD14 expression. However, it significantly (p = 0.018) increased the proportion of cells in the G1 phase of the cell cycle, indicating that the cells were no longer proliferating.

In HL60G and U937 cells, similar effects were observed, but significance was not reached with only three experiments.

It was previously shown that the early stage of 1,25D-induced differentiation of HL60 cells is characterized by an active Erk1/2 pathway. Thus, further work will be directed to examination of the question whether COT1 has a role in controlling the Erk pathway. Additionally, the interaction of COT1 with JNK and p38 MAP kinase pathways will be examined in relation to the known control of Kinase Suppressor of Ras (KSR1/2) by COT1.

Conclusion:

It is clear that, in HL60 ATCC cells, 1,25D-induced differentiation is enhanced only when COT1 inhibitor is added simultaneously with 1,25D. This may apply also to the other cell types studied, but evidence is not conclusive at this stage of our investigation.

Our data suggest that, in combination with 1,25D, the cell-permeable naphththyridine compound (COT1 inhibitor) may have potential for clinical use in the treatment of myeloid leukemias.

References:


Acknowledgments:

Dr. George P. Studzinski, Xiangwen Chen-Deutsch, Xuening Wang, Ela Gocek, Jing Zhang, S.M. Saad Hussain, UMDNJ-New Jersey Medical School.

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20. PAUL THERATTIL (NJMS 2013)

EFFECT OF 5-AZA-2’-DEOXYCYTIDINE ON STAT3 PHOSPHORYLATION IN DU-145 CELLS

Mentor: Beverly E. Barton, PhD (Surgery)

Objective:
In the United States, prostate cancer remains the most common type of cancer diagnosed in men, as well as the second leading cause of cancer death among men only behind lung cancer (1). Advanced prostate cancer remains unresponsive to current chemotherapeutics. With an expanding proportion of elderly males in the population, prostate cancer will become an even more pervasive medical problem. Therefore, it is imperative to develop new therapies for advanced prostate cancer.

5-aza-2’-deoxycytidine (5-AZAC) is a cytosine analog that decreases DNA methylation. Many tumor types show abnormal hypermethylation in the CpG island promoters of certain tumor suppressor genes, potentially reducing levels of expression of them (3). Decreasing hypermethylation in cancer cells has shown to decrease proliferation or induce apoptosis (4,5,6). 5-AZAC treatment of ALK-positive anaplastic large cell lymphoma and HT29 colon adenocarcinoma cells reduced cell proliferation, while treatment of multiple myeloma cells increased apoptosis (4,5,6). 5-AZAC (Decitabine) is currently used to treat hematological malignancies. Modest responses occurred when treating solid tumors and non-small cell lung cancer, but only one preliminary trial has been performed for hormone-refractory prostate cancer (2).

STAT3 (Signal transducer and activator of transcription) is constitutively activated in prostate cancer cells, enhancing their growth and promoting their metastatic phenotype (7,8). Direct inhibition of STAT3 in prostate cancer cells leads to apoptosis (9). In multiple myeloma, negative regulators of the Jak/STAT pathway were found to be hypermethylated and treatment with 5-AZAC induced subsequent demethylation and down-regulation of STAT3 activity (10). However, the methylation status and epigenetic role of STAT3-related genes in prostate cancer has not been examined. Therefore, we wanted to determine whether inducing hypomethylation with 5-AZAC treatment decreases STAT3 activation in prostate cancer cells.

Methods:
Cell culture
DU-145 prostate cancer cells were grown in DMEM/Ham’s F12 + GlutaMAX (Invitrogen) supplemented with 10% newborn calf serum (HyClone) and HEPES buffer (Invitrogen), and were incubated at 37°C in an atmosphere containing 5% CO₂. Cell viabilities were determined using fluorescein diacetate. 96-well plates were assayed using the CyQuant Cell Proliferation Assay Kit (Invitrogen) and a fluorescence microplate reader (PerkinElmer VICTOR X2 1420).

Cell Culture Treatment With 5-AZAC
Experiments were performed to determine appropriate concentrations of 5-AZAC (Alexis Biochemicals) with which to treat DU-145 cells. 1 x 10⁴ cells were seeded overnight in 96-well plates in quadruplicate to a total volume 200 µl in each well. Quadruplicates were assigned to control, vehicle control, and 5-AZAC treatment groups. 5-AZAC was dissolved in dimethyl sulfoxide (Fisher Scientific) and diluted in Dulbecco’s phosphate buffer saline (Invitrogen). 5-AZAC was added to wells at final concentrations of 1 µM, 10 µM, 30 µM, 100 µM, and 300 µM. 5-AZAC in fresh medium was replenished daily to maintain constant concentration. At 24, 48,
and 72 hours, medium was removed from wells and plates were stored at -20°C or -80°C until all samples were ready to be assayed using the CyQuant Cell Proliferation Assay Kit.

**Immunoprecipitation and Western blot analysis for P-STAT3**
2 x 10^5 DU-145 cells were seeded in 6-well plates overnight. Wells were assigned to control, vehicle control, and 100 μM 5-AZAC treatment groups. Treatment was performed for 48 hours, after which cell lysates were prepared using standard procedures. Protein concentration in STAT3 lysates was determined using a spectrophotometer (Thermo Scientific Nanodrop 1000) calculating concentration using a mass extinction coefficient of 13.7 at 280 nm. STAT3 was detected by Western blot analysis using anti-STAT3 antibodies (Santa Cruz Biotechnology). Immunoprecipitation was performed for P-STAT3 lysates using Protein A/G PLUS-Agarose (Santa Cruz Biotechnology). P-STAT3 lysates were pre-cleared by rotating with beads and normal goat IgG in cold (Santa Cruz Biotechnology). Pre-cleared samples were incubated with P-STAT3 antibody, and then rotated with beads in cold overnight. After washing, beads were heated with sample buffer and loaded onto precast gels. P-STAT3 was detected by Western blot analysis using anti-P-STAT3 antibodies (Santa Cruz Biotechnology). After transferring proteins from gels to PVDF membranes (Millipore) using a Hoefer apparatus, membranes were blocked with 5% non-fat dry milk prior to probing with antibody. Blots were read on a Typhoon imager (Molecular Devices).

**Summary:**
Treatment of DU-145 for 48 and 72 hours with up to 300 μM 5-AZAC had no significant effect on cell proliferation. However, treatment of DU-145 cells for 24 hours with as little as 30 μM 5-AZAC decreased cell proliferation. 100 μM 5-AZAC treatment over 24 hours decreased cell proliferation by up to ~37%. Inhibition of cell proliferation by 300 μM 5-AZAC over 24 hours was similar to inhibition induced by 100 μM treatment. These results are shown in Figure 1.

![Figure 1. Effect of 5-AZAC on DU-145 cell proliferation](image)

Treatment of DU-145 cells with 100 μM 5-AZAC for 48 hours followed by immunoprecipitation and Western blot analysis revealed that there was no change in the levels of P-STAT3 relative to the levels in cells treated with vehicle (dimethyl sulfoxide). Western blot for P-STAT3 displayed bands of similar intensity at ~90 kD for cells with no treatment, vehicle treatment, and 100 μM 5-AZAC treatment.

Western blot for STAT3 displayed doublet bands at ~90 kD for cells with no treatment, vehicle treatment, and 100 μM 5-AZAC treatment. The doublet band for vehicle-treated cells had the highest intensity. The doublet band for cells with no treatment was slightly less intense than that of vehicle-treated cells. The doublet band for cells with 100 μM 5-AZAC treatment was
significantly less intense than that of both non-treated and vehicle-treated cells. The protein concentration in STAT3 lysates was measured (Table 1).

Table 1. Protein concentration in STAT3 lysates

<table>
<thead>
<tr>
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<th>Protein concentration</th>
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<tr>
<td>Cells with no treatment</td>
<td>0.22 mg/ml</td>
</tr>
<tr>
<td>Cells with vehicle treatment</td>
<td>0.37 mg/ml</td>
</tr>
<tr>
<td>Cells with 100 μM 5-AZAC treatment</td>
<td>0.24 mg/ml</td>
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Conclusion:
We observed that treatment of DU-145 cells with up to 300 μM 5-AZAC decreased the proliferation of DU-145 cells by ~37% (Figure 1). Although 5-AZAC incorporation into DNA and role as a methyltransferase inhibitor are known, the actual mechanism by which 5-AZAC reduced proliferation of cancer cells remains unknown (11). 5-AZAC has the potential to cause cell death by direct cytotoxicity or through changes in DNA methylation (11). As both cell-cycle arrest and apoptosis have been induced in other cancer cell lines treated with 5-AZAC, further experiments are necessary to determine the specific effect of 5-AZAC on DU-145 cells (4,14).

Western blot analysis revealed that treatment of DU-145 cells with 100 μM 5-AZAC for 48 hours did not affect levels of P-STAT3, however, treatment with 100 μM 5-AZAC decreased the levels of total STAT3 protein without decreasing total protein concentration in the cell lysate (Table 1). These results differ from those obtained in published experiments with multiple myeloma and anaplastic large cell lymphoma cells, in which both cell lines had a reduction in P-STAT3 levels, but no change in total STAT3 protein levels when treated with 5-AZAC (4,10). Therefore, further experiments must be performed to determine the specific mechanism by which DU-145 cell proliferation is inhibited by 5-AZAC. The results from these experiments may indicate a STAT3-independent mechanism for 5-AZAC action. Analyzing downstream targets of STAT3, such as cyclin D3, survivin, Bcl-2 and SOCS-3, may help to determine whether the effects of 5-AZAC are truly STAT3-independent.

References:

Acknowledgments:

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I would like to thank Adetola Shodeinde for her assistance with immunoprecipitation and Western blot analysis and H. Dan Lewis for his assistance with general laboratory techniques and cell culture. I would also like to thank Dr. Beverly Barton for inviting me into her laboratory for the summer, and mentoring me along each step of this process.
21. FRANK WANG (NJMS 2013)

THE ROLE OF OSTEOBLASTS IN MAINTAINING DORMANCY IN BREAST CANCER CELLS

Mentor: Robert Wieder, MD, PhD (Medicine)

Objective:
We investigated the capacity of a rat calvarium pre-osteoblast cell line, MC3T3-E1, to support the dormancy of human breast cancer cell line MCF-7 in vitro in a co-culture model. The rationale was based on our prior observations in a simplified in vitro dormancy model which consisted of the bone marrow stromal elements fibronectin and FGF-2. In the model, FGF-2 partially re-differentiates estrogen receptor positive breast cancer cells and induces re-expression of integrins lost with malignant transformation. Among these, we demonstrated that integrin α5β1 specifically initiates survival signaling by ligation of fibronectin. FGF-2 induces growth arrest and phenotypic changes in the breast cancer cell morphology, resulting in dormant clone formation. Data from the literature strongly support a hypothesis that stromal elements that support dormancy of breast cancer micrometastases in the bone marrow are members of the pre-osteoblast population in the endosteum. The reasoning for this is two-fold: 1) hematopoietic stem cells have been found to localize within niche populations of osteoblasts; 2) breast cancer micrometastases have been within the bone marrow and evidence has shown breast cancer micrometastases to reduce the overall number of hematopoietic stem cells. We tested the hypothesis that a pre-osteoblast cell line is capable of supporting dormant breast cancer colony formation and that this support requires ligation of integrin α5β1.

Methods:
Cell lines
MCF-7 breast cancer cells – Estrogen Receptor/Progesterone Receptor Positive
MC3T3-E1 rat calvarium pre-osteoblast cell line
NIH 3T3 fibroblast cell line – murine cell line (positive control for growing clones)

Osteoblast differentiation
90% confluent MC3T3-E1 cells were induced to differentiate with 50 μg/mL ascorbic acid and 10mM β-glycerol phosphate. Cells were partially differentiated for 6 days as determined by positive staining for alkaline phosphatase and completely differentiated with mineralization by 28 days as determined by Alizarin red S staining.

Dormancy assay
Breast cancer cells were co-incubated on confluent osteoblast monolayers in 24 well tissue culture dishes that were either undifferentiated or partially differentiated for six days with ascorbate and phosphate at a clonogenic density of 1,000 cells/well that ensured the principle contact of each breast cancer cell was with the substratum and not each other. Colonies were counted after 6 days. Breast cancer cells were incubated on fibronectin-coated 24 well tissue culture plates at clonogenic density with and without 10 ng/ml FGF-2 to demonstrate the dormancy efficiency in our model, as controls. Growing and dormant clones were counted after 6 days.
Determination of the role of integrin \( \alpha_5 \beta_1 \) in dormancy on osteoblasts

A total of 2 mg/well monoclonal anti-integrin \( \alpha_5 \beta_1 \) blocking antibody, the fibronectin ligand, 2 mg/well monoclonal anti-integrin \( \alpha_2 \beta_1 \), a collagen ligand, or 2 mg/well of mouse IgG, as controls, were added to co-cultivated cells on day 3 to determine their role in maintaining the capacity of osteoblasts to support dormant clone formation.

**Summary:**

In order to be sure that differentiation of MC3T3-E1 cells occurred, staining for alkaline phosphatase was performed. Preosteoblasts (Fig. 1 left), produced a negative result, while staining differentiated MC3T3-E1 cells (Fig. 1 middle) on day 6 for alkaline phosphatase indicates differentiation of our MC3T3-E1 cells. As further evidence of differentiation, mineralization of hydroxyapatite occurs in differentiated osteoblasts around day 28 (Fig. 1 right) as demonstrated by Alizarin Red S staining.

Antibody treatments were introduced on day 3, with MCF-7 clonogenic growth being observed on day 6. Growing colonies (Fig. 2 left) were \( \geq 30 \) cells whereas dormant colonies (Fig. 2 right) were \( \leq 10 \) cells. Colonies in between were considered of indeterminate classification.

Blocking antibody to integrin \( \alpha_5 \beta_1 \) had a marginal but significant effect on growing clones and had no effect on dormant clones on undifferentiated pre-osteoblasts. Integrin \( \alpha_2 \beta_1 \) and IgG had a suppressive effect on both types of colonies. Blocking antibody to integrin \( \alpha_5 \beta_1 \) resulted in an increased ratio of growing to dormant clones compared to untreated or control treated plates. The significance remains to be determined with repeated assays and appropriate controls.
Conclusion:
Our data suggest that the ratio of growing to dormant clones increases with blocking the interaction of this integrin with osteoblast but the system is sufficiently complex that a large series of control experiments need to be conducted to support this conclusion or define its relevance.

Preliminary observation that blocking antibody to integrin α5β1 may increase the ratio of growing to dormant clones needs to be validated. Appropriate and extensive controls need to be done to determine the nature of the interaction of the osteoblast with breast cancer cells.

The capacity of dormant breast cancer cells recovered from co-culture to regrow into growing clones must be evaluated and the phenotypic nature of these cells needs to be characterized.
We derived an initial working capability with this co-cultivation system that will enable us to pursue these relevant questions.

References:
22. PRASANTHI YELAVARTHY (NJMS 2014)

CELLS THAT EVADE SENESCENCE IN BREAST CANCER HYPERPLASIAS: POTENTIAL CANCER PROGENITOR/STEM CELLS?

Mentors: Terri Wood, PhD (Neurology), Deborah Lazzarino, PhD (Neurology), Utz Herbig, PhD (Microbiology and Molecular Genetics)

Objective:

The molecular events leading to the initiation and early progression of human breast cancer growth are incompletely understood. Although it is evident that breast cancer initiates from cells located in the ducts or lobules of the breast, the exact identity of the tumor initiating cell types has not been identified. Two models, the cancer stem cell (CSC) and the clonal evolution model, have been put forward to account for these cells. Cancer stem cells are believed to be a subset of tumor-initiating cells that are derived from transformation of a normal stem and/or progenitor cell. On the other hand, the clonal evolution model hypothesizes that mutant tumor cells of any lineage origin with a growth advantage are selected and expanded, with these cells having a potential for regenerating tumor growth. Interestingly, recent work has confirmed that different breast tumor types have gene expression profiles that are similar to the normal early stem and progenitor epithelial cell lineages in human breast. Yet still, little is known about when and how these cells arise.

Cellular senescence is a stable and irreversible growth arrest that eukaryotic cells encounter in response to various stresses. Studies in mice have shown that cellular senescence can function as a tumor suppressing mechanism by limiting proliferation of cells that could become cancerous. Previously, it was demonstrated that almost all of the cells within ductal hyperplasias of the breast contained elevated levels of macroH2A, a marker of cellular senescence, suggesting that cells within these breast cancer precursor lesions were stably and irreversibly arrested. Cells also displayed telomere dysfunction induced DNA damage foci (TIF) suggesting that the reason for cellular senescence was due to dysfunction of telomeres. However, one or very few macroH2A negative cells were also observed within the hyperplastic ducts, suggesting that some cells had escaped telomere dysfunction induced senescence (TDIS) and retained the ability to proliferate. These cells were typically observed to reside in a suprabasal position suggesting that they may be stem/progenitor cells.

The objective of this project was to determine with the macroH2A-negative cells are likely to be developmentally immature stem/progenitor cells that have evaded senescence and retain the ability to proliferate. Therefore, in this study, we have used lineage and other potential stem cell markers to characterize the identity of non-senescent cells within breast cancer precursor lesions.

Methods:

Patient samples were taken of representative DCIS and ductal hyperplasias. 4 μm paraffin embedded tissues were baked in the oven at 60-65°C for 2 hours and deparaffinized in xylene. The tissues were hydrated with ethanol and antigens were retrieved in a sodium citrate buffer. The tissues were then immunolabeled with combinations of the following antibodies and nuclear DNA was counterstained with DAPI:
- cytokeratin 14 (ck14): marker for myoepithelial cells
- cytokeratin 18 (ck18): marker for luminal cells
- macroH2A: a heterochromatin protein that marks sites of DNA damage, has been demonstrated to be absent in proliferating cells and up-regulated in senescent human fibroblasts and HMECs (preliminary work in Herbig Lab)
- ALDH: aldehyde dehydrogenase, an enzyme expressed at high levels in normal and malignant human mammary stem cells

Analysis was performed using 4-color immunofluorescence (IF) microscope with a Zeiss Axiovision 200M. Images were taken at 40X and 63X using the apotome.

Summary:

In this experiment, we used immunostaining to identify cells that were macroH2A negative and combined these with other markers, including lineage and luminal markers, to further classify these cells. We found that the macroH2A negative cells are also negative for epithelial cell lineage markers (ck14 and ck18), supporting the hypothesis that these cells may be stem and/or progenitor cells. In addition, we found that, in general, there were fewer macroH2A negative cells with stem cell-like properties found in the DCIS stage than in the hyperplasia stage. This may have been observed because these “stem cells” could have differentiated into their lineages (luminal or myoepithelial) by then. Furthermore, these macroH2A, ck14, and ck18 negative cells were found in multiple hyperplasias in thirteen different patient samples, indicating that these cells are found throughout. When ALDH, an enzyme in high concentrations in stem cells, was combined with macroH2A, there were certain cells that stained extremely positively, showing that these cells could be early stem/progenitor cells.

Conclusions:

The macroH2A negative cells were also negative for epithelial cell lineage markers (ck14 and ck18), supporting the hypothesis that these cells may be stem or progenitor cells. These “stem cells” were found in multiple hyperplasias in different patients. In order to get more accurate data, a larger sample size should be used to determine if these results are found within all patients. In addition, we need to obtain more definitive markers or combination of markers to identify cancer stem cells since none of the current markers are exclusively expressed by the CSCs. Using triple immunostaining will help us gain a better understanding of these cells, since we can cross out more possibilities at once.