2014
The Rutgers New Jersey Medical School Cancer Summer Research Program Abstracts

Supported by a training grant from the National Institutes of Health, National Cancer Institute R25CA019536
FORWARD

The Cancer Summer Student Research Program, in existence at NJMS since 1969, is supported through an NCI Cancer Education Program Grant (Principal Investigator, Gwendolyn Mahon, PhD, R25CA019536). This program, which has been continuously funded by the NCI for 44 years, provides a unique eight-week research experience for New Jersey Medical School first and second-year medical students as well as undergraduate students enrolled in our combined BS/MD seven-year program. This year 29 students participated in biomedical research activities in both laboratory and clinical settings at either the New Jersey Medical School Cancer Center, or other facilities on the Newark Campus, while developing a close working relationship with their faculty mentors. All students were required to present their research at a poster session during the concluding symposium, one of the program highlights. Further information about the program is available for viewing at the Rutgers New Jersey Medical School Cancer Center website which can be found at the following URL:

http://njms.rutgers.edu/cancercenter/summer_program.cfm

Awardees of the 2014 Cancer Summer Research Program Poster Session (from Left to Right): Robert Grant (2nd place), Neelam Upadhyaya (2nd place), Renuka Reddy (2nd place), Stacy Pan (1st place), and Dr. Edmund Lattime (closing symposium guest speaker, Rutgers Cancer Institute of New Jersey, Associate Director for Education and Training).

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**FACULTY EXECUTIVE ADVISORY COMMITTEE**

The faculty executive advisory committee is responsible for reviewing and selecting the projects submitted by faculty, as well as reviewing the structure of the forthcoming Cancer Summer Research Program. We appreciate the time they took out of their busy schedules to evaluate the program and help make it a success. Department affiliations are in NJMS unless otherwise stipulated.

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CANCER EDUCATION PROGRAM FACULTY

While the Cancer Center building currently houses 16 laboratories, the broader Cancer Center Community (CCC) is composed of 104 investigators from several departments of Rutgers NJMS, the Rutgers School of Dental Medicine, the Rutgers School of Public Health, and the Rutgers School of Health and Related Professions (SHRP). 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 40 years. The 2014 participating faculty and their respective departments are listed below. Department affiliations are in NJMS unless otherwise stipulated.

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# Table of Contents

<table>
<thead>
<tr>
<th>Student Name</th>
<th>Page #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amer, Kamal</td>
<td>6</td>
</tr>
<tr>
<td>Chang, Andrew</td>
<td>11</td>
</tr>
<tr>
<td>Biondi, Breanne E.</td>
<td>15</td>
</tr>
<tr>
<td>Deshpande, Kaivalya</td>
<td>21</td>
</tr>
<tr>
<td>Elias, Marcus</td>
<td>25</td>
</tr>
<tr>
<td>Elias, Michael</td>
<td>28</td>
</tr>
<tr>
<td>Elsekhely, Aya</td>
<td>31</td>
</tr>
<tr>
<td>Goldman, Jordana</td>
<td>35</td>
</tr>
<tr>
<td>Grant, Robert</td>
<td>41</td>
</tr>
<tr>
<td>Haque, Maryam</td>
<td>45</td>
</tr>
<tr>
<td>Kim, Julia J.</td>
<td>50</td>
</tr>
<tr>
<td>Kim, Mitchell</td>
<td>54</td>
</tr>
<tr>
<td>Kim, Sangsoo</td>
<td>60</td>
</tr>
<tr>
<td>Kothari, Pankti</td>
<td>65</td>
</tr>
<tr>
<td>Lambda, Richa</td>
<td>68</td>
</tr>
<tr>
<td>Marcel, Christian</td>
<td>71</td>
</tr>
<tr>
<td>Mendelson, Zachary S.</td>
<td>75</td>
</tr>
<tr>
<td>Nawrocki, Tomer</td>
<td>78</td>
</tr>
<tr>
<td>Pan, Stacey</td>
<td>82</td>
</tr>
<tr>
<td>Parikh, Bijal</td>
<td>87</td>
</tr>
<tr>
<td>Patel, Viral</td>
<td>92</td>
</tr>
<tr>
<td>Pinho, Gabriella</td>
<td>96</td>
</tr>
<tr>
<td>Reddy, Renuka</td>
<td>99</td>
</tr>
<tr>
<td>Schaap, Ariel</td>
<td>103</td>
</tr>
<tr>
<td>Solanki, Jay</td>
<td>107</td>
</tr>
<tr>
<td>Sun, Michael</td>
<td>111</td>
</tr>
<tr>
<td>Upadhyaya, Neelam</td>
<td>113</td>
</tr>
<tr>
<td>Wu, Lesley</td>
<td>118</td>
</tr>
<tr>
<td>Yeom, Richard</td>
<td>120</td>
</tr>
</tbody>
</table>

Abstract Format:

**JOHN SMITH (NJMS 2014) [Student Name, (school, class year) Bold All Caps] (space)**  
**CELL TRANSFORMATION BT THE CRK ONCOGENE (Title Bold, All Caps) (space)**  
Jane Doe, PhD, (Microbiology) [Mentor, Degree, (Department) mixed case]  
Names of co-mentors and other contributing authors follow in order of appearance on the poster.
Objective:
Aging of the human skeleton has been characterized by a decrease in bone mass and bone formation, both of which are more pronounced in patients with osteoporosis. While the underlying mechanism of osteoporosis comes from an imbalance between osteoclast and osteoblast activity, previous studies indicate mesenchymal stem cell differentiation plays a role as well. Bone marrow stem cells can differentiate into a variety of other cells including myocytes, adipocytes, and osteocytes. Patients with osteoporosis display an increase of adipocyte cell volume (Justesen, 2001). Since both adipocytes and osteoblasts come from a common precursor in bone marrow, possible regenerative therapies could include selective differentiation of these bone marrow mesenchymal stem cells (BMMSCs) into osteoblasts, as opposed to adipocytes.

Differentiation of BMMSCs is highly dependent upon the chromatin remodeling complex, SWI/SNF. SWI/SNF alters gene expression, but has not been considered as a target for pharmaceutical medications due to its ubiquitous role in almost every cell lineage pathway. The SWI/SNF complex, however, is complemented by two independent ATPases, BRM and BRG-1. BRG-1 impedes differentiation of various models of differentiation (de la Serna et al., 2006). However, the individual role of BRG1 BRM have not been fully addressed.

In a pre-osteoblast cell model of differentiation, MC3T3-E1, siRNA depletion of BRM revealed the role of BRM as being a repressor of osteoblast differentiation (Flowers et al., 2009). In contrast, depletion of BRM in the 3T3-L1 pre-adipocyte model resulted in the failure of differentiation (Xu, 2013). Furthermore depletion of BRM in both the mesenchymal stem cell line, CH310T1/2 and primary bone marrow stem cells from BRM null mice favored differentiation to the osteoblast lineage over adipogenesis. The data from these studies strongly suggest that BRM is a key regulator of differentiation whose depletion causes a shift in lineage selection towards osteoblastogenesis over adipogenesis. The end result of this shift is seen in aged BRM null mice. While wild type mice show an increase in osteoporosis during aging (18 month), little to no loss of bone density was seen in BRM null mice(Nguyen et al., in prep).

The main purpose of this study is to address the underlying mechanisms in BRM regulation of a stem cell to a pre-osteoblast. In the pre-osteoblast model, loss of BRM resulted in constitutive up-regulation of the early differentiation marker, alkaline phosphatase, and of late stage markers such as osteocalcin (Flowers, 2009). However, with loss of BRM in the CH310T1/2 stem cell model, induction of only the early marker alkaline phosphatase was seen (Nguyen et al., in prep). This suggests that loss of BRM in the CH310T1/2 cells is not sufficient to bring the cells to a pre-osteoblast state, but somewhere in between a stem cell and a pre-osteoblast.
Selective osteoblast differentiation of BMMSCs is dependent upon the transcription factor RUNX2. RUNX2 induces the differentiation of multi-potent mesenchymal cells into immature osteoblasts (Komori, 2010).

We questioned whether induction of RUNX2 by an exogenous factor such as retinoic acid could combine with BRM-depletion to drive BRM-depleted cells all the way to mature osteoblasts, marked by expression of high levels of osteocalcin. Previous data indicate the role of retinoic acid (RA) in the induction of mesenchymal stem cells into pre-osteoblasts (Skillington et al., 2002).

A second objective addressed in this study concerns the metabolic state of BRM-depleted cells in terms of fatty acid metabolic activity, namely through expression analysis of Acaa2, Agpat4, and Hadha (genes encoding key enzymes of lipid metabolism).

**Methods:**
CH310T1/2 mesenchymal stem cells were maintained using BME media. BRM knock down cell lines were maintained using BME media plus puromycin (4ug/ml). Retinoic acid was applied independently to both media at a concentration 1 μM RA. Note that cells reached confluence on day 0 before introducing RA.

Cells were fixed with 100% methanol, and assessed for ALP activity in a colorimetric assay via NCB and BCIP substrates overnight. The stained cells were photographed using an optical microscope. Photos were taken in 10x magnification.

RNA was isolated using RNeasy Mini Kit (Quigen) following manufacturer’s instructions. cDNA was made using SuperScript III First-Strand (Invitrogen). Quantitative PCR was used to determine expression levels of RUNX2, OCN, and the common adipolytic enzymes Acaa2, Agpat4, and Hadha. The primer pairs utilized were: RUNX2: sense 5’-CGC CCC TCC CTG AAC TCT-3’, antisense 5’-TGC CTG CCT GGG ATC TGT A-3’; OCN: sense 5’-CTG ACA AAG CCT TCA TGT CCA A-3’, antisense 5’-GCC CCG GAG TCT GTT CAC TA-3’; Acaa2: sense TCT GCT GGC AAA GTT CCA CCT G-3’, antisense 5’-ACA GAG CCT GTT GAG GGT AAG G-3’; Agpat4: sense 5’-GGG TCA TTG TCA ACG CCA TCC A-3’, antisense 5’-CCA CTC CAG AAG CAT CAC CAA C-3’; Hadha: sense 5’-GTG TGA GGT GCC CCT CGG TGT AAA GC-3’, antisense 5’-GAG AGC AGA TGC TGT GGT GCG A-3’. Fate of lipid enzymes gene expression was suggested by RNAseq performed in collaboration with Dr. Robert Donnelly at the NJMS Molecular Resource Facility.

**Summary:**
- Proliferating CH310T1/2 cells treated with RA for 7 days showed increased expression of RUNX2 as assayed by qPCR at day 3 and day 7. (Figure 1).
- ALP colorimetric assay of CH310T1/2 parental mesenchymal stem cells induced with RA displayed increased activity of the enzyme alkaline phosphatase by day 3, indicative of responsiveness to RUNX2 (Figure 2).
- qPCR expression of OSC was higher in Day 3 RA induced CH310T1/2 cell lines (Figure 3a). But this level of induction is not indicative of a mature osteoblast (Figure 3b). Moreover, induced OSC levels were not sustained and declined by day 7.
- ALP colorimetric assay of BRM knockdown cells showed increased expression of ALP for both RA induced and non RA induced cell lines when compared with CH310T1/2 mesenchymal stem cells (Figure 4).
- This establishes a system in which we can assay the long term effects of RA on BRM-depleted cells in respect to progression to a mature osteoblast phenotype marked by
high levels of OSC expression. This is not yet complete due to lack of time in the present program.

- Preliminary qPCR analysis shows significantly lower expression of genes required for lipid metabolism (Hadha, Acaa2, and Agpat4) in BRM knockdown cells relative to CH310T1/2 mesenchymal stem cell lines (Figure 5a-c) and BRM expression (d).

Conclusion:

- Retinoic acid induction of CH310T1/2 mesenchymal stem cells causes their differentiation into pre-osteoblast cells as opposed to adipocytes. This is similar to the constitutive status of BRM knockdown cells. OSC expression was induced slightly in parental cells, supporting the suggestion that a combination of RA and BRM knockdown will drive progression to a mature osteoblast phenotype. This will be tested in future experiments.

- Uninduced BRM knockdown cells displayed significantly lower expression of Acaa2, Hadha, and Agpat4; each of which is involved in fatty acid metabolism. Cells focused onto the osteoblast lineage (such as BRM knockdown cells) should suppress fatty acid metabolism enzymes, and these results indicate that fatty acid metabolism is slowing down very early after commitment to the osteoblast lineage.

Tables and Figures:

- Figure 1. RUNX2 expression in RA induced and uninduced CH3 10T1/2 cells as displayed via qPCR analysis.

- Figure 2. 10x magnification of RA induced and uninduced CH310T1/2 mesenchymal stem cells. Dark staining is indicative of ALP expression.

- Figure 3. qRT-PCR of Osteocalcin expression in (a) induced and uninduced CH310T1/2 cells and (b) in MC3T3E1.
<table>
<thead>
<tr>
<th>Retinoic Acid</th>
<th>CH310T1/2</th>
<th>BRM Knockdown</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
</tbody>
</table>

**Figure 4.** Parallel alkaline phosphatase (ALP) staining of CH310T1/2 and BRM (CD606) cells induced and uninduced with retinoic acid (RA).

![Graphs](image5.png)

**Figure 5.** qRT-PCR Expression of genes involved in lipid metabolism (Agpat4 (a), Hadha (b), and Acaa2 (c)) in uninduced CH310T1/2 cells and BRM knockdown cell lines. BRM expression was also assayed (d).
References:


7. Xu, F. 2013. Role of the SWI/SNF chromatin remodeling complex in transcriptional regulation of osteoblast and adipocyte differentiation. A Dissertation Submitted to the Graduate School of Biomedical Science, University of Medicine and Dentistry of New Jersey in Partial Fulfillment of the Requirements for the Ph. D Degree.
Objective:

Kaposi’s Sarcoma-associated Herpesvirus (KSHV) is a DNA tumor virus implicated in the etiology of Kaposi's sarcoma and lymphoproliferative diseases like primary effusion lymphoma and multicentric Castleman's disease. The reactivation of KSHV from latency and production of mature, viral progeny is necessary for the progression of these diseases. Autophagy is a cellular survival mechanism that contributes to the resistance of transformed cells to cytotoxic treatments and has been closely linked to the control of KSHV reactivation. KSHV latency proteins inhibit autophagy while histone deacetylase inhibitors (HDACi) and other known inducers of KSHV reactivation have been found to induce autophagy. Given that autophagy can regulate cell secretion, we hypothesize that autophagy links KSHV reactivation to cancer by altering the secretome and promoting the expression of viral, pro-inflammatory genes. Moreover, the ability of HDACis to induce reactivation of KSHV is troubling, as many of these inhibitors are used in cancer therapeutics. The overall goal of this project is to define the mechanisms by which HDAC6 regulates KSHV reactivation through its regulation of autophagy. Elucidation of these mechanisms is critical to determining whether or not HDACis belong in cancer treatments. Additionally, a better understanding of the role HDAC6 plays can allow for the manipulation of autophagy to restore chemosensitivity and make tumor cells amenable to cytotoxic treatments.

Methods:

Tissue Culture

Vero rKSHV.294, 293 MSR tet-OFF, and Vero rKSHV.219 cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100u/mL penicillin, and 100 µg/mL streptomycin. BCBL-1 cells were propagated in Roswell Park Memorial Institute medium supplemented with 12% fetal bovine serum, 2mM L-glutamine, 100u/mL penicillin, 100 µg/mL streptomycin, and β-mercaptoethanol. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Determining the effect of Valproic Acid (VPA) on autophagy

BCBL-1 cells latently infected with KSHV were treated with 1mM valproic acid (VPA) or 2µM of rapamycin (Rap) and incubated for 4h or 24h. After treatment, cells were fixed using methanol and stained for LC-3B. Nuclear DNA was stained using DAPI. LC-3B puncta were detected using indirect immunofluorescence.

Determining the effect of HDAC6 inhibition of autophagy

BCBL-1 cells latently infected with KSHV were treated with 1mM VPA, 2.5µM tubacin, or both. Cells treated with both VPA and tubacin were first treated with tubacin and incubated for 4 before adding VPA and incubating overnight. After treatment, cells were fixed using methanol and stained for LC-3B. Nuclear DNA was stained using DAPI. LC-3B puncta were detected using indirect immunofluorescence.

Quantitation of infectious virus

Vero rKSHV.294 cells latently infected with KSHV containing the secreted alkaline phosphatase (SeAP) gene under the control of a tetracycline-responsive promoter were transfected with one of three amounts of HDAC6 plasmid alone (0.25, 0.5, or 1.0µg) or with 1.25...
µg of Rta plasmid. Cells were incubated for 72h. The media used to grow the Vero rKSHV.294 cells, containing virus, was transferred to 293 MSR tet-OFF cells, which express the tetracycline transactivator, and incubated for 72h. SeAP reporter assay was performed using the 293 cell media with a spectrophotometer to quantify fluorescence of SeAP.

**Overexpression of HDAC6 and Rta expression in Vero rKSHV.219 cells**

Vero rKSHV.219 cells latently infected with recombinant KSHV containing the RFP gene under the control of a delayed early promoter were transfected with 1.0, 1.5, or 2.5µg of HDAC6 plasmid or 1.0, 1.5, or 2.5µg of replication and transcription activator (Rta) plasmid. Cells were incubated for 48h and fixed using methanol. Cells were then visualized directly to score for for RFP signal.

**Overexpression of HDACs in BCBL-1 and Western Blotting**

BCBL-1 cells were electroporated with 30µg of HDAC1, HDAC3, or HDAC6 plasmid, fused to the FLAG epitope, or pcDNA3 (empty vector). Cells were incubated for 72h and lysed. The cell lysate was treated with a protease inhibitor cocktail, and cellular debris was cleared by centrifugation. Supernatants were collected, and a Bradford assay was performed to determine total protein concentration. One hundred micrograms of protein from each lysate were separated by SDS-PAGE. Proteins were electrophoretically transferred to a nitrocellulose membrane in transfer buffer and tested using Western for expression of Rta.

**Determining the use of toll-like receptors (TLRs) in BCBL-1 cells**

BCBL-1 cells latently infected with KSHV were treated with 1mM VPA or 10µM 3M003, an agonist for TLR7, and incubated for 24h. After treatment, cells were fixed using methanol and stained for LC-3B. Nuclear DNA was stained using DAPI. LC-3B puncta scored detected using indirect immunofluorescence.

**Summary:**

BCBL-1 cells treated with VPA showed an increase in the induction of LC-3B puncta after 4h and 24h relative to the mock-treated cells. Relative to the rapamycin treatment, the VPA treatment showed comparable LC-3B induction after 4h and higher magnitude induction after 24h (Fig. 1A). The VPA-treated cells displayed much more intense LC-3B signal as well. Treatment of BCBL-1 cells with tubacin prior to a VPA treatment decreased LC-3B puncta induction from 7.0 fold to 4.4 fold induction (Fig. 2). Quantitation of infectious virus using SeAP reporter assay found that ectopic HDAC6 and Rta cooperate at low amounts to reactivate virus (0.25µg and 0.5µg HDAC6) better than ectopic Rta alone. However, at a higher amount of 1.0µg HDAC6, viral reactivation is decreased when compared to Rta (Fig. 3). Overexpression of HDAC6 in Vero rKSHV.219 cells showed no increase in Rta expression, resulting in little to no RFP expression (Fig. 4). However, overexpressing HDAC6 in BCBL-1 led to an increase in Rta expression relative to tubulin expression (Fig. 5A, 5B). BCBL-1 cells treated with 3M003 showed an increase in the induction of LC-3B puncta relative to both the mock-treated cells and the VPA-treated cells (Fig. 6A). The 3M003-treated cells displayed a greater number of more intense and distinct LC-3B puncta signals comparatively (Fig. 6B).

**Conclusion:**

- VPA is sufficient to initiate autophagy in KSHV-infected BCBL-1 cells.
- Tubacin decreases LC-3B induction by VPA, suggesting a role for HDAC6 in the initiation of autophagy.
- HDAC6 cooperates with Rta to reactivate virus up to a certain concentration, emphasizing the importance of the stoichiometry of the complex containing HDAC6.
- Overexpression of HDAC6 shows no increase in Rta expression in Vero rKSHV.219 cells, but overexpression of HDAC6 increases Rta expression in BCBL-1 cells, suggesting that the biology of the viral host cell is important to the reactivation of virus.
TLR signaling is sufficient to induce autophagy in BCBL-1 cells. TLR7 is a known KSHV reactivator, suggesting that the initiation of autophagy may be a general mechanism to control KSHV reactivation. Overall, our data provide evidence that HDAC6 plays a prominent role in autophagy and KSHV reactivation, potentially serving as a key player in the initiation of autophagy as a mechanism for KSHV reactivation.

**Figure 1. VPA induces the initiation of autophagy.** (A) KSHV-infected BCBL-1 cells were treated with 1mM VPA or 2µM Rapamycin (Rap) and incubated for 4 or 24h. Cells were then fixed and stained for LC-3B puncta. (B) LC-3B puncta were detected using indirect immunofluorescence. Note the increase in number and intensity of puncta in VPA treatment.

**Figure 2. Tubacin inhibits HDAC6 and decreases the initiation of autophagy in BCBL-1 cells.** KSHV-infected BCBL-1 cells were treated with 1mM VPA, 2.5µM tubacin, or both. Cells with VPA and tubacin treatments were first incubated with tubacin for 4h before VPA was added and incubated overnight.

**Figure 3. HDAC6 cooperates with Rta to reactivate virus.** HDAC6 and Rta cooperate when ectopic HDAC6 is at lower concentrations to reactivate virus better than ectopic Rta alone.

**Figure 4.**
Overexpression of HDAC6 in Vero rKSHV.219 cells shows an increase in Rta expression. Three different amounts of HDAC6 plasmids transfected in Vero rKSHV.219 cells show little to no effect on Rta expression.

Figure 5. Overexpression of HDAC6 in BCBL-1 cells increases Rta expression. (A) Ectopic HDAC6 expression increases Rta expression in BCBL-1 cells relative to tubulin expression. (B) Ectopic HDAC6 increases Rta expression better than HDAC1, HDAC3, and empty pcDNA3 vector.

Figure 6. BCBL-1 cells can use TLRs to initiate autophagy. (A) Treatment of BCBL-1 cells with 3M003 shows that cells are capable of utilizing TLR signaling and that TLR signaling is sufficient to induce autophagy in BCBL-1 cells. (B) 3M003 induces strong LC-3B puncta signal relative to VPA and mock-treated cells.
Objective:

In 2014, there will be over 12,000 new cases of cervical cancer in the United States. Minority populations, specifically blacks and Hispanics, are at greater risk for developing cervical cancer. The Surveillance, Epidemiology, and End Results Program (SEER), of the National Cancer Institute, reports incidence rates (per 100,000) of 9.4 and 10.3 respectively for blacks and Hispanics, whereas the rate for non-Hispanic whites is only 7.2. Other race/ethnicities range from 6.4-7.6 (SEER, 2007-2011, Age-Adjusted)\(^1\). Cervical cancer mortality has been declining in all racial/ethnic groups, attributable both to screening (with consequent earlier detection) and treatment advances. However, outcome disparities persist, particularly for blacks. Cancer stage is determined at the time of diagnosis, and is prognostic of mortality. Lower socioeconomic status (SES) is associated with poorer screening rates.

Our analysis includes cervical cancer registry data from University Hospital (UH) in Newark, NJ, from 2000-2012 to examine survival in an urban hospital setting. UH draws from inner city urban areas as well as the broader surrounding community.

We compared survival at UH to national treatment data from patients comprising the National Cancer Data Base (NCDB) to examine quality of care. The NCDB captures almost 70% of newly diagnosed U.S. cancer cases.\(^2\) Stage distribution and cancer survival at each stage was compared between these two datasets.

Our research group has previously found great similarity in SES and in health measures among residents of four neighboring cities: Newark, East Orange, Orange, or Irvington, which we term “Greater Newark”. Since all patients are treated at the same hospital, with the same clinicians and treatment protocols, this presents an interesting opportunity to test whether mortality varies based on where people live. We compared survival by stage of those living in the Greater Newark area to those outside of it. We also examined age, race/ethnicity, and several SES variables. In particular, we wanted to examine whether living in the Greater Newark area was merely a proxy for lower SES. This could very well be true, as Newark has a much large population living below the poverty level (28%) compared to all of New Jersey (9.9%).\(^3\) It is also possible that living in Greater Newark serves as a unique attribute that is not addressed by various SES indicators.

Methods:

As the initial goal to examine and help improve data quality within the UH, tumor registry, we attempted to identify potentially problematic data issues. Given the registrars’ limited resources, we prioritized their efforts towards data more likely to be in error. Putative total cervical cancer cases at UH between 2000-2012 were identified by the UH Tumor Registry. All cases of stage 0, cases with stage 1 who died (as overall stage 1 mortality is quite low, those who died may have been miscoded), and those missing A or B subclasses were rechecked by the tumor registrars at UH, and reclassified as appropriate. Stage distribution data for cervical cancer from the NCDB five-year survival reports with half year intervals from 2003-2006 were used for comparison to UH, as that time period had consistent coding and is the latest with full 5-year follow-up. Cases from UH had included exact dates of death (and sometimes of loss to follow up). However, for comparison to the NCDB, date values were rounded to half year intervals in survival calculations. The NCDB does not report A and B substages, so for the comparisons of UH to the NCDB, we could not use substage.
For the analysis comparing UH to the NCDB, our exclusions from UH included 1 case with an unknown stage and 1 whose address was in the U.S. Virgin Islands. This left a dataset of 313 domestic, staged cervical cancer cases. For the lifetest analysis, all UH cases were censored at five years to match the NCDB analysis. Kaplan-Meier survival curves were developed to compare survival by stage.

After all attempts at data review and corrections were completed, for the intra-UH analysis, 9 additional cases were excluded. Two exclusions were due to geographic issues (one where the address was a correctional facility and one where only a post office box number was available). We also excluded 7 cases with unusual histologies that have distinct clinical features and known higher mortality (4 neuroendocrine carcinoma, 1 carcinosarcoma, 1 spindle cell carcinoma, and 1 signet ring cell carcinoma), so that we could specifically analyze only the common forms of cervical cancer.

Kaplan-Meier survival curves were used to compare survival by stage for UH cases versus the NCDB stages. Race/ethnicity was divided into 4 non-overlapping categories: Hispanic; black non-Hispanic, white non-Hispanic, and other.

Analyses were performed using Microsoft Excel and SAS v 9.4 (SAS Institute, Cary, NC). Each person’s address was matched to a census tract using SAS Proc Geocode. Initially, non-matches were rechecked for common errors and problems, such as street abbreviations. For example, an address with “Ave” instead of “Avenue” led to non-matches. After a series of corrections, those still unmatched to a census tract were looked up via the U.S. Census Bureau’s address search tool, and the census tract manually entered. These census tracts were used to find ecologic estimates of socioeconomic status (% below poverty line, % using the Supplemental Nutrition Assistance Program (SNAP), % unemployed, and average income). These SES indicators were acquired from the American Community Survey (ACS) database for 2006-2010, the most recent SES data stratified by race/ethnicity for each census tract. Our 304 UH cases resided in 202 different census tracts, 76 (37.6%) of which are from the Greater Newark area. For the 4 (1.3%) census tracts from the ACS database without a breakdown by race/ethnicity, as well as race/ethnicity that was other, the overall census tract data were used as the best available estimate.

Cox proportional hazard models were created to evaluate what factors may influence death from cervical cancer. There were a large proportion of stage 1 cases that did not have sub stage information; therefore there were 9 stage groups in the model. Predictors included in the model were stage, living in the Greater Newark area, race group (Hispanic or non-Hispanic), age, and poverty. In the models, all stages were broken down into A and B subclasses. Hispanics and non-Hispanics were also examined in separate models.

Summary of Results:
Since A and B sub stages were not available from the NCDB, 4 stages were used for comparing national statistics from the NCDB to UH. Compared to the NCDB, UH patients from the Greater Newark area were significantly more likely to be diagnosed at a later stage (Jonckheere–Terpstra trend test, p=.0002). Patient five year survival for stages 1, 2, 3 and 4 at UH each are better than national statistics (Figure). For stage 3, the improved survival at UH approaches statistical significance even despite our limited numbers (Log Rank: p=0.107, Wilcoxon: p=0.087).

For intra-UH analyses A and B subtypes were available and used. Survival by stage among the UH patients tended to decline with advancing stage. Those with stage 1 due to insufficient data to sub-stage) did slightly worse than 1B. Stage 2A is often grouped as an “early stage” cancer whereas 2B as an “advanced stage,” but the difference between 2A and 2B did not reach statistical significance.

Survival for those in the Greater Newark area was worse than for those outside of the Greater Newark area for all 8 stages, and better just for stage 3A. The survival difference was statistically significant only for stage 2B (Log rank: p=.005, Wilcoxon: p=.034).
Survival for those in the Greater Newark area compared to those outside of the Greater Newark area was significantly worse among non-Hispanic blacks (Hazard Ratio=2.83, 95% CI [1.23, 6.54]). The increased hazard was similar among non-Hispanic whites (Hazard Ratio=1.97, 95% CI [.468, 8.33]), and among all non-Hispanics combined (blacks, whites, and other), (Hazard Ratio=1.96, 95% CI [1.15, 3.32]). There was no evidence of this geographic disparity, in contrast.

Blacks and whites showed similar trends for survival in Greater Newark. Therefore they were combined together into a single stratum in models incorporating SES. We also examined including “other” race in that stratum. Controlling for stage, no model demonstrated a significant association with survival with respect to any of the four SES variables either among Hispanics or all non-Hispanics combined.

Conclusions:

Patients at UH tend to be diagnosed at later stages compared to NCDB statistics. This might be due to factors such as a lack of early screening options, access to primary care, insurance issues, or cultural issues. Since cases at UH were diagnosed at systematically later stages than nationally, a residual uncontrolled effect likely also remains within each stage stratum. The impact would be to tend to underestimate the UH survival advantage for each stage. In other words, adjusting for stage is likely an incomplete adjustment.

Nevertheless, UH had better survival outcomes for patients at all stages, with stage 3 survival significantly better. Stage 4 survival remains poor for both UH and the rest of the nation. This re-emphasizes the importance of early detection, so that treatment can begin before the most advanced stage has been reached. Our data demonstrate that, despite all of the expected challenges in caring for a poor, urban, and largely minority community, the clinical care at our institution excelled.

We had conducted extensive error checking with the tumor registrars. These reviews ultimately led to all cases initially retrieved as stage 0 cervical cancer as being reclassified into other stages or as non-cervical cancers. We posit that it is likely that some of the 230 stage 0 cases in the NCDB (0.7% of their cases) are erroneous, as the procedures we used exceed the required general error checking procedures conducted by cancer registries. In our experience, data outliers, such as these, are more likely to represent miscoding; therefore we recommend routine targeted checking of all outlier records.

Within the UH population, there were a number of interesting trends in survival. First, those diagnosed with stage 1 that were not categorized into subclasses had higher mortality than stages 1A, 1B, and even 2A. This suggests that those with stage 1 but without substage may have been understaged at diagnosis, consistent with there being insufficient clinical data to fully stage them.

There was no significant difference in survival at UH between those in stage 2A and stage 2B. However, this may in part merely reflect our limited sample size as there were just 14 stage 2A patients and 40 with stage 2B. Interestingly, the only patient with a clear cell carcinoma tumor was stage 2A and died after 32 months; she was born in 1949. This histology is strongly and causally associated with prenatal diethylstilbestrol (DES) exposure, a common exposure until 1971 when DES was banned for use in pregnant women. If this unusual case were excluded, the 5-year survival for stage 2A increased from 72% to 83%, as compared to stage 2B survival of 57%, but the survival difference still did not attain statistical significance (Log rank: p=.347).
Stage 2A and 2B cervical cancer differ in that stage 2A has no parametrial invasion while stage 2B does. It is important to note that cancer treatment tends to greatly differ among early invasive cancers (Stage 1-2A), advanced local disease (2B-4A), and distant disease (4B). For this reason, we would urge the NCDB to revise its procedures to include dissemination of substage data, at least for stage 2 cervical cancers. No other stage is so distinctively different between the A and B substages.

All patients had equivalent access to the same clinicians and treatment facilities. Nevertheless, our survival by race and ethnicity mirrors the national trend of worse survival for non-Hispanic blacks compared to non-Hispanic whites.

Our analysis suggests that where one lives can be strongly associated with mortality from cervical cancer. Living in Greater Newark, which were those living closest to our hospital, was associated with higher overall mortality. This phenomenon was significant for both non-Hispanic whites and non-Hispanic blacks, with a nearly two and three-fold greater risk, respectively, for those living in Greater Newark compared to those residing outside of Greater Newark. The lack of a similar finding among Hispanics may reflect high ethnic heterogeneity among Hispanics.

Our analysis of SES markers did not explain this phenomenon; surprisingly, socioeconomic status was not associated with cervical cancer survival. This may represent a limitation of our methodology, since we could only use ecological estimates for SES. However, SES data from individuals is susceptible to substantial reporting biases. Census tracts are periodically redrawn, and can change from year to year. We standardized the data by using only one time frame. An advantage of our approach is that our census tract and SES information were comparable across the large time span of our study. A disadvantage is that the neighborhoods can change over time.

The reasons are not known for our observation of a geographic survival disparity. Given our dramatic finding, we posit that the inner city residents of Greater Newark may benefit from enhanced social services to complement the medical therapies UH offers. A prospective research study that examines compliance with therapy, delays in therapy, loss to follow-up, and such may be of value. Our data suggest that increased resources may be needed to ameliorate problems that are especially common within Greater Newark. For example, more patient navigators as well as more support for purchasing medications and travel facilitation may help.

The American Joint Committee on Cancer (AJCC) revised their staging guidelines twice during our study period. The 5th edition was used from 2000-2002, 6th edition from 2003-2009, and 7th edition from 2010 onwards; the impact, if any, of potential stage reclassifications in our study would require further examination. However, the AJCC noted that reclassification might neither be possible nor feasible. The NCDB stage information used for our stage comparison analyses was from 2003-2006, which represented the latest available data including 5-year survival, and used only a single edition (6th) of the AJCC staging manual.

The registry rarely had data classifying the cause of death, so we could only perform all-cause mortality. It would have been of interest to analyze cancer-related mortality. However, we doubt that violence or other non-health factors per se would account for our mortality finding associated with Greater Newark.
Figure. Comparison of UH to NCDB for five year survival at each stage

Acknowledgements:
I would like to thank Ms. Luz Ortiz-Romero and Ms. Judy George-Palmer, CTR from the UH Tumor Registry for their assistance in checking data. I would also like to thank those from the research group I worked with for their assistance during this project, and especially Arjun Gupta and Dr. Daniel Rosenblum.
References:

6. Personal communication, Katherine Mallin, Ph.D. to Arjun Gupta, July 9, 2014.
Insulin and IGF receptors in Glioblastoma

Steven Levison, PhD, (Department of Neurology and Neurosciences)

Introduction:
Glioblastoma multiforme (GBM) is one of the most aggressive tumors in the CNS and it accounts for 78% of all intracranial tumors in adults. Presumably, the recurrence of GBM in any individual patient is because of invasive self-renewing cancer “stem” cells that initiate tumor formation, which are resistant to chemo- or radiotherapy.

It has been demonstrated that the insulin receptor (IR) is abnormally expressed in breast, colon, lung and thyroid malignancies. IR is expressed as IR-A and IR-B splice variants, and their relative level depends on the tissue type as well as the stage of cell development and differentiation. IR-A homodimers specifically bind to insulin and IGF-II. The IGF-1R will bind IGF-I, IGF-II and also insulin.

Objective:
IR-A is prevalent in many types of solid tumors. A recent study in human hepatocellular carcinoma showed that epidermal growth factor receptor (EGFR) signaling increased levels of RNA splicing factors that are responsible for producing IR-A. The goal of this study was to determine if IR-A is overexpressed in a particular subtype of GBM. In particular, we hypothesized that the IR-A isoform will be highly expressed in the classical form of GBM, which is characterized by high levels of EGFR.

Methods:
In vitro methods for IR knockdown—
Neural stem cells (NSCs) were isolated from P4/P5 IR^fl/;^ mice and allowed to grow into neurospheres (NS) for 7-10 days. The NS were dissociated and plated onto 6 wells plates. These cells were infected at 1000 MOI (multiplicity of infection) with adenoviruses containing Cre recombinase and green fluorescent protein (GFP), GFP virus, and no virus. These cells were grown in a biochemically defined medium with 5 μg insulin and 20 ng/ml EGF. Twenty-four hours after infection, the total number of GFP+ cells were counted using an inverted epifluorescence microscope. The total number of GFP+ cells/total number of cells translates to transfection efficiency. These cells were allowed to grow into spheres for 7-10 days and images were obtained on days 1, 4 and 6.

In silico methods to evaluate relative levels of Insulin/IGF and EGF system mRNAs, and transcription factors involved in either self-renewal or inhibiting differentiation in GBMs—
RNAseqV2 data matrix was obtained from the level 3 data of all batches available for public access in the TCGA database. Then for each TCGA barcode, the RSEM normalized genes file was downloaded. All these files were consolidated onto one excel spreadsheet. Using the supplemental information in Bernan et al. 2013, the files from each TCGA barcode were classified to the subtype of GBM that they belonged to such as classical, neural, proneural, and mesenchymal. Gene expression for each subtype was calculated by averaging all the normalized
values in the files that belonged to that particular subtype. Gene expression of the Insulin/IGF
and EGF systems, and of the transcription factors involved in self-renewal and inhibiting
differentiation was compared across the different subtypes of GBM. A table was constructed
using excel. The heat map was made by using conditional formatting in excel, which ranks the
numbers in the table and assigns the colors accordingly.

Summary:
Experiments in the Levison lab had previously demonstrated that the self-renewal of primitive
mouse neural precursors was promoted by the action of the IR and not the IGF-1R or IGF-2R
and that the ratio of IR-A to IGF-1R was much higher in the primitive neural precursor cells as
opposed to more differentiated cells such as oligodendrocytes, astrocytes and neurons. To
establish whether the insulin receptor was necessary for the growth of the primitive neural
precursors into neurospheres, experiments were conducted to determine the consequences of
decreasing the expression of the IR. Single cell suspensions from the subventricular zones
(SVZs) of IR^{fl/fl} mice were propagated as neurospheres for 9 days and then dissociated into
single cells. These cells were then infected with either Ad-GFP, Ad-Cre-GFP or no-virus
(control) at 1000 MOI. Cells were grown under neurosphere-producing conditions. As
hypothesized, knocking down the IR using IR^{fl/fl} neural precursors that were infected with Ad Cre virus decreased neurosphere number and size.

The IR-A: IGF-1R ratio was calculated from RNAseq data for normal human neurospheres and
for human Glioblastoma neurospheres. Data were gathered by Dr. Nikolaos Tapinos of the
Geisinger Clinic, PA. Averaging the RNAseq data from neurospheres generated from 3 different
GBMs (GB2, WCR8, and GB9) and from 2 normal human brains (HNSC and HNSC2), we
observed that the IR-A: IGF-1R ratio was 3.48 higher in GBM neurospheres than in normal
human neurospheres (Table 1). Dr. Tapinos also evaluated these neurosphere samples to detect
the presence of IR-B, but IR-B was virtually absent in these primitive neural cells. Thus, it can
be concluded that the IR-A pathway is one of the main pathways used by these primitive human
neural precursors for growth.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td></td>
<td>Average of GB2, WCR8, and GB9 GBM Neurospheres</td>
<td>Average of HNSC and HNSC2 Neurospheres</td>
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<tr>
<td>INSR/IGF-1R Ratio</td>
<td>0.6128</td>
<td>0.1759</td>
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</table>

Since the experiments thus far showed that IR-A was relatively high in peripheral tumors and in
GBM neurospheres, the TCGA database was queried to determine whether the IR-A and other
related genes were more expressed in one particular subtype of GBM. The subtypes of GBM,
defined by Verhaak et al., 2010 are the classical, proneural, mesenchymal and neural types.
RNAseqV2 data were collected and expression levels of genes in the insulin family and EGF
pathways were evaluated. Numbers obtained from TCGA represent quantified gene expression
from RNA sequencing data that were normalized by total read length and the number of
sequence reads. Next, a heat map was created by ranking the values for genes across the four
subtypes. This analysis revealed that the IR-A splice factors (CUCGBP1, HNRNPH1,
HNRNPA1, HNRNPA2B1, SFRS1) that act downstream of the EGF pathway to generate the IR-A form of the insulin receptor, were not different among the subtypes of GBM. Interestingly, even though EGFR expression levels were high enough to serve as a differential for the classical subtype, this difference in expression was not reflected in the levels of the splice factors for IR-A (Table 2). Similarly, there was no significant difference in the expression of IGF-II, IR-A or IGF-1R among the GBM subtypes. An interesting result emerged for the expression levels of IGF-II and IGF-2R. Compared to the other subtypes, proneural GBMs showed relatively higher levels of IGF-II, and this was not counteracted by an increase in IGF-2R. Expression levels of other transcription factors that either promoted self-renewal or inhibited differentiation including TCF4, ID2 and ID4 were also expressed at similar levels across the different GBM subtypes (Table 3).

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Average of Classical</th>
<th>Average Neural</th>
<th>Average of Proneural</th>
<th>Average of Mesenchymal</th>
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<tr>
<td>IGF1</td>
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<td>IGF1R</td>
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<td>596.74294</td>
<td>953.72653</td>
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<tr>
<td>IGF2R</td>
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<td>1221.06784</td>
<td>1454.21586</td>
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<tr>
<td>INSR</td>
<td>3643</td>
<td>998.07436</td>
<td>815.20911</td>
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<tr>
<td>EGFR</td>
<td>1956</td>
<td>26885.81065</td>
<td>7511.90156</td>
<td>4346.86922</td>
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<tr>
<td>(CUCGBP1) CELF1</td>
<td>10658</td>
<td>1660.82663</td>
<td>1326.94946</td>
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<tr>
<td>HNRNPH1</td>
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<td>5649.12367</td>
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<td>HNRNPA2B1</td>
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<td>16012.25043</td>
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<td>3696.09203</td>
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</table>

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<th>Gene ID</th>
<th>Average of Classical</th>
<th>Average Neural</th>
<th>Average of Proneural</th>
<th>Average of Mesenchymal</th>
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<tr>
<td>ID2</td>
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<td>4303.40200</td>
<td>5383.43466</td>
<td>4190.54391</td>
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<tr>
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<td>TCF4</td>
<td>6925</td>
<td>4433.10894</td>
<td>3548.74777</td>
<td>4744.65740</td>
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**Conclusion:**
From experiments completed in the Levison lab it is evident that IGF-II promotes the growth of primitive neural precursors by stimulating IR-A receptors that are highly expressed. We found that reducing IR decreases growth of primitive neural precursors. In addition, we found that the IR-A: IGF-1R ratio in human GBM primitive neural precursors was increased when compared to normal human primitive neural precursors suggesting that the IR-A pathway is much more significant than the IGF-1R pathway. However, the RNAseqV2 data obtained from TCGA failed
to reveal robust differences in IGF-II, IR and IGF-1R between the different forms of GBMs. Moreover, these data suggest that unlike hepatocellular carcinomas, the IR-A splice factors do not appear to be regulated by EGFR in GBM. Again, the uniquely high IGF-II to IGF-2R ratio remains a point of interest for proneural GBMs, suggesting that the excess IGF-II could be acting through the IR-A pathway to promote the growth of this subtype. As discussed by Verhaak et al (2010) the proneural subtype of GBM is the most deadly type of GBM. Querying the TCGA database affirmed that several transcription factors that inhibit differentiation and promote self-renewal are highly expressed in all subtypes of GBM, but again, no differences were seen between the different forms of GBMs. These data suggest that IR-A promotes the growth of the tumor initiating cells across the various subtypes for GBM.

References:

2) Verhaak et al. Integrated Genomic Analysis Identifies Clinically Relevant Subtypes of Glioblastoma characterized by Abnormalities in PDGFRA, IDH1, EGFR, and NF. Cancer Cell. 2010 Jan 19;17(1):98-110
4) Ziegler et al. IGF-II Promotes Stemness of Neural Restricted Precursors. Stem Cells 2012;30:1265-1276
Objective:

Traumatic brain injury (TBI) occurs when an external force alters normal brain function. A TBI consists of primary and secondary injuries. Primary injury occurs with the initial force. It results in brain movement within the skull and displacement of cells. Secondary injury occurs gradually over hours to months, and involves an array of responses such as inflammation and cell death.

Chronic inflammation is a main component of secondary injury in TBI as well as a major component of gliomas. Neuroinflammation involves the release of many inflammatory mediators including cytokines and activation of microglia and astrocytes. Studies have shown that excessive or chronic neuroinflammatory can be toxic to neuronal cells.

The objective of this study was to reduce inflammation after TBI. We focused on Pannexin 1 which indirectly activates the P2X7 receptor of neural and glial cells—receptors that are activated by ATP. In order to abate this response, we used Brilliant Blue FCF (BBFCF, Figure 1)—blue food dye—to antagonize Pannexin 1. The Calderon lab hypothesized that treatment with BBFCF would reduce inflammation after TBI. If correct, the prediction is that treatment with BBFCF would reduce the level of cytokines in the injured tissue as compared to vehicle-treated animals.

Figure 1. Chemical structure of brilliant blue FCF (BBFCF)
Methods:

Sprague-Dawley rats were anesthetized with isoflurane. The concentration for induction was 4% at a flow of 0.8 L/min. For maintenance, it was 3% at a flow of 0.7 L/min. We used the controlled cortical impact (CCI) model of contusion. First, we performed a craniotomy drilling the skull with a 5 mm trephine maintaining the integrity of the dura. After removing the flap, CCI was performed with a pneumatic controlled piston with a 2.5 mm diameter. The coordinates for the impact were -2.20 Anterior/Posterior, +2.00 Medial/Lateral, -1.00 Dorsal/Ventral.

Twenty minutes after the surgery, we administered the BBFCF 50 mg/kg by intraperitoneal injection. The rat’s feet, tail, and eyes turned a blue hue afterwards—confirming successful administration and distribution through the circulatory system.

Four hours afterwards, the rats were sacrificed by decapitation and brains were dissected to obtain the ipsilateral injured cortex. The samples were later homogenized, the RNA isolated with Trizol and quantified by absorbance. The mRNA was reverse transcribed to cDNA then, the samples were placed in a quantitative thermocycler (Applied Biosystem 7300 Real-Time PCR System) to quantify the cDNA. The data was normalized with beta-actin to compare changes in mRNA expression among different tissue samples.

Summary:

Figure 2. Real-time qPCR quantification. Treatment was done 20 minutes after injury and samples were obtained 4 hours after injury. Each bar represent mean +/- SE.
The levels of expression of cytokine mRNAs were comparable to what has been previously obtained in the lab. This preliminary data indicates that treatment with BBFCF significantly reduced the expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. In addition, NLRP3 and caspase-1, both components of inflammasome signaling were significantly reduced.

**Conclusion:**

Our results support the hypothesis that BBFCF drastically reduces pro-inflammatory responses caused by TBI. Implications are that BBFCF can be an easy and non-toxic addition—as it is approved by the FDA as a food dye—to aid in treatments when excessive or chronic inflammation is problematic. This study on TBI most likely has broader application in reducing inflammation of gliomas or other neurodegenerative diseases—topics for future studies. Other avenues for future research could involve the dosage and administration regime for treatments. Behavioral studies could also examine the physical or cognitive states of animals with and without treatment.

**References:**

Objective:

Monocytes can be recruited to sites of inflammation and at tissue injury. The attractants include chemokines. Once monocytes enter the tissue, they differentiate into macrophages. Tissue factors can polarize the macrophages (M into M1 and M2 types).\(^1\,^2\) Classical activation is a macrophage response to pro-inflammatory Th1-type cytokines (interferon gamma and lipopolysaccharide) while alternative activation is a macrophage response to anti-inflammatory TH2-type cytokines (IL-4,IL-10 or IL-13).\(^3\)

M2 macrophages are part of the bone marrow microenvironment where dormant breast cancer cells survive, suggesting a role in breast cancer dormancy. Mesenchymal stem cells (MSCs) have been shown to influence macrophage polarization, indicating a role in the macrophage-breast cancer axis. The overarching objective is to study how MSCs interact with macrophage to cause polarization. The lab has shown MSCs induce the differentiation of monocytes to M2 macrophage whereas activated MSCs differentiate monocytes to M1 type.

We and others have reported on dormancy by cancer stem cells (CSCs) in the bone marrow. Dormancy is defined as the stage when the cancer cells are in cycling quiescence. It is during this phase, that the cancer cells become resistant to chemotherapies. Overtime, the cells can resurge and metastasize to other organs.\(^4\,^5\)

While the CSCs can form gap junctional intercellular communication with bone marrow stroma, it’s unclear how the other cancer cell subset can adapt quiescence. To test this, this study test the **hypothesis** that the M2 macrophage are responsible for sustained dormancy of breast cancer whereas M1 macrophage facilitates the exit of dormant breast cancer cells into metastatic cells.

Materials and Methods:

**Macrophages:**

Peripheral blood monocytes were differentiated into M1 macrophages with 10 nM of IFN\(_\gamma\) and LPS. M2 macrophage was prepared with 20 nM IL4. Both types of macrophages were maintained using RPMI-1640.

**Cell lines:**

Two breast cancer cell lines from ATCC: MDA-MB-231 (triple negative and highly metastatic) and T47D (triple positive; low metastatic).
Co-culture:

Breast cancer cells were co-cultured with M1 or M2 macrophages at a 1:1 ratio for 48 and 72 hours.

Cell Proliferation Assay:

Two methods were used to examine proliferation of BCC, CyQuant direct proliferation assay (Invitrogen) and propidium iodine staining, which enables the analyses of cell cycle phase. The CyQuant assay was analyzed on a fluorescence micro-plate reader at 480 nm excitation/520 nm emission.

Cell cycle analyses:

Propidium iodine (PI) staining determined DNA content in our proliferating cell population. The BCCs were gated by labeling with a pan anti-cytokeratin-FITC. Data was collected using the FACS caliber.

Summary:

Increased proliferation was observed when the breast cancer cells were co-cultured with M1 macrophage. Time course studies (not shown) indicated optimal proliferation at 72 hours. In contrast, M2 macrophage did not show a significant increase in the proliferation of breast cancer cells.

In general, reversal of dormancy transitions the breast cancer cells out of G0/G1 phase. Indeed, our findings showed M1 transitioning the cancer cells into S and G2 phases of the cell cycle whereas M2 retained cycling quiescence.

The cell cycling studies correlated with the studies using the CyQuant proliferation assay. M1 macrophages induced the proliferation of breast cancer cells.

Conclusion:

The results based on the CyQuant proliferation assay and propidium iodine staining, M1 macrophage seems to have the ability to reverse dormancy of breast cancer cells while M2 macrophage might sustain dormancy. These findings form the impetus for future studies on the role of macrophagewill aim to confirm this finding.

Future Research: Studies are needed to study different subsets of breast cancer cells and to determine the mechanisms by which both M1 and M2 macrophage affect the behavior of breast cancer cells. Also, the link to MSCs (as per the hypothesis diagram) needs to be investigated.
References:

AYA ELSEKHELY (NJIT 2015)

THE EFFECTS OF EXERCISE ON CANCER PATIENTS: A META-ANALYSIS AND SYSTEMATIC REVIEW

Syed S. Haque, PhD, Shankar Srinivasan, PhD (Department of Health Informatics – School of Health Related Professions)

Objective:
Physical exercise has been identified as a potential intervention to improve Quality of Life (QOL) in women with breast cancer. Cancer patients frequently suffer from fatigue and loss of physical performance; this is due to the cancer itself and the treatment. Exercise is not only targeted to better the QOL in women with breast cancer but the QOL in cancer patients and survivors as a whole. In general, we know that exercise is helpful in many ways to achieve a better QOL. Not only does exercise better the health of individuals, but also better the mood and energy of the individuals. Published data has shown that physical activity has a positive role on the primary prevention of specifically, breast cancer risk, however the role of physical activity on breast cancer outcome has had inconsistent data. It has been studied that lifestyle changes such as exercise may decrease physical and psychological issues associated with cancer, thus improving QOL. Many cancer patients/survivors may find it difficult to adapt to an exercise lifestyle due to symptoms and treatments that can cause exercise to become more difficult. However, with the appropriate help from healthcare professionals, a modified lifestyle, the implantation of exercise can benefit the patient thus making it successful. After a wide-ranging literature review, this study compares two studies that have been done showing the effects of exercise and physical activity on cancer patients with randomized controlled trials. Extensive research was done to compare two different studies. Effects of exercise on breast cancer patients and survivors: a systematic review and meta-analysis by Margaret L. McNeely and Randomized Controlled Trial of Exercise Training in Postmenopausal Breast Cancer Survivors: Cardiopulmonary and Quality of Life Outcomes by Kerry S. Courneya. These two studies were chosen due to the similarities in surveys that were presented, both studies were randomized controlled trials (RCTs), and both patient populations were the same.

Methods:
In McNeely’s study on the Effects of exercise on breast cancer patients and survivors: a systematic review and meta-analysis, 14 studies were considered eligible for inclusion because they were RCTs comparing exercise with a placebo, controlled comparison or standard care. Participants in the study were included if they involved women with early to later stage (Stage O-III) breast cancer or who had undergone breast cancer surgery with or without adjuvant cancer therapy; the studies were also required to have cardiorespiratory fitness or physical functioning as a primary quality of life. In order to determine the quality of each RCT, an assessment was followed using the following questions: Was there adequate concealment of allocation? Was the method of randomization well described and appropriate? Was the outcome assessment described as blinded? Was the method of blinding of the assessment of outcomes well described and appropriate? Was there a description of withdrawals and drop-outs? Was the analysis intention-to-treat? Were withdrawals and drop-outs less than 10%? These questions were then answered with either +/-? indicating positive (+), negative (-) or unclear (?). Studies were then defined as high quality if they had met 4 or more the quality criteria.

In Velthuis’s study on The Effect of Physical Exercise on Cancer-related Fatigue during Cancer Treatment: a Meta-analysis of Randomised Controlled Trials 18 studies were met the inclusion
criteria and were analyzed; of the 18 studies, 12 of which were breast cancer patients four in prostate and two in other cancer patients. Like McNeely’s study, the inclusion criteria included randomized controlled trials only and participants that evaluated the effects of exercise with cancer related fatigues (CRF) in adults of any age regardless of their type of cancer. The studies compared exercise with no exercise, a usual care group without emphasis on physical exercise or a different non-exercise intervention. Velthuis measured quality assessment using the PEDro scale. This scale was chosen as it is valid for assessing the quality of randomized controlled trial and has been widely used in other rehabilitation and physical therapy reviews. The PEDro scale consists of 11 items and one point was awarded when an item was present which resulted in a range of scores between 0-8. If a study fell below 4, it was considered to be of low methodological quality.

In Courneya’s study on *Randomized Controlled Trial of Exercise Training in Postmenopausal Breast Cancer Survivors: Cardiopulmonary and Quality of Life Outcomes* 53 postmenopausal breast cancer survivors were randomly assigned to an exercise (n=25) or control group (n=28). The exercise group trained on cycle ergometers three times per week for 15 minutes and the control group did not train. The measureable difference between each group was the changes in peak oxygen consumption and the overall QOL from baseline to postintervention. The overall QOL was measured using the Functional Assessment of Cancer Therapy – Breast (FACT-B) scale. A random sample of female breast cancer survivors was obtained from the Alberta Cancer Registry and the referring physician was contacted for approval. The patient was then sent a recruitment letter and contacted the project director if interested. The participants were randomly assigned to one of the two groups using a random-numbers table. Exercise training was supervised and if the participant was unable to make the exercise session, it was rescheduled to when they were most available. The control group did not train, however, the control group participants were offered the intervention after the trial.

Summary:
In McNeely’s study, within the 14 studies that were used in the meta-analysis, 717 participants were involved. From the 14 studies, 4 of them were considered of high quality. There were shortcomings that were examined throughout the studies and the most common shortcoming was the failure to blind the outcome assessment. Three studies involved 194 patients compared exercise with usual care. Exercise was superior to usual care for both the Functional Assessment of Cancer Therapy – General (FACT-G) and the FACT-B quality of life scales. The data from these three studies demonstrated that exercise led to significant improvements in quality of life using both the FACT-G (WMD 4.58%, 95% CI 0.35 to 8.8) and FACT-B (WMD 6.62%, 95% CI 1.21 to 12.03). Three other studies measured the cardiorespiratory fitness that reported peak oxygen consumption; the pooled results from the three studies demonstrated a significant improvement in peak oxygen consumption with exercise (WMD 3.39%, CI 1.67 to 5.10). Only three studies provided adequate data to assess quality of life. The pooled estimate showed that a statistically significant increase of greater than 4.0 points on the FACT scale represents a clinically meaningful improvement in quality of life from exercise. Because of the 14 studies that were used in this meta-analysis and of which only 4 studies were considered high quality, conclusions are then tempered by this fact.

Velthuis measured the study using a PEDro scale, which is commonly used for assessing the quality of randomized controlled variable. When the study results were pooled, statistical heterogeneity among the studies was assessed using a chi-squared test. A P-value <0.10 indicated significant heterogeneity and if this occurred, a post-hoc sensitivity analysis was carried out. The study was summarized using the standardized mean difference (SMD). Out of the 18 articles that were used in Velthuis’s meta-analysis, 12 of the articles pertained to breast cancer patients. These 12 studies investigated the effect of exercise during adjuvant treatment of breast cancer. The supervised exercise group met two or three times a week and aerobic...
exercises were conducted for 10-30 minutes; the control group was given advice. The completion rate was reported in all but one of the studies with 100% completion.\textsuperscript{4} The pooled results of three high quality studies (340 patients) showed a medium sized and significant reduction in CRF in favor of the exercise groups (SMD 0.30, 95% confidence interval 0.09 to 0.51).\textsuperscript{4}

The study done by Courneya measured the changes that occurred in the QOL of the patients. The baseline value for overall QOL did not differ between the exercise and control groups (P=.286).\textsuperscript{3} Overall QOL increased by 9.1 points in the exercise group compared with 0.3 points in the control group (P=0.001). Differences between groups in changes from baseline to post intervention were also observed for happiness (P=.019), self-esteem (P=.010), fatigue (P=.006), FACT-B components (P=.016) and the breast cancer subscale (P=.001).\textsuperscript{3}

\textbf{Conclusion:}
As of now, these findings suggest that exercise may have a small beneficial effect on cancer patients. The evidence of the studies suggests that exercise is an effective intervention to improve quality of life, cardiorespiratory fitness, physical functioning and symptoms of fatigue in breast cancer patients and survivors.\textsuperscript{5} These findings also suggest that exercise should become part of the usual care of breast cancer patients during their adjuvant treatment.\textsuperscript{4} Central funding should be made available to train more exercise professionals specifically in cancer rehabilitation. Also, because many different exercise regimes were prescribed\textsuperscript{5}, future research should explore the effects of a standard exercise for all cancer patients or survivors to follow.

![Figure 1: Pooled effects of exercise on quality of life from clinical trials involving breast cancer patients. FACT-G = Functional Assessment of Cancer Therapy – General, FACT-B = Functional Assessment of Cancer Therapy - Breast.](image-url)
Table 3. Effect of Exercise Training on Quality of Life Outcomes

<table>
<thead>
<tr>
<th>Variable (scale range)</th>
<th>Baseline Mean ± SD</th>
<th>Post-intervention Mean ± SD</th>
<th>Change Mean ± SD</th>
<th>Difference between Groups Mean Change ± SD</th>
<th>V6% CI</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Primary outcome</td>
<td></td>
<td></td>
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<tr>
<td>FACT-B (10-40)</td>
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<td></td>
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<tr>
<td>Exercise group</td>
<td>110.5 ± 19.0</td>
<td>119.6 ± 16.9</td>
<td>+9.1 ± 16.1</td>
<td>+8.8 ± 16.1</td>
<td>3.6 to 14.0</td>
<td>&lt; .001</td>
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<tr>
<td>Control group</td>
<td>115.5 ± 12.4</td>
<td>115.8 ± 14.9</td>
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<tr>
<td>Secondary outcomes</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Happiness, % time happy</td>
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<td></td>
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<tr>
<td>Exercise group</td>
<td>48.1 ± 27.1</td>
<td>65.4 ± 24.6</td>
<td>+17.3 ± 26.1</td>
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<td>Control group</td>
<td>57.5 ± 21.3</td>
<td>58.3 ± 22.4</td>
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<tr>
<td>Self-esteem (10-50)</td>
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<td></td>
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<tr>
<td>Exercise group</td>
<td>32.2 ± 5.6</td>
<td>34.8 ± 4.7</td>
<td>+2.6 ± 3.7</td>
<td>+2.7 ± 3.7</td>
<td>0.7 to 4.7</td>
<td>.019</td>
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<tr>
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<td>36.7 ± 4.5</td>
<td>36.6 ± 4.4</td>
<td>+0.1 ± 3.5</td>
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<tr>
<td>Fatigue, 0-32</td>
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<tr>
<td>Exercise group</td>
<td>17.6 ± 11.5</td>
<td>8.3 ± 7.9</td>
<td>+9.3 ± 10.2</td>
<td>+7.3 ± 10.2</td>
<td>12.2 to 7.3</td>
<td>.005</td>
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<tr>
<td>Control group</td>
<td>10.8 ± 8.8</td>
<td>8.8 ± 8.1</td>
<td>+2.0 ± 7.5</td>
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<tr>
<td>Composite of FACT-B</td>
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<tr>
<td>Exercise group</td>
<td>85.5 ± 12.4</td>
<td>91.3 ± 11.0</td>
<td>+5.7 ± 7.4</td>
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<tr>
<td>Control group</td>
<td>88.8 ± 9.0</td>
<td>89.3 ± 10.9</td>
<td>+0.6 ± 7.4</td>
<td>+5.2 ± 7.4</td>
<td>1.0 to 9.3</td>
<td>.016</td>
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<td>TOL, 0-100</td>
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<tr>
<td>Exercise group</td>
<td>70.8 ± 13.7</td>
<td>77.0 ± 12.0</td>
<td>+6.2 ± 7.2</td>
<td></td>
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<tr>
<td>Control group</td>
<td>76.9 ± 9.4</td>
<td>74.8 ± 10.1</td>
<td>+0.1 ± 5.1</td>
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<tr>
<td>Physical wellbeing (0-28)</td>
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<td></td>
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<td></td>
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<tr>
<td>Exercise group</td>
<td>23.3 ± 3.6</td>
<td>25.3 ± 2.5</td>
<td>+2.0 ± 2.0</td>
<td>+2.1 ± 2.0</td>
<td>0.9 to 3.3</td>
<td>.001</td>
</tr>
<tr>
<td>Control group</td>
<td>25.4 ± 2.6</td>
<td>25.3 ± 2.8</td>
<td>+0.0 ± 2.2</td>
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<tr>
<td>Functional wellbeing (0-28)</td>
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<td></td>
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<tr>
<td>Exercise group</td>
<td>22.3 ± 4.3</td>
<td>23.4 ± 4.0</td>
<td>+0.9 ± 2.8</td>
<td>+0.9 ± 2.8</td>
<td>1.0 to 2.1</td>
<td>.468</td>
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<tr>
<td>Control group</td>
<td>22.8 ± 3.8</td>
<td>23.1 ± 4.0</td>
<td>+0.6 ± 2.7</td>
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<td>Emotional wellbeing (0-24)</td>
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<td></td>
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<td>Exercise group</td>
<td>19.2 ± 3.9</td>
<td>21.5 ± 3.4</td>
<td>+2.3 ± 3.1</td>
<td>+2.4 ± 3.1</td>
<td>0.9 to 3.1</td>
<td>.090</td>
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<tr>
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<td>19.4 ± 2.8</td>
<td>20.3 ± 3.0</td>
<td>+0.8 ± 2.9</td>
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<tr>
<td>Social/family wellbeing (0-24)</td>
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<tr>
<td>Exercise group</td>
<td>20.5 ± 3.7</td>
<td>21.1 ± 3.5</td>
<td>+0.6 ± 2.4</td>
<td>+0.5 ± 2.8</td>
<td>0.5 to 2.8</td>
<td>.180</td>
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<tr>
<td>Control group</td>
<td>21.1 ± 3.3</td>
<td>20.7 ± 3.6</td>
<td>+0.4 ± 3.3</td>
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<tr>
<td>Breast cancer subscale (0-36)</td>
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<tr>
<td>Exercise group</td>
<td>25.0 ± 7.6</td>
<td>28.4 ± 6.7</td>
<td>+3.3 ± 6.0</td>
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<tr>
<td>Control group</td>
<td>26.6 ± 4.6</td>
<td>26.8 ± 5.1</td>
<td>+0.2 ± 3.6</td>
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</tr>
</tbody>
</table>

Figure 2: Effects of Exercise Training on Quality of Life Outcomes

References:

JORDANA A GOLDMAN (NJMS 2017)

REPRESSING THE PRO-ONCOGENIC BONE MORPHOGENETIC PROTEIN (BMP) 2 GROWTH FACTOR

Melissa B Rogers, PhD, (Department of Biochemistry and Molecular Biology)

Objectives:

Lung cancer is the leading cause of deaths due to cancer in the United States, with an astonishingly low 5-year survival rate of 15% (1). BMP2 is a powerful growth factor, involved in differentiation, modeling, and regeneration, that is often elevated in non-small cell lung cancer (NSCLC). The 3’untranslated region (UTR) of BMP2 messenger RNA (mRNA) post-transcriptionally regulates expression of BMP2. An ultra-conserved sequence (UCS) in the first half of the 3’UTR greatly represses expression of BMP2 in non-transformed lung cells. MicroRNAs (miRs) that bind to the UCS further reduce BMP2 expression (2). Therefore, we hypothesized that mechanisms preventing synthesis of BMP2 synthesis are impaired in lung cancer, and methods used to repress BMP2, such as miR replacement therapy, may effectively treat lung cancer patients.

In this project, we intended to develop a model for repressing BMP2. Our first aim was to transfect mutated plasmids bearing the BMP2 3’UTR into BEAS-2B cells and measure the effects of mutating predicted miR binding sites on the activity of reporter gene luciferase. BEAS-2B cells were used because they are immortalized bronchial epithelial cells which do not constitutively express BMP2. This phenomenon is likely due to active repression of BMP2 by the UCS. MiRs 374a, 1200, 486, and 34c-3p are predicted to bind sites in the 3’UTR. We have previously shown that the abundance of miRs 486 and 34c-3p is decreased in transformed lung cells, and can repress reporter genes bearing the BMP2 3’UTR (Fotinos et al., 2014). Consequently, we hypothesized that binding of miRs 374a, 1200, 486, and 34c-3p represses expression of BMP2 in BEAS-2B cells. If miR binding to these sites represses BMP2 expression in BEAS-2B cells, then mutation of these sites should increase the luciferase activity of the BMP2 reporter gene.

Our next aim was to compare the heart valve and lung morphology of day 13.5 embryos bearing the UCS to those lacking the UCS. The UCS is conserved across distantly related species, including mammals and fishes, suggesting that it is crucial for survival. Previous results indicated that the UCS represses a BMP2 reporter gene in the heart valves, vasculature, and lungs. Cre-recombinase/loxP deletion was used to delete the UCS, resulting in a short (sh) allele. The breeding scheme was predicted to generate female pups with two genotypes: sh/sh and sh/+ at a 1:1 ratio. 25 sh/+ and 4 sh/sh were observed at birth ($\chi^2 (1) = 15.207$, $p < 0.0001$), suggesting that the sh/sh pups die in utero. We hypothesized that UCS deletion causes a lethal morphological anomaly during gestation. If so, morphological abnormalities should be observed in the heart valves and lungs of the sh/sh, but not the sh/+, embryos.

Next, we aimed to measure levels of BMP2, its downstream signal phosphoSMAD (pSMAD), and its antagonist, Noggin, in klotho mice, by Western blots. Absence of the klotho gene results in kidney disease and calcification of the heart valves, aorta, and kidneys in both humans and mice. BMP2 leads to this calcification, making klotho mice a potential BMP2 inducible model. As a result, we hypothesized that BMP2, BMP2 signaling via pSMADs 1, 5, and 8, and Noggin (a BMP induced antagonist of BMPs) are induced in the heart valves, aorta, and kidneys of sick, klotho null homozygous (kl/kl) mice, relative to healthy, heterozygous (kl/+ ) control mice. If so,
greater BMP2, pSMAD, and Noggin levels should be observed in tissue from kl/kl mice than in kl/+ mice.

Lastly, we aimed to use Alizarin Red and β-Gal staining to compare the calcium levels and number of blue nuclei, respectively, in kl/kl mice and kl/+ mice bearing a BMP2-driven lacZ transgene. We hypothesized that the kl/kl physiology induces BMP2 transgene expression. If this is true, more calcification and blue nuclei from β-Gal staining should be observed in tissue from kl/kl mice relative to kl/+ mice.

**Methods:**

We transfected mutated SGG-NE-3'UTR-BMP2 Genscript plasmids into BEAS-2B bronchial epithelial cells using FuGene, a lipid-based transfection reagent. Upon incubation for 24 hours, the cells were lysed. Then, using the Promega Luciferase Reporter Assay System, the activity levels of BMP2 reporter gene luciferase were measured to determine the effects of the mutations on BMP2 expression. A Nanodrop spectrophotometer was used to measure protein levels which served as a control.

Next, we took day 13.5 embryos that were extracted from a litter mated to strains expressing the Cre-recombinase gene. In this breeding scheme, a male with the X-linked Cre-recombinase gene, having the sh/+ genotype, was mated with a female having the neo/neo genotype. The embryos were genotyped as sh/sh using PCR analysis. They were fixed in 3.7% formaldehyde, dehydrated in 25%, 50%, 75%, 95% methanol, and stored in 100% methanol. Then, the embryos were washed with xylenes, embedded in paraffin, sectioned, stained with Hematoxylin and Eosin, and mounted onto slides. The slides were visualized with a Nikon microscope and images were captured at 40x magnification. Embryo morphology of the heart valves and lungs of the sh/sh pups was compared to that of healthy controls.

We then extracted the heart, aorta, and kidneys from klotho mice. To measure BMP2, pSMAD, and Noggin levels by Western blot analysis, the tissue was lysed in Laemmli SDS buffer, run on SDS PAGE, and probed with antiBMP2, antiSMAD, and antiNoggin antibodies. Images of the gels were captured using a GelDoc apparatus, and the relative protein levels were determined by measuring relative band intensity with AlphaView software. Relative band intensity was normalized to the lowest value in each dataset for BMP2, pSMAD, and Noggin.
Lastly, we extracted additional hearts, aortas, and kidneys from klotho mice bearing the BMP2-driven lacZ transgene. As a positive control, a Cayman Chemical Calcium Assay of the tissue was performed. Protein levels were measured with a Nanodrop spectrophotometer, and calcium per mg protein was determined (± SEM. Aorta & valves: kl/kl n=1, kl/+ n=2; kidneys kl/kl n=2, kl/+ n=3). Tissue from littermates was stained for beta-gal activity, paraffin sectioned, and stained with Eosin counterstain or Alizarin Red for calcium. The slides were visualized with a Nikon microscope and images were captured at 200x magnification. Both calcification and number of blue nuclei were compared between klotho null homozygous mice and heterozygous positive control mice.

Summary:

When testing miRNA binding effects, luciferase activity of mutated plasmids was compared to that of the wild-type parent plasmid. Data was normalized to the parent plasmid ± SEM (n = 12). As evidenced below, all mutations repressed expression of the luciferase reporter gene (Fig. 1). Regarding the effect of deleting the UCS on embryo morphology, thus far, no obvious congenital anomalies were observed in the sh/sh embryos, but the analysis is in progress. In the Klotho model of BMP2 induction, BMP2 and pSMAD levels were not significantly different between kl/kl and kl/+ mice. However, Noggin was induced in the aorta and heart valves of the kl/kl mice. Relative signaling of BMP2, pSMAD, and Noggin is shown (Fig. 2). In the study of BMP2 reporter gene activity, more calcification and blue nuclei were observed in tissue from kl/kl mice relative to kl/+ mice. Calcium levels were measured with the Cayman Chemical Calcium Assay. Average mg calcium per mg protein is shown ± SEM (Fig. 3).

Fig. 1. Repression of luciferase activity in mutated plasmids.
Fig. 2. Relative levels of BMP2, pSMAD, and Noggin in kl/kl and kl/+ mice.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Genotype</th>
<th>BMP2</th>
<th>pSMAD</th>
<th>Noggin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>kl/kl</td>
<td>1</td>
<td>2</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>kl/+</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>kl/kl</td>
<td>3</td>
<td>2</td>
<td>24</td>
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<tr>
<td></td>
<td>kl/+</td>
<td>3</td>
<td>2</td>
<td>11</td>
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<td>Heart Valves (base)</td>
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<td>54</td>
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<td>kl/+</td>
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<td>kl/+</td>
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<td>1</td>
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<tr>
<td></td>
<td>kl/+</td>
<td>1</td>
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Conclusion:

Our initial hypothesis for the transfection failed. One possible explanation is that only one plasmid preparation was used. If the concentration of the control to which other plasmids were normalized was too high, the results could have been skewed. However, direct examination of the plasmid set by gel electrophoresis ruled this out (A Fotinos, unpublished results). Another possible explanation is that the miRs tested might bind additional sites that mediate the observed repression. For example, some miRs, such as miR374a, have multiple sites, and mutation of all sites may be required to induce BMP2 expression. Alternatively, the structure of the 3'UTR may have changed, preventing the binding of an activator or facilitating the binding of a repressor. As a future direction, a Luciferase assay may be performed upon plasmids mutated at alternative sites.

The results of our analysis of embryo morphology are inconclusive at this point, as the work is still in progress. On the other hand, our hypothesis concerning the klotho model failed, perhaps due to activation of the autoregulatory loop in which Noggin suppresses BMP2 signaling. This explanation seems plausible based on the observed elevated Noggin levels in kl/kl mice. We also showed that regulatory elements that respond to klotho are present in the transgene, leading to more calcification and blue nuclei in kl/kl mice relative to kl/+ mice. This increase in indirect measures of BMP2 levels suggests that the klotho model is effective in inducing BMP2 expression. In the future, immunohistochemistry can be used to see if nuclear SMAD phosphorylation is increased in kl/kl mice relative to kl/+ mice. Additionally, RNA, Western blot, and histological analyses of BMP2 levels in lung cancer BMP2 inducible mouse models may be performed to quantify their effectiveness. Lastly, miRs that reduce BMP2 expression can be identified and their efficacy as therapeutics for lung cancer and vascular pathology can be tested on in vivo models.
References:


LIPID COCHLEATES ARE A POTENTIAL INTRACELLULAR DRUG DELIVERY SYSTEM IN PLASMACYTOID DENDRITIC CELLS

Patricia Fitzgerald-Bocarsly, PhD (Department of Pathology & Laboratory Medicine)

Background:
Lipid cochleates, first described in 1975\(^1\), are a potential novel drug delivery nanotechnology. Cochleates are phosphatidylserine based lipid bi-layers which form a spiral hydrophobic structure in the presence of high concentrations of ionic calcium\(^{1,2}\). Additionally, hydrophobic chemicals can be integrated into cochleates\(^{2-5}\) and cochleate uptake by macrophages is seen\(^2\). In the presence of low calcium, the cochleate spiral structure unwinds, exposing the integrated molecule\(^3\). Advantage can be taken of the vast difference in plasma vs. intracellular calcium concentration whereby internalized cochleates open and release their cargo intracellularly.

Plasmacytoid dendritic cells (pDC) are the primary type I interferon alpha (IFN\(\alpha\)) producing cell\(^6\). IFN\(\alpha\) is an immune system mediator that serves many roles in both the innate and adaptive immune response. It interferes with cellular machinery takeover in viral infection and modulates antigen processing and presenting cells\(^7\). Interferons in general have also been shown to play a major role in the body’s natural defense of certain types of cancer\(^8\).

Objective: Our objective was to determine if cochleates associate with pDC, by what mechanism, and whether cochleate integrated molecules are released intracellularly into pDC.

Methods:

**Cochleates:** Cochleates at a concentration of 165\(\mu\)gsiRNA/mL in 5mM aqueous CaCl\(_2\) were produced by and obtained from Raphael Mannino, Ph.D. and Aquarius Biotechnology. Non-specific small interfering ribonucleic acid (siRNA) bound to Cy3 fluorochrome was embedded in the cochleate membrane.

**Macrophage:** Cell line U937 macrophages (macrophages) were used as a positive control in certain experiments. Cells were cultured in RPMI 1640 (Life Technologies) containing 10% Fetal Calf Serum (FCS), 2mM L-glutamine, 100U/mL penicillin, 100\(\mu\)g/mL streptomycin, and 25mM HEPES (collectively, “RPMI media”), and stored at 37°C in 5% CO\(_2\) environment.

**Flow Cytometry:** Flow cytometry was performed on LSRII (BD Biosciences) flow cytometer. Data analysis was performed on FlowJo software (Tree Star, Inc.).

**Imaging Flow Cytometry:** Imaging flow cytometry was performed on ImageStream and data analysis was performed on IDEAS software (Amnis Corporation).

**Peripheral Blood Mononuclear Cell (PBMC) Isolation:** Peripheral human blood was collected from consenting healthy donors into heparinized collection tubes. Tubes were spun at 1200 RPM for 10 minutes, plasma was discarded, the white cell layer was collected and mixed with Hanks balanced salt solution (HBSS) (Life Technologies), and PBMC were isolated using Ficoll-Hypaque density centrifugation (Lymphoprep; Accurate Chemical and Scientific Co.). The buffy coat was collected, resuspended, and washed twice in HBSS. Cells were then resuspended in RPMI media and counted with Series Z1 Coulter Counter (Coulter Electronics, Inc.).
Surface Staining: Isolated PBMC were washed with phosphate-buffered saline (PBS) (Life Technologies). Five µL 5% heat-inactivated pooled human serum (PHS) was added, followed by fluorochrome conjugated antibody specific for BDCA2 and CD123 pDC cell surface proteins, incubated at 4°C for 20 minutes, washed with PBS, fixed with 300µL 1% paraformaldehyde (PFA), and stored at 4°C overnight.

Intracellular Staining: Cells were first surface stained. After overnight fixation in PFA at 4°C, cells were washed in 2% FCS in PBS (wash buffer), resuspended in 500µL 0.5% saponin solution for permeabilization and incubated at room temperature for 15 minutes. After incubation, cells were spun down in saponin, and 5µL PHS was added followed by fluorochrome conjugated antibody specific for IFNα. Cells were then incubated at room temperature for 30 minutes, washed once more in PBS, and fixed with PFA.

pDC Enrichment: After PBMC isolation, pDC were negatively enriched using the Human Dendritic Cell Enrichment Kit (StemCell Technologies). Cells were washed in cold MACS buffer containing PBS, 0.5% Bovine Serum Albumin (BSA), and 2mM EDTA (collectively, “MACS buffer”), spun and resuspended in MACS buffer, and incubated with biotin antibody cocktail at 4°C for 10 minutes. Cells were washed, spun, and resuspended again with MACS buffer, and incubated with anti-biotin microbeads at 4°C for 15 minutes. Cells were washed, spun, and resuspended in MACS buffer, and run through a MACS LS negative selection column (Miltenyi Biotec) in a magnetic field, with a tube in place to collect the cellular flow-through. Cellular flow through was then counted, washed and resuspended in RPMI media.

Statistics: Statistical analysis was done using either one or two-way analysis of variance with Bonferroni post test on Prism software.

Summary:

Experiment #1, Determining if cochleates associate with pDC: Our main objective was to determine if cochleates associate in any way with pDC. After PBMC isolation, 3M-003 (final concentration=10µM) was added to aliquots of 2 million cells/mL and incubated at 37°C for 2 hours to activate pDC. 10µL cochleates were then added and incubated at 37°C in batches for 1, 3, or 5 hours. Following each time point, samples were washed twice in 10mM EDTA in PBS at pH 7.4 and once in PBS alone. Cells were then surface stained with either FITC, APC, PECy5, or PercpCy5.5 fluorochrome conjugated antibody specific for BDCA2 and CD123. Other samples were subjected to the same conditions without 3M-003 to control for pDC activation. Samples +/-3M-003 were incubated at 4°C after addition of cochleates to control for association. Macrophages in 1mL aliquots at 1 million cells/mL were activated with 10µg phorbol 12-myristate 13-acetate (PMA) and incubated overnight at 37°C; 10µL cochleates were added and again incubated at 37°C overnight. The macrophages were used as a positive control. After pDC surface staining, flow cytometry analysis was performed to measure Cy3 fluorescence present in pDC. See results in Figure 1 below.

Figure 1: Cochleate association with pDC increases with time and activation by 3M-003.

[*p=<0.0001; **p=<0.05; ***not significant; MFI=mean florescence intensity]
Starting with a 1 hour incubation period, the percent of pDC expressing Cy3 fluorochrome as well as the fluorescence intensity increased with time for both activated and resting pDC (although more so in activated pDC) as compared with the 4°C negative control.

Experiment #2, ImageStream analysis to elucidate if pDC – cochleate association is extracellular or intracellular: After learning that cochleates do indeed associate with pDC, we sought to determine whether association was simply surface binding or if cochleates were reaching the intracellular space. After PBMC isolation, pDC enrichment was performed. After enrichment, 3M-003 was added (final concentration 10µM) and incubated at 37°C for 2 hours. 10µL cochleates were then added and incubated at either 37°C or 4°C for 5 hours. Samples were then washed, surface stained, and imaging flow cytometry analysis was performed. See results in Figure 2 below.

Figure 2: Cochleates release embedded molecules intracellularly into pDC.

After ImageStream analysis intracellular Cy3 fluorescence intensity was measured with a digitally generated mask placed on the cell membrane to exclude it from fluorescence measurement. ImageStream images of pDC and macrophages depicted intracellular Cy3 indicating intracellular release of siRNA embedded cochleates.

Experiment #3, Evaluation of pDC – cochleate association mechanism: Following our determination that cochleates do reach the intracellular space in pDC, we attempted to elucidate the mechanism by which this occurs by inhibiting actin polymerization and clathrin dependent endocytosis. After PBMC isolation, 3M-003 was added to 1mL aliquots of 2 million cells/mL and incubated per experiment #1. Samples were either treated with cytochalasin D (cytoD) in dimethyl sulfoxide (DMSO), an actin polymerization inhibitor, (final concentration 4.9µM), or monodansylcadaverine (MDcad) in DMSO, a clathrin dependent endocytosis inhibitor (final concentration 150µM). All samples were incubated at 37°C for 30 minutes. 10µL cochleates were added and incubated at 37°C for 5 hours. Samples were washed and surface stained. PBMC with only cytoD, MDcad, or DMSO at the same concentration as experimental samples were used to control for toxicity of those substances. After pDC surface staining, flow cytometry analysis was performed. See results in Figure 3 below.

Figure 3: Cochleate association with pDC is actin and clathrin independent

[***not significant; MFI=mean fluorescence intensity]
Neither a decrease in the percentage of pDC expressing Cy3 fluorochrome, nor a decrease in the fluorescence intensity was observed after use of inhibitors indicating that the endocytic pathway is not required for uptake of cochleates by pDC.

**Experiment #4, Evaluation of pDC function after cochleate exposure:** Finally, we sought to determine if cochleate exposure affected normal pDC function. After PBMC isolation, 10µL cochleates were added to 1mL aliquots of 2 million cells/mL and incubated at 37ºC for 1 hour. Samples were then either treated with 35µL HSV, MOI 1, or 2µL SeV, 16 hemagglutinatingU/mL, and incubated at 37ºC for 6 hours. Two hours prior to incubation completion we added 10µL of 5mg/mL brefeldin A (BFA), a protein transport inhibitor, diluted 1:10 in RPMI media. Samples were then washed, surface stained and stored at 4ºC overnight. Cells were then stained intracellularly for IFNα. Virus treated PBMC samples without cochleates were positive controls for IFNα production, and +cytoD or +MDcad samples were negative controls since virus induced IFNα production requires endocytosis. After intracellular IFNα staining, flow cytometry analysis was performed. See results in Figure 4 below.

**Figure 4:** Cochleates do not affect pDC function

There was no appreciable difference in IFNα production between pDC exposed to HSV or SeV alone and pDC pre-incubated with cochleates before virus exposure, indicating no negative effects of cochleates on pDC function.

**Conclusions:**

Lipid cochleates associate with pDC and molecules embedded into cochleates are released into pDC intracellularly. The process of association and intracellular release is actin and clathrin independent. Additionally, cochleates do not inhibit pDC function. Future studies will look at cochleate delivery mechanisms and if cochleates can deliver drug molecules that induce a physiological response in pDC.

**References:**

MARYAM HAQUE (NEW JERSEY INSTITUTE OF TECHNOLOGY, 2014)

INVESTIGATION OF CLINICALLY DERIVED P210 BCR-ABL RHO-GEF MUTATIONS AND THEIR EFFECTS ON CHRONIC MYELOGENOUS LEUKEMIA DISEASE PROGRESSION

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Objective/Hypothesis:

It has been seen in a murine bone marrow transplantation model, that the activity in the RHOGEF domain in the Break point Cluster Region (BCR) of BCR-Abl is intimately involved in the disease progression of chronic myelogenous leukemia. The experiment featured three different isoforms of the fusion gene BCR-Abl; p190 BCR-ABL, p210 BCR-Abl, and p210 BCR-Abl (S509A). The latter was of the p210 isoform where a mutation was made in the RHOGEF domain to inhibit the activity of the RHOGEF domain. The results were that the p210 BCR-Abl isoform yields CML. The p190, and p210(S509A) BCR-Abl isoforms had the same phenotype of the more aggressive ALL/CML Blast crisis stage. This suggests that inhibiting RHOGEF activity will yield a more aggressive type of leukemia. All of this was shown in a murine bone marrow transplantation model. (1)

Another study used RT-PCR DNA amplification and sequencing on patient specimens from CML blast crisis and ALL patients that showed many mutations and deletions in the Dbl homology in the RHOGEF domain. (2)

The objective of this study is to link the Rho Guanine-nucleotide Exchange Factor (RHOGEF) activity to the disease progression chronic myelogenous leukemia (CML) for human based mutations.

Most patients with CML are treated with a TK inhibitor called Imatinib. Imatinib targets the constitutively active TK activity of Abl by competitively binding to the ATP binding site and inhibiting downstream phosphorylation. There are several other proposed mechanisms of Imatinib resistance (e.g., duplication of Bcr-Abl, drug efflux pumps, and extracellular depletion of Imatinib by P-glycoprotein), yet, none of these have been shown to account for a significant portion of this unexplained resistance. Bcr is necessary for the constitutive activity of the Abl TK domain; it has also been shown that mutations in Abl but outside of the TK domain can cause Imatinib resistance. With these issues in mind, we wondered if mutations in the Rho-GEF domain could also contribute to this resistance. To that end, in the coming months, we will determine if clinically derived mutations in Bcr can result in Imatinib resistance using in vitro growth assays. We will use 3 subtypes of Abl mutations as positive controls. The first is the T315I “gatekeeper” mutation, which we will use because it is the most common clinical mutation within the TK domain and results in strong Imatinib resistance. The next is the E255K mutation, which is located outside of the Abl TK domain in the P-loop; this will be used as a control because it allosterically contributes to a moderate amount of Imatinib resistance. The last control is K51Q mutation, located in the CAP domain of Abl. It was chosen because of its close proximity to Bcr and because it provides a low level of Imatinib resistance. We are currently creating these three mutations in a retroviral vector containing p210 Bcr-Abl by site-directed mutagenesis, and in addition to the F547L and T654K mutants, we will use them to transduce the 32D murine myeloblast cell line. Next, we will screen all of the mutations for TK inhibitor resistance by exposing the cells to different amounts of Imatinib and monitoring their subsequent growth via in vitro growth assays. After this experiment, we hope to be able to
determine if clinically derived Bcr Rho-GEF mutations may confer resistance to TK inhibitor drugs.

**Materials and Methods:**

Molecular constructs and cell culture: Put the f547L,T654K, F547L+T654K, and three Abl mutations into the p210 plasmid using site directed mutagenesis. The MSCV-IRES-gfp retroviral vector has been previously described (Addgene, Cambridge, MA, USA). MSCV-bcr-abl/p190-IRES-gfp, MSCV-bcr-abl/p210-IRES-gfp and MSCV-bcr-abl/p210(f547L)-IRES-gfp contain full length p190 BCR/ABL, p210 BCR/ABL and a p210 BCR/ABL RhoGEF mutant, respectively. Phoenix-Ecotropic cells (ATCC, Manhassas, VA, USA) were maintained in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum (FBS; Gemini, Woodland, CA, USA). Ba/F3 cells were cultured in RPMI supplemented with 10% FBS and interleukin (IL)-3-conditioned WEHI media. High-titer retrovirus was generated using Phoenix-Ecotropic packaging cells as previously described. (1)

Sangre sequencing and restriction endonuclease analysis to confirm the mutation was a success. Used Alkaline lysis maxi prep, and cesium chloride gradient centrifugation.

Cell proliferation and apoptosis assays: Ba/F3 cells were infected by retroviral particles that encode MSCV-bcr-abl/p210-IRES-gfp, MSCV-bcr-abl/p210(f547L)-IRES-gfp, MSCV-bcr-abl/p190-IRES-gfp, MSCV-bcr-abl/p210(T654K)-IRES-gfp, MSCV-bcr-abl/p210(f547L+T654K)-IRES-gfp, MSCV-bcr-abl/p210(T315I)-IRES-gfp, and MSCV-bcr-abl/p210(E255K)-IRES-gfp or cognate vector. At 48 h post-infection cells that express green fluorescent protein (GFP) were sorted by fluorescence activated cell sorting (FACS), seeded in media with and without IL-3, and then grown for 24, 48 and 72 h time points. For proliferation assays, cells were counted using a hemocytometer. For cell cycle analysis, cells were washed with phosphate buffered saline, fixed with ethanol for 20 min, then resuspended in propidium iodide (PI)-RNase solution (50 mg/ml PI¡p100 mg/ml RNase A in phosphate-buffered saline), incubated for another 20 min and analyzed by flow cytometry. Quantification of apoptotic cells was performed using the Annexin V-Biotin Apoptosis Detection Kit according to the manufacturer’s instructions (Calbiochem, La Jolla, CA, USA). All experiments were performed on a minimum of three independent sorts. (1)
Summary/Results:

Used two single nucleotide point mutations which were found in human specimens with CML blast crisis from a prior study and found out where they actually laid in the BCR-Abl sequence (figure 1 and figure 2). We mapped the two mutations to an actual sequence of BCR and compared the two mutations to the known single nucleotide polymorphisms (figure 1). This was done to make sure that the mutations do not represent genetic background since the study is old and it was necessary to verify that these two mutations are novel somatic mutations. In order to determine that the mutations (Figure 2) were in fact missense mutations, the sequence of the mutations, BCR-Abl (F547L) and BCR-Abl (T654K), were compared to a consensus sequence of the DH domains from 21 different RhoGEF models family (adapted from Whitehead IP (1997) BBA, 1332:F1-23)(3). This comparison showed that the mutations were made in a strongly conserved residue which indicated that the function of the RHOGEF domain was affected. Mutation F547L and T654K were categorized as missense mutations that inactivated the activity of the RHOGEF domain.

<table>
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<tr>
<th>Patient [Status]</th>
<th>Nucleotide Position</th>
<th>Nucleotide Change</th>
<th>Mutation Type</th>
<th>Amino Acid Position</th>
<th>Amino Acid Change</th>
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<tbody>
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<td>T➔C</td>
<td>Missense</td>
<td>547</td>
<td>F➔C</td>
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<tr>
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<td>C➔A</td>
<td>Missense</td>
<td>654</td>
<td>T➔K</td>
</tr>
</tbody>
</table>

Figure 1: Indicates the nucleotide position of the p210 BCR-Abl (F547L) and p210 BCR-Abl (T654k) mutations found in the study that sequenced patient specimens.

Figure 2: Alignment of the RhoGEF domains from representative members of the mammalian RhoGEF family (adapted from Whitehead IP (1997) BBA, 1332:F1-23). CR2 and CR3 indicate segments of Conserved Regions 2 and 3 respectively. ★ indicate strictly conserved residues; ◇ indicate very high sequence homology; ▼ indicate high sequence homology. The position of the F547L and T654K substitutions are indicated; the F547L mutation involves a strictly conserved residue in the RhoGEF domain of a blast crisis patient.

The BCR-Abl (F547L) mutant was synthesized into the p210 plasmid through the use of site directed mutagenesis. Then this was put into the MSCV- IRES gfp retroviral vector. Confirmation that the p210 BCR-Abl (F547L) was correctly synthesized was done by using restriction endonuclease analysis and sangre sequencing (Figure 3).
Figure 3: Successful Creation of the F547L Mutation in the Mig p210 Plasmid Construct via Site-Directed Mutagenesis

Then, large quantities were made by using alkaline lysis mini prep and cesium chloride centrifugation.

Figure 4: Mutations K51Q, E255K, and T315I and their proximity to BCR

This figure shows the location of the T315I “gatekeeper mutation,” which will be used as a control because it is the most common clinical mutation in the TK domain and resulting in a strong Imatinib resistance. The next is the E255K mutation located outside of the Abl tyrosine kinase domain in the P-loop; this will be used as a control because it allosterically contributes to a moderate amount of Imatinib resistance. The last control is K51Q mutation, located in the CAP domain of Abl. It was chosen because of its close proximity to Bcr and because of the two other controls provides lowest Imatinib resistance.

We then made virus by Calcium phosphate transfection with Phoenix-Ecotropic packaging cells for all four mutations. The next step is infecting 32D cells invitro cultures with the virus.
References:


Objective:
Telomeres are the protective end regions of linear chromosomes containing G-rich repetitive DNA sequences that shorten with every cell division. Because of the end replication problem, telomeres inevitably shorten causing the effects of aging and eventually, cell death. Therefore, the maintenance of telomere length is crucial in a cell’s survival.

To maintain telomere homeostasis, cells can regulate telomerase, the enzyme responsible for adding TTAGGG sequences to elongate chromosomal ends. It has been proven that telomere length is heterogeneous and telomerase processivity is independent of this length. Cells can also shorten the telomere region by inhibiting telomerase activity. Other proposed mechanisms include the formation of a “D- and T-loop” or the replication fork barrier which both resolve the ends to a shortened telomere sequence.

A third mechanism involves DNA double-strand breaks (DSB) within a telomere sequence. Past studies have shown that DSBs are induced after replication by the presence of telomere sequences. However, the mechanisms of the DSBs remain unknown. The overall objective of this study was to understand the role and involvement of specific genes in regulating this phenomenon. I developed the system to screen such genes and started off with URA3 marker loss in helicase mutant strains.

Methods:
Plasmid Manipulation:
The DNA fragment containing the TG250 repeat sequence was obtained through PCR and integrated into chromosome 7. Both fragments were integrated into the tested strains through transformations with lithium salts. The cells were treated with lithium acetate making the cell wall permeable for the DNA and then PEG to allow the cell to coprecipitate. After a brief heat shock, the cells were washed, left overnight, and plated on corresponding selective medium.

Cell Culture:
Yeast colonies were studied through standard transformation, pre-culture, dilution and spotting techniques. After transformations of TG250, cells were pre-cultured in liquid Synthetic Dextrose media, containing casamino acid which contain all amino acids, with dropouts of amino acids uracil, thymine, and adenine (SDC-UWA) overnight in order to marginalize the number of cells for each yeast strain. 4 µl of cells was then transferred to 2 ml of Yeast Extract Peptone Dextrose (YPD) and grown overnight. 10 µl of dilutions of 10^-3 and 10^-5 for each strain was plated out on YPD plates as a control and on fluoroorotic acid (FOA) media which select for URA3- cells because they convert to the toxic compound 5-fluorouracil.

Southern Blotting without using radioisotope probe:
Previously used membrane was stripped of the probe by incubating in 0.2 M NaOH, 0.1% SDS at 37°C twice for 20 minutes then washing with 5xSSC. The membrane was then hybridized using anti-digoxigenin antibodies suspended in Hybridization buffer of 5% skim milk in 5xSSC and placed in a 60° water bath overnight. The membrane was washed twice in 2xSSC, 0.1% SDS for 5 minutes and twice with 0.5xSSC, 0.1% SDS for 15minutes at 65° in water bath. The membrane was washed with Tween-TBS then incubated with antibodies using 1/5000 vol. of Anti-Dig-POD for 1.5 hours in 37°C. After three 15 minute washes in Tween-TBS, DNA was detected with ½ diluted Super signal with film exposure time of 3 minutes.
**Summary:**

*DSB Induction*

All five helicase mutant strains with TG250 transformations grew on both YPD and FOA plates at varied frequencies (Fig 1). For instance, the colonies with the Elg1 gene mutation showed a significant effect. In addition, the frequency of DSBs was lower for the Srs2 gene and higher for the Chl1 gene.

*Telomere Addition & Southern Blot*

The kanMX probe was successfully hybridized based on the reproduction of data from previously used membrane using Southern blotting and film exposure (Fig 3).

**Figure 1.** Expected result: DSB induced at TG250 sequence leading to addition of telomere repeats. Cells lose URA3 marker. Proliferation on FOA plates indicates selection of URA3- colonies.

**Figure 2.** Comparison of YPD (left) and FOA (right) plates. URA3 + cell served as negative control for FOA plate. Elg1 cells were diluted to $10^{-2}$ and $10^{-4}$. Used mutations of genes Elg1 – subunit of an alternative replication factor C complex involved in telomere homeostasis, Srs2 – DNA helicase and DNA-dependent ATPase, Chl1 – involved in DNA helicase activity with mutants defective in aging, Mph1 – 3’-5’ DNA helicase involved in error-free bypass of DNA lesions, and Pso2 – nuclease required for DNA single- and double-strand break repair.
**Conclusion:**
In conclusion, it was shown that the frequencies of induced DSBs varied for the 5 tested helicase mutant strains. For future studies, the Elg1 mutation seems to be a likely candidate because of its higher rate compared to the other strains. Data remains inconclusive because of the lack of a wild type. In order to further investigate the mechanism of telomere shortening through DSBs, other mutants such as nuclease or polymerase mutants should be tested through the same screening process. Moreover, the addition of telomeres at the correct TG sequence has yet to be confirmed so a combination of western and Southern blot needs to be done using the successfully hybridized kanMX probe. For future direction, this probe would be used on these 5 mutations in order to monitor telomere addition depending on the different frequencies of the mutant strains.
Participation Description:

For my research project, I was responsible for doing the experiments involved in better understanding the mechanism of telomere shortening. I was able to amplify and fuse fragments using the PCR machine, run these fragments through a gel, and purify them for further investigation. Using the strains picked by Dr. Sugimoto, I cultured, transformed, and plated out the colonies and collected data as shown in my results. I was also able to hybridize a probe following a protocol given to me by Dr. Sugimoto and use it to perform a southern blot on a previously used membrane given to me by a fellow lab member. With Dr. Sugimoto’s help I was able to analyze and interpret the data for my project.
Evaluation of the Community Baseline Knowledge on Gynecologic Cancers and the Efficacy of an Education Program for a Lay Audience

Daniel M. Rosenblum, PhD (Department of Preventive Medicine and Community Health), Stanley H. Weiss, MD (Department of Preventive Medicine and Community Health)

Objective:

The five main types of gynecologic cancers are cervical, ovarian, uterine, vaginal and vulvar. Each year, about 80,900 women in the United States are diagnosed with gynecologic cancer and approximately 28,000 women die from them (1). Due to the widespread impact of these cancers, the Centers for Disease Control and Prevention (CDC) implemented in 2007 its Inside Knowledge: Get the Facts About Gynecologic Cancer campaign to help educate women and healthcare providers about the signs, symptoms, risk factors, and prevention strategies related to the five types of gynecologic cancer. The CDC campaign's objectives are to: 1) raise awareness of the five main types of gynecologic cancer; 2) encourage women to pay attention to their bodies and know what is normal for them so they can recognize warning signs and symptoms and seek treatment; and 3) encourage women to see a gynecologic oncologist when appropriate (2). In Fall 2013, the CDC competitively funded six sites to implement educational sessions for laywomen in part to gauge what impact, if any, their education materials have: Dr. Weiss and his team in New Jersey, plus sites in Alaska, Tennessee, Wisconsin, Puerto Rico, and Yap (in the Federated States of Micronesia).

For this project, there were two objectives. The first was to assess the current baseline levels of knowledge regarding the five main types of gynecologic cancers in a New Jersey community. We hypothesized that level of knowledge of both the Inside Knowledge campaign and gynecologic cancers in general are low. The second was to evaluate the CDC’s Inside Knowledge: Get the Facts About Gynecologic Cancer campaign in terms of effectiveness in teaching a lay public audience. Only at our site has data collection and analysis used paired pre- and post-surveys to examine whether there are improvements from the baseline. This can in turn demonstrate whether these materials may be useful public health tools to educate the general public to improve health knowledge and to increase early detection and the use of prevention methods such as the HPV vaccine. Because many of the deaths associated with these cancers are preventable, with increased early detection, screening, and prevention, mortality can be reduced both in New Jersey and nationally.

Methods:

Education Program and Data Collection

We organized an educational symposium in Montclair, NJ and invited members of the general public in order to present the Inside Knowledge materials in a forum to a lay audience. Participants were self-selected. We invited them through various media outlets including newspaper ads, e-mail lists, posted flyers, the e-mail list maintained by the Essex-Passaic Wellness Coalition (which Dr. Weiss directs), various community groups, etc. A free online ticketing service, http://eventbrite.com, tracked RSVPs from guests. Walk-ins were welcome.

Before the session began, a pre-survey, designed by the CDC, was administered. We used this to assess baseline levels of knowledge within the community. Then the educational session was
implemented through a series of lectures by medical experts, including a local gynecologic oncologist and a nurse practitioner with the Division of Gynecologic Oncology at University Hospital. After the lectures, the audience participated in a series of round-table discussions in which they engaged in dynamic dialogue and question sessions with both the experts and gynecologic cancer survivors in small groups of about 10 persons. A post-survey, also designed by the CDC and identical to the pre-survey, was administered after the session concluded. At the end of the session, we gave each participant a “fact sheet” with the correct answers to help them solidify their knowledge.

Addendums to both the pre- and post-survey were designed by us. All forms were approved by the Rutgers Health Sciences Institutional Review Board (IRB) – Newark. The pre- and post-surveys contained factual, opinion, and demographic questions. The addendums mainly contained demographic questions such as gender, county of residence, and key other information. The pre- and the post-surveys, with the addendums attached, were printed on different color paper and distributed at the outset as paired sets, linked with an ID label so that we could analyze paired pre- and post- data while maintaining subjects’ anonymity. Because our data were paired, instead of collected only in aggregate as in the CDC’s own protocol, we can conduct more powerful analyses and track changes based on individual paired samples rather than overall aggregate scores that can mask internally divergent trends.

A total of 68 pre-surveys and 67 post-surveys were collected, leaving us with a sample of 67 linked pairs. The participant who did not hand in her post-survey was excluded from data analysis. All the paired surveys were stratified into three separate subgroups: staff/speakers (n=16), males who were not staff (n=3), and the rest – female audience members (n=48).

Data Analysis
Data entry and initial analyses were done using Epi Info, freely available from the CDC at http://www.cdc.gov/epiinfo/. We designed a scoring system for all of the factual questions on both of the surveys. Each part of each question was assigned a weight based on our assessment of the importance of knowing that fact, with right answers scored positive, wrong answers scored negative, and no answer scored zero. With one exception, getting all parts of a question correct was worth +10, and all parts wrong was worth -10. Potential maximal and minimal overall scores were +104 and -94 (Table 2). Scores were calculated for each individual factual question, as well as total score, separately for both the pre- and post-surveys, and each subject’s score change was calculated for both each individual question and overall. Paired Student’s t-test was used to test the individual question score changes and the total score change for statistical significance. For the two factual questions where potential answers were simply “Yes” or “No”, paired pre- and post- scores were compared using McNemar’s test.

In a separate analysis, the 67 paired samples were also stratified into staff and non-staff, and the latter was further split into cancer survivors and non-survivors (Table 3). We hypothesized that staff and cancer survivors would score higher on the pre-surveys, demonstrating higher baseline knowledge and thus have less improvement than females without a history of cancer.

Summary:

Table 1 shows, separately for program staff, males, and audience members, the demographic characteristics of the 67 who filled out both pre- and post- surveys at the educational symposium and whether they had heard of the Inside Knowledge campaign or not.
The majority of the respondents from the audience were from Essex County (52.1%), where this event was held. Only 8.3% of the female audience members and none of the males remembered previously hearing about the Inside Knowledge campaign.

Table 2 shows the mean pre-survey score and the mean change, as well as the p-value for statistical significance (calculated either with Student’s t-test or McNemar’s test) for each of the individual factual questions (identified by the CDC question number) and for the total scores for the 48 paired samples among the female audience subgroup, the primary target for educational outreach.

For each of these 11 factual questions (Table 2) there was an improvement in knowledge for the female audience. For five (Q10, Q11, Q13, Q14, Q17), there was statistically significant improvement (p<0.05). Q10 and Q13 dealt with risk factors, Q11 & Q14 with screening and prevention, and Q17 with signs and symptoms. For two other questions (Q9 & Q16) the improvement approached statistical significance; these dealt with risk factors and treatment options, respectively. The four (Q7, Q8, Q12, Q15) without significant improvement dealt with screening & prevention (Q7 & Q12), risk factors (Q8), and signs & symptoms (Q15).

Analysis of the questions by category (Table 2) reveals that for all but one of the Risk Factors questions there was significant improvement Questions from Screening/Prevention (Q7, Q11, Q12) and Signs/Symptoms sets show limited improvement. However, the three questions from those sets that did not significantly improve also had the highest baseline scores, so the room for improvement was minimal. Furthermore these topics may already be well known among the public.

The overall mean pre-score for the female audience subgroup was 34.56 (SD = 19.75). The mean score significantly improved by +15.5 points (SD = 18.43).

Table 3 compares the mean pre-survey scores stratified into staff (n = 16) and lay audience (n = 51). The non-staff were further stratified to compare cancer survivors (n = 8) and those without a history of cancer (n = 43).

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Table 3 compares the mean pre-survey scores stratified into staff (n = 16) and lay audience (n = 51). The non-staff were further stratified to compare cancer survivors (n = 8) and those without a history of cancer (n = 43).
As expected, the staff had significantly higher pre-survey scores, indicating higher baseline knowledge. All three subgroups had statistically significant improvements from pre- to post-scores. Unexpectedly, the mean pre-survey scores and mean score changes were lower in the cancer survivor non-staff subgroup than the non-cancer survivor non-staff subgroup. Further analysis of this group revealed a bimodal distribution in the pre-survey score with extremes of either very low or very high values. This intra-group heterogeneity might reflect survivors with vs. without gynecologic cancer.

**Conclusion:**

The CDC-designed pre-survey tests baseline knowledge of the risk factors, signs and symptoms, screening/prevention, and treatment options for gynecologic cancers. The post-survey repeats the exact same questions but is completed after an educational session incorporating materials from the *Inside Knowledge* campaign. Our design strengthened and expanded this study with paired analyses. The baseline knowledge among women in our community was limited (mean score 34.56 with a wide range). Our educational session for the general public was effective, with statistically significant improvements for many factual questions and the overall scores. However, the overall post-survey mean scores of 50.66 indicate that there is still room for improvement. Future public health efforts should be designed to improve the campaign’s scope and materials to educate the public on gynecologic cancers. Follow-up with individual participants may be worthwhile to assess knowledge retention and further improve their knowledge.

The results from the individual questions provided insight into which specific educational areas have much room for improvement. Four individual factual questions did not show significant improvement (Q7, Q8, Q12, & Q15). Q7 and Q12, concerning screening and prevention of cervical cancer and Pap smears, had high mean pre-survey scores (6.32 and 7.81), indicating an already high baseline knowledge. Thus, room for improvement was limited, and there was very limited power to detect improvement. Similarly, Q15, on signs and symptoms of all the gynecologic cancers, had a relatively high pre-survey score (6.00). In contrast, Q8, dealing with uterine cancer risk factors, had relatively low pre-survey scores. The limited change may indicate a need to refine the educational materials on this subject. Other areas of potential improvement suggested in our analyses include more emphasis on HPV vaccine recommendations and when to seek medical care with symptoms of gynecologic cancer. These questions (Q11 & Q16 respectively) still had relatively low post-survey scores despite showing statistically significant improvement.

The sample size of female audience members \((n=48)\) limited the statistical power for our single site. However, serving an audience of this size made it possible to hold multiple small group
sessions to maximize personal interactions. Our post-survey addendum question asked whether participants were cancer survivors, not specifically gynecologic cancer survivors. Thus the cancer survivor group (in Table 3) may be too inclusive for an assessment of gynecologic cancer knowledge. Further analyses are planned, such as stratifications into different subgroups (e.g. race/ethnicity) to see how the scores varied among these groups and whether certain groups may have greater needs for outreach. The opinion and attitude questions on the CDC surveys, many of which were presented as Likert-scale questions, will be analyzed for direction of change. This can only be done at our site, where the pre- and post-surveys were linked to create paired data.
References:


Objective:

Diagnosis of cancer and subsequent cancer treatment are both emotionally and physically demanding on patients. Emotional distress, anxiety, and depression in turn are highly correlated with sleep disturbances, particularly insomnia, which in turn further contributes to the fatigue level of the cancer patient. Despite the prevalence of fatigue and sleep disturbances in cancer patients, these relevant issues are often not addressed by healthcare providers.

A promising adjunct to conventional cancer treatment is aromatherapy. Aromatherapy is based upon the use of essential oils, which are the concentrated aromatic extracts of plants or parts that are claimed to possess therapeutic properties. Previous studies have reported that cancer patients show improvements of reduced anxiety levels, relief of emotional stress, pain, muscular tension, and fatigue following aromatherapy. In particular, the use of lavender essential oil can reduce psychosocial stress and perceived pain.

For this study, it is believed that lavender essential oil aromatherapy would significantly improve the quality of life for cancer patients by alleviating fatigue and promoting good sleep behavior. Considering that the practice of aromatherapy is considered to be limited to the use of essential oils, we also hypothesize that lavender essential oil will be more effective than a synthetic fragrance (a bergamot-scent) or an oil placebo (olive oil) in affecting fatigue and sleep.

Methods:

A convenience sample of adult patients who were receiving outpatient treatment at NJMS Cancer Center was recruited for the study.

This 3-week study employed a repeated measures design in which patients would serve as their own controls. The aim was to compare aromatherapy (lavender essential oil) with a synthetic fragrance (a bergamot-scent) and an unscented oil (olive oil). Over the course of three weeks, patients would receive a different aromatherapy kit once each week for nightly home use for a one-week period. At baseline (prior to treatment) and at the end of each study week, patients filled out surveys that measure fatigue and sleep patterns. This provided a total of four time points at which survey data was collected.

Functional Assessment of Chronic Illness Therapy (FACIT-F)

The FACIT-F is a 13-item fatigue scale designed to evaluate fatigue in patients with chronic illnesses including patients with cancer. The FACIT-F contains positive (e.g. “I have energy”) and negative (e.g. “I have to limit my social activity because I am tired”) questions. These questions are answered using a Likert-type scale ranging from 0 to 4. The answers are re-coded by reverse-coding the negative questions. Then the answers for all questions are added together to provide a total fatigue score. Although it is termed a fatigue assessment, a higher score is considered optimal and indicative of better quality of life.
**Newark Sleep-Ability Survey (NSA)**

The survey contains 55 questions on sleep habits, sleep hygiene and ability to sleep for the past seven days to give information on general sleep hygiene and what factors—if any—interfere with quality of sleep. Prior to any initiation of study procedures, the protocol was reviewed and approved by the Rutgers Health Sciences Institutional Review Board.

**Summary:**

A total of 13 patients were recruited and retained for the three-week study. Out of the 13 patients, 5 were male and 8 were female, with a mean age of 51 and an age range of 27-65. The patients recruited were also from diverse ethnic backgrounds and showed a wide range of cancer diagnoses.

In the quasi-experimental design employed in this study, patients served as their own controls. The effect of treatment over time and the effect of different types of treatment were evaluated in comparison to the baseline data obtained in the first time point of the study. These comparisons and trends were observed in both dimensions of fatigue (FACIT-F survey) and quality of sleep (NSA survey).

**Fatigue**

Figure 1 shows FACIT-F scores for lavender (essential oil), bergamot (synthetic fragrance), and olive oil (placebo) in comparison to the FACIT-F score obtained at baseline. Higher FACIT-F scores indicate higher quality of life and the mean FACIT-F value for lavender across all 13 patients [38.46] was the highest. While bergamot [35.23] and olive oil [35.46] FACIT-F score values were also higher than that of baseline [30.08], they were nevertheless lower than the value observed for lavender.

Application of treatment over time, regardless of the type and order the treatments were given, also shows a consistent increase in FACIT-F score values (Figure 2). The longer the patients were in the aromatherapy study, the better quality of life they experienced. By the time that the fourth time point (TX Week 3) was reached, patients displayed a FACIT-F value of 38.31, which is close to the FACIT-F score value of 38.46 reported by patients who used only lavender for an entire week (Figure 1).

**Sleep**

The NSA survey inquires about various factors that contribute to quality of sleep. Physical and psychological factors that interfered with the ability to fall asleep or that caused the patient to wake include if the patient: were nauseous, were in pain, needed to use the toilet, had muscle cramps, had difficulty breathing, or were anxious or worried. Insomnia was evaluated by the survey question of “How many nights did you take more than 30 minutes to get to sleep?”
The resulting NSA survey data values as shown in Figure 3 and Figure 4 represent the average number of nights over the past seven nights that the patient had trouble falling asleep. In other words, as shown in Figure 3, patients displayed poor sleep behaviors (i.e. taking drugs or watching television to fall asleep) for more than two nights (2.32) out of the past seven nights as baseline behavior. In comparison, treatment with lavender for a whole week resulted in a decrease of poor sleep behaviors, down by almost a day [1.39]. Lavender treatment particularly shows a marked decrease from baseline values in psychological factors that interfere with sleep, by almost a day and a half. Overall, baseline data showed the most disturbance of sleep while lavender showed the biggest improvement in sleep in five out of the six parameters that were examined (Figure 3). The results of applying either bergamot or olive oil were a consistent improvement over baseline quantity of sleep but both were also less effective than treatment with lavender essential oil (Figure 3).

Figure 4 demonstrates the overall effect of the aromatherapy over the three weeks during which the study was conducted. In particular, a marked decrease in insomnia is seen from baseline [3.38 nights/week] to 3 weeks post-baseline [1.23 nights/week]. Psychological factors that interfere with a patient’s ability to fall asleep was also reduced dramatically by aromatherapy, showing a decrease from an average of 2.38 nights/week at baseline to 0.54 nights/week at the end of the study (Figure 4). Overall, based on Figure 4, every investigated parameter that contributed to poor quality of sleep decreased from baseline with the application of aromatherapy over the three-week study period.
Conclusion:

As predicted by our hypothesis, lavender as an essential oil aromatherapy treatment was consistently the most effective in alleviating fatigue and promoting sleep in cancer patients. However, it is also interesting to see that bergamot synthetic fragrance and olive oil placebo contributed to better performances in the fatigue and sleep parameters compared to baseline. This implies that there may be more than just the inherent benefits of the aromatherapy oil itself affecting better fatigue and sleep. One possibility is that application of the aromatherapy to one’s pillow is akin to introducing a bedtime ritual behavior, very much like brushing one’s teeth. This preparation may prime the patients for sleep, which would cut down on poor sleep behaviors and psychological factors interfering with sleep.

This study employed a counterbalanced treatment order to control for any order effects in the repeated measures design but the end time point (3 weeks post-baseline) consistently showed the most improvement for all parameters examined. This raises the thought that any aromatherapy treatment is preferable to having no treatment whatsoever. Considering that cancer patients show deterioration over time due to aggressive treatment and the continued progression of cancer, it would be of interest to survey patients on the same parameters of fatigue and sleep over the course of three weeks without administering aromatherapy. Obtaining progressive baseline data would better elucidate the actual degree as to which aromatherapy preserves and further improves the patient’s quality of life by promoting better fatigue and sleep.

It is important to note that the average values for FACIT-F and NSA surveys obtained from the thirteen patients in the study were not statistically significant. However, the resulting trends were remarkably consistent; lavender essential oil was the best in promoting better fatigue/sleep quality while bergamot synthetic fragrance and olive oil placebo were similar in effect but were both considerably better than baseline.
Also, although not shown, FACIT-F and NSA data comparing first baseline time point to the 3 weeks post-baseline time point (Figure 2 and Figure 4) closely approached statistical significance. This furthers the notion that the study should aim to recruit more patients, especially from locations other than NJMS-UH Cancer Center for a broader demographic.

A challenge with this study was the small sample size, due to the limited number of patients that met the inclusion criteria at the recruitment site. Future research should explore the protocol in a larger sample of patients. Additional exploration between fragrances and aromatherapy protocols may help to identify if there are differences detectable in essential oil treatments.

References

Objective:

Multiple myeloma is a disease that accounts for 10% of blood cancers and 1% of all cancers. The American Cancer Society estimates that 24,050 adults will be diagnosed with multiple myeloma and 11,090 adults will die of the disease in the year 2014. Current multiple myeloma therapy involves the use of the FDA-approved proteasome inhibitor bortezomib (Btz). The median survival in multiple myeloma patients with such treatment is 3-5 years, the 5-year survival rate being 40%. These statistics bring to light the urgent need to develop treatments to improve outcomes of multiple myeloma patients. Naltrindole (Nti), a delta opioid receptor antagonist, has been shown to inhibit multiple myeloma cell proliferation \textit{in vitro} and \textit{in vivo} via a non-opioid receptor-dependent mechanism (Mundra et al., 2012). While the mechanism of action of naltrindole is not as well understood as that of bortezomib, data from previous experiments show that naltrindole has an inhibitory effect on proliferation of multiple myeloma cells. The objective of this study, therefore, is to determine if naltrindole would enhance the efficacy of bortezomib to create a stronger drug that has greater therapeutic effect due to synergy between bortezomib and naltrindole.

Methods:

\textit{Culturing human U266 multiple myeloma cells}: Human U266 multiple myeloma cells are cultured in RPMI 1640 media containing 10% fetal calf serum and are used as the test subject throughout the study. Analysis of the synergy between naltrindole and bortezomib requires that we determine each drug’s EC50 value, also known as the concentration of the drug at which cell proliferation is inhibited by 50%.

\textit{Determining dose response curves of naltrindole and bortezomib}: 12-well plates are prepared such that wells are filled with 2 ml U266 cells at 100,000 cells/ml media. One well serves as the control and receives no drug treatment. The remaining wells receive one of the following drug treatments: 1.5 µM naltrindole, 5 µM naltrindole, 15 µM naltrindole, 50 µM naltrindole, or 5 nM, 6 nM, 7 nM, 8 nM, 9 nM, or 10 nM bortezomib. These plates are incubated at 37 °C for 72 hours (which has been experimentally determined to be the time at which there is optimal inhibition of cell proliferation for data collection purposes.) After 72 hours, a ViCell instrument (Beckman-Coulter) is used to determine the number of viable cells/ml in each sample, which when compared to the number of viable cells/ml in the control, provides a value that is reflective of the percentage inhibition of proliferation by that particular concentration of drug administered. Once the EC50’s of both drugs have been determined, the second part of the experiment begins, where the U266 multiple myeloma cells are exposed to varying concentrations of one drug combined with a fixed concentration of the other. These samples are also incubated for 72 hours and then analyzed via the ViCell instrument to determine the inhibitory effect of the combination of the drugs on cell proliferation.

\textit{Using combinations of naltrindole and bortezomib to investigate synergistic effects on multiple myeloma cell proliferation}:
Once the EC50 values are determined for each drug separately, we test drug synergy by adding a fixed amount of one drug to varying amounts of the other, and vice versa. In one experimental set up, 10 \( \mu \)M of naltrindole is added to 6 nM, 7 nM, 8 nM, and 9 nM concentrations of bortezomib. These combined drugs are each added to 2 ml of U266 cell cultures each with an approximate cell count of \( 1.0 \times 10^5 \) cells/ml in a 12 well cell culture plate. The controls consist of a control well that received no drugs, and four wells that received only the varying concentrations of Btz and no naltrindole. The second experimental set up consists of 7 nM of Btz added to 1.5, 5, 15, and 50 \( \mu \)M Nti in a similar fashion, with one control well that receives no drugs and four wells that receives only the varying dosages of Nti and no Btz. These two setups combined are able to provide us with data indicating the effect of combining the two drugs on cell proliferation. This can help tell us if Nti and Btz are synergistic, additive, or neither. As done previously, both experimental set ups are also incubated for 72 hours and then analyzed via ViCell to determine the inhibitory effect of the combination of the drugs on cell proliferation.

Summary:

Results from this study show that the EC50 of naltrindole, the concentration at which naltrindole inhibits cell proliferation by 50%, is 13 \( \mu \)M, while the EC50 of bortezomib is 7.7 nM (Fig. 1). Furthermore, results also indicate that adding Nti, in this case so that the solution is 10 \( \mu \)M Nti, to varying dosages of Btz, leads to increased inhibition of cell proliferation compared with the effect of either drug alone (Fig. 2), particularly at the two lowest concentrations of Btz (6 and 7 nM). When a fixed concentration of 7 nM Btz is added to varying concentrations of Nti, the combination of drugs is more effective at a concentration of Nti close to its EC\text{50} (Fig. 3).

![Fig. 1](image_url)  
**Fig. 1** Dose-response curves of the effect of naltrindole and bortezomib on human U266 multiple myeloma cell proliferation

![Fig. 2](image_url)  
**Fig. 2** Synergy of Bortezomib and Naltrindole toward Inhibition of Human U266 Multiple Myeloma Cell Proliferation
Conclusion:

Since the combination of Btz and Nti clearly led to increased inhibition of cell proliferation of multiple myeloma cells, this drug combination is of even more interest for potential therapeutic use. Addition of Nti to a drug cocktail could lower the amount of Btz given to a patient, which simultaneously reduces the patient’s risk of developing harmful side effects associated with Btz. Further experimentation that investigates the combinatory effect of Nti and Btz is needed. Such further experimentation includes testing other combinations of dosages to determine optimal combinations, and also testing the in vivo effect on tumor growth in mouse xenograft models. It is also important to determine the toxicity of the drug combination by testing the effect of the drugs on normal, non-cancerous cell types to ensure that they are less sensitive and that their cell growth is not impacted significantly.

References:

NEW INSIGHTS INTO MECHANISM OF VITAMIN D ACTION REVEALED THROUGH THE STUDY OF TRANSGENIC, KNOCKOUT AND AGING MICE

Sylvia Christakos, PhD (Department of Biochemistry and Molecular Biology)

Objectives:

The intestine plays the major role in the action of vitamin D on calcium homeostasis. Yet the mechanisms involved remain incompletely understood. The established model of regulation of intestinal calcium absorption by the active form of vitamin D, 1,25(OH)₂D₃, postulates a critical role for the duodenum. However it is the distal intestine where 70 -80% of the ingested calcium is absorbed. Therefore, one of the objectives this study was to test directly the role of 1,25(OH)₂D₃ and the vitamin D receptor (VDR) in the distal intestine. In this study we compared the expression of two vitamin D target genes, the calcium binding protein calbindin and the enzyme involved in the catabolism of 1,25(OH)₂D₃, CYP24A1 in the distal intestine of the transgenic mice (Tg) to levels in VDR KO mice and wild type (WT) mice.

Another objective of this study was to observe differences in 1,25D₃ mediated gene expression that are related to calcium homeostasis, with increasing age. Additionally, the differences in regulation of vitamin D target genes between treatment with vitamin D and an analog, Hectorol, were also observed. Hectorol is used for the treatment of hyperparathyroidism and has been suggested for the treatment of osteoporosis.

We hypothesize that the distal intestine plays a crucial role in regulating the body's calcium levels under regulation by 1,25(OH)₂D₃ and with increasing age, 1,25(OH)₂D₃ mediated gene expression changes which leads to changes in calcium homeostasis. We also hypothesize that treatment with 1,25(OH)₂D₃ and Hectorol leads to comparable changes in vitamin D target gene regulation and expression but Hectorol, which is less toxic than 1,25(OH)₂D₃ at higher doses, may be a better alternative to 1,25(OH)₂D₃ treatment to maintain calcium homeostasis with age.

Methods:

To test the role of 1,25(OH)₂D₃ and VDR in the distal intestine, mice expressing VDR exclusively in the ileum, cecum, and colon were generated by breeding VDR knockout (KO) mice with transgenic (TG) mice expressing human VDR (hVDR) under the control of the 9.5kb cdx2 promoter (which targets gene expression specifically to the distal intestine). After this step, these mice were bred with VDR knockout mice that expressed no hVDR or mouse VDR (mVDR) anywhere. The progeny of these mice only expressed hVDR and its expression was limited to the distal intestine. To confirm the progeny generated expressed hVDR exclusively in the distal intestine, two methods were utilized. First, the same 9.5kb cdx2 promoter was used to transduce the lac operon into the distal intestine exclusively and then the expression of β galactosidase was tested. Second, the expression of β actin was compared to the expression of hVDR in the same mice tissue samples. β actin was used for the comparison because it is a cytoskeletal component expressed uniformly in all tissues.
For the second component of this study concerned with the effects of aging, wild type C57BL6 mice were utilized to study the effects of aging on VDR expression and expression of VDR target genes in kidneys and in the intestines. These mice were kept on a regular chow diet and sacrificed to harvest the tissues of interest at appropriate time points.

Finally, the component of the study concerned with comparing the effects of vitamin D with its analog on the expression of VDR and VDR target genes, C57BL6 mice were used which were fed a vitamin D deficient diet for 1 week and injected with 1 ng/g body weight Hectorol, 1,25D$_3$ or vehicle; 48, 24 and 3 hours prior to sacrifice.

For all these studies, the appropriate mice were sacrificed to obtain their intestines and kidneys. The intestines were rinsed with saline. Consequently, the tissues were frozen in liquid nitrogen and utilized as needed. RNA and protein needed to be isolated from these tissue samples. In order to isolate protein, the tissue was ground in liquid nitrogen and added to a buffer consisting of protease inhibitor and phosphate buffered saline (PBS) and incubated for about ten minutes on ice. Then, the mixture was sonicated and incubated on ice once again for five minutes. After centrifugation at 12,000 rpm, 4°C, the supernatant was collected and re-centrifuged for ten minutes at 12,000 rpm, 4°C and stored at -80°C.

Similarly, for RNA isolation, the ground tissue was added to empty tubes, to which 1 ml Ribozol/tube was added. These were incubated for ten minutes at room temperature. 200 µL chloroform was added to each tube and thoroughly mixed, after which the tube was incubated for 2-3 minutes at room temperature. This mixture was then centrifuged at 12,000 g for 15 minutes at 4°C. Doing so will result in the formation of 3 phases. 80% of the top aqueous phase was collected. RNA was precipitated out by adding 500 µL isopropanol followed by an incubation period of ten minutes at room temperature. A white pellet was formed at the bottom of the tube at the end of the incubation. After the supernatant was removed, the pellet as washed with 1ml of 75% ethanol prepared in DEPC H$_2$O. After this, the sample was centrifuged at 7500 g for five minutes at 4°C. The ethanol was removed and the pellet was allowed to air dry for five to ten minutes. Before the pellet dried completely, it was dissolved in 30 µL of DEPC H$_2$O and the pellet was passed several times through a pipette tip to make sure it was well dissolved. This mixture was incubated at 55-60°C for ten minutes to completely dissolve the pellet. After this, the mixture was immediately placed on ice till it was needed.

For all parts of the study, cDNA needed to be created from the isolated RNA which was then used for PCR. For cDNA synthesis, the following technique was used: 1µL of 50µM oligo(dT)$_{20}$ was added to 1µL of a 10 mM dNTP mixture with upto 5µg of RNA. The volume of this mixture was then brought upto 10µL with the addition of DEPC-treated water. This mixture was then incubated at 65°C for five minutes and then placed on ice for at least one minute. These initial steps prime the RNA for the cDNA synthesis process. Next, the cDNA Synthesis Mix is made by adding 2 µL 10X RT buffer, 4 µL 25mM MgCl$_2$, 2 µL 0.1 M DTT, 1 µL RNaseOUT™ (40 U/ µL) and 1 µL SuperScript™ III RT (200 U/ µL) for each reaction mix. After adding 10 µL of the cDNA Synthesis Mix to each RNA/primer mix, the mixture was mixed gently and collected by brief centrifugation. This was followed by incubation at 50°C for 50 min. To terminate the reactions, they were incubated at 85°C for five minutes and then chilled on ice. The reactions were collected by brief centrifugation. 1 µL of RNase H was added to each tube and incubated for 20 minutes at 37°C. This cDNA synthesis reaction was then used for PCR. Different PCR settings were used depending on the gene to be amplified.
For RT-PCR, gel electrophoresis was conducted on these samples. In order to do this an agarose gel was made with 1.5g agarose added to 100 mL of 1X TAE buffer. This solution was microwaved to make sure that the agarose dissolved completely. Upon cooling for a couple minutes, 6 µL of ethidium bromide was added. The gel was then poured into a gel tray and allowed to cool completely until the gel was set. Before running the samples on the gel, they were prepared by adding 3 µL of 6X DNA dye to each sample. The tubes were centrifuged to bring all the dye down. The gel was added to the running buffer and then appropriate volumes of the samples and a ladder were added. After running the gel electrophoresis, the gels were imaged to visualize the bands produced.

Summary:
VDR mRNA was undetectable in all segments of the intestine in VDR KO mice and was 1.7 fold elevated in ileum, cecum and colon of Tg mice compared to WT mice. The calcium binding protein calbindin-D$_{9k}$ mRNA was decreased in VDR KO mice and in Tg mice levels of calbindin-D$_{9k}$ mRNA in the distal intestine were equivalent to WT. mRNA for CYP24A1, an enzyme regulated by 1,25(OH)$_2$D$_3$, was undetectable in intestine of VDR KO mice and was expressed at equivalent levels in the distal intestine of Tg and WT mice. Decreasing expression of VDR target genes was found with increasing age. Finally, Hectorol was as effective as 1,25(OH)$_2$D$_3$ in inducing calbindin-D$_{9k}$ mRNA in all regions of the intestine of vitamin D deficient mice.

Conclusion:
Since expression of the VDR only in the distal intestine reversed the rickets observed in VDR KO mice, these findings provide evidence for the first time using VDR transgenic mice, about the importance of the distal intestinal segments in vitamin D mediated calcium and bone homeostasis. Our findings indicate that expression of vitamin D target genes in the distal intestine was equivalent to WT.

These findings are significant because they can help us understand the impact of gastric bypass surgery on intestinal calcium absorption and how regional bowel disease, such as Crohn's disease and colon cancer, may disrupt calcium absorption.

In addition, our findings have provided evidence for age related changes in VDR target gene expression. Our findings also provide evidence for the efficacy of Hectorol as an alternative to 1,25 (OH)$_2$D$_3$ in maintaining calcium homeostasis.
ANALYSIS OF BREAST CANCER INCIDENCE AND MORTALITY IN THE UNITED STATES

Syed Haque, PhD, Dinesh Mital, PhD, Shantha Bethusamy, Riddhi Vyas, Riaz Basha Shaik, (Rutgers Biomedical and Health Sciences- School of Health Related Professions, New Jersey Medical School & Graduate School of Biomedical Sciences)

Objective:

Our main objective is to model cancer incidence in the U.S and tri-state area in order to examine potential environmental factors. Accurate prediction models can inform future disease burdens, health policies and individual decisions. The objective of this study is to investigate incidence of breast cancer and find potential patterns or clusters in some specific regions that may arise awareness. If so, this will permit us to investigate spatial patterns within the geographical regions, and give us the relationships between incidence and mortality of breast cancer and other health, socioeconomic, and environmental variables. The combination of statistical modeling and mapping is a powerful tool for visualizing disease risk in a spatial-temporal analysis. With the increased use of statistical modeling and mapping, epidemiologic research and studies have been able to examine lifetime exposure and statistical data sets. We will perform statistical analyses incorporating spatial information, and utilize some statistics algorithms to do disease mapping based on certain environmental factors.

Methods:

As stated above, the overall goal of this study was to generate disease (breast cancer) maps to display cancer density in the regions selected for this study in order to improve cancer control program. This study is an integration of spatio-temporal perspective into breast cancer research with a focus on the relationship, if any, between environmental exposures and breast cancer risk. Breast cancer cases data from 2003-2010, was obtained from the Health Cost and Utilization Project, National Inpatient Sample (HCUP-NIS), the largest database for the collection of multi-year hospital care (inpatient, outpatient, and emergency department) data in the United States. In order to analyze data sets and generate reports, statistical analysis was performed by using Statistical Analysis Software (SAS).

Codes and Variables:

The International Classification of Diseases (ICD) is the classification used to code morbidity data from the inpatient and outpatient records and physician offices. ICD codes are for all neoplasms of the breast. HCUP is possible due to the following 38 state data collection partners from across the United States.

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<tr>
<td>198.81</td>
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</tbody>
</table>

Summary:

SAS:

![The FREQ Procedure](image)

**Table 1. Distribution of race vs. mortality for breast cancer hospitalization by year**

*RACE*  
(1) White  
(2) Black  
(3) Hispanic  
(4) Asian/Pacific Islander  
(5) Native American  
(6) Other  

*DIED*  
(0) Did not die  
(1) Died  

![Table 1](image)
Figure 1. Distribution of breast cancer-census driven across states in 2006

*est_06 SUM = incidence rate = (# of cases in state/ state census population) * 100,000

Conclusion:

Some conclusions based on the results solely using SAS include:

- Much of the cases concentrated in the age group 50 to 60 years for women and between 40 to 50 years in men in the years 2006 and 2008.
- The white population had the maximum number of observations in all years contributing 75% of the total observation. Second, were blacks followed by Hispanic.
- The top five states with highest incidence in 2006: Connecticut, South Carolina, Minnesota, Tennessee and Iowa.
- States with lower reported incidence were Nebraska, Nevada, Kansas, Massachusetts and Michigan.

Cancer incidence rates will be examined by the spatial analysis power of GIS. We plan to map with aggregate data and mapping with individual datasets and plan different strategies in dealing with these various environmental and/or other factors.
References:


Objective:

Currently, there is a debate surrounding the best method in order to gain access to anterior skull base (ASB) tumors. Both endoscopic and open procedures have been successfully employed to resect ASB lesions.\textsuperscript{1,2,3} Although the endoscopic endonasal approach (EEA) for resection of pituitary and parasellar lesions have become an accepted treatment option, controversy still exists about EEA for anterior midline skull base lesions.\textsuperscript{4} Traditionally, these lesions have been treated via a transcranial or craniofacial approach. These approaches are not only cosmetically undesirable but are also associated with frontal lobe edema, venous infarction, hematoma, CSF leak, bone flap infection, pneumocephalus, and mortality.\textsuperscript{3,4} Alternatively, the endoscopic endonasal transcribriform approach (EEA-TC) is an extracranial approach that provides direct visualization and exposure of the ventral ASB without brain retraction or manipulation of neurovascular structures.\textsuperscript{1,2} However, some have advised against EEA-TC due to an association with a higher incidence of postoperative CSF leak and infection rate.\textsuperscript{4}

In this study, we review our experience with EEA-TC and detail our multi-layer reconstruction technique. We focus on rates of gross total resection (GTR), intraoperative CSF leak, and postoperative complications.

Methods:
A retrospective chart review was performed from July 2009 to July 2014 for patients who underwent EEA-TC. Data were extracted regarding patient demographics, clinical presentation, pathologies, postoperative complications, and outcomes. This study received approval form the Institutional Review Board.

Surgical Technique
The initial endoscopic endonasal approach to the anterior skull base was performed using a 30-degree high definition Storz endoscope. In cases in which there was lesion invasion into the nasal cavity a microdebrider was used to debulk the tumor mass, and the mass was subsequently peeled away from the nasal septum. A medial maxillectomy and a medial turbinectomy were performed along with a total ethmoidectomy and frontal sinusotomy. This
allowed excellent exposure of the anterior skull base. Using the 30-degree angled endoscope
the frontal sinus and sphenoid sinus mucosae were inspected. In cases in which a mass was
visualized meticulous bipolar cautery and suction were employed to remove the mass.

A high-speed drill was used to drill out the base of the skull floor, typically extending
partly to cross the midline. The dura was then incised around the skull base defect and brought
down from anterior to posterior until the olfactory tract was identified, which was then
coaugulated and divided.

Multilayer closure was performed and secondary repair of the dura was used. Repair
materials included AlloDerm and/or a pedicled nasoseptal flap, gelfoam, surgicel, and Mercocel
packs.

Summary:

Thirty-two patients were indentified who underwent EEA-TC. Twenty-three received a pure
endonasal approach. Nine underwent a combined cranionasal approach (EEA-TC plus transbasal
craniotomy) because of significant intracranial tumor extension. The pathologies included
olfactory groove meningioma (9), esthesioneuroblastoma (6), encephalocele (4), sinonasal
melanoma (2), small cell neuroendocrine carcinoma (2), olfactory schwannoma (1),
inflammatory pseudotumoral lesion (1), sinonasal teratocarcinosarcoma (1), osteoblastoma (1),
renal cell metastasis (1), adenoid cystic carcinoma (1), basaloïd carcinoma (1), ossifying fibroma
(1), and nonspecific adenocarcinoma small blue cell type (1). Patients underwent either
nasoseptal flap, pericranial flap, and/or alloderm reconstruction of large cribriform skull base
defects.

Gross-total resection was achieved in 90.6% (29 cases), and near-total resection was achieved
in 9.4% (3 cases). Primary repair materials included a nasoseptal flap (71.9%), AlloDerm alone
(12.5%), pericranial flap with a nasoseptal flap (9.4%), pericranial flap with AlloDerm (3.1%),
and pericranial flap alone (3.1%). Postoperative complications included pneumocephalus
(6.2%), CSF leak (3.1%), hematoma (3.1%), delayed hypertension hemorrhage (3.1%) and visual
loss (3.1%). Mean follow-up was 27 months (range: 2 to 56 months).

Conclusion:

The EEA-TC is a safe and viable approach for resection of benign and malignant ASB tumors
involving the cribriform plate. This can be combined with a transcranial approach in cases with
significant intracranial extension. Meticulous multi-layer reconstruction with a nasoseptal flap,
pericranial flap, and/or AlloDerm can minimize the risk of CSF leakage.
References:
Objective:

The project presented here adds novel functionality to an existing multi-port low-fluence alpha-particle irradiator. A graphic user interface (GUI) and control electronics were built that enable the user to control this multi-port alpha irradiator to deliver a number of dose patterns. These different dose delivery patterns simulate dose rate patterns observed in targeted alpha particle therapy as well as in environmental exposures.

Methods:

A system of shutters constructed by Neti et al. (1), sits on top of three α-particle irradiator ports (Figure 1). The current project established an interactive communication between this shutter system and a graphic user interface (GUI), which was coded in Visual Basic. To establish communication, a microcontroller (Arduino Uno Rev3) was programmed to interpret input it receives from the user via the GUI, and send corresponding signals to relays which activate the shutters. A custom-made electronic compartment was built (Figure 2) to house the Arduino and electronic relays which establish independent connections to the three α-particle irradiator shutters. Specialized circuitry was also developed to filter the EMF noise generated by the high currents used to activate the shutter electromagnets.

Figure 1. Detailed enlargement of one of the ports involved in producing the α-particle beam.

Figure 2. Circuitry designed and assembled in this project. A PC communicates with the microcontroller via a USB port. The microcontroller actuates relays which power the shutters.
Summary:

The GUI provides a number of dose delivery options that are available in several tabs.

The first tab, ‘Single Fraction’, allows the user to specify the dose to be delivered to samples of cells in each of the three ports, independently. This dose, delivered in a single fraction, may be chosen explicitly by the user and the corresponding percentage of cell nuclei that receive 0, 1, or 2 α-particle traversals is calculated (2).

The second tab, ‘Multiple Fractions,’ allows the user to divide the dose into multiple fractions (Figure 3). After user input of the overall treatment time, total dose, and number of fractions, the other fields on the GUI are automatically populated. Once the program is set to run, on-screen graphics shows the progress of the experimental run in real-time (Figure 4).

Figure 3. Graphic user interface for the Multiple-Fraction tab. Parameters have been set to show functionality capacity.
Figure 4. Graphics showing the timing of the opening and closing of the three shutters.

The third and fourth tabs offer exponentially varying functions of dose delivery with respect to time (Figure 5). For each function selected, the user provides the overall treatment time, dose, and number of fractions that will be used to deliver the dose. The GUI compiles these inputs, determines the temporal spacing of the fractions, and graphs the planned dose delivery.

Exponentially increasing/decreasing and multi-exponential functions were chosen for this GUI as they have wide reaching application in cancer research (see discussion).

Discussion:
The pharmacokinetics of radiopharmaceuticals in the tumor and normal tissues conform to an exponential increase followed by an exponential decrease thereby delivering a complex dose rate pattern. The ability to simulate these complex dose rate patterns in the laboratory may lead to new understanding of how the dose rate pattern affects the relative biological effectiveness (RBE) of radiopharmaceuticals labeled with α- versus β-particle emitters.

The graphic user interface also enables the user to study dose rate patterns that are encountered in circumstances where humans are irradiated chronically by very low fluences of α-particles such as:

1. Radon-222, a naturally occurring radioactive gas that is found in the soil, leaks into homes across the country. It decays to several daughter radionuclides that can be inhaled. The inhaled radon-222 and daughters lodge in airways whereupon they decay to release α- and β-particles that can cause lung cancer. In fact, it is estimated that radon gas is the second leading cause of lung cancer (after smoking) according to the National Cancer Institute at the NIH.

2. High Z energy ions (HZE) irradiate astronauts when they travel in space. These HZE particles cause biological damage similar to α-particles. Each cell is traversed by about 1 HZE particle per month and each traversal causes substantial DNA damage.

In the context of these scenarios, this system can be used to study how dose rate affects bystander effects which arise from nearby irradiated cells (3).

References:


IDENTIFICATION OF PHOSPHOTYROSINE EVENTS INDUCED BY IONIZING RADIATION IN BREAST CANCER CELLS

Tong Liu, PhD (Center for Advanced Proteomics Research), Hong Li, PhD (Center for Advanced Proteomics Research), Edouard I. Azzam, PhD (Department of Radiology)

Objective:

Tyrosine phosphorylation is an important post-translational modification (PTM) that plays a significant role in cell development, division, and oncogenesis [3]. Changes in tyrosine phosphorylation can be induced by damaging rays of ionizing radiation (IR). Its dysregulation, specifically due to exposure to IR, may interfere with critical signaling pathways that affect cell survival and development of long term health effects. Studies show that dysregulation of protein tyrosine phosphorylation is linked to the cause of more than 80% of human cancers [1]. Understanding the modulation of tyrosine phosphorylation in proteins and the ensuing effect on signaling pathways is relevant to devising novel therapeutic interventions in cancer treatment. Since protein tyrosine phosphorylation is extremely low in abundance, making up only 0.05% of total protein phosphorylation in human cells, it is very difficult to detect [2]. Immunoprecipitation of novel proteins is necessary to enrich phosphoproteins and phosphopeptides prior to nano-liquid chromatography and tandem mass spectrometry (LC-MS/MS) analysis. The goal of the project is to identify tyrosine phosphorylated (P-Tyr) sites on the digested peptides of control and irradiated MCF-7 breast cancer cells. This is crucial to achieve a global understanding of posttranslational signal transduction pathways that may affect DNA repair, apoptosis, and cell cycle checkpoints, among other processes. The outcome may help formulate new strategies that enhance the efficacy of cancer radiotherapy.

Methods:

Cell Culture
MCF-7 breast cancer cells were cultured in Eagle’s minimal essential medium (MEM) supplemented with heat inactivated fetal bovine serum. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. For experiments, they were exposed to 0 or 4 Gy (3 Gy/min) of 137Cs g rays. They were incubated for 15 min or 1 h, respectively, post-irradiation.

Cell Lysis
Cells from each of the three treatments of control, 15 minutes following irradiation, and 1 hour following irradiation were washed three times with 1X PBS. 1 mL of lysis buffer containing 50 mM Tris-HCl, 1% NP40, 1mM EDTA, 150 mM NaCl, 2 mM Na3VO4, 2 mM Na4P2O9, and 1X protease inhibitor cocktail, was used for resuspension and lysis of the different samples of cells through ultrasonication (15 seconds with 10 seconds interval). Lysed cells were centrifuged, and supernatant was transferred to new tubes. The total protein concentration was estimated using Bradford protein assay.
**SDS-PAGE Western Blotting**
50 μg of proteins from each sample were separated on 12% SDS-PAGE. The proteins were either stained with Coomassie Brilliant Blue or transferred to a nitrocellulose membrane. The membranes were blotted with 5% milk and washed three times with TBS-T. The membrane was incubated with PY99 mouse monoclonal antibody overnight and washed three times with TBS-T. Then the membrane was incubated with anti-mouse antibody in 2% milk for 1 hour. The signals of phosphorylated proteins were detected using ECL chemiluminescence.

**Phosphotyrosine Protein Enrichment**
10 mg of extracted proteins from each sample was incubated with 4G10 and PT66 anti-phosphotyrosine agarose beads in a 1:1 ratio for phosphotyrosine protein enrichment at 4°C overnight. After washing the beads three times with the lysis buffer, enriched proteins were eluted from the beads by Reducing Sample Buffer for SDS-PAGE separation. The gel lanes were cut for in-gel trypsin digestion. Proteins were reduced by DTT and alkylated by iodoacetamide, then digested with trypsin. The peptides were extracted for TiO2 enrichment.

**Phosphotyrosine Peptide Enrichment**
Peptides extracted from in-gel digestion were subjected to TiO2 enrichment. Phosphotyrosine peptides were eluted with 5% ammonium hydroxide and then desalted using C18 ZipTips.

**LC-MS/MS Analysis & Bioinformatics**
Peptides from TiO2 enrichment were analyzed using LC-MS/MS on Q Exactive tandem MS instrument. The MS/MS spectra were searched against the Swiss-Prot human database using Mascot search engine (V2.3). The results were displayed in and filtered with Scaffold software. Ingenuity Pathway analysis was performed for identified phosphotyrosine proteins.

![Figure 1: Workflow for phosphoprotein and phosphopeptide enrichment prior to LC-MS/MS analysis.](image-url)
Summary:

Western Blot Analysis of Phosphotyrosine Levels
The Coomassie Blue Stain in Figure 2 shows equal loading of proteins for each of the three 50 μg samples. Relative to control, immunoblots revealed a significant number of proteins with enhanced tyrosine phosphorylation in the samples that were irradiated with recovery times of 15 min and 1 h, respectively. Often, tyrosine phosphorylation levels were greater when the samples were incubated at 37 °C for 1 h rather than 15 min after irradiation. These increase in phosphorylation were not apparently due to increase in protein levels (Figure 2, right panel).
LC-MS/MS & Pathway Analysis

Following LC-MS/MS analysis, we found 40 proteins to be tyrosine phosphorylated (Table 1). Irradiation followed with 1 h recovery time showed the most P-Tyr proteins, which was consistent to that of the Western Blot. Both batches of irradiated cells expressed proteins differing from that of the control group, showing that exposure to ionizing radiation induced proteome changes. Many of these proteins encoded by different genes are known to function in the regulation of important biological processes like cell division, such as SMEK2, POGZ, ASPM, and DYRK2. Others, including ALDH1A3, SBF2, MAPK14, and PPP1R13L, play an important role in regulating apoptosis. Several of the P-Tyr proteins, such as SMC3, FMO3, PRKDC, RBM39, and NUP160, also govern DNA repair pathways and contribute to genomic stability. Pathway analysis using Ingenuity showed that many of the P-Tyr proteins were linked to carcinoma (Figure 3). Sufficient damage to DNA repair pathways used by cells to recover from IR induced DNA breaks can lead to abnormal cells that may escape immune surveillance and apoptosis. Dysregulation of the PTMs and the proteins in Table 1 interfere with the cell’s ability to regulate cell cycle checkpoints, a primary event in the carcinogenic process.
Conclusion:

When exposed to IR, several stress-responsive pathways are rapidly activated. These are likely to involve PTMs, including tyrosine phosphorylation. Aberrant PTMs can lead to dysregulation of cell cycle checkpoints and apoptosis, leading to enhanced genomic instability. As predicted, relative to control cells, irradiated MCF-7 cells experienced altered PTMs that affect regulation of important signaling pathways. Further experiments, comparing the results with those occurring in normal cells, together with deeper analyses of the affected signal transduction pathways may lead to novel or improved anti-cancer therapies.

References:


Objective:
Medical treatment for cancer patients can, and does interfere with quality of life. Cancer-related symptoms (CRS) and cancer-treatment related symptoms (CTRS) include an array of symptoms such as pain, nausea, anxiety, and fatigue, which impede the patient’s ability to function. With increasing frequency, patients with cancer and their family members are turning to the Internet to explore treatment options, including complementary and alternative medicine (CAM) to manage the physical, psychological, and emotional consequences of cancer. Non-pharmacological interventions like massage can play an important role to improve quality of life and relieve CRS/CTRS in oncology patients.

Research on massage as a complementary therapy for patients undergoing active cancer treatment has indicated even a small amount of regional massage or bodywork modality can offer overall relaxation and wellness. Although evidence supports that massage is a low risk/high benefit treatment for cancer patients, it is unclear how outpatient oncology clinics incorporate massage into services for patients. Massage does not seem to be a standard offering in oncology care; therefore, it is important to investigate the delivery of massage services in outpatient oncology clinics.

Massage offers significant potential for symptom relief when applied with proper understanding of the adaptations needed to accommodate the needs and vulnerabilities of cancer patients. Our hypothesis was that massage is underutilized at oncology centers, and if it is offered, it is not fully integrated into health care delivery for cancer patients. The goal of this study is to examine the prevalence and extent of the clinical application of massage therapy in outpatient oncology clinics in the northern New Jersey/New York City metropolitan area. This project will report on if and where massage is being offered, who is providing it, and the different modalities being practiced. A descriptive analysis was chosen for this study. There is opportunity for the medical field to be educated on what is lacking in our integrative oncology model and to take steps to provide comprehensive care to our patients.

Methods:
A mixed methods approach was used to gather information about massage at outpatient oncology clinics in the Northern New Jersey/NYC metropolitan area. All clinics within a fifty-mile radius of the Rutgers University Biomedical and Health Sciences Campus in Newark, NJ were included in analysis. The websites for the American College of Osteopathic Surgeons (ACOS) Commission on Cancer (COC) and National Cancer Institute (NCI)-designated cancer centers were used to compile a list of cancer clinics for inclusion in the analysis.
A systematic review of patient services information on facility websites was undertaken. Areas searched included general patient services and treatment, integrative oncology, and patient support. Information was abstracted and entered into a database. A follow up survey was conducted to verify website information and obtain more detailed information. A survey tool was specifically developed for this project containing 14 questions. Information from the survey was transcribed and pooled with the web-obtained information into a Microsoft Excel database for coding and analysis. General coding categories included type of facility, massage availability as well as information about the massage providers and styles of massage.

The protocol was reviewed and approved by the Rutgers Biomedical and Health Sciences Newark Campus Human Subjects Institutional Review Board. Data were analyzed using SPSS version 20. The goal is to identify the percent of outpatient oncology clinics that offer massage and describe how oncology massage programs are structured.

**Summary:**
A total of 78 cancer centers were included in this analysis. Information was available via websites for 100% of the outpatient oncology centers. Web information was verified by telephone survey for 29 centers (37.2%). Information was collected from either the web or by telephone for an additional 34 centers (43.6%). No information about massage was available by phone or web for 15 centers (19.2%), including 3 centers that refused to participate in the phone survey.

Approximately half of the centers (51.3%) offered massage therapy for cancer patients undergoing treatment (Figure 1). Of these, 18 (23.1% of total) centers employed or contracted with a Licensed Massage Therapist (LMT). Other providers were used to provide massage, including nurses, physical therapists, and occupational therapists. A variety of massage modalities were offered including general massage therapy and energy-based therapies (Reiki and reflexology.). Manual lymphatic drainage, a specific modality used to treat inflammation and lymphedema was also included. Physical or occupational therapists as well as nurses and LMTs can perform this therapy. Other therapies were listed as being available that would not be suitable for cancer patients for example: deep tissue, hot stone, kinesio taping, prenatal, repetitive stress injury, and sports massage.

Contradictory information was gathered for nine centers when comparing website and phone survey data. Although many centers did not offer massage therapy, they offered massage information for patient education. Three centers requested free massage services for their patients and eight centers refer patients out to external establishments for therapy. This indicates recognition for the value of massage therapy, but it makes us question what hurdles exist for offering the service. A mixed methods approach was necessary for data collection and to yield a more complete and comprehensive analysis.
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<th>No (%)</th>
<th>Information not available (%)</th>
<th>Total</th>
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<td>51.3%</td>
<td>21.8%</td>
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</tbody>
</table>

*No information available on the website, could not be verified by phone (includes three centers that refused to participate in the phone survey.)*

**Table 1: Massage availability at each type of cancer center**

**Figure 1: Massage availability distribution and breakdown of massage providers.**
Conclusion:
Despite promising evidence that suggests massage is an effective treatment for CRS and CTRS, findings reveal massage is only utilized at approximately half of oncology centers surveyed. When massage is available, there are discrepancies on massage providers and types available. LMTs are not overwhelmingly represented as massage providers. Comprehensive cancer centers seem to be the most progressive in terms of have massage therapy and information available.

The clinics that support integrative medicine may generally offer massage, but it is not fully integrated into health care delivery for cancer patients. This was very evident when trying to collect data. Many of the hospitals that were called were unaware whether massage was offered or which department would be able to enlighten the situation. Effective development and redesign of many of the websites is needed to better inform and empower patients and families seeking CAM and supportive care information.

Future research should explore what barriers exist besides cost and insurance reimbursements for providing massage. There is an opportunity for hospitals to market a new dynamic concept of treatment. Raising awareness about the underutilization of massage in clinical cancer settings is essential to its integration into oncology health care delivery. Clinical care and the physician-patient relation would benefit from an enhanced understanding of massage therapy as it applies to a patient’s needs.
References:


VIRAL PATEL (NJMS 2017)

IMPROVING THE STANDARD MEDICAL THERAPY FOR ACUTE MYELOID LEUKEMIA USING VITAMIN D DERIVATIVES AND CARNOSIC ACID

George Studzinski, MD, PhD (Department of Pathology)

Background:
Vitamin D is a fat-soluble vitamin derived from diet and synthesized by skin when exposed to sufficient sunlight. Its primary functions are to stimulate calcium and phosphate absorption from the intestines and regulate bone remodeling. Recent evidence is pointing to a new role of vitamin D in treating leukemia, such as acute myeloid leukemia (AML). Several in vitro and clinical studies have provided encouraging results as vitamin D supplementation with chemotherapy prolonged remission in AML patients. However, vitamin D use has been severely limited by its hypercalcemic side effects, which led us to study vitamin D derivatives (VDD) and plant polyphenols with anti-oxidant properties. In this study, we treated HL60 and U937 cell lines with arabinocytosine (AraC), a vitamin D2 derivative (VD2D) doxercalciferol (1α-OH-D2), and carnosic acid, an anti-oxidant polyphenol extracted from rosemary and common sage plants, to determine the therapeutic potential of VD2D and carnosic acid.

Objectives:
Previous experiments have studied AraC, VD2D, and carnosic acid on cells directly derived from AML patients. Therefore, we:
1) Use cultured cell lines HL60 and U937 to study cell death and viability to determine if we can use a cell line model for further experiments, where obtaining large number of cells (e.g., for additional molecular studies) is more feasible than directly extracting them from AML patients.
2) Vary the VD2D concentration to determine if lower concentrations can achieve similar results to higher concentrations, thus avoiding hypercalcemia in patients.

Methods:
HL60 and U937 cells were propagated with RPMI-1640 and six 15mL aliquots were transferred to sterile cell culture flasks. Cells were either untreated or treated with 100uM AraC and incubated for 72 hours at 37°C in 5% CO2. Subsequently, we added VD2D doxercalciferol and carnosic acid at final concentrations of 10nM D2 + 10uM carnosic acid, 100nM D2 + 10uM carnosic acid, or no treatment for additional 72 hours at 37°C in 5% CO2. Cells were harvested and viability counts were performed using trypan blue exclusion. Apoptosis and cell viability were also studied by flow cytometry using annexin V and propidium iodide stains. This experiment was repeated twice.

Summary:
The following bar charts average the results from our two experiments. The error bars indicate standard error. The summary of our findings are:
Adding VD_{2}D and carnosic acid decreased cell viability in both cell lines as determined by trypan blue exclusion and flow cytometry. As we increased VD_{2}D concentration, cell viabilities decreased.

Adding VD_{2}D and carnosic acid increased proportion of apoptotic cells in both cell lines as determined by flow cytometry. As we increased VD_{2}D concentration, percent apoptotic cells increased.

Figure 1 - shows trypan blue viabilities for HL60 and U937 cell lines. As we increased VD2D concentration, cell viability decreased for both cell lines in both AraC and non-AraC groups.

Figure 2 – shows cell viabilities as determined by flow cytometry (cells negative for both annexin V and propidium iodide). Cell viabilities decreased with addition of increasing concentration of VD2D in both AraC and non-AraC groups.
Figure 3 – shows % apoptotic cells as determined by flow cytometry (annexin V positive cells). Apoptosis increased with increasing concentrations of VD2D in both AraC and non-AraC groups.

**Conclusions:**
Adding VD2D and carnosic acid to a chemotherapeutic agent such as AraC led to more cell death and increased apoptosis for both cell lines, than AraC alone. We observed significant differences between our experimental and control groups to warrant the use of cultured cell lines such as HL60 and U937 for further research. It is more feasible to use cultured cell lines to obtain larger number of cells for further research, such as molecular studies, than to repeatedly extract cells directly from AML patients. Furthermore, VD2D concentration at 100nM produced greater effects than 10nM. Therefore, higher VD2D concentrations are superior to lower concentrations as adjunct therapy for AML. This research was novel as it used a VD2D, a polyphenol anti-oxidant, and AraC in two cell lines. Previous studies have used much higher vitamin D3 concentration (which is more hypercalcemic than vitamin D2) to show differences in cell kill when combined with a chemotherapeutic agent. Therefore, this research indicates that we can use a lower vitamin D concentration and still achieve significant results, when combined with a polyphenol such as carnosic acid. Further studies are being performed by our colleagues to elucidate the mechanism of action of VD2D and carnosic acid in potentiating cell death and increasing overall apoptosis. Additionally, more cell lines need to be used, such as THP1, to determine if we can extrapolate these results across other cell lines.
Visual Abstract:

1) AML Cells + Vitamin D & Carnosic Acid → Differentiation

2) AML Cells + AraC → Cell Death

3) AML Cells + Vitamin D, Carnosic Acid, AraC → Increased Cell Death

4) Normal Cells + Vitamin D, Carnosic Acid, AraC → No Increase In Cell Death

References:
IS UNIVERSITY HOSPITAL FOLLOWING THE RECOMMENDED GUIDELINES FOR COLONOSCOPY SURVEILLANCE

Steven Keller, PhD, (Department of Psychiatry), Michael Demyen, MD, (Department of Gastroenterology)

Objective:
Colon cancer affects over a hundred thousand men and women across the country each year and is the third most common cancer diagnosed in men and women (American Cancer Society, 2014). The American Gastroenterological Association (AGA) publishes guidelines for the recommended surveillance time for patients that have had a colonoscopy for colon cancer screening. The surveillance guidelines ensure that people at higher risk repeat their colonoscopies earlier than those at average risk (Lieberman et al., 2012). Patients who are at average risk are brought back in 10 years, thus conserving resources and reducing exposure to the risk of colonoscopy (Lieberman et al., 2012). The purpose of this study will be to determine whether or not University Hospital is following the accepted guidelines for colonoscopy surveillance set forth by the American Gastroenterological Association (AGA).

Methods:
In order to test the null hypothesis that University Hospital is not following the guidelines for colonoscopy surveillance correctly for each patient, a retrospective analysis of patient charts was done. One hundred and ten patient charts from the year 2012 were selected based on numbers generated by Random.org, a free computer assisted random numbers generator. Using Random.org, 150 random numbers between 1 and 365 were generated in random order. The numbers were used to correspond to calendar dates from 2012, for example if the number 1 had been generated it would correspond to January 1, 2012. Each date was used only once so that data from the same chart was not duplicated. Weekends and holidays were not used since patients were not seen on those days. The first 89 random dates from the list were used and every colonoscopy report that met the inclusion criteria for the study from those dates were accepted, which was 110 colonoscopy reports. The inclusion criteria for the study was male and female patients referred to University Hospital specifically for colorectal cancer screenings within 2012 and the exclusion criteria was patients referred for inflammatory bowel disease surveillance and those patients having colonoscopies for other reasons than colorectal cancer screenings. The following data was collected from each of the 110 colonoscopy reports: age, sex, endoscopist, prep quality, number of polyps removed, number of adenomatous polyps removed, size of the largest polyp removed, was a surveillance time recommended, what was the surveillance time, and did this time meet the accepted guidelines set forth by the AGA. The guidelines for colonoscopy screening used in this study were the same guidelines used by the AGA described in the paper Guidelines for Colonoscopy Surveillance After Screening and Polypectomy: A Consensus Update by the US Multi-Society Task Force on Colorectal Cancer (Lieberman et al., 2012). In order to analyze the data, Vassarstats.net was used.
Summary:
The patients in this study ranged in age from 40-79 years, the mean age was 57.8 years, and the median age was 56 years. The patient population was made up of 37.30% male patients and 62.70% female patients. Guideline 1, no adenomas or polyps, has a recommended 10 year repeat interval; 45 patients fell in this category, 3 were lost at follow up and 42 were given surveillance times. Within this guideline category UH is following this guideline 9.52 % of the time, 95% CI [0.0376, 0.2206]. Guideline 2, no adenomas; distal small (<10 mm) hyperplastic polyps, has a recommended 10 year repeat interval; 29 patients fell in this category, 2 were lost at follow up and 27 were given a surveillance time. Within this guideline category UH is following this guideline only 3.70% of the time, 95% CI [0.0066, 0.1828]. Guideline 3, finding 1–2 tubular adenomas (<10 mm), has a recommended 5-10 year repeat interval; 24 patients fell in this category, 1 patient was lost at follow up and 23 patients were given a surveillance time. Within this guideline category UH is following this guideline 26.09% of the time, 95% CI [0.1255, 0.4647]. Guideline 4, finding 3-10 adenomas, has a recommended 3 year repeat interval; 4 patients fell into this category and none were lost at follow up. Within this guideline category UH is following this guideline 25.00% of the time, 95% CI [0.0456, 0.6994]. Finally, guideline 6, one or more tubular adenomas (>10mm), has a recommended 3 year repeat interval; 5 patients fell into this category and none were lost at follow up. Within this guideline category UH is following this guideline 20.00% of the time, 95% CI [0.0362, 0.6245]. Three patients (2.73%) were excluded from the data due to receiving barium enemas. The prep quality of the patients varied with excellent prep 1.82% of the time, good prep 33.64% of the time, mediocre prep 10.91% of the time, fair prep 8.18% of the time, and no prep recorded 0.91% of the time.

Discussion:
Overall, the lifetime risk of developing colorectal cancer is about 1 in 20 (American Cancer Society, 2014). Colorectal cancer is the third leading cause of cancer-related deaths in the United States when men and women are considered separately, and the second leading cause when both sexes are combined; it is expected to cause about 50,310 deaths during 2014 (American Cancer Society, 2014). However, the death rate from Colorectal cancer has been dropping over the past 20 years partly due to the improvement of polyp detection rates through colonoscopy surveillance (American Cancer Society, 2014). Thus, it is clearly evident that colonoscopies are extremely important in helping patients stay a step ahead.

Based on the results of this study, University Hospital is not following the AGA guidelines correctly; however, this is most likely due to the prep quality of most of the patients. If the prep is not done well it is advised for physicians to bring patients back sooner for a follow up colonoscopy in case something was missed (Lieberman et al., 2012). The prep for UH patients is 4 liters of polyethylene glycol 3350 a laxative (colyte, trilyte), which is typically given in one dose. A further study may be to look into giving different preps to patients at UH, for example, there are newer preps that require less volume to be ingested and even split preps where one dose is taken at home and a second dose is taken at the hospital prior to the procedure. It is possible that with a change in prep at UH patients would go into their procedures with better preps, there would be less risk of missing a polyp or adenoma, and the hospital could better follow the correct surveillance times confidently.
References:


RENUKA REDDY (NJMS 2018)

MICROARRAY ANALYSIS REVEALS CONCOMITANT E2F2 DELETION RESCUES KEY PATHWAYS TO PROMOTE NORMAL ERYTHROID DIFFERENTIATION IN RB KNOCKOUTS

Lizhao Wu, PhD (Department of Microbiology and Molecular Genetics)

Objective:

The retinoblastoma tumor suppressor protein (Rb) regulates the cell cycle by binding to and inhibiting a family of activator E2f transcription factors, E2f1, E2f2, E2f3. We have previously shown that inactivation of Rb in mouse erythroid cells leads to ineffective erythropoiesis due to failed terminal differentiation (inability to exit cell cycle) at the orthochromatophilic stage, resulting in mild anemia and splenomegaly. Furthermore, we have shown that concomitant inactivation of E2f2 in the erythroid cells of Rb knockout (KO) mice significantly rescues the anemic phenotype of the knockouts. This rescue is specific to E2f2, as concomitant inactivation of E2f1 or E2f3 in Rb KOs does not rescue the anemic phenotype. However, the molecular mechanism by which inactivation of E2f2 prevents the erythropoietic defects in the Rb KO mice is not known. In order to identify the dysregulated genes and signaling pathways that potentially mediate this E2f2 specific rescue of the Rb KO anemic phenotype, we conducted a gene expression microarray analysis of late-stage (orthochromatophilic) erythroblasts from WT (Rb\(^{loxP/loxP}\)), Rb KO, Rb/E2f1 double knockout (DKO), Rb/E2f2 DKO, Rb/E2f3 DKO, and E2f2 KO mice.

Methods:

Mice: An EpoR-GFPCre-mediated conditional knockout system (Figure 1) was utilized to generate the erythroid-specific knockout mice utilized in this study.

![EpoR-GFPCre transgenic mouse conditional knockout system for erythroid-specific gene deletion (Rb KO system shown here)](image)

**Figure 1.** EpoR-GFPCre transgenic mouse conditional knockout system for erythroid-specific gene deletion (Rb KO system shown here)

**Erythroid cell staging and sorting:** Orthochromatophilic erythroblasts (OrthoE) (Figure 2) were isolated using CD44-FSC-based erythroid staging on cells harvested from the bone marrow.

![Stages of erythroid development with CD44 expression scale (Rb KO mice display differentiation block in the OrthoE stage of erythropoiesis, which is rescued in Rb/E2f2 DKOs)](image)

**Figure 2.** Stages of erythroid development with CD44 expression scale (Rb KO mice display differentiation block in the OrthoE stage of erythropoiesis, which is rescued in Rb/E2f2 DKOs)
Gene expression microarray analysis: RNA was extracted from the sorted OrthoE cells of WT (Rb<sup>loxP/loxP</sup>), Rb KO, Rb/E2f1 double knockout (DKO), Rb/E2f2 DKO, Rb/E2f3 DKO, and E2f2 KO mice using a Qiagen RNeasy Plus kit. The RNA was subsequently utilized to synthesize cDNA. Microarray analysis was completed using an Affymetrix Mouse Gene 1.0 ST array. Raw intensities from CEL files were converted to expression values on a log-2 scale and normalized. Fold changes compared to WT expression were calculated for each genotype studied. Any fold change ≥ 1.5 was considered significantly dysregulated. Among the genes significantly upregulated in Rb KOs, any fold change < 1.2 for those genes in the DKO groups was considered a significant ‘rescue’. Further expression analysis was completed using Ingenuity Pathway Analysis and Multiexperiment Viewer to identify significantly dysregulated cellular and molecular pathways, identify gene clusters, and generate heatmaps with Euclidean correlation-based hierarchical clustering.

Summary:

Fold-change analysis demonstrated that 83.5% of the genes significantly upregulated (≥ 1.5 fold change) in Rb KOs were rescued in the Rb/E2f2 DKO mice. The extent of expression level rescue achieved in the Rb/E2f2 DKO was unmatched by the other two DKO groups (Figure 3). Ingenuity Pathway Analysis revealed that the top 5 most significantly upregulated (p < 1E-10) pathways in Rb KOs, such as gene expression, cellular growth and proliferation, cell death and survival, cell cycle, and DNA replication, recombination, and repair were rescued in the Rb/E2f2 DKOs. Candidate genes that were significantly upregulated in Rb KOs but demonstrated distinct rescue in the Rb/E2f2 DKO were also identified (Figure 4). Once again, several of these candidate genes normally play key roles in cell cycle regulation, particularly in relation to G1/S phase transition and DNA replication, and are involved in signaling for cellular differentiation.

Figure 3. E2f2 deletion is uniquely capable of significantly rescuing genes upregulated in Rb KO mice. (A) Venn diagram depicting the number of upregulated genes unique to or shared among Rb KOs (R), Rb/E2f2 DKO (R2), and E2f2 KOs (2). (B) Venn diagram depicting the number of genes upregulated in R that are uniquely rescued or overlappingly rescued by concomitant E2f1 (R1), E2f2 (R2), or E2f3 (R3) deletion. (C) Scatterplots demonstrating R2 rescue of genes upregulated in R (right) and the relative lack of differences between R2 and W for genes that are upregulated in R (left).
In addition to the gene expression data analyzed, data from previously completed immunoprecipitation assays determining the degree of binding between Rb and the various E2f activators were included in the current analysis. These data revealed that Rb interacts with and binds to E2f2 significantly more than it does with E2f1 and E2f3, likely due to the high levels of E2f2 expressed in late-stage erythroids (data not shown). We determined that E2f2 is more abundant than E2f1 and E2f3 in late-stage erythroids not only through qRT-PCR, but also through immunoprecipitation/Western blots that compared the level of the three activator E2f transcription factors between mouse embryonic fibroblasts (control) and late-stage erythroids (data not shown).

Conclusion:

We have previously shown that erythroid-specific Rb KO mice develop an anemic phenotype due to failed terminal differentiation and inability to exit cell cycle at the orthochromatophilic stage. This phenotype is rescued specifically in the Rb/E2f2 DKO, but not in the Rb/E2f1 or Rb/E2f3 DKO s. Through global gene expression profiling we demonstrated that there is a significant and specific rescue of upregulated genes in the Rb/E2f2 DKO, which was not observed in the other DKO s. These key upregulated, rescued pathways include DNA replication, damage, and repair as well as cell cycle, cell proliferation, and gene expression pathways. Combining the current results with previous immunoprecipitation findings that Rb interacts with and binds to E2f2 significantly more than it does with E2f1 and E2f3 (due to a specific increase in E2f2 expression in late-stage erythroids) we proposed a model (Figure 5) to explain the potential molecular interactions between Rb, the three activator E2fs, and target gene promoters under E2f control in OrthoE cells of the various genotypes tested:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>p21</td>
<td>Cyclin-dep kinase inhibitor</td>
</tr>
<tr>
<td>Ccng1</td>
<td>Cyclin G1, G2/M arrest - DNA damage</td>
</tr>
<tr>
<td>Ccne2</td>
<td>Cyclin E2, G1/S</td>
</tr>
<tr>
<td>Slfn9</td>
<td>Hematopoietic cell differentiation</td>
</tr>
<tr>
<td>Apol11a</td>
<td>Lipoprotein, gene transcription, signal transduction</td>
</tr>
<tr>
<td>Mybl1</td>
<td>TF, cell cycle, differentiation, apop</td>
</tr>
</tbody>
</table>

Figure 4. Upregulated candidate genes that demonstrated striking and oftentimes specific rescue by loss of E2f2.
**Figure 5.** Theoretical molecular interactions between Rb, E2f1, E2f2, E2f3, and E2f target gene promoters in OrthoE cells of the genotypes investigated.

At this late stage of differentiation, OrthoE cells are normally preparing to exit cell cycle, enucleate, and complete differentiation to become reticulocytes and later mature red blood cells. In the model above, in the wild-type OrthoE cells, Rb binds and sequesters the three E2f activators, predominantly E2f2, to prevent the aberrant transcription of target genes that trigger cell cycle entry. In part through the effective transcriptional repression by Rb, the OrthoE cells are capable of exiting cell cycle and normally differentiating. However, in the Rb KO OrthoE cells, Rb is no longer present to repress these activator E2fs, which results in deregulation of activator E2f activity and the untimely transcription of target genes. Even in the Rb/E2f1 and Rb/E2f3 DKO s the levels of aberrant transcription remain intermediate/high as E2f2 is present at high levels and is free to promote transcription of target genes. However, in the Rb/E2f2 DKO, aberrant transcription is dramatically decreased as E2f2 is no longer present. Although E2f1 and E2f3 are present without Rb repression, they are at such low levels that their effects are minimal. In this manner, concomitant deletion of E2f2 in Rb KOs results in a gene expression rescue.

Overall, these results suggest that E2f2 has a tissue-specific, non-redundant role in its interaction with Rb and in regulating normal erythroid differentiation/cell cycle exit. Nevertheless, further tests such as qPCR and ChIP to validate the microarray results and understand the specific interaction of Rb and E2f2 at the promoter regions of candidate genes are required to further our current understanding. Going a step beyond expression validation, functional validation of candidate genes can be achieved through knockdown of these genes in Rb KO hematopoietic stem cells, which can then be used in adoptive transfer experiments with lethally irradiated mice to determine if phenotypic rescue is achieved. These future directions will help expand the basic molecular model of Rb/E2f2 interactions to include the effects of key E2f2 target genes and their products.

**References:**

ONCOLOGY PATIENT AWARENESS OF DISEASE

Rashi Aggawal, MD, (Department of Psychiatry), Jason D. Domogauer, MD/PhD student, (Rutgers-NJMS)

Objective:

Physicians often use medical language that patients may not understand, leading to occurrences where patients fail to fully comprehend their disease and/or its prognosis. Such an occurrence has been documented in patients with diabetes, where physicians overestimate patient understanding by asking questions such as “do you understand?” as opposed to asking, “what do you understand?” Patients were found to frequently respond “yes” to the former question without a true understanding (1). Such lack of knowledge can lead to a lowered involvement of patients within their own care, potentially contributing to poorer outcomes (2).

Unfortunately, this occurrence is also observed in oncology patients. In the UK, 37% of the lay people were not aware that the term ‘metastasis’ meant the spread of cancer (3). On the other side of the spectrum, women with breast cancer felt that when doctors properly informed them about their treatment, they were able to take better control of their care and become more responsible patients (4). Furthermore, research has shown that, in women with breast cancer, the level of involvement in care is highly correlated with the intensity of treatment (5). A sense of loss of control in one’s life can also be due to the overwhelming nature of receiving a diagnosis of cancer (6). It is important that the doctor establish good communication with the patient to restore that sense of control to the patients’ life both by making sure they understand their diagnosis and by being of aware of the impact a cancer diagnosis may have on them.

This leads to another concern, which is the effect of an oncology diagnosis and treatment on a patients’ mental health. Depression has a higher prevalence in oncology patients than in the general population, with a possible prevalence as high as 20-50% of patients depending on the cancer (7,8) versus approximately 6.7% in the general population (9). There are many causes of depression in oncology patients, but one possible cause is denial of illness, which may lead to less active participation in treatment (10). In addition, some patients may not admit to feelings of depression because of the stigma associated with mental illness (11). This study is a part of a larger study examining prevalence of untreated depression in English-speaking patients at an urban oncology outpatient cancer center. Here, we assessed the correlation between patient level of education, depression status, and self-perceived depression stigma, and the patients’ level of disease awareness.

Methods:

This project is part of a larger questionnaire-based study examining prevalence of untreated depression and its associated stigma in cancer patients. Participants consisted of oncology patients at New Jersey Medical Cancer Center outpatient oncology clinics (medical oncology clinic, chemotherapy infusion clinic, and radiation oncology clinic). Sample questionnaire questions that were analyzed included:

1. What is your level of education?
   a. There were five options to check off ranging from ‘eighth grade or less’ to ‘college graduate or higher’.
2. What stage was your cancer in when you were diagnosed?
   a. Patients who did not check off any of the listed options were assumed to not
      know their staging.
3. Currently is your cancer localized to a single area or has it metastasized (spread) to
   other areas?
   a. Patients who did not check off any of the listed options or checked ‘do not know’
      were assumed to not know whether their cancer had spread.
4. Depression symptoms were assessed using the CES-D depression scale (12).
   a. Patients who scored 16 or higher were categorized as showing depressive
      symptoms.
5. Self-stigma was assessed using Depression Stigma Scale (13).
   a. Patients answered 9 questions each on a scale of 0-5 and the sum of those five
      questions gave the patients self-perceived stigma score, with higher scores
      correlating to higher levels of stigma.

### Summary:

An increased level of education is associated with a significantly increased level of awareness
of cancer staging, linear by linear association (1, N=223)=9.353 p=0.002.

<table>
<thead>
<tr>
<th>Level of Education</th>
<th>Unaware of Staging/Metastasis</th>
<th>Aware of Staging and/or Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>8th grade or less</td>
<td>16%</td>
<td>4%</td>
</tr>
<tr>
<td>Some high school</td>
<td>25%</td>
<td>18%</td>
</tr>
<tr>
<td>High school graduate or GED</td>
<td>33%</td>
<td>31%</td>
</tr>
<tr>
<td>Some college</td>
<td>14%</td>
<td>29%</td>
</tr>
<tr>
<td>College graduate or higher</td>
<td>12%</td>
<td>18%</td>
</tr>
</tbody>
</table>

31% of patients do not know their cancer staging and/or whether or not it has metastasized. Out of the
people who are aware of staging only 28.5% are depressed, whereas 34.8% of people who are
unaware of staging and/or metastasis are depressed, (fisher exact test p=0.308). While this is not significant,
the trend is an important one to be aware of and to examine in future research.

Patients who are aware of their staging and metastasis have a lower mean self-perceived stigma than patients who are unaware of staging and/or metastasis, 14.87 and 15.19 respectively, 
\( t(216)=.32, p=0.031 \)
Patient lack of knowledge about their disease is also evident from a question on the questionnaire that asked, “What type of cancer do you have”? Patients asked the study staff how to spell lymphoma, prostate, throat, and lung. The patient who asked how to spell lung, thought he had lung cancer but in fact had lymphoma. Some patients reported never being told what type of cancer they had or what stage their cancer was in.

**Conclusion:**

As standard medical ethics dictates that patients be fully informed of their disease, it should clearly follow that patients would be aware of their cancer staging and know of if it has metastasized. However, in clinics in New York, 65% of Latino patients did not know their staging and 38% were unaware of the metastatic state of their disease (13,14). This can perhaps be attributed to difficulty in communication, as explanations may get lost in translation. But since our study was looking only at English speaking patients, language issues were apparently not the only factor contributing to lack of awareness in oncology patients about their disease.

Therefore, doctors must recognize that patients, in urban areas, with low levels of education, such as Newark, may require specialized attention, which includes expanded evaluation of their understanding of their disease. The doctors in the Newark oncology clinics attribute lack of patient knowledge about their disease to such things as: lack of patient desire to be involved in their own care, uncertainty in cancer diagnoses, overwhelming nature of finding out one has cancer, language barriers, and patient denial. Further research should be focused on analyzing the factors underlying this lack of patient knowledge.

In addition, the correlation between lack of knowledge and depression should continue to be explored as patient control and autonomy can improve patient outcomes. Doctors should recognize that patients might have a high self-perceived stigma associated with depression, which can act as a barrier to treatment. Future studies should examine ways to dispel such stigma. A potential solution could consist of a psychiatrist and/or social worker dedicated to the outpatient clinics who can discuss mental health issues with patients and who can take more time to go over and discuss patients’ disease as needed. Additionally, a future study may consist of questionnaires provided to doctors asking if they believe their patient is depressed, what stage the patients’ cancer was in when they were diagnosed, and whether the cancer has metastasized. This would serve as an important comparison to see if the patients’ knowledge regarding their disease is accurate and/or provide an opportunity to identify and correct any miscommunication. Furthermore, it would provide an opportunity to determine the staging for patients who did not report a staging, as well as allow for full analysis of the correlation between severity of staging and patients’ knowledge of staging.
References:

CELLULAR RESPONSE TO EXPONENTIALLY VARYING DOSE RATES ENCOUNTERED IN TARGETED RADIONUCLIDE THERAPY

Roger W. Howell, PhD, (Department of Radiology)

Objectives:

The ionizing radiations emitted by radiopharmaceuticals deliver radiation absorbed doses to various organs and tissues over protracted times that depend on the biological uptake and clearance half-times of the radiopharmaceutical in the tissue and the physical half-life of the radionuclide. The corresponding dose rate patterns that emerge are continuously variable and uniquely different than most encountered in other diagnostic and therapeutic modalities that usually involve either single or multiple fractionated acute exposures. There is a wealth of knowledge that has been gained regarding the biological effects of single or multiple fractionated acute exposure patterns as well as the effects caused by chronic irradiation at constant dose rate. Research has shown that responses to continuous radiation exposures are markedly different from responses to an acute exposure. Depending on the tissue irradiated, the underlying reasons behind these differences have been attributed to a multitude of factors such as cell cycle dependence of radiosensitivity, proliferation, cell signaling, inflammatory responses, adaptive responses, compensatory responses, and many other factors such as linear energy transfer (LET). The relative importance of such factors depends on the dose rate and total absorbed dose. Yet, there is a paucity of data available regarding how the exponentially varying dose rates encountered in nuclear medicine affect response, especially under conditions that are not influenced by the spatial nonuniformities of dose that are often inherent in nuclear medicine. This is relevant not only for diagnostic nuclear medicine, but also for radioimmunotherapy (RIT) utilized in the treatment of cancers. More data concerning biologic responses to the dose rate patterns associated with these modalities could lend insight into the effectiveness and toxicity of new and existing procedures.

Methods:

Cell Cultures

Chinese Hamster V79 cells were maintained as monolayers in culture flasks (37°C and 5% CO₂-95% air) containing minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomycin, and 2 mM L-glutamine.

Experimental Setup

Triplicate Corning 25 cm² culture flasks were seeded with either 3.0 x 10², 3.0 x 10³, or 3.0 x 10⁴ V79 cells and incubated for 4 h to attach to flasks. After 4 h, flasks were capped tightly and irradiated chronically with ¹³⁷Cs γ-rays for 168 h with different dose rate increase half-times (Tᵢ = 0, 24 h) while maintaining a fixed dose rate decrease half-time (T₉₀ = 64 h). At the end of 168 h, cell colonies were fixed with ethanol and stained with crystal violet. Cells that were exposed acutely to ¹³⁷Cs γ-rays (2.61 Gy/min) were capped tightly and were left in an incubator for 168 h.
at 37°C. At the end of 168 h, cell colonies were fixed with ethanol and stained with crystal violet. Cell colonies were counted, surviving fractions relative to controls were calculated, and cell survival curves were plotted. Dose rate profiles for cells varied depending on their distance from the $^{137}$Cs gamma ray source. These profiles are graphed below:

**Figure 1:** Dose rate patterns for a fixed dose rate decrease half-time ($T_d = 64$ h) and different dose rate increase half-times ($T_i = 0, 24$ h). Dose rates are plotted on a linear-linear scale on the left and a log-linear scale on the right to emphasize the exponential nature of the profiles. Note that the maximum dose rate decreases as a function of the distance of the flask from the $^{137}$Cs γ-ray source.
Results:

- Cell survival curves for irradiated Chinese hamster V79 cells are shown in Figure 2.
- A least-squares fit to the data using the linear-quadratic model $S = \exp(-\alpha D - \beta D^2)$. Results are shown in Table 1.

**Figure 2:** Cell survival curve showing survival of Chinese hamster V79 cells following irradiation. Error bars represent the standard deviation of the mean of triplicate colony counts. Solid lines represent a least squares fit to the data using the linear quadratic model.

- Cell survival curves for irradiated Chinese hamster V79 cells are shown in Figure 2.
- A least-squares fit to the data using the linear-quadratic model $S = \exp(-\alpha D - \beta D^2)$. Results are shown in Table 1.

**Table 1.** Linear quadratic parameters obtained with a least squares fit to the survival data.

<table>
<thead>
<tr>
<th>Radiation Profile</th>
<th>$\alpha$ (Gy$^{-1}$)</th>
<th>$\beta$ (Gy$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td>0.022 ± 0.072</td>
<td>0.029 ± 0.007</td>
</tr>
<tr>
<td>Chronic ($T_{d_2} = 64$ h)</td>
<td>1.80 x 10$^{-10}$ ± 0.0769</td>
<td>0.0061 ± 0.0037</td>
</tr>
<tr>
<td>Chronic ($T_i = 24$ h, $T_{d_2} = 64$ h)</td>
<td>3.9 x 10$^{-10}$ ± 0.0036</td>
<td>0.00060 ± 0.00020</td>
</tr>
</tbody>
</table>
Conclusions:

- For a given dose, acute radiation exposure was most effective at killing cells.
- Cell survival was highest for cells when exponentially increasing dose rate preceded exponentially decreasing dose rate. This suggests that exponentially increasing dose rates can cause cells to become more resistant to low-LET ionizing radiation. Molecular mechanisms that are responsible for this finding remain unclear.
- Rapid targeting is preferred for radiopharmaceuticals that emit low-LET radiations to avoid decreased effectiveness caused by an initial exponential rise in dose rate.
- Further research is needed to dissect the effect of uptake half-time on the biological effectiveness of radiopharmaceuticals in order to predict their therapeutic efficacy and guide their development.

References:


INTERFERON-λ RECEPTOR EXPRESSION IN CANCEROUS AND NON-CANCEROUS EPITHELIAL CELLS

Sergei Kotenko, PhD, (Department of Biochemistry and Molecular Biology)

Objective:

Interferons are a group of cytokines, signal-transmitting proteins, important in activating the body’s immune system to ward off viral attack. Their very name derives from the observation that they interfere with viral replication. There are three classes of interferons: type I IFNs (including IFN-α and IFN-β), type II IFN (IFN-γ), and type III IFNs (IFN-λ). Type I IFNs are the oldest, discovered in the 1960s, while type III IFNs are the newest members, discovered in 2003. Type I and III IFNs signal through the JAK-STAT pathway. Binding of IFN to the receptors results in receptor dimerization. Janus kinases (JAKs) associated with the receptors are activated and then mediate the phosphorylation of STAT1 and STAT2. STAT1 and STAT2 join with IRF-9 to form the ISGF3 transcription factor complex. Upon activation, this complex relocates from cytoplasm to nucleus and binds to IFN-stimulated response elements (ISRE) in promoter regions and activates target genes. Interestingly, in addition to antiviral properties, interferons have antitumor properties. IFN signaling can increase protein levels of p21, a cyclin-dependent kinase inhibitor, and caspases involved in cell-death pathways. It also can prevent the inactivation of the tumor-suppressing protein Rb. Consequently, IFN-α is used clinically to treat renal cell carcinoma, melanoma, and chronic myelogeneous leukemia. IFN-λ has been shown to restrict the growth of glioblastoma, colon cancer, esophageal cancer, and melanoma. The objective of my summer research project was to investigate any possible changes in expression of IFNLR1, the receptor for IFN-λ, between cancerous and non-cancerous cells. As cells underwent malignant transformation, expression of IFNLR1 could potentially change.

Methods:

ARPE-19 (retinal epithelial cell line), HT-29 (colon cancer cell line), and NCM-356 (colon epithelial cell line) cells were cultured in DMEM supplemented with 10% fetal bovine serum. RNA was harvested when cells were at 90% confluence using TRIzol reagent. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) according to the protocol provided. The primers used amplified ifnlr1. Gene expression was normalized to the human β-actin gene using the ΔCt method. IFNLR1 expression at the mRNA level was compared between the three cell lines.

Summary:

IFNLR1 mRNA levels appeared to be almost three times higher in NCM-365 than in HT-29 cells, suggesting that IFNLR1 mRNA levels decrease as colon cells become cancerous.

Conclusion:

IFNLR1 expression may decrease in colon cancer cells. Additional replicates will be performed to assess whether this finding is statistically significant. Other colon cancer cell lines will also be tested to determine if reduced levels of IFNLR1 expression represent a general pattern in colon
cancer. IFNLR1 has the potential to be a marker of malignant transformation in intestinal epithelial cells.

References:

IRF5 IS A NOVEL REGULATOR OF CYTOKINE AND CHEMOKINE EXPRESSION IN BREAST CANCER THAT INCREASES TRAFFICKING OF SPECIFIC T CELL SUBSETS TO THE TUMOR

Betsy Barnes, PhD, (Department of Microbiology)

Objective:

The main cause of fatality from breast cancer is the metastasis of cancer cells. For this to occur, a permissive microenvironment must be coordinated by the tumor in which normal cells, both resident and recruited, are deregulated. Indeed, decreased recruitment of tumor infiltrating leukocytes (TILs) to the site of breast tumors is a poor prognosis indicator for patients.

Recent data from our lab and others shows that the transcription factor interferon regulatory factor 5 (IRF5) plays a major role in recruiting TILs. It is an important regulator of cytokines and chemokines that help create a tumor-suppressive environment by recruiting specific subsets of immune cells to form tertiary lymphoid structures near the tumor. IRF5 has also been shown to revert invasive MDA-MB-231 cell growth in 3D cultures to normal duct structure. In addition, expression of IRF5 in a breast cancer molecular signature has recently been shown to predict lower frequency of metastasis and higher survival. Data from our lab has shown that IRF5 expression is lost in ~80% of patients with invasive ductal carcinoma in situ with Stage II-IV disease (Figure 1).

Based on these data, we hypothesize that loss of IRF5 expression in human mammary epithelial cells stimulates breast cancer cell metastasis by 1) deregulating breast cancer cell migration and 2) failing to recruit tumor-suppressive immune cells into the tumor microenvironment. In this study, we specifically aim to characterize the specific subsets of immune cells recruited by IRF5(+) breast tumor cells. Based on the array of cytokines/chemokines identified to be regulated by IRF5 in human breast cancer cells, we expect to find anti-tumor immunogenic cells, such as Th1 and Tfh cells, to infiltrate IRF5(+) tumors, as opposed to immunosuppressive cells, such as Tregs or Th2 cells.

Methods:

1. Healthy donor PBMC will be isolated.
2. Migration assays will be performed using 24-well Boyden chambers.
3. 5 x 10^5 purified PBMC will be seeded on inserts in 100 µl of 10% FBS DMEM.
4. Bottom chambers will contain TCM from IRF5(+) and (-) MDA-MB-231 cells. (TCM is prepared from 90% confluent cells with fresh media containing 10% serum).
5. Transwells containing cells and TCM will be incubated at 37°C in 5% CO2 for 2 hrs.
6. Number of cells migrating into bottom chamber will be determined using Vi-CELL (Bechman Coulter).
7. Isolated, migrated PBMC will be stained for B and T cell subsets using antibodies.
8. Cells will be fixed and analyzed on a Becton Dickinson LSRII flow cytometer.

Summary:

Using highly invasive MDA-MB-231 breast cancer cells grown in 3D culture, our lab had previously identified a number of deregulated cytokines and chemokines between IRF5(+) and
negative(-) MDA-MBA-231 cells. Using tumor-conditioned media (TCM) from these cell lines, IRF5-231 and EV-231, as the chemoattractant for human healthy primary peripheral blood mononuclear cells (PBMC), we found a significant increase in anti-tumor T cell migration and a decrease in immunosuppressive T cell migration to IRF5-231 TCM.

The PBMCs that had migrated to the bottom chamber in the assay were collected, counted, and analyzed using flow cytometry. Lymphocytes were first gated for CD3+ T cells and CD+19 B cells. CD3+ gated T cells were then grouped into CD4+ and CD8+ populations. CD4+ gated T cells were then further grouped into Th1 cells (CXCR3+), Th2 cells (CCR4+), Tregs (CD25+), and Th17 cells (CCR6+) (Figure 2).

Flow cytometry data was statistically analyzed, and it was found that the total number of PBMCs migrating to TCM from IRF(+) MDA-MB-231 cells was significantly higher than the number of PBMCs migrating to TCM from EV-231 cells (Figure 3a). Furthermore, there was a statistically significant increase in the number of CD3+ T cells and CD19+ B cells that migrated to IRF5-TCM relative to the EV-TCM control. Additionally, there was a significant increase in migration of CD3+CD4+ and CD3+CD8+ T cells to the IRF5-TCM (Figure 3b), as well as Th1 and Tfhl cells. Lastly, there was a significant decrease of migration of Th2 cells to the iRF5-TCM (Figure 3c).

Conclusion:

1. IRF5 loss in breast cancer reduces the levels of immuno-stimulatory chemokines/cytokines in the tumor microenvironment.
2. Restoring IRF5 expression increases the number of immunogenic T cells that migrate to TCM.
3. IRF5 expression specifically increases migration of cytotoxic CD8+ cells and anti-tumor immunogenic Th1 and Tfhl cells.
4. IRF5 expression also shows decreased migration of Th2 cells, an immunosuppressive cell.

Figure 1.

Stable expression of IRF5 in MDA-MB-231 cells. In 3D culture, the presence of IRF5 allows for a more normal cell morphology.
Figure 2.
References:
LESLEY WU (NJMS 2018)

NOVEL MECHANISM OF CELL INVASION BY CRK AND ABI1 IN METASTATIC GBM

Sushil Kumar (Department of Microbiology, Biochemistry, and Molecular Genetics), Leszek Kotula (Department of Biochemistry & Molecular Biology, SUNY Upstate Medical University) Raymond B. Birge, PhD (Department of Microbiology, Biochemistry, and Molecular Genetics)

Abstract:

The Crk family of adaptor proteins acts as a molecular bridge between tyrosine kinases and their downstream effectors in order to regulate cell motility and proliferation. Dysregulated Crk oncogene has been enumerated in numerous cancers including human lung, breast, sarcoma, ovarian, hematopoietic, and glioblastoma (GBM). In addition, recent findings implicate that the level of Crk overexpression correlates with the severity of malignant cell transformation. The mechanism of cell motility has been identified as a Crk-mediated pathway leading to lamellipodia formation. Currently we are interested in a novel invasion pathway in which Crk promotes invadapodia formation. Unlike motile cells that are impeded by the basement membrane, invasional cells can secrete metalloproteinase to degrade surrounding extracellular matrix (ECM), penetrate the basement membrane, and metastasize. Metastasis represents the major problem in cancer treatment and has dramatic effects on the survival of patients.

Abl tyrosine kinase plays a role in cell proliferation, motility, and apoptosis via a canonical pathway, but constitutively active Abl has a pathological role in driving invasion. Recently, our lab’s research has investigated a non-canonical pathway involving Crk II Y251 phosphorylation on the SH3C domain which can transactivate Abl kinase and lead to tumor invasion and progression to metastatic disease. While phosphorylated Crk II binds to closed conformation Abl, another adaptor protein Abi1 iso2 has been shown to bind to open conformation Abl and down-regulate Abl kinase activity. Using an in vitro kinase assay, we showed that Abi1 and Crk II compete for Abl using their SH3 domains. We compared our findings with next-genome sequencing data bases and observed abnormal expression patterns of Abi1 iso2 and Crk II in GBM patients. We also used western blot to further show that Crk Y251 phosphorylation is correlated with Abl kinase activation in EGF-stimulated GBM when Abi1 iso2 is lost.

Our findings lead us to believe that Abl kinase is reciprocally regulated by Crk II and Abi1 iso2, and that this fine-tuned balance is defective in GBM. Specifically, we propose that Crk II Y251 phosphorylation drives invasion and can be used as a marker for metastasis. In order to test our hypothesis in GBM invasion, we developed a method for optimizing a spheroid invasion assay in order to observe invasion past a basement membrane ECM. We then applied our optimized conditions to an XCELLigence assay that studied the invasion of HS683 cells with a vector containing Crk II, Crk II Y251F, and Abi1 iso2 overexpression. We hypothesize that compared to control GBM, Crk II overexpression will result in the greatest cell invasion, Crk II Y251F overexpression will result in second highest invasion, and Abi1 iso2 overexpression will result in the lowest invasion. Future studies will explore the structural basis of Abl kinase regulation using solution NMR and continue to investigate the tumor suppressor capabilities of Abi1 iso2.
My participation

I was involved in developing a method for optimizing the spheroid invasion assay. I designed part of the protocol in which I decided what percentage fetal bovine serum (FBS) I should use to create an effective FBS gradient for attracting the glioblastoma (GBM) cells out of the spheroid and into the higher FBS solution. I also tested for the ideal concentration of matrigel to add to develop a representative basement membrane for the GBM cells to invade out of. I was responsible for taking care of my human glioblastoma MSCV-EYFP-HS683 cell line with a Crk overexpression vector. In addition, I learned how to freeze down some of my original cells and eventually thaw them out to use 4 weeks later when I needed more cells for the experiment. I learned how to change the media and add puromycin to select for stably expressed EYFP. I learned how to use the cell counter machine and take photos of my spheroid invasion assays. I also conducted a spheroid invasion assay with 4 cell lines: empty vector, Crk II overexpression, Crk II Y251F overexpression, and Abi1 iso2 overexpression. On my poster, I created the following figures myself, including Figures 1, 2, 6, and 8.
Objective:

Cancer, in its most simple definition, is uncontrolled proliferation of cells. When the cell cycle is not regulated normally, due to various reasons, cells start to proliferate abnormally and form what we know commonly as neoplasias. Melanocytic nevi and colon polyps are examples of such lesions. It is thought that every individual, regardless of their age, has a some form of abnormal cellular growth; however, not everyone has cancer. Most of the abnormally growing cells either stop proliferating by undergoing cellular senescence or alternatively, become eliminated by some other type of cell intrinsic or extrinsic tumor suppressing mechanism (1).

Cellular senescence is a cell intrinsic tumor suppressing mechanism that permanently turns off the ability of a cell to divide. We previously demonstrated that benign human neoplasias, including melanocytic nevi and colonic adenomas are comprised of cells displaying features of a telomere induced-senescence response. Cells in their malignant cancer counterparts, however, did not and instead, displayed features of proliferation (2). These data demonstrate that telomere induced-senescence is not a foolproof tumor suppressing mechanism. Indeed, some of benign tumors such as adenomatous polyp and dysplastic nevi, progress to the more advanced cancer stages colon cancer and melanoma, respectively. This shows that some cells are somehow bypassing telomere induced-senescence and regain the ability to proliferate. Using senescent and proliferative markers, our lab recently demonstrated that some regions in early neoplastic lesions display features of both telomere induced-senescence and proliferation. This suggests that these regions represent areas of senescence bypass in which some cells had inactivated telomere-induced senescence. One possible scenario how this may be achieved is that cells had reactivated telomerase, a reverse transcriptase that prevents telomere induced-senescence by various mechanisms. This hypothesis, however, has never been tested directly.

To test the hypothesis that telomerase reactivation is one of the earliest events that promotes progression of early and benign lesions to malignant cancers in humans, we wished to characterize and compare gene expression profiles in senescent and proliferating cells residing in regions of senescence bypass. Formalin-Fixed-Paraffin-Embedded (FFPE) human tissue samples were used as a source of mRNA. We first tested our ability to isolate mRNA from FFPE samples, a task that is not trivial due to fragmentation of RNA that occurs with storage in FFPE tissue (2). Upon optimization of the method, we discovered a 30 fold increase of telomerase gene expression in colon carcinoma tissue compared to that of benign colon adenoma. In addition to these studies, we also wished to characterize the mechanisms by which reactivated telomerase promotes proliferation of cancer cells. A previously generated, but never tested system to study telomerase reactivation was characterized and used to determine whether
telomerase prevents or repairs dysfunctional telomeres. TRE3G-hTERT TET3G GM21 cells, in which telomerase expression can be turned on and off by modulating doxycycline level in the culture medium, were used for these studies. After performing a series of experiments to confirm that the hTERT-dox system works as designed, we went on to test whether telomerase can repair or prevent telomere dysfunction, one of the pathways that cause cells to enter senescence.

Our preliminary data suggest that expression of telomerase suppresses formation of double stranded DNA breaks in response to DNA replication stresses.

Methods:

We performed deparaffinization of the tissue sample to remove paraffin, which was followed by RNA extraction on various FFPE tissue slides. We then performed PCR to amplify the cDNA enough for q-PCR for measuring gene expression of different genes of interest. We used different deparaffinization methods to determine the most efficient method of RNA extraction. Upon optimization, I measured the gene expression of MacroH2A, p16, p53, HP1 \( \beta \), and hTERT.

To evaluate the role of telomerase in senescence bypass, we transfected GM21 human foreskin fibroblasts, with TRE3G-hTERT, which expresses hTERT when activated, and TET3G, which activates TRE3G plasmid in presence of doxycycline. We performed a series of q-PCR experiments to evaluate the validity of the inducible hTERT construct. We tested for the best doxycycline exposure period, the ability to turn off the system, and the leakiness of the system.

TRE3G-hTERT TET3G cells (telomerase inducible cells) were treated with hydroxyurea to cause double strand breaks in DNA. Subsequently those cells were treated with doxycycline and were ImmunoFISH stained for 53BP1 to quantify the double strand breaks.

Summary of the results:

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<tr>
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<th>MacroH2A</th>
<th>p16</th>
<th>p53</th>
<th>HP1 ( \beta )</th>
<th>hTERT</th>
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<td>1</td>
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<tr>
<td>Colon carcinoma</td>
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<td>5.19</td>
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<table>
<thead>
<tr>
<th></th>
<th>MacroH2A</th>
<th>p16</th>
<th>p53</th>
<th>HP1 ( \beta )</th>
<th>hTERT</th>
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<tbody>
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<td>1</td>
<td>2.5</td>
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<table>
<thead>
<tr>
<th></th>
<th>MacroH2A</th>
<th>p16</th>
<th>p53</th>
<th>HP1 ( \beta )</th>
<th>hTERT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ductal hyperplasia</td>
<td>1.09</td>
<td>1</td>
<td>2.69</td>
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<tr>
<td>Breast invasive cancer</td>
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<td>3.96</td>
<td>1</td>
<td>1.57</td>
<td>1.35</td>
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As seen in Figure 1, there was a 31 fold increase in induction of hTERT mRNA in colon carcinoma in comparison to its benign counterpart, colon adenoma. In other benign-carcinoma pairs, the hTERT induction level difference is less than 5 folds.
Figure 2. A) Induction of hTERT mRNA in presence of different concentration of doxycycline for 72 hours in comparison to the untreated control GM21 cells. B) Optimization of hTERT mRNA induction with doxycycline treatment at 100 ng/ml for varying periods of time. C) Induction of hTERT mRNA in the presence or absence of 100 ng/ml doxycycline for 24 hours.

When TRE3G-hTERT TET3G GM21 cells are treated with different concentration of doxycycline, there is a 65 fold increase in hTERT induction compared to untreated TRE3G-hTERT TET3G GM21 cells. The concentration of doxycycline above 100ng/ml did not show difference of hTERT induction (Figure 2A). At 100ng/ml of doxycycline, the exposure time in between the range of 24 hours to 72 hours did not show a difference in hTERT induction in TRE3G-hTERT TET3G GM21 cells. When removing doxycycline after 24 hour exposure, hTERT induction decreases 17 folds in comparison to when doxycycline was present in the media (Figure 2B). TRE3G-hTERT TET3G GM21 cells have 1500 fold increase in hTERT induction even in the absence of doxycycline in comparison to untransfected control GM21 cells. TET3G cells have 3 fold increase in hTERT induction in the absence of doxycycline; however, even without the exogenous hTERT gene, hTERT induction increases 170 folds in the presence of doxycycline. Untransfected cells do not increase hTERT induction in presence of doxycycline. This indicates that doxycycline-activated-TET3G plasmid is increasing the induction of endogenous hTERT. This is a novel finding, and the mechanism is yet to be explained (Figure 2C).

Figure 3. ImmunoFISH analysis using antibodies against double stand breaks marker γH2AX shown in green and cell nuclei with DAPI shown in blue. a) GM21 untreated control. b) GM21 treated with 100µM hydroxyurea for 24 hours, recovered with fresh media for 48 hours. c) GM21 treated with 100µM hydroxyurea for 24 hours, treated with 100 ng/ml doxycycline for 48 hours. d) GM21 TET3G untreated control. e) GM21 TET3G treated with 100µM hydroxyurea for 24 hours, recovered with fresh media for 48 hours. f) GM21 TET3G treated with 100µM hydroxyurea for 24 hours, treated with 100 ng/ml doxycycline for 48 hours. g) GM21 TRE3G-hTERT TET3G untreated control. h) TRE3G-hTERT TET3G treated with 100µM hydroxyurea for 24 hours, recovered in fresh media for 48 hours. i) GM21 TRE3G-hTERT TET3G treated with 100µM hydroxyurea for 24 hours, treated with 100 ng/ml doxycycline for 48 hours. Images were taken at 63x magnification.
Conclusion:

In previous studies, our lab has shown that colon adenoma on FFPE slide has both senescent regions and proliferative regions, suggesting that senescent bypass occurs. To determine the first event that triggers senescence bypass, we want to compare the gene expression of both regions from the same tissue. However, our lab has never extracted RNA from FFPE tissue slides in the past. I was able to successfully extract RNA from FFPE tissue slides, subsequently used the extracted RNA for gene expression analysis of hTERT, and found a 31 fold increase of hTERT induction in colon carcinoma in comparison to colon adenoma (Figure 1).

To further study the role of hTERT in senescence bypass, I optimized a previously transfected, but never tested, telomerase inducible system containing TET3G and TRE3G-hTERT plasmids. Upon optimization of concentration and duration of doxycycline treatment, I measured the amount of DNA double strand breaks to quantify the effect of reactivation of telomerase. In comparison to the control GM21 cells, the percentage of cells with double strand breaks decreases by 50% in TRE3G-hTERT TET3G GM21 cells, which have constitutive high induction of hTERT, the percentage of cells with γH2AX foci is significantly lower than that of GM21 or TET3G GM21. In TET3G cells, which are also hTERT inducible, but not leaky (Figure 2C), the percentage of cells with γH2AX foci decreases when subsequently treated with doxycycline after being treated with hydroxyurea (Figure 4).

Further experiments will have to be conducted to test whether the decreased amount of DNA double strand breaks represents a reduction in dysfunctional telomeres.

Figure 4. Percentage of cells with γH2AX foci in each of the 9 treatments from figure 3. γH2AX foci represent double strand breaks. Percentage of cells with γH2AX foci increases when treated with hydroxyurea as expected. In TRE3G-hTERT TET3G GM21 cells, which have constitutively high induction of hTERT, the percentage of cells with γH2AX foci is significantly lower than that of GM21 or TET3G GM21. In TET3G cells, which are also hTERT inducible, but not leaky (Figure 2C), the percentage of cells with γH2AX foci decreases when subsequently treated with doxycycline after being treated with hydroxyurea.
References:


2. Herbig, Utz. Unpublished Data
