2015
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
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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 19 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 website at the following URL: http://njms.rutgers.edu/cancercenter/summer_program.cfm

Awardees of the 2015 Cancer Summer Research Program Poster Session and Invited Speaker for the Concluding Symposium (from Left to Right): Kevin Chen, 2nd, Brian Liu, 1st, Megan Campbell 1st, Michael Koscuiszka, 2nd place awardee.

Guest Speaker: Edmund Lattime, PhD, Associate Director for Education and Training
Rutgers, Cancer Institute of New Jersey

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

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CANCER EDUCATION PROGRAM FACULTY

While the Cancer Center building currently houses 14 laboratories, the broader Cancer Center Community (CCC) is composed of 104 investigators from several departments of the NJMS, the New Jersey Dental School and the School of Public Health, as well as the 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 2015 participating faculty and their respective departments are listed below. Department affiliations are in NJMS unless otherwise stipulated.

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The authorship format for the abstracts is as follows:

**JOHN SMITH (NJMS 2015)** [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]

Co-mentors and other contributing authors follow in order of appearance on the poster.

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IDENTIFICATION AND CHARACTERIZATION OF POST-TRANSLATIONAL MODIFICATIONS OF INTERFERON-λ3

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

Objective:

The interferon-λ family was recently discovered and has not yet been fully characterized. Interferon-λs have been shown to have antitumor and antiviral properties, making them a prime target for study. Previous work has indicated that interferon-λs, in addition to being secreted from cells, may be incorporated into the extracellular matrix. In order to further characterize the latter process, we aimed to determine the details of the 3-dimensional structure of interferon-λ3, including its post-translational modifications (PTMs) and the locations of its cysteine disulfide bridges. A PTM on the protein would increase the protein’s mass above the theoretical mass predicted by its sequence.

Although interferon-λ3 was previously crystallized, the protein used in that study was produced in *E. coli*. Based on previous work, we predicted that a PTM on the protein would be a glycosylation, a modification that would not be present on protein produced in prokaryotes. Additionally, during the purification of that protein from *E. coli*, the disulfide bridges were broken and reformed, potentially changing the location of the bridges from the biologically active form. We used proteomic analysis techniques to analyze IFN-λ3 produced in mammalian cells, aiming to determine the location and character of the PTMs and disulfide bridges.

In addition, we wished to run a pilot study to determine whether an in vitro luciferase assay could be used to determine the activity of interferons in cells without lysing the cells, as is called for by protocol. This would allow us to measure the activity in these cells across a period of time, as we would be able to take more than one reading from a given sample of cells.

Methods:

We used IFN-λ3 produced in HEK293 cells that we purchased from PBL Assay Science. The protein was further purified in-house via a molecular weight filter and transferred from a glycerol/PBS buffer to an acetonitrile/formic acid buffer. The intact protein was then analyzed for purity and molecular weight via LC-MS/MS.

The protein was then separated from its buffer via gel electrophoresis. The intact protein was then labeled with a heavy-chain iodoacetamide that would bond to free cysteine residues – i.e. any cysteines not part of a disulfide bridge. The disulfide bridges were then reduced with dithiothreitol (DTT), and the protein was labeled with a light-chain iodoacetamide that would bond to the newly free cysteine residues – i.e. the cysteines that had been part of a disulfide bridge. Samples of the labeled protein were then digested with trypsin and chymotrypsin. The resulting fragments were analyzed via LC-MS/MS. In this software-aided analysis, the measured molecular weights of the fragments were compared against the molecular weights of the fragments predicted based on the cleavage sites of the proteases used.

For the luciferase assay, we used mouse epithelial cells transfected with the luciferase gene regulated by the interferon-response promotor Mx2. Cells were cultured for several days in black tissue-culture plates. The cells were then exposed to titrations of different interferons.
(murine IFN-alpha and IFN-λ2, and human IFN-λ3) for 2, 6, or 24 hours. Luciferin diluted in media was then transferred into the wells just prior to measurement via luminometer.

Summary:

With LC-MS/MS, the mass of the intact protein was measured to be 20,690.7 Da. The theoretical mass of the protein is 19,630.8 Da; the difference was 1,059.9 Da, which was attributed to the presence of a post-translational modification. The small molecular weight of the modification agreed with the prediction that this modification would be a glycosylation. Based on previous computer studies, we predicted that this would be an O-linked glycosylation on either Thr58, Thr121, or Thr126 of the protein.

The results of the protease analysis are presented here:

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The underlined portions of the sequence were covered in this analysis. A single asterisk indicates a cysteine that was labeled with the light chain iodoacetamide, and a double asterisk indicates a cysteine that was labeled with the heavy chain.

We had approximately 75% coverage of the protein. For a sequence to be covered, there must be a fragment measured to have the same molecular weight as a predicted fragment. If there were a post-translational modification attached to a particular residue, any fragment containing that residue would not match its theoretical mass; thus, coverage of a residue via this analysis rules out that residue as a potential PTM location. Since Thr121 was covered, Thr58 and Thr126 are the remaining potential sites for O-glycosylation. Future work will involve deglycosylation of the protein via beta-elimination, allowing for characterization of the sugar moiety and better determination of the location of the PTM.

We had coverage of every cysteine residue except for Cys50. Cys148 was the only cysteine covered that was labeled with a heavy chain, indicating that it was free in the intact protein. However, only about 60% of the Cys148 fragments measured were labeled with the heavy chain; the remaining fragments were labeled with the light chain. This is likely the result of one of two situations. The folding of the intact protein could have sterically hindered the heavy-chain iodoacetamide from completely labeling the free cysteine residues; or, there could be several isoforms of the protein with different arrangements of cysteine disulfide bridges. The latter hypothesis would account for the spread of hydrophobicity that was measured in the LC-MS/MS
of the intact protein. In future study, the protein will be unfolded with SDS, which will denature all of its secondary and tertiary structure other than the cysteine disulfide bridges, allowing for better labeling of the free cysteines at the beginning of the analysis.

The luciferase assay pilot study was successful. The measured luminescence of cells decreased with the titration of cytokines, indicating that this technique could be used quantitatively in future study of cell response to interferons.

**Conclusion:**

Interferon-λ3 was studied via proteomic analysis that used LC-MS/MS to compare the measured mass of the protein and its fragments to the theoretical masses. The results indicated that there is likely a post-translational modification on the protein. Other work has suggested that this modification is an O-linked glycosylation on Thr58, Thr121, or Thr126 of the protein, and this study has ruled out Thr121 as a glycosylation site. Future work will further narrow down the character and location of this modification. Our study has also given some indication as to the cysteine disulfide bridge sites within the protein, but due to limited coverage and an inconsistent labeling of one of the cysteines, we are unable to conclusively say which cysteines are bonded to which. Future work will allow for better determination of which cysteines are part of a disulfide bridge and of whether there are multiple isoforms of the protein with different bridges. The luciferase assay pilot study indicated that *in vitro* luciferase analysis with live cells is a viable technique for future study of cellular response to interferons.
DYSREGULATION OF CALCIUM HOMEOSTASIS WITH AGE: EFFECT OF 1,25(OH)₂-VITAMIN D₃ AND HECTOROL

Sylvia Christakos, PhD, (Molecular Biology, Biochemistry and Microbiology)
Vaishali Veldurthy, PhD candidate (Molecular Biology, Biochemistry and Microbiology)
Puneet Dhawan, PhD, (Molecular Biology, Biochemistry and Microbiology)

Objective:

The active form of vitamin D, 1,25(OH)₂-vitaminD₃, is a steroid hormone which plays an important role in the endocrine system, especially in regard to the maintenance of calcium and phosphorus homeostasis. Through different calcium channels and other target proteins, vitamin D modulates a wide range of homeostatic functions mainly in the bone, kidney, and intestine. Vitamin D, cholecalciferol, is either synthesized by sunlight in the skin or ingested as a fortified dairy product, and is then hydroxylated twice to generate the active form of vitamin D. In this state 1,25(OH)₂-vitaminD₃ can either induce biological changes or be degraded by CYP24A1 (24-hydroxylase). When the body detects a calcium deficit, additional 1,25(OH)₂-vitaminD₃ is synthesized in order to generate downstream effects. As a steroid hormone, 1,25(OH)₂-D₃ translocates into the nucleus of a cell, binds to its receptor VDR, and dimerizes with RXR. Upon dimerization, the complex can then bind to DNA at a VDRE site, a Vitamin D Response Element site, in order to modulate transcriptional activity of its gene products. This complex depends heavily on the presence of primary coactivator GRIP1, which recruits a number of secondary activators such as CARM1 that function as histone methyltransferases associated with increased epigenetic expression of VDR function, to make such transcriptional changes available. With increased synthesis of 1,25(OH)₂-D₃, additional VDR, calbindin D9k, and TRPV5/6 are synthesized in order to promote calcium absorption and retention.

In aging, unknown molecular mechanisms cause dysregulation in the normal actions of 1,25(OH)₂-vitamin D₃. Less 1,25(OH)₂-D₃ is synthesized and intestinal calcium absorption decreases through decreased production of both the calcium channel TRPV6 and the calcium binding protein calbindin. Additionally, increased CYP24A1 and therefore increased catabolism of 1,25(OH)₂-D₃ is found. This results in increased age-related bone loss, intestinal dysfunction and renal imbalance. Vitamin D deficiency and abnormal calcium homeostasis have been also linked to osteoporosis and colon cancer, and have also been correlated to an increased risk of developing breast, ovarian, and prostate cancer. We sought to investigate the molecular mechanisms behind the altered 1,25(OH)₂-vitamin D₃ metabolism and calcium homeostasis during the aging process. In pursuit of such a goal, we also explored the effects of Hectorol (1αOH-vitamin D₂), a 1,25(OH)₂-D₃ analog. Hectorol is currently being used clinically in patients with chronic kidney disease (CKD) and has been suggested to be a promising therapy in osteoporotic conditions. This led us to compare the implications of injecting both young and old mice with 1,25(OH)₂-D₃ and Hectorol on gene product induction.

Methods:

In order to achieve this end, we utilized a variety of methods to determine levels of RNA of target genes. To begin the process, wild type male C57BL6 mice were raised until 2 and 20 months of age and were fed a strontium-rich diet for a week to make the mice D-deficient in order to simulate the human aging population’s deficiency. The mice were injected with 1 ng/ g
of body weight of either vehicle, 1,25(OH)2-D3 or Hectorol three times: 48, 24 and 6 hours prior to sacrifice. After killing the mice (via cervical dislocation in order to maintain the integrity of the tissues), plasma and urine were collected and the intestine (duodenum, jejunum, ileum, cecum and colon) and kidneys were harvested. Tissues were frozen and ground using liquid nitrogen with a mortar and pestle. Both protein and RNA were isolated from each tissue.

To isolate RNA, ground tissue was added to empty eppendorf tubes, to which 1 ml Ribozol was added per tube. These were incubated for ten minutes at room temperature. 200 µL chloroform was added to each tube, mixed, and incubated for 2-3 minutes at room temperature. The tubes were then centrifuged at 12,000 g for 15 minutes at 4°C which led to a separation of liquids by density in each tube. 80% of the top aqueous phase was retained and placed in a fresh eppendorf tube. RNA was precipitated out by adding 500 µL isopropanol to each fresh tube and incubating for 10 minutes at room temperature. A pellet remained in each tube after incubation, which was washed with 1mL of 75% ethanol (prepared in DEPC H2O) after the supernatant was removed. Samples were centrifuged at 7500 g for five minutes at 4°C, after which the ethanol was removed and the pellet was allowed to air dry or ten minutes. Before drying completely, 30 µL of DEPC H2O was added to each pellet and the pellet was passed several times through a pipette tip and incubated at 55-60°C to ensure it was completely dissolved. The mixture was placed on ice until needed.

In order to measure RNA levels of target genes, cDNA needed to be created from the isolated RNA to be used for PCR. For cDNA synthesis, RNA primer mix was created by the following procedure: 1µL of 50µM oligo(dT)20 was added to 1µL of a 10 mM dNTP mixture with 2µg of RNA. The volume of this mixture was then brought up to 10µL with the addition of DEPC-treated water. This mix was incubated at 65°C for five minutes and then briefly placed on ice. The cDNA synthesis mix was made by adding 2 µL 10X RT buffer, 4 µL 25mM MgCl2, 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 combination was mixed gently and briefly centrifuged. This was followed by a 50 minute 50°C incubation, and then by a five minute 85°C incubation to terminate the reaction. Tubes were centrifuged and 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. PCR settings were dependent on the particular gene being amplified and varied as such. Gel electrophoresis was used to then visualize PCR products and Image J calculated the fold induction present from those gel bands via densitometry.

**Summary:**

**Calbindin D9k intestinal mRNA:**

Results from calbindin D9k studies exhibited a few trends. First, that 20 month old mice are less responsive to both 1,25(OH)2-D3 and Hectorol than 2 month old mice, which could provide a mechanism behind some of the changes which occur during the aging process. Secondly, that overall both young and old mice are more responsive to 1,25(OH)2-D3 than Hectorol. Thus the highly calcemic response which accompanies the injection of 1,25(OH)2-D3 does not analogously occur with injection of Hectorol. This would suggest Hectorol may prove to be an alternative to 1,25(OH)2-D3 but would result in lower serum calcium levels. Additionally, it appears given these results that the distal intestine is more responsive to 1,25(OH)2-D3 with aging as opposed to the proximal intestine. The distal intestine is where 70-80% of ingested calcium is absorbed, and this would suggest that there is a possibility for targeted calcium absorption therapy.
Figure 1. RTPCR using total RNA from intestine of 2 and 20 month old C57BL6 mice. Results showed that 20 month old mice are less responsive to both 1,25(OH)$_2$-D$_3$ and Hectorol than 2 month old mice, and that overall, both young and old mice are more responsive to 1,25(OH)$_2$-D$_3$ than Hectorol. Additionally, it appears the distal intestine is more responsive to 1,25(OH)$_2$-D$_3$ with aging as opposed to the proximal intestine (PI; Ileum, Ile; Cecum, Ce; Colon, Co).

CYP24A1 renal mRNA:

Results from 24-hydroxylase mRNA are the following: the first observation was that young and old mice were nearly equally responsive to 1,25(OH)$_2$-D$_3$ in induction of CYP24A1 mRNA. Given that during aging, levels of 1,25(OH)$_2$-D$_3$ decrease, the continuously high induction of 24-hydroxylase would contribute to an even higher deficit of 1,25(OH)$_2$-D$_3$ as it is the enzyme entirely responsible for the breakdown of 1,25(OH)$_2$-D$_3$. In contrast, both young and old mice did not respond to Hectorol with induction of CYP24A1 mRNA. This finding is particularly interesting, especially in light of our findings with Hectorol in the calbindin mRNA results. This would suggest that Hectorol is able to maintain physiological levels of 1,25(OH)$_2$-D$_3$ by acting as an analog, yet not induce the proportional levels of 24-hydroxylase, resulting in a net increase in serum 1,25(OH)$_2$-D$_3$. Such an implication would be of significant therapeutic potential for osteoporosis and aging patients. Further *in vivo* studies must be performed to get an accurate picture of the genomic implications under each of these conditions.

Figure 2. RTPCR using total RNA from kidney of 2 and 20 month old C57BL6 mice. Young and old mice were equally responsive to 1,25(OH)$_2$-D$_3$ (+D) in induction of CYP24A1 mRNA. Both young and old mice did not respond to Hectorol (+H) with induction of CYP24A1 mRNA.

Conclusion:

Our results showed that 20 month old mice are less responsive than 2 month old mice to 1,25(OH)$_2$-vitaminD$_3$ or Hectorol with regard to induction of calbindin-D9k mRNA. This finding suggests a mechanism for lower calcium status in aging. In addition, the distal intestine is more responsive to 1,25(OH)$_2$-vitamin D$_3$ than the proximal intestine with age, suggesting translational potential of targeting the distal intestine to maximize calcium absorption. Since the
distal intestine is responsible for 70-80% of ingested calcium absorption, this suggests a promising avenue of targeting the distal intestine in those who are calcium deficient.

Hectorol induced less CYP24A1 mRNA in C57BL6 young and aging mice as compared to 1,25(OH)₂-vitamin D₃. Hectorol may therefore be an effective analog of 1,25(OH)₂-vitamin D₃ which may result in less catabolism of 1,25(OH)₂-vitamin D₃ and therefore increased availability of calcium. Further in vivo studies are needed to investigate these effects.

References:

REGULATION OF AUTOPHAGY AND THE SECRETOME BY HISTONE DEACETYLASE 6 IN KAPOSI’S SARCcoma-ASSOCIATED HERPESVIRUS REACTIVATION

Hyejin Shin, Ph.D. (Department of Microbiology, Biochemistry, and Molecular Genetics)
David M. Lukac, Ph.D. (Department of Microbiology, Biochemistry, and Molecular Genetics)

Objective:

Kaposi’s Sarcoma-associated Herpesvirus (KSHV) is a DNA tumor virus implicated in the etiology of Kaposi’s sarcoma (KS) and lymphoproliferative diseases like primary effusion lymphoma (PEL) and multicentric Castleman’s disease. The reactivation of KSHV from latency and production of mature, viral progeny are 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. Despite this link between reactivation and autophagy, a question that remains unclear is whether autophagy is an inducer of viral reactivation or autophagy is a response to viral reactivation. KSHV latency proteins inhibit autophagy while histone deacetylase inhibitors (HDACi) and other known inducers of KSHV reactivation have been found to induce autophagy, even within uninfected cells. The ability of HDACis to induce reactivation of KSHV is troubling, as many of these inhibitors are used in cancer therapeutics. Given that autophagy can regulate cell secretion, we hypothesize that autophagy links KSHV reactivation to cancer by reprogramming the host secretome and promoting the expression of viral paracrine, pro-inflammatory genes; in our model, the reprogrammed secretome also promotes viral reactivation. The overall goal of this project is to investigate the secretome of KSHV-infected cells to refine our understanding of how autophagy influences viral reactivation and ultimately the development of cancers. Elucidation of these mechanisms is critical to determining whether or not HDACis belong in cancer treatments. Additionally, a better understanding of HDAC6 and KS-related autophagy can allow for the manipulation of autophagy to restore chemosensitivity and make tumor cells amenable to cytotoxic treatments.

Methods:

Tissue Culture:

Vero rKSHV.294 and 293 MSR tet-OFF 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.

**Determining the effect of VPA on the secretome:**

BCBL-1 cells latently infected with KSHV were incubated in serum free media or treated with 1mM VPA in serum free media. Cells were incubated for 24 or 48h. The media that cells were grown in (supernatant, S) were isolated, and cells were lysed (pellet, P). The cell lysate was treated with a protease inhibitor cocktail, and cellular debris was cleared by centrifugation. Total S and P protein concentrations were determined using Bradford assay. Eight hundred micrograms of protein from each group were separated by SDS-PAGE. Proteins were electrophoretically transferred to a nitrocellulose membrane in transfer buffer and tested using Western for expression of HMGB1.

**Determining the effect of serum starvation on autophagy:**

BCBL-1 cells latently infected with KSHV were treated with media containing FBS, media without FBS, or media containing FBS and 1mM valproic acid and incubated for 24h or 72h. After treatment, cells were fixed using methanol/acetone. Cells incubated for 24h were stained for ORF59 or LC-3B, and cells incubated for 72h were stained for K8.1. Nuclear DNA was stained using DAPI. ORF59, LC-3B puncta, and K8.1 were 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). HMGB1, a non-histone, DNA-binding protein suggested as a secreted protein involved in the pathogenesis of KSHV, was found to be secreted in BCBL-1 cells at 48h regardless of treatment with VPA. Serum-
starvation induced HMGB1 secretion whereas treatment with VPA inhibited serum-starvation induced HMGB1 secretion (Fig. 4A, 4B, 4C). Serum-starved cells showed comparable levels of ORF59 and K8.1 induction and LC-3B induction to cells grown in media containing serum (Fig. 5A, 5B, 5C).

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 in a concentration-specific manner, suggesting that HDAC6 promotes autophagy in response to reactivation.
• Treatment of VPA can alter the secretome, supporting the idea that reactivation of KSHV can reprogram the secretome through autophagy.
• Serum starvation induced HMGB1 secretion, but VPA treatment (reactivation) inhibited serum-starvation-induced HMGB1 secretion.
• Serum starvation is insufficient to overcome the inhibition of autophagy by KSHV oncoproteins in latently infected cells, supporting the co-regulation of reactivation and autophagy.

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 and ultimately resulting in reprogramming of the secretome.

Figures:

**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, suggesting that HDAC6 promotes autophagy in response to reactivation.

Figure 4. Reactivation of KSHV using VPA ultimately reprograms the secretome. (A) HMGB1 is secreted at 48h regardless of treatment with VPA. (B) Treatment with VPA decreased serum-starvation induced HMGB1 secretion. (C) Cell lysates (pellets) were tested for HMGB1 expression and compared to α-Tubulin expression.
Figure 5. Serum starvation is insufficient to overcome the inhibition of autophagy by KSHV oncoproteins in BCBL-1 cells. KSHV-infected BCBL-1 cells were grown in media containing FBS, media without FBS, or media containing FBS and 1mM VPA and incubated for 24h ((A) ORF59 and (B) LC-3) or 72h ((C) K8.1). ORF59, LC-3B puncta, and K8.1 were detected using indirect immunofluorescence. Nuclear DNA was stained using DAPI. Note the increase in number and intensity of LC-3B puncta and expression of ORF59 and K8.1 in VPA treatments.
KEVIN CHEN, (NJMS 2019)

ALTERNATIVE POLYADENYLATION OF CANCER-RELATED GENES IN JEG3 AND HTR8 CELLS

Bin Tain, Ph.D., (Microbiology, Biochemistry and Molecular Genetics)

Objective:

The 3’ end of most eukaryotic precursor mRNAs is cleaved and polyadenylated during transcription. Since a large percentage of human genes contain more than one polyadenylation site (pA), there are many different possible locations for adding the poly(A) tail, resulting in alternative cleavage and polyadenylation (APA) [1][4]. APA can produce isoforms that differ in coding (CDS) or non-coding regions (3’ UTR), with variable protein functions and/or regulations by microRNAs or RNA-Binding proteins (RBPs). As such, APA is a very important regulator of gene expression. APA regulation is especially relevant for cancer cells. It has been reported that cancer cells tend to have short 3’UTRs due to high proliferation rate [2]. In addition, the APA profile has been shown to help categorize tumor subtypes with differing survival consequences, which would make it a very important factor in cancer development and possibly a future marker for cancer diagnosis [3]. We focused on APA differences between HTR8 and JEG3 cell lines in this study. JEG3 is a primary clone of choriocarcinoma (malignant, trophoblastic cancer that commonly occurs in the placenta) and HTR8 is a transformed extravillous trophoblast cell line that is frequently utilized to model the first trimester extravillous trophoblasts[5]. These cells show characteristics of stem or progenitor cancer cells yielding a greater cause for study since they may have a large impact on tumor initiation, drug resistance, and tumor aggressiveness. We hypothesize that cancer-related genes will show a higher degree of APA regulation between the JEG3 and HTR8 cell lines.

Methods:

We used the Cancer Gene Census (CGC) [6] from Catalogue of somatic mutations in cancer (COSMIC) to define cancer-related genes. The CGC cancer Gene Census consists of 571 genes for which mutations have already been causally implicated in cancer. This data was then intersected with JEG3 and HTR8 3’READS data to create two subsets, the cancer related gene list and non-cancer related gene list. The data acquired through 3’READS was analyzed and the significance score of APA (APA SS) between JEG3 and HTR8 cell lines was calculated using Fisher’s exact test on the relative abundance of APA isoforms. These were then sorted to find the genes with significant APA regulation differences between the JEG3 and HTR8 cell lines, and the top 4 genes with greatest absolute value of APA SS were selected for in depth study.

Fisher’s exact test is a test for statistical significance generally based on a contingency table and is utilized for categorical data when the sample size is too small for a chi-squared test for independence. It yields an exact p-value rather than an approximation that improves in accuracy as sample size approaches infinity.

Cell culture - JEG-3 was maintained at 37°C in complete growth medium made of 90% Eagle’s Minimum Essential Medium(EMEM) and 10% Fetal Bovine Serum (FBS). Subculturing of the cells consisted of removing the medium, rinsing with 0.25% trypsin, 0.03% EDTA solution, removing the solution and adding an additional 1-2 ml of trypsin-EDTA solution. Then the cells were allowed to sit until they detached after which, fresh culture medium was added, then aspirated, and finally dispensed into new culture flasks. The recommended subcultivation ratio is between 1:4 and 1:6 with the medium renewed 2 to 3 times per week[7]. HTR-8 was
maintained at 5% CO₂ and 37°C in RPMI 1640 solution with 5% FBS and no antibiotics. It was split using Trypsin 0.05% EDTA twice a week was not allowed to grow past 90% confluency[8].

3’ READS stands for 3’ Region Extraction And Deep Sequencing. This method is designed to circumvent the internal priming and oilgo(A) tail issues that are generally associated with identifying poly(A) sites [9]. The use of 3’READS avoids false positives from mistaking internal A-rich sequences for the actual poly(A) tail, the internal priming issue, that is found in other methods by eliminating the use of oilgo(dT) in reverse transcription and sequencing. The oilgo(A) tail issue is alleviated by setting a condition that distinguishes RNA with long and short A-tails [9]. When compared to other deep sequencing methods that use oilgo(dT) in reverse transcription such as PolyA-seq[22] and PAS-seq[23], 3’READS generated >10-fold fewer reads mapped to rRNA, snoRNA, and snRNA genes [9], proving significant alleviation of issues caused by internal A-rich sequences and oilgo(A) tails.

The procedure begins after fragmentation of RNA, the poly(A)-containing RNA fragments are captured onto magnetic beads coated with a chimeric oligonucleotide (oligo), which contained 45 thymidines (Ts) at the 5’ portion and 5 uridines at the 3’ portion (CU₅T₄₅). A condition is set which enriches RNAs with 60 terminal As by ~12-fold as compared to those with 15 As (Figure 1b). RNase H digestion is used to release RNA from the beads and to remove most of the As of the poly(A) tail. Eluted RNA was ligated to 5’ and 3’ adapters, followed by reverse transcription, PCR amplification, and deep sequencing[9].

Summary:

**Figure 1.**

**Alternative polyadenylation Analysis**

(a) The figure is a stacked bar graph depicting the percentage of genes with APA significance score greater than or equal to a designated cut off value at 3, 5, 10, 15, and 20. The comparison of this statistic in non-cancer and cancer genes show significance at all but APA SS>=20.

(b) This figure depicts the side by side comparison of the poly(A)site percentage with APA SS scores greater than a specific cut off value for cancer and non-cancer genes. This showed significant p values at cut offs 5, 10, and 15 but not the lowest or highest values at 3 and 20. (c) The bottom most figure shows a graph of the –log(P Value) of each APA SS cut off value from 3 to 20 inclusive. This shows a marked high point at APA SS>=14 with a fairly consistent loss of significance as the cut off value increases towards APA SS>=20.
The top 4 genes selected based on absolute value of APA SS calculated through Fisher's exact test from the intersected data set from Tian lab of genes studied in cell lines HTR8 and JEG3 and the cancer Gene Census[4]. WHSC1, NONO, XPO1 genes have somatic tumors while EXT1 gene shows a germline tumor type.

**Figure 2. COSMIC Gene Descriptions**

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<th>Tumor Type: Somatic</th>
<th>Tumor Type: Germline</th>
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<td>Multiple myeloma</td>
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<td>Exportin 1 (CRM1 homolog, yeast)</td>
<td>Chronic lymphocytic Leukemia</td>
<td></td>
<td>24</td>
</tr>
</tbody>
</table>

**Figure 3. UCSC Browser Genome Tracks**

These figures depict the most significant alternative polyadenylation sites of the top 4 genes. The difference in relative peak height between the poly(A)sites in each cell line was compared to identify significant differences in APA. The top most picture of each figure shows the full gene and directly below that lies the zoomed in section of that gene to scale with the peaks below. The two areas showing peaks are the cell lines HTR8 and JEG3 cell lines respectively running from 3' to 5'. The peaks are measured in reads per million, or RPM, and can represent isoform expression. The track below shows the conservation of the gene, a measure of how similar the sequence is across species, measured against 100 species in this case. The most bottom label on the figures show the distance between poly(A)sites in nucleotides. The tumor type is also shown to the right of the chart.
Conclusion:

The analysis of the 3’ READS data through Fisher’s exact test shows a significant difference in the regulation of alternative polyadenylation between cancer affiliated genes and non-cancer genes in cell lines JEG3 and HTR8. These results coincide well with our hypothesis that the highly proliferative cancer cells would exhibit stronger APA regulation in cancer genes compared to that of non-cancer genes. We further compared the genes by calculating the percentage of genes in both cancer and non-cancer data sets with APA significance score greater than varying cut offs. Using a significance level of alpha = 0.05, the p-values calculated show that all cut off values except for APA SS>=20 are indeed significant. The lack of significance at a higher APA SS could be due to the much smaller data set used for cancer genes as opposed to the non-cancer genes and the greater variability of APA SS at higher levels allowing the selection of cut off score to be more influential. The use of 3’READS as opposed to other deep sequencing methods yielded results due to the lack of false positives from (A) rich areas and alleviated the issues in internal priming and oligo(A)tails.

The cell lines JEG3 and HTR8 both show progenitor/stem cancer cell characteristics that make them especially of interest. A stem cancer cell plays a large role in the initiation and progression of tumors. We have seen a marked APA regulation difference between cancer and non-cancer genes in these cell lines and previous studies have already shown that APA profiles could possibly be utilized in future diagnostic markers.

References:

ANIRUDH K. GOYAL (TCNJ 2018/NJMS 2021)

COMBINATION OF METFORMIN + ORLISTAT PREVENTS TUMOR GROWTH

Mariana S. De Lorenzo, Ph.D. (Department of Cell Biology and Molecular Medicine)

Objective:

The only non-pharmacological intervention known to date to combat cancer is caloric restriction (CR). CR has been shown to increase longevity as well as reduce adiposity levels. Lower adipose levels correlate with lower rates of obesity and lower risk for the development of malignancies like cancer (Fontana 2007). Dr. De Lorenzo's group has reported recently for the first time the impact CR has on metastases and ECM formation. They showed that tumor metastasis were significantly reduced by CR. Additionally, it was observed that there was a significant decrease in the levels of matrix metalloproteinase-9 upon CR, essential to the formation of a viable ECM for cells and hence needed for proliferation and cancer (De Lorenzo 2011).

However, CR can be hard to adopt in a daily regimen and so CR mimetic drugs are also available. Metformin is an anti-diabetic drug and mimics CR. In addition, Orlistat is an anti-obesity drug which inhibits the fatty acid synthase (FAS) and reduces tumor cell proliferation (Kridel 2004). It prevents cell proliferation and colonies from developing by arresting breast cancer cells in G1 (Alimova 2009). Orlistat blocks the fatty acid synthesis and indirectly constrains tumor growth.

Our hypothesis is that the combination of Metformin, and Orlistat enhances the anti-cancer properties of each drug alone, leading to a beneficial reduction in 4T1 mammary tumor progression. Tumor volume, incidence of metastasis to the lungs, adhesion capacity, migration and angiogenesis were measured to see the impact on 4T1 cellular growth upon various treatments. Our objective was to elucidate whether the combination of Metformin and Orlistat suppresses tumor cell properties and the secretion of ECM molecules and adipokines. To this end, the levels of vascular endothelial growth factor, insulin-like growth factor-1, metalloproteinase-9, and fibroblast growth factor 21 were immunoassayed to study the effects of treatments on the secretion of these molecules to the 4T1 cells-conditioned medium.

Methods:

In vivo-Induced Angiogenesis. Mice were fed regular chow food and administrated: vehicle, oral MET (3.3 mg/ml), OR injection (240mg/kg/d) or MET+OR. Mice (n=3/group) were treated for 3 weeks previous to the 4T1 cells injections (10⁵ cells/mouse) and then will continue on treatments for 7 days. At that time, mice were sacrificed and angiogenesis was photographed using a magnification microscope. Pictures were taken for quantification purposes.

Cell Culture. Medium from plates was aspirated and rinsed with 5 mL of phosphate buffered saline (PBS). Another 4.0 mL of PBS was added to plates along with 1.0 mL of Trypsin-EDTA (10X) to promote the disaggregation of cells from the plate. Light tapping was implemented to promote further separation. Each plate was added to a separate tube with 5.0 mL of growth medium and centrifuged. Upon breaking and mixing the resulting pellet, a 40 μL sample was taken and stained with trypan blue dye. The average number of cells per mL was calculated by multiplying the average number of cells in one unit of a 9x9 array by 9 (for total number of units in array) by 2 for the dilutions and then by 1000 to adjust the volume for 1 mL.

Cell Adhesion Assay. Upon determining the number of cells per mL, adjustments were made accordingly to obtain 25,000 4T1 mammary cells per well for two 96 well plates. Six treatments
were established with groups as follows: vehicle (control), 1 mM of MET, 2.5 μM OR, 5 μM OR, 1mM MET + 2.5 μM OR and 1mM MET + 5 μM OR. Each of 32 wells had one of the above treatments added as well as the appropriate amount of cell supernatant. The wells were incubated for 90 minutes and then the medium was aspirated out. Cells were stained with 100µL of MTT (Thiazolyl Blue Tetra-zolium Bromide)/PBS mixture. After another 3-4 hours of incubation, 100 µl of DMSO was added to each well. After 30 minutes incubation at 37 °C, the absorbance was then measured at 560 nm using a spectrophotometer.

Wound Healing Cell Migration Assay. 600,000 4T1 mammary tumor cells were seeded in each well of a 6-well plastic plate. They were left to proliferate and adhere for 24 hours in an incubator before being treated. Afterwards, two straight lines were drawn in the confluent monolayer and cells were treated with either basal medium or 1% FBS in the presence of vehicle, Metformin, Orlistat or a combination of both. Cells were once again incubated for 24 hours. After the final incubation period, a control line was drawn as a representative of zero migration. Then confluent monolayers were fixed and stained with crystal violet. Photographs were taken of the areas free of cells as an indication of no-migration area.

Immunoassays. FGF-21, MMP-9, VEGF, CXCL1, IL-6 and IGF-1 levels were measured by Quantikine immunoassay kits. Prior to ELISA, 4T1 cells were treated with doses of 1mM Metformin, 2.5μM and 5μM Orlistat and combination for 24 hours. Then, the medium were collected and centrifuged.

Tumor Collagen Concentration. 4T1 tumor samples from mice treated with water, vehicle, 3mg/ml metformin, 240mg/kg/day Orlistat or combination were fixed in a 10% formalin solution; to identify collagen deposits, the samples were stained with picric acid Sirius Red (Sigma Aldrich Corp). Extracellular collagen deposits were photographed and recorded by a Nikon E800 Eclipse; each image from a treatment was broken down into twenty pictures. Each picture was analyzed through the software Image J and the collagen volume fraction was measured by percentage of pixels staining red in each image.

Summary:

Tumor Volume. Volume of tumor was significantly lower in mice that had a combination of Metformin plus Orlistat than any other treatment with drugs acting independently (data not shown).

Angiogenesis. It is seen that a combination of the drugs with Metformin and Orlistat showed the significantly smallest proportion of vessel formation around the tumor relative to the control treatment and a lower levels of VEGF secreted by the 4T1 cells treated with Metformin + Orlistat (Figures 1 and 2).

Metastases. Metastases were assessed by measuring incidence of tumor nodules developing in the lungs that spread from the tumor on the mammary gland fat pad. There were significantly fewer nodules on lungs dissected from mice treated with a combination of Metformin and Orlistat when compared to any other treatment.

Intra-Tumoral Collagen Deposition. Upon extracting and staining 4T1 mammary tumors, treatments with a combination of MET and OR showed, on average, the lowest level of collagen deposition (Figure 3).

Adhesion. Upon performing adhesion assays to plastic, treatments with a combination of 1 mM Metformin and 5 μM Orlistat had cells with a significantly lower adhesion to the plastic than the vehicle. However, 5 μM of Orlistat alone seemed to minimize the affinity to plastic most significantly. However, the combination treatment above, on average, decreased the 4T1 mammary tumor cells adhesion to collagen IV the most compared to any other treatment (Figures 4).
Migration. The area free of cells, an indicator of the migration capacities of 4T1 cells, was significantly greater when the treatments with drugs were applied. However, the data suggest that Orlistat independently at 5 μM or 1 mM of Metformin and 2.5 μM of Orlistat showed the most significant greatest inhibition to migration (Figure 5).

ELISA Analysis. Analyzing the data from the immunoassays indicated that there was no significant difference in FGF-21, IGF-1 or MMP-9 levels at the doses of drugs used for the treatments. However, VEGF levels were found to be significantly lower in the combination of both Metformin and Orlistat (1 mM and 5 μM respectively). Levels of cytokines IL-6 and CXCL1 were also found on average be the same in each group.

Conclusion:
In conclusion, the experiment did strongly suggest that tumor growth is inhibited by a combination of both drugs as tumor volume was drastically lower with the drugs. Other aspects of the tumor were confined by the drugs such as reduced angiogenesis as well as reduced metastasis throughout the body, specifically the lungs. Additionally, the migration data supported that the cells in the tumor were less mobile and less readily able to spread. Hence, the drugs definitely worked to combat the mammary tumor on the external level. However, the cellular mechanic is still unknown. It has been shown that adhesion to collagen IV has decreased with the drugs together indicating a possible interference with this protein and attachment. Additionally, lower levels of collagen and fibrosis were observed in the tumors which may be tied back to less viable adhesion and anchorage of the cancer cells and further establishes vulnerability. When studying certain proteins involved in the regulatory pathways of the two drugs such as IGF-1 and FGF-21, no notable difference was observed at the doses used of Metformin and Orlistat. Prior research even suggested how CR can work and affect MMP-9 and yet no differences in concentrations of all the above molecules could be observed. This could very well be due to employing such a low dosage. Other studies suggested use of MET hovers around 10 mM but the intent was also to try to minimize the dosage delivered while still trying to elicit results (Kridel 2004). The low dosage may not have been sufficient enough in disrupting the respective cell pathways for the above proteins. Yet, VEGF levels, important in vessel formation, were shown to be reduced significantly with a combination of the drugs, further suggesting that VEGF plays a role. Additionally, proinflammatory agents like CXCL1 and IL-6 were tested and no significant difference was shown in the treatments, which suggests that these drugs do not work to target the inflammatory response. As for now, a combination of Orlistat and Metformin does seem to inhibit tumor growth and data strongly suggest it is via angiogenesis and alterations in collagen deposition. However, further trials need to be run along with more immunoassays to better elucidate the cellular disruptions involved.
Figure 4: Collagen IV adhesion capacity of 4T1 mammary tumor cells. 25,000 4T1 mammary tumor cells were seeded per collagen IV-coated well in two 96 well plates in basal medium containing vehicle or the drug(s). ***P<.001 vs vehicle; n=32 for each group.

Figure 5. Effect of the drugs Orlistat and Metformin on 4T1 mammary tumor cell migration. 4T1 cells were pre-treated in 1640-RPMI Serum containing either the vehicle or the drug(s). n=9-15; *P<.05, **P<.01 and ***P<.001 vs vehicle; n=8-12.

References:

Objective:

Most cancer cell lines are characterized by increased rates of aerobic glycolysis. This finding is known as the “Warburg effect” and serves as a potential target of anti-cancer therapy. This study examines how the growth of human bladder and colon cancer cell lines can be inhibited by the following anti-glycolytic compounds: oxamate, isosafrole, and 1-(3-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PQP). Oxamate and isosafrole are inhibitors of lactate dehydrogenase (LDH) which catalyzes the conversion of pyruvate to lactate in the final step of glycolysis. PQP is an inhibitor of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3). PFKFB3 catalyzes the conversion of fructose-6-phosphate to fructose-2,6-bisphosphate, which is an allosteric activator of phosphofructokinase-1 (PFK1). PFK1 catalyzes the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate which is an important regulatory step in glycolysis.

The present study also observed for potential additive effects on growth suppression by exposing tumors to oxamate or isosafrole in combination with PQP or phenformin. Phenformin is a biguanide, like metformin, that has been used in the past to treat type II diabetes and has been found to have anti-cancer effects. The mechanism of action by which phenformin suppresses tumor growth remains ambiguous. One theory is that phenformin inhibits complex I of the electron transport chain.

In a previous experiment, Lea et al. observed the inhibitory effects of 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) on the growth of bladder and colon cancer cell lines. Like PQP, PO is an inhibitor of PFKFB3. The potency of PQP in the present experiment was compared to that of 3PO in past experiments.

Methods:

Cell culture. 5637, SW780, T24, UM-UC-3, HT1197, RT4, TCCSUP, and HT1376 human bladder cancer cell lines and HT29 and Caco-2 human colon cancer cell lines were used in the present study. They were maintained in 96-well plates at 37°C in 5% CO₂ in RPMI-1640 medium with 5% fetal calf serum. After 24 hours, the medium was then changed to include different doses and combinations of reagents. Cells were then incubated for another 72 hours.
Reagents. PQP was administered as a single agent and in combination treatments with oxamate and isosafrole at 5 μM. Isosafrole was administered as a single agent and in combination treatments with PQP and phenformin at 0.25 mM and 0.5 mM. Oxamate was administered as a single agent and in combination treatments with PQP and phenformin at 10 mM and 20 mM. Phenformin was administered as a single agent and in combination treatments with oxamate and isosafrole at 25 μM and 50 μM.

Determination of cell proliferation, pH, and glucose uptake. Cell proliferation was measured via staining of protein with sulforhodamine B followed by measuring light absorbance at 510 nm with a microplate reader. Medium pH was determined via phenol red light absorbance at 560 nm with a microplate reader. Glucose uptake was determined using Kit GAGO-20 from Sigma-Aldrich, St. Louis, MO, USA.

Statistical evaluation. Data are presented as means and standard deviations. Statistical significance was assessed using a two-tailed Student’s t-test or Dunnett’s test for multiple comparisons. A P-value of <0.05 were considered statistically significant.

Summary:

PQP had significant growth inhibitory effects on all cancer cell lines, except for RT4 in 1 of 2 trials. Isosafrole had significant growth inhibitory effects on all cancer cell lines, except for UM-UC-3 in 2 of 3 trials and SW780 and HT1197 in 1 of 2 trials. PQP and isosafrole in combination had significant growth inhibitory effects on all 10 cancer cell lines. Additive effects on growth inhibition were observed in the following cell lines when treated with both PQP and isosafrole: UM-UC-3, RT4, and HT1376. Additive effects were also seen with T24 in 1 of 2 trials.

There was significant growth inhibition when all cells, except for Caco-2, were incubated with phenformin. Combination treatment with phenformin and isosafrole had significant growth inhibitory effects on all cell lines and additive effects on Caco-2 and RT4. Isosafrole appeared to reverse decreases in medium pH and increases in glucose uptake associated with phenformin.
Oxamate had significant growth inhibitory effects in all cells, except for SW780 and RT4. Growth inhibition was significant in all cells with the combined treatment of oxamate and PQP. Additive effects were observed with the following cell lines: UM-UC-3, HT1197, RT4, and T24.

Changes in medium pH and glucose uptake were more readily detected in the more rapidly growing HT29, T24, and UM-UC-3 cells. Oxamate, isosafrole, and PQP as single agents and all combination treatments generally decreased medium acidification and glucose uptake, while phenformin as a single agent essentially had the opposite effects.
Conclusion:

Our results demonstrated that PQP, oxamate, isosafrole, and phenformin are effective growth inhibitors of most colon and bladder cancer cell lines when administered both as single agents and in combination. Combination therapy is widely considered the best approach because it reduces the likelihood of drug resistance. In addition, administering drugs at lower concentrations decreases the possibility of toxic side effects. In the present study, we found that the PFKFB3 inhibitor PQP had growth inhibitory effects at lower concentrations than its counterpart 3PO in all cancer lines assessed, except for RT4. Similarly, the LDH inhibitor isosafrole had growth inhibitory effects at lower concentrations than oxamate in most cancer lines used in the present study.

The therapeutic potential of combined treatment with inhibitors of PFKFB3 and LDH merits further investigation. Future work in this lab will include use of the LDH inhibitor methyl 1-hydroxy-6-phenyl-4-((trifluoromethyl)-1H-indole-2-carboxylate (NHI-2), which has been proven to have cytotoxic effects on cancer cells at lower concentrations than isosafrole. PFK-15 and PFK-158 are also known PFKFB3 inhibitors and the latter is currently in phase I clinical trials for patients with advanced solid tumors. It would also be interesting to examine how these small molecule inhibitors compare to PQP with respect to growth inhibition of bladder and colon cancer cell lines.

References:


Acknowledgements:

This research was supported by the NJMS Hispanic Center of Excellence Health Resources and Services Administration through Grant D34HP26020 and the Alma Toorock Memorial for Cancer Research.
Objective:

Cancer patients are known to have a high comorbidity with depression, with studies indicating ranges between 20-50% depending on the type of cancer, as compared to an incidence of 6.7% in the general population (2, 5, 7). In this study we looked for demographic correlations between those patients that screened positive for depression and social support factors, including marital and employment status.

The importance of social support in dealing with traumatic health events is well understood, as individuals in the general population with a social network, including spouses, friends and family are typically in better health than those without as many social contacts (3). Cancer can be a difficult diagnosis for patients, and the support of a robust social network can help patients navigate the course of treatment (11). Patients in urban hospitals often talk less with their doctor about their emotional state, so existing social networks are especially important (1).

Existing studies evaluating depression levels and different social support factors in oncology patients have found mixed results, with variation between types of cancer and time since diagnosis. A systematic review of studies on patients with head and neck cancer found that being unmarried or living alone correlated with higher levels of depression in five studies, but was not correlated in two others (4). In breast cancer, studies have found that marriage is often associated with depression (p =0.041), which may be due to physical consequences of breast cancer and sexuality concerns with marital partners (10). A study in colon cancer patients found that lower levels of social support are associated with higher levels of depression, but disease progression modulates the association (9).

The objective of this study was to look for an association between depression level and social support factors, including marital and work status among outpatients at the New Jersey Medical Cancer Center, regardless of patients’ diagnosis or prognosis.

Methods:

This project is part of an ongoing questionnaire-based study to evaluate prevalence of depression symptoms in outpatient English-speaking oncology patients at New Jersey Medical Cancer Center, as well as personal and perceived stigma of depression. The study questionnaire includes the CES-D depression screener, for which a score of 16 or greater is positive for clinically significant depression symptoms (6). This project leveraged the data from the CES-D depression screener as well as demographic identifiers, including marital and work status to evaluate social support.

1) What is your current marital status?
   a. Married
   b. Marriage like relationship
   c. Separated/Divorced
   d. Single
   e. Never Married
   f. Widowed
2) What is your current work status?
   a. Working full-time (35 or more hours/week)
   b. Working part-time
   c. Unemployed
   d. Disabled

Summary:

226 patients were surveyed for this study. Patient ages ranged from 21 to 86; the mean age of patients surveyed was 54.68 (SD 11.74). 54.2% of the patients surveyed were female, and 85% of patients had a household income under $34,999. 30.4% of patients had completed high school or obtained a GED, and another 26.3% had completed some college but had no degree. 50.5% of patients surveyed identified as Black or African American, 25.2% identified as White and 18% identified as Hispanic or Latino.

Table 1
Oncology patient demographics (N=226)

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<th>Category</th>
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<td></td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eighth grade or less</td>
<td>13</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Some high school</td>
<td>42</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>High school or GED</td>
<td>68</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Some college</td>
<td>59</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>College graduate or higher</td>
<td>42</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Household Income</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Under $25,000</td>
<td>133</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>$25,000 to $34,999</td>
<td>40</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>$35,000 to $49,000</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>$50,000 to $74,999</td>
<td>9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>$75,000 to $99,999</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>$100,000 to $149,999</td>
<td>1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>$150,000 or more</td>
<td>1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Cancer Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>16</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>50</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>62</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Stage IV</td>
<td>41</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Localized</td>
<td>105</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Metastasized</td>
<td>64</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

Of the 226 patients surveyed, 32 ± 6 % were found to have clinically significant levels of depression, as indicated by a score of 16 or higher on the CES-D questionnaire. The mean score for all respondents on the CES-D scale was 13.02 (SD: 9.78). Gender was found to be significantly associated with depression ($\chi^2 = 5.047$, p=0.025): more women screened positive for depression (38%) than men (24%).

Marital status was found to be a statistically significant predictor of a score of ≥ 16 on the CES-D Depression Screener by a Likelihood Ratio Chi-Squared test ($\chi^2=12.307$, p=0.031). Within marital status, patients who identified as being “married” or in a “marriage-like relationship” had significantly lower rates of depression (p=0.003, p=0.043). Other marital status categories were insignificant predictors of depression. A binary logistic regression was performed to find the odds ratio for each marital status category, using “married” as the control group to demonstrate the increased odds of a patient screening positive for depression in other categories.
Mean CES-D screener scores varied between marital status groups. An independent-samples t-test was used to compare CES-D scores in married and widowed patients. There was a significant difference in the mean scores for married (M=10.5, SD=8.8) and widowed patients (M=15.4, SD=9.5) patients; t(96)= -2.253, p=0.027.

Among 215 patients who completed the survey question on employment status, there was no statistically significant correlation to the number of patients screening positive for depression, as determined by a Likelihood Ratio Chi-Squared test (χ²=8.182, p=0.147).

Conclusion:

The total percentage of oncology patients who screened positive for depression (32 ± 6 %) falls within the documented range of 20-50% for depression in cancer patients (2,5,7). It is clear that oncologists should be aware of potential depression symptoms in their patients. In an urban center such as New Jersey Medical Cancer Center, physicians should appreciate that patients may not be as willing to discuss feelings about their diagnosis, and should pursue a

---

**Table 2**

<table>
<thead>
<tr>
<th>Oncology Patient Marital Status</th>
<th>N</th>
<th>CES-D score ≥ 16</th>
<th>Percent positive for depression symptoms</th>
<th>Significance</th>
<th>Exp(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>222</td>
<td>71</td>
<td>32%</td>
<td>0.031*</td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>76</td>
<td>16</td>
<td>21%</td>
<td>0.003*</td>
<td>0.267</td>
</tr>
<tr>
<td>Marriage-like relationship</td>
<td>12</td>
<td>2</td>
<td>17%</td>
<td>0.043*</td>
<td>0.750</td>
</tr>
<tr>
<td>Separated/Divorced</td>
<td>47</td>
<td>19</td>
<td>40%</td>
<td>0.274</td>
<td>2.545</td>
</tr>
<tr>
<td>Single</td>
<td>45</td>
<td>15</td>
<td>33%</td>
<td>0.100</td>
<td>1.875</td>
</tr>
<tr>
<td>Never Married</td>
<td>20</td>
<td>7</td>
<td>35%</td>
<td>0.207</td>
<td>2.019</td>
</tr>
<tr>
<td>Widowed</td>
<td>22</td>
<td>12</td>
<td>55%</td>
<td>0.670</td>
<td>4.500</td>
</tr>
</tbody>
</table>

---

**Table 3**

<table>
<thead>
<tr>
<th>Oncology Patient Employment Status</th>
<th>N</th>
<th>CES-D score ≥ 16</th>
<th>Percent positive for depression symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working full-time</td>
<td>9</td>
<td>4</td>
<td>44%</td>
</tr>
<tr>
<td>Working part-time</td>
<td>19</td>
<td>4</td>
<td>21%</td>
</tr>
<tr>
<td>Unemployed</td>
<td>74</td>
<td>17</td>
<td>22%</td>
</tr>
<tr>
<td>Disabled</td>
<td>110</td>
<td>43</td>
<td>39%</td>
</tr>
<tr>
<td>Total</td>
<td>215</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
conversation regardless, as it has been shown that a good doctor-patient relationship leads to better health outcomes (1).

Marital status was shown to be a significant predictor of depression, although not all marital status categories were found to be statistically significant. The insignificant results for marital status categories other than “married” or “in a marriage-like relationship” may be due to small sample sizes, or may be due to the fact that the survey did not include any questions to address other areas of emotional support. There may be other family members or friends in patients’ lives that offer emotional support not included in the measure of marital status (11). Future analyses should also delve further into the correlation between gender and depression, to evaluate potential association between gender and depression with marital status. Employment status was found to be insignificantly associated with depression, but this result may be due to the small number of patients working full- and part-time (N=9, 19). Furthermore, it is unclear from the survey whether patients’ employment level was impacted by their cancer diagnosis.

Future analyses will examine depression levels among types of cancer diagnoses, so that results could be compared to the existing literature on social support in breast and colon cancer patients. Future analyses should also examine the time between initial cancer diagnosis and survey completion date, to look for a correlation between time between diagnosis and depression rate.

Sources:
Objective:

1α,25-dihydroxyvitamin D3 (1,25D) is a steroid hormone involved in diverse biological processes. In acute myeloid leukemia (AML) cells, it has been shown to induce cell differentiation and block cell cycle progression.1 Akt, a serine/threonine protein kinase, has been shown to play a role in cell survival and proliferation.2 It has been suggested that 1,25D may induce cell differentiation and cell cycle arrest in part via Akt through Raf/MEK/ERK MAPK signaling.3,4 While the effect of 1,25D on Akt expression was studied,5 the effect of Akt inhibition in conjunction with 1,25D treatment has not been investigated. Considering 1,25D’s potential in the treatment of AML, and current interest in Akt inhibitors as potential treatments in other types of cancers, it is of great interest to elucidate the signaling mechanisms by which this occurs as it may prove to be a viable option in treating patients with AML.

Methods:

Cell culture. HL60-G cells (FAB M2) were subcloned from HL60 cells (ATCC, Manassas, VA) and maintained in Iscove’s Modified Dulbecco’s Medium (IMDB) with 10% heat-inactivated, iron-enriched bovine calf serum (HyClone, Logan, UT). These cells were kept at a temperature of 37°C in an incubator supplying 5% CO2. The cells were passaged and fed with medium 2-3 times per week.4 Experimental groups were treated with Akt1/Akt2/Akt3 inhibitor MK2206 (0.1µM, 1.0µM) and 1,25D (1.0nM) and incubated for 72 hours (Selleck Chemicals, Houston, TX).

Cell lysis and Western blotting. Cell pellets were washed with PBS and then treated with cell lysis buffer (CST, Danvers, MA) and protease inhibitor cocktail (Hoffman-La Roche, Germany) for 30 min at 4°C. The crude extract was then centrifuged at 14,000g for 30 min. The supernatant was collected, quantified, and suspended in an equal volume of Laemmli sample buffer (BioRad, Hercules, CA). 40µg quantities of protein were separated in a 10% polyacrylamide gel and transferred to nitrocellulose membranes. Protein signals were developed using the ECL technique.6

Summary:
Figure 1. Akt inhibition both decreases cell differentiation and promotes cell cycle arrest. HL60 cells were treated with a low concentration (1.0nM) of 1,25D and various concentrations (0.1µM, 1.0µM) of MK2206 for 72 hours. (A) Flow cytometry was performed CD14, a monocytic marker, and analyzed by a Beckman-Coulter EPICS XL-MCL machine. (B) Flow cytometry was performed for CD11b, a general myeloid marker, and analyzed by a Beckman-Coulter EPICS XL-MCL machine. (C, D) Cells were stained with propidium iodide and cell cycle parameters determined by flow cytometry and analyzed by MultiCycle AV (Phoenix Flow Systems) for G1/S and G2/S ratios.
Figure 2. Akt inhibition leads to an increase in p27Kip1 protein, which plays an important role in cell cycle progression. HL60 cells were treated with a low concentration (1.0 nM) of 1,25D and various concentrations (0.1 µM, 1.0 µM) of MK2206 for 72 hours. Western blot of p27Kip1 was performed. Optical density ratios were calculated using ImageJ (NIH) and normalized against CrkL optical density.

Figure 3. MK2206 inhibits the fully-activated form of Akt. Western blots of pAkt (Ser473), Akt1, and CrkL (used as a loading control) were performed. Akt has two phosphorylation sites, but only phosphorylation on Ser473 is required for full activation. MK2206 inhibits Akt1/Akt2/Akt3. Akt1 was probed for as it is the form primarily involved in cell survival and protein synthesis pathways. Optical density ratios were calculated using ImageJ (NIH) and normalized against CrkL optical density.

Figure 4. Inhibition of Akt results in activation of ERK5. Western blots of pERK5, ERK5, and CrkL (used as a loading control) were performed. Optical density ratios were calculated using ImageJ (NIH) and normalized against CrkL optical density.
Figure 5. Inhibition of Akt results in activation of ERK1/2. Western blots of pERK1/2, ERK1/2, and CrkL (used as a loading control). Optical density ratios were calculated using ImageJ (NIH) and normalized against CrkL optical density.

Conclusion:

It was found that treatment of HL60 cells with 1,25D modestly activates the Akt pathway, which may contribute to the increase in cell differentiation markers CD14 (a monocytic marker) and CD11b (a general myeloid marker) in cells treated solely with 1,25D. In addition to this, it was also found that inhibition of the Akt pathway results in decreased expression of CD14 and CD11b markers. Inhibition of Akt kinase also results in cell cycle arrest. This was shown through increased G1/S and G2/S ratios and increased levels of p27Kip1 protein as concentration of MK2206 increased. Inhibition of Akt kinase, which plays an important role in cell cycle regulation and differentiation, increases the activated, phosphorylated forms of both ERK5 and ERK1/2. This suggests that this is a compensatory increase, further implying that these pathways have overlapping functions. Additional research needs to be performed both to determine the statistical significance for these findings and to determine points of crosstalk between the Akt, ERK5, and ERK1/2 pathways. Potential targets for further experimentation may include the downstream targets of ERK5 (ex.: MEF2C) and ERK1/2 (ex.: Elk1).

Objective:

Approximately 30 years ago, two large prospective cohorts of injection drug users (IDU) were formed. The first was initiated in September 1984 [referred to below as the New Jersey Health Study (NJD)], reflecting a collaboration of the National Cancer Institute (NCI), the National Institute on Drug Abuse (NIDA), and the NJ Department of Health (NJDOH). This cohort of 1,040 IDU received care at 10 drug treatment centers located in six New Jersey cities. The Women’s Study, an extension of NJD, consists of 126 women (seven of which had previously enrolled in NJD) enrolled in 1990 from two cities, two drug treatment programs and one HIV treatment program. While several individuals in the Women’s Study were not IDU, many were at risk with HIV+ male sexual partners. Written informed consent including permission for long-term follow-up was given. Extensive baseline information was obtained in a questionnaire including essential contact information, past and current drug use, sexual behavior, and relevant health data. Subjects were tested for human immunodeficiency virus (HIV) and human T-lymphotropic virus (HTLV types I and II), other laboratory tests were performed, specimens stored, and clinical data obtained. This study was the first of its kind to test patients for HIV in New Jersey and serves as an invaluable source of data for long term follow-up, such as cancer outcomes over time. Given the size of the cohort, extensive data collection, and the long-term follow-up, this study could elucidate key risk factors for a variety of chronic diseases and for drug users, including understanding relationships between HIV and various cancers. Some subjects were seen multiple times. All data, consent forms and biological specimens not already in Dr. Weiss’s possession have been transferred to him and are currently stored by Dr. Weiss in appropriate Rutgers Biological and Health Science (RBHS) facilities.

In collaboration with NIDA, beginning in 1989 a cohort of 7,542 IDU was recruited from 9 cities across the country including Trenton, Asbury Park, Los Angeles, Miami, Baltimore, New York, San Antonio, Chicago and Philadelphia. The 944 persons that were enrolled from the two NJ sites, Asbury Park and Trenton, will be the focus of this analysis and referred to as the NID study. Similar surveys and data were collected as in NJD. Some subjects were seen more than once.

The value of our now conducting a 30-year follow-up is underscored by statistics showing age-specific incidence rates for various cancers from 1992-2000 (Fraumeni & Schottenfeld, 2006). As illustrated in figure 1, the incidence rates for many cancers tend to increase with age, especially after age 50. Based on the mean age and year of enrollment (table 1), the current mean age would now be over 60. Thus, we expect that this study will be able to identify numerous cancer outcomes due to both the longer observation time and higher incidence rates in recent years as compared to similar analyses by Dr. Weiss et al. fifteen years ago. With high rates of HIV and drug use among the cohorts, using these linkage data has the potential to find predictors of certain HIV-related cancers. A previous study drawn from these cohorts was the first of its kind to show high prevalence of hepatitis C virus (HCV) in injection drug users (table 1). The HCV rates in the NJD and NID imply a high risk for end-stage liver disease and hepatocellular carcinoma. In an earlier analysis, HIV infection was not associated with end-stage liver disease (Hisada et al., 2005). Early detection of hepatocellular carcinoma is crucial for favorable outcomes. Its incidence is expected to increase in the coming decades. The HCV rate in the Women’s Study (n=126) of 41.9% is lower, but some subjects were not drug users. These elevated rates of hepatitis C illustrate the importance of studying biomarkers in infected persons that are associated with an increased risk of developing hepatocellular carcinoma. Similarly, previous analyses
have shown significantly elevated rates of HIV-associated cancers in these cohorts such as non-Hodgkin’s lymphoma. With now a 30-year follow-up period, we will be better able to assess whether or not other cancers, such as cervical cancer, are significantly increased from the expected rates.

![Figure 1: Age-specific incidence rates for certain cancers, 1992-2000](from Fraumeni & Schottenfeld, 2006)

**Methods:**

In collaboration with the New Jersey State Cancer Registry (NJSCR), identifying information in these cohorts (including first and last name, social security number, gender, race, and date of birth) will be linked to registry cancer data using the Centers for Disease Control and Prevention (CDC) software Link Plus. Accessing information in the NJSCR database requires that a set of strict requirements be met so as to avoid the misuse of data and maintain patient confidentiality. Similarly, there are constraints related to the cohort data itself. Data review in preparation for construction of the datasets to be shared with NJSCR was commenced.

As an initial step, discussions were held with the NJSCR related to study merit and design. The NJSCR worked with us on submissions to the Rutgers Cancer Institute of New Jersey (CINJ) Scientific Review Board (SRB) and the Rutgers Institutional Review Board (IRB), as well as a scope of work between Dr. Weiss and CINJ/NJSCR.

Link Plus is a probabilistic record linkage program, which generates a match score as a quantitative measure of the certainty of a potential match. There are a number of complicated issues that arise when preparing the study patient data for matching with the NJSCR. For instance, many subjects in
these cohorts provided discrepant information, particularly first name and last name (related to aliases and colloquial names). Also, differences in last name due to change in marital status cause many discrepancies. Multiple records for each such case will be submitted to the NJSCR for linkage so as to account for all likely variations of the identifying information and to increase the likelihood of not missing a valid match. A subsequent manual review of putative matches will be a critical component in assessing the likelihood that the match is valid. In addition, some individuals in the cohorts are highly transient and oftentimes visit drug and medical treatment facilities outside of NJ, so their cancer information will sometimes not be found in the NJSCR. This is of concern as there are large numbers of participants from Jersey City and Newark in these cohorts who visit New York for care, and those from Camden and Trenton traveling to Philadelphia, PA. Although these cancer cases diagnosed and/or treated in other states are supposed to be reported back to New Jersey (to the NJSCR), it is known that these data are not always reported back to NJ. Thus, we anticipate performing a National Death Index search so as to minimize this problem of missing cancer information through examination of national data sources that include cause of death.

Link Plus offers two integrated phonetic systems for matching of names, Soundex and the New York State Identification and Intelligence System (NYSIIS). Soundex is the older, and more simplistic, of the two systems; it helps to account for minor misspellings in first or last name. The NYSIIS algorithm is reportedly 2.7% more accurate than the Soundex algorithm. The NYSIIS system yields a significant improvement in matching hyphenated names, which are common among Hispanics; since Hispanics comprise a substantial portion of our subjects from NJ (table 1), this is a major issue for our studies.

Another challenging aspect is the software’s heavy reliance on the Social Security number in the linkage algorithm. In this study, we do not have Social Security number recorded for approximately 8% of the subjects. In our test trials of that software, we found a glaring difference in linkage scores for those with and without Social Security numbers. Thus, to assess the linkage scores, we plan to create two linkage datasets, one for those that we have Social Security numbers recorded and another for those with missing Social Security numbers. Further, cancer information for these cohorts can be obtained using a search of the National Death Index, supplemented by the death certificate information. A wealth of information regarding the outcomes in the NID cohort was obtained from a match performed for the years 1989-1998.

Summary:

Cancer outcomes were tabulated based on the most recent matches with the New Jersey State Cancer Registry and a National Death Index Search through December 1998. Those tabulations indicate high counts of cervical cancer, non-Hodgkin’s lymphoma (NHL), and lung cancer in the NJD group (Table 2). Although there were only 2 cases of hepatocellular carcinoma in NJD, this was a statistically significant elevated rate of occurrence. Given that over 99% of this group is HCV+, we expect now to observe a significantly higher count of liver cancer with a longer latency period. This expectation is supported by the fact that it takes an average of 30 years for hepatocellular carcinoma to develop following an HCV infection. Previous matching performed on the NJD cohort through 1999 confirmed that total cancers were significantly elevated and we expect that this result will become even more apparent as we pursue this study. We also show in Table 2 the counts of previously accrued cancers in the other two NJ-based cohorts.

Conclusion:

The cancer counts in table 2 were determined about 15 years ago. We expect to observe a superlinear increase due to the long-term follow-up and the higher cancer incidence rates that are expected with increasing age (figure 1). Some subjects in these cohorts died prior to getting cancer, which will be accounted for statistically through life-table analyses. (This will be particularly true for those already infected with HIV at study outset.) Overall, we expect that the outcomes may more than
double for many cancers, as the higher cancer incidence at older ages is likely to outweigh the diminished cancer counts due to death. The data previously collected for this study constitute a preliminary step towards our end goal of performing a long-term follow-up and determining how cancer outcomes have changed since the prior 15-year benchmark.

Table 1: Baseline statistics for NJD study, Women’s study, and NID study
*Data based on sub-study of NJD study (Weiss et al., 1995)
**Data based on sub-study of NID study and assumed to be equivalent for NJ group (Hisada et al., 2005)

<table>
<thead>
<tr>
<th>TYPE of CANCER</th>
<th>Cervical</th>
<th>NHL</th>
<th>Lung</th>
<th>Skin</th>
<th>Liver</th>
<th>Urinary</th>
<th>Oral</th>
<th>Breast</th>
<th>Myeloma</th>
<th>Colorectal</th>
<th>CNS</th>
<th>Unknown</th>
<th>Total Cancers</th>
</tr>
</thead>
<tbody>
<tr>
<td>NJD (n=1,040)</td>
<td>15</td>
<td>9</td>
<td>10</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>51</td>
</tr>
<tr>
<td>Women’s (n=126)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>NJ-NID (n=898)</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
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<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 2: Cancer counts by site for NJD, Women’s and proportional estimate for NJ subset of NID studies

References
MECHANISMS INVOLVED IN CELLULAR RESPONSES TO VARIABLE DOSE RATES IN TARGETED RADIONUCLIDE THERAPY

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

Objective:

External beam radiation therapy utilizes x-rays, gamma rays, or protons to deliver large doses of ionizing radiation to clinically identifiable tumors and involved tissue(s). Unfortunately, even when combined with surgical intervention and chemotherapy, the lack of tumor specificity of chemotherapy agents does not always sterilize circulating tumor cells, disseminated tumor cells, and micrometastases which have a significant impact on patient survival outcomes. First described in the early 1950’s, radioimmunotherapy (RIT) has gained renewed interest for targeting systemic disease. RIT draws upon aspects of immunology and radiation to direct the delivery of ionizing radiation to tumor cells by conjugating radionuclides to a tumor-specific antibody. The radiation doses delivered to the targeted cells depend in part on the biological uptake and clearance half-times of the agent, and the physical half-life of the radionuclide.

The dose rate patterns that the targeted cells receive are exponentially varying and are markedly different than those corresponding to the fractionated acute exposures used in external beam radiotherapy. Acute fractionated and chronic dose rate irradiation are known to elicit different biological responses. However, little is known regarding how multi-exponential dose rates encountered in RIT affect cell response (e.g. survival). Further understanding of the responses to these varying dose rate patterns could provide novel insight on the effectiveness and toxicity of existing modalities and allow for appropriate assessment of clinically administered procedures, such as radionuclide therapies, that could provide the advantages of tumor-specificity and normal tissue sparing.

Objectives:

1. Confirm and expand upon the biological responses to varying dose rate patterns first seen by J. Solanki (NJMS 2017). Irradiate Chinese hamster V79 lung fibroblasts and MDA-MB-231 human breast cancer cells with $^{137}$Cs $\gamma$-rays using a custom irradiator and obtain survival curves for different dose rate patterns. 

Figure 1. Mechanism of RIT for metastatic prostate cancer (Simone and Hahn, 2013).

http://clincancerres.aacrjournals.org/content/19/18/4908.figures-only
2. Understand whether the underlying mechanisms that mediate responses to exponentially varying dose rates of ionizing radiation depend upon dose rate kinetics.

**Methods:**

**Cell Cultures:**

MDA-MB-231 and V79 cells were grown as monolayers in culture flasks (37°C, 5% CO₂ - 95% air) containing minimum essential medium (MEM) supplemented with 10% fetal calf serum, 2mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, and 10 mg/ml non-essential amino acids.

**Irradiation and Determination of Cell Survival**

Cells were trypsinized and seeded into 25 cm² flasks in triplicate. After 4 h incubation, flasks were capped tightly and chronically irradiated with $^{137}\text{Cs}$ γ-rays for 168 h with different dose rate patterns. MDA-MB-231 were incubated for another 168 h. Cells were fixed with ethanol and stained with crystal violet. Cell colonies were counted, surviving fractions were calculated relative to controls, and cell survival curves were plotted. Distances from the $^{137}\text{Cs}$ gamma ray source were modified for MDA cells in order to accommodate for sensitivity to chronic radiation.

**Figure 2.** Experimental set up inside the $^{137}\text{Cs}$ γ-irradiator that delivers time-varying dose rates.

The $^{137}\text{Cs}$ source is mounted on top of the irradiator (above the field of view).

**Figure 3.** Comparison of dose rate profiles of exponentially decreasing dose rate, multi-exponential dose rate with $T_i = 8$ h, and $T_d = 64$ h, and multi-exponential dose rate with $T_i = 24$ h, $T_d = 64$ h. The profiles represent dose rates delivered to the top position with a maximum dose rate of $R_0 = 33$ cGy/h.
Proteomics:
Cells were set up for irradiation as described above and flasks were removed from the irradiator at \( t = 8, 24, \) and 48 h after initiating the irradiation. Cells were trypsinized and frozen at -80°C. Cell lysates were analyzed by the Center for Advanced Proteomics Research and protein identification and analysis was through LC-MS/MS (LTQ Orbitrap Velos) and Ingenuity Pathway Analysis.

Summary:
- Cell survival curves are for chronically irradiated Chinese hamster V79 and MDA human breast cancer cells are shown in Figure 4.

**Figure 4.** Comparison of surviving fractions of V79 and MDA-MB-231 cells irradiated with exponentially decreasing and multi-exponential dose rates. Data were fitted to a linear-quadratic model, \( S = e^{(-\alpha x - \beta x^2)} \). All data points except MDA-MB-231 multi-exponential and the two highest doses for V79 were averages of 2 trials.

Conclusion:
- Sensitivity to chronic radiation was markedly different for V79 Chinese Hamster and MDA-MB-231 human breast cancer cells.
- Cell survival was higher for multi-exponential dose rates compared to exponentially decreasing dose rates.
- Dose rate increase half-time should be considered when predicting responses to radioimmunotherapy and other radionuclide therapies.
Future Directions:

- Irradiate with additional dose rate patterns to further characterize findings.
- To explore underlying mechanisms, perform proteomic analyses of chronically irradiated V79 and MDA-MB-231 cells and confirm through western blot.

Figure 5. Preliminary proteomics analysis of varying multi-exponential increase half times of 8 and 24 h. Samples were taken at 8, 24, and 48 h after initiation of irradiation. Shades of orange represent upregulation of pathway while blue represent downregulation in comparison to controls taken at each corresponding time point.

References:

INVESTIGATION OF BCR-ABL RHOGEF ACTIVITY IN THE INVASIVENESS OF CHRONIC MYELOGENOUS LEUKEMIA.

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Objective:

The Philadelphia chromosome is formed by a balanced, reciprocal translocation that pairs sequences from BCR on Chromosome 22 with sequences from ABL on Chromosome 9 and results in the production of constitutively active tyrosine kinase Bcr-Abl (Nowell & Hungerford, 1961; Rowley, 1973). Depending on the location of the breakpoint within BCR, three different sizes of Bcr-Abl may form (i.e., p230, p210, p190) and each is associated with distinct clinical sequelae [Figure 1] (Melo, 1996; Pane et al., 1996; Sawyers, 1999). p210 and p190 are the best characterized: p210 is observed in greater than 95% of chronic myelogenous leukemia [CML], while p190 is present in approximately 2/3 of Philadelphia-positive [Ph+] acute lymphoblastic leukemia [ALL]. We previously identified a functional domain within the Bcr sequence retained by p210, but not by p190, that exhibits a Rho GTPase-specific guanine nucleotide exchange [RhoGEF] factor activity (Korus, Mahon, Cheng, & Whitehead, 2002; Sahay et al., 2007; Whitehead, Campbell, Rossman, & Der, 1997). We also demonstrated that this activity is important for Bcr-Abl-mediated leukemogenesis and disease progression using in vivo and in vitro murine models (Tala et al., 2012). To the best of our knowledge, however, this relationship has yet to be thoroughly evaluated in clinical specimens. We recently learned that the Bcr-Abl RhoGEF domain was reported to contain several in-frame deletions and two point mutations (i.e., F547L, T654K) in two blast crisis CML patients and a single p210 Bcr-Abl-positive ALL patient (Telegeev, Dubrovsk, Dybkov, & Maliuta, 2004). Given the advanced status of their disease, the presence of these variants strongly suggested that the Bcr-Abl RhoGEF domain may affect the course of human leukemia as well. Beginning with F547L and T654K, and a double-mutant F547L+T654K, we have examined the effects of clinically derived Bcr-Abl RhoGEF domain mutations in vivo using a murine bone marrow transplant model (mBMT) of Ph+ leukemia. In addition to altered disease latency [Figure 2], mice transplanted with cells expressing these variants are characterized by a dramatic increase in the invasiveness of their disease, as evidenced by increased penetration into typical organs, the involvement of atypical organs, and the heretofore unreported appearance of chloromas [Figures 3-6]. To gain more-specific insight into the origin of the invasive phenotypes associated with each mutant, we have recently begun to study them in vitro using retrovirally transduced, murine hematopoietic stem cell (HSC) lines. Here, we present our results on the altered degradative and angiogenic capabilities of these cells.

Methods:

The three mutations (i.e., F547L, T654K and F547L+T654K) were first introduced into the MSCV-bcr-abl/p210-ires-gfp retroviral vector containing full-length p210 Bcr-Abl via an optimized site-directed mutagenesis protocol that allows for rapid throughput. The resulting retroviral constructs were then used to transfect the Phoenix ecotropic packaging cell line in order to produce high-titer retroviral supernatants. Finally, the supernatants were used to transduce the 32Dcl3 murine myeloid HSC line.
In order to determine if the clinical mutations affect the Bcr-Abl RhoGEF domain, we next interrogated the transduced 32Dcl3 cells using affinity purification assays to measure the endogenous levels of the active, GTP-bound RhoA and Cdc42 GTPases.

To examine the degradative abilities of the cell lines, gel zymography studies were performed by utilizing transduced 32Dcl3 cells grown in IL3- and serum-free RPMI for 24 hours at a density of $2 \times 10^5$ cell/mL, after which the culture media was collected, clarified and used in experiments. Both whole cell lysates and supernatants were run, individually, on Western gels prepared with 10% SDS page gels copolymerized with gelatin at a final concentration of 0.15%. To quantify the transduced 32Dcl3 cells’ secretion of angiogenic factors, we also used the clarified culture media to perform ELISA studies for the detection of FGF-2 and VEGFA in duplicate.

Summary:

*Bcr-Abl RhoGEF Domain Activity:*

All three clinical mutants resulted in decreased detection of active RhoA compared to p210 [Figure 1A]. They did so with the gradient F547L>T654K>F547L+T654K, perhaps indicating that the individual mutations have unique effects on the Bcr-Abl RhoGEF domain and together they are synergistic.

Based on these findings, we also assessed downstream activity to confirm the reduction of RhoA-mediated signaling [Figure 1B]. Rho-associated protein kinase, the preferred effector for RhoA, is known to phosphorylate the myosin-associated protein MLC2 on Serine 19. When we evaluated the level of phospho-MLC2 in the mutant cells, we observed a significant reduction compared to p210 with a trend that closely mirrored that of the decrease in RhoA activation. This suggests that all three clinical mutants have impaired RhoA activation in myeloid stem cells.

*MMPs and Angiogenic Factors:*

All three mutants secreted considerably more MMP9 than p210 – no other MMPs were observed [Figure 2A]; the blot shown is representative. Data presented are the average, p210-normalized intensity values for 3 experiments ($^*P<0.05$ rel. to p210). We also saw qualitative differences in the F547L and T654K lines when we assessed whole cell lysates - representing either membrane-associated or intracellular MMPs- from the same cells [data not shown]. The approximately 25 kDa band is likely membrane-associated MMP3 or 7.

Similar to MMP9, we observed an increase in the levels of both FGF1 and VEGFA among all three mutants, compared to p210 (the coefficient of variation was $<2\%$ for each set of replicates) [Figure 2B].

Conclusion:

*Bcr-Abl RhoGEF Domain Activity:*

Given that all three clinical mutants reduced RhoA activation, an obvious question brought about by these results is, why do they result in different in vivo phenotypes compared to p210, to each other and to S509A. With that in mind, we also plan to determine the effects of the clinical mutations on the activation of the other canonical Rho GTPases – Cdc42 and Rac1.
We do not yet have a full set of data for Cdc42 and Rac1, however, what we have been able to acquire is thought-provoking (data not shown). For both GTPases, we compared the following types of 32Dci3 cells: MIG (vector), p210, F547L, T654K, S509A, and p190. In the case of Cdc42, there were significant differences from the pattern observed for RhoA activation. While p210 also resulted in Cdc42 activation compared to vector, it was much more modest than for RhoA. In addition, where F547L and T654K both caused a drop in active RhoA versus p210, here, F547L displayed a slight increase in GTP-bound Cdc42 and T654K once again diminished. Although Rac1 was highly expressed in all of the 32Dci3 cells tested, we were unable to detect any of the active form in any of the lines.

Similar to RhoA, B and C, we also have the ability to investigate Rac2 via the same affinity purification assay used to pull down Rac1. The expression of Rac2 is restricted to hematopoietic cells, and it has been linked to both neutrophil and macrophage function, so we plan to probe for this potentially important GTPase in the near-future.

Cross-talk between the different Rho GTPases is a well-documented phenomenon and this collateral regulation plays a role in wide variety of cellular processes. That said, going forward, we anticipate that we will continue to see differences in the activation of the individual GTPases similar to what we have already noted. Based on our preliminary studies, it seems likely that their relative levels may ultimately be responsible for the phenotypic differences we have observed in vivo.

**MMPs and Angiogenic Factors:**

Much work remains to be done. In addition to repeating the assays, we are also planning to determine how much of the MMPs identified in the whole cell lysates are membrane-associated by using a detergent to separate the lipid-soluble portion. It is also important to examine the MMPs in the context of their inhibitors, so we will also assess their relative production of the TIMPs using reverse zymography.

An interesting potential connection between the clinical mutants and the MMPs has already been outline in the literature by us and others. The transcription factor NF-kB is a major mediator of immune and inflammatory processes, and an increase in its activity has been linked to upregulation of MMP2 and MMP9. We previously published that the isolated Bcr-Abl RhoGEF domain is capable of strongly activating NF-kB. What's more, others have shown that the Rho GTPases can either activate or suppress it. Taken together, if would be very interesting to see if NF-kB activity correlates with the presence of the clinical mutations, and, in turn, the levels of the active MMPs.
Figure 1: The presence of clinically derived Bcr-Abl RhoGEF mutations results in down-regulation of RhoA activity and a parallel decrease in downstream signaling in the 32Dcl3 murine HSC line.

A. GTP-RhoA/Total-RhoA Norm. to p210

B. Phospho/Total MLC2 Norm. to p210

Figure 2: The presence of clinically derived Bcr-Abl RhoGEF mutations increases secretions of angiogenic and degradative factors by the 32Dcl3 murine HSC line.

A. Levels of Secreted FGF-2

B. Levels of Secreted VEGFA
IDENTIFICATION OF PROTEIN CHANGES IN CANCER ASSOCIATED FIBROBLASTS FOLLOWING CHRONIC HYPOXIA

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Objective:
Cancer Associated Fibroblasts (CAF) are an integral component of many solid tumors, comprising up to 50-90% of the tumor volume. Studies have demonstrated a role of CAF in tumor development and metastasis [1], with additional evidence suggesting a strong involvement in therapy failure [2]. However, very little is known of the role of in vivo-like tumor partial oxygen tension (e.g. 7 mm Hg; 0.5% O₂) on CAF development and their subsequent role within the tumor microenvironment (TME).

In this study, we adapted a transwell insert co-culture system to generate a pure population of CAF. We co-cultured normal human fibroblasts (AG1522) with triple-negative breast cancer cells (MDA-MB-231) for 120 h under ambient (155 mm Hg; 21%) or in vivo-like (i.e. 7 mm Hg; 0.5%) oxygen conditions. CAF proteins were extracted, identified, and quantified using Liquid Chromatography Tandem-Mass Spectrometry (LC-MS/MS) along with pathway analyses to identify target proteins and pathways potentially involved in CAF development and their role in therapy resistance.

The goal is to identify altered protein expression and signaling networks in CAF under ambient or hypoxic conditions. The identification of pro-proliferative and/or pro-survival cascades may suggest opportunities to disrupt TME development and improve therapy outcomes.

Methods:

Cell Culture
AG1522 normal diploid human skin fibroblasts and MDA-MB-231 human triple-negative breast cancer cells were used for co-culture to generate pure CAF population. Cell populations were cultured on a transwell insert separated by 10 μm-thick polyester membrane, yet in communication through 1.0 μm pores. Oxygen concentration was titrated from 21% to 0.5% over 12h and kept at 0.5% for 5 days. Inserts were rapidly refed at 24 and 72 hours with growth medium maintained at 0.5% oxygen.

Cell Lysis
Cells were removed by accutase and washed three times with 1X PBS. Cells were resuspended in 8 M urea, 50 mM ammonium bicarbonate, and protease inhibitor cocktail (1:100) and ultrasonicated for 10 seconds twice. Bradford assay was used to measure protein concentration.

In-solution Digestion
DTT and iodoacetamide were added for protein reduction and alkylation. Proteins were digested by Lys-C and trypsin. Protein digestion was desalted using C18 spin column.
LC-MS/MS analysis
Digested peptides were analyzed by LC-MS/MS on Q Exactive tandem MS instrument using technical triplicates for each sample.

Protein Database Search
MS/MS spectra data was searched against Swissprot Human Protein Database. Database search criteria consisted of: acetyl (protein N-Terminus), carbamidomethyl (Cys), and oxidation (Met) modifications, 10 ppm for precursor mass tolerance, 0.1 daltons for fragment mass tolerance.

Ingenuity Analysis
The average spectra counts of AG co-cultured with MDA were compared to AG control spectra counts under ambient and hypoxic conditions. Only ratio changes greater than 1.5 or less than 0.68 were included in analysis.

Summary:
AG1522 CAF co-cultured with MDA-MB-231 breast cancer cells show alterations in protein expressions through proteomics. Although there were distinct protein expression changes in either ambient or hypoxia, the changes in total number of proteins are similar in both groups (Figure 1). Literature analysis revealed several proteins with known associations with CAF and/or cancer cells (Figures 1 & 2), validating the effectiveness of proteomic approach. Through Ingenuity, a heat map was generated to highlight novel differences in protein signaling pathways between ambient and hypoxic conditions (Figure 3), including known cell cycle regulation pathways involved in tumor growth.

Figure 1: Hypoxia Does Not Affect Total Number of Proteins, but Alters Protein Expressions

Majority of ratio changes for proteins were between the thresholds of 0.68 and 1.5, which was considered ‘no change’ in protein expression. Regardless of down or upregulation, total number of proteins in ambient or hypoxia were approximately equal.

Proteins were grouped based on ratio changes under ambient or hypoxic conditions. Highlighted rows indicate potential proteins of interest affected by MDA or hypoxia.
**Figure 2: Identified Proteins with CAF Relevance**

<table>
<thead>
<tr>
<th>Identified Proteins</th>
<th>Function</th>
<th>Ratio Change for MDA_AG Ambient</th>
<th>Ratio Change for MDA_AG Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin subunit beta-1 (LAMB1)</td>
<td>Vascular Basement Membrane Structure</td>
<td>3.78</td>
<td>3.95</td>
</tr>
<tr>
<td>High mobility group protein B1 (HMGB1)</td>
<td>Chromatin-Association Nuclear Protein</td>
<td>3.5</td>
<td>3.15</td>
</tr>
<tr>
<td>Caveolin-1 OS=Homo sapiens (CAV1)</td>
<td>Integral Membrane Protein</td>
<td>0.65</td>
<td>0.36</td>
</tr>
<tr>
<td>Signal transducer and activator of transcription 1 alpha/beta (STAT1)</td>
<td>Cytoplasmic Transcription Factor</td>
<td>1.96</td>
<td>0.39</td>
</tr>
<tr>
<td>Insulin-like growth factor 2 mRNA-binding protein 3 (IGF2BP3)</td>
<td>RNA-Binding Factor</td>
<td>1.65</td>
<td>0.47</td>
</tr>
<tr>
<td>Actin, gamma-enteric smooth muscle (ACTG2)</td>
<td>Cell Migration</td>
<td>0.01</td>
<td>159.73</td>
</tr>
</tbody>
</table>

Shown are examples of 6 proteins with documented association to CAF and/or cancer cells. These proteins were not between the ratio change thresholds of 0.68 and 1.5, and had spectral counts above 2 for the triplicates from the mass spectra analysis. LAMB1 and HMGB1 levels were upregulated in ambient and hypoxic conditions, as ratio change was above 1.5 threshold. CAV1 is downregulated in both environments, as ratio change was below the 0.68 threshold. STAT1 and IGF2BP3 had upregulation in ambient conditions, but downregulation of protein expression in hypoxia, while ACTG2 was opposite.

**Figure 3: Enriched Canonical Pathways Indicate Altered CAF Signaling Cascades**

A comparison analysis generated in Ingenuity. The heat map compares two different data sets: MDA_AG in ambient and hypoxic conditions. Each enriched canonical pathway shown has differences in upregulation or downregulation in ambient or hypoxic conditions. Z-scores are assigned to each data set by comparing it to the known pathway and determining the level of signaling based on the ratio changes.
Conclusion:

Alteration in CAF protein expressions and signaling pathways under ambient (21%) or reduced oxygen concentrations were analyzed through proteomics. Several proteins were identified that had known involvement in CAF and/or cancer cell biology, with varying differences being attributed to oxygen culture conditions. Ingenuity bioinformatics analysis enabled us to identify possible novel pathways involved in pro-proliferative and/or pro-survival cascades of CAF that were altered between ambient and hypoxic environments. Further experiments will be conducted to validate proteomics data and identify novel pathways involved in CAF development in order to further our understanding of TME evolution and gain insight into potential therapeutic targets.

References:

Objective:

Multiple myeloma occurs when plasma cells become malignant and proliferate to form a tumor in the bone marrow. It represents 10% of blood cancers and 1% of all cancers. Multiple myeloma occurs slightly more frequently in men than in women, and the median age of diagnosis is 66 years. In 2015, the American Cancer Society estimates that 26,850 adults will be diagnosed with multiple myeloma in the US, and that 11,240 will die of the disease\(^1\). Current treatment for multiple myeloma depends on if the patient is eligible for autologous stem cell transplantation. If eligible, the patient undergoes induction therapy of bortezomib-adriamycin-dexamethasone prior to transplantation. If ineligible, the patient undergoes therapy involving bortezomib-melphalan-prednisone. Patients diagnosed with multiple myeloma have a particularly dismal 5-year survival rate of 47% with current treatment options. Consequently, there is a research need to develop novel therapeutic treatments for multiple myeloma in order to improve the survival rate and quality of life for patients.

Our laboratory has reported that naltrindole, a delta opioid receptor antagonist, inhibits MM cell proliferation \textit{in vitro} and \textit{in vivo} via a non-opioid receptor-dependent mechanism\(^2\) (Mundra et al., 2012). In the xenograft model, naltrindole (Nti) inhibited the tumor growth in SCID mice infused with human RPMI 8226 multiple myeloma cells. Given the promising effects of naltrindole, the goal of this study was to determine the efficacy of synthetic structural analogs of naltrindole\(^3\) in inhibiting the \textit{in vitro} proliferation of human U266 multiple myeloma cells. The hope was that a structural analog of naltrindole would prove to be more effective than its parent compound in inhibiting multiple myeloma proliferation, which would potentially pave the way for an innovative therapeutic treatment.

Methods:

Human U266 multiple myeloma (MM) cell cultures were grown in RPMI 1640 media with 10% fetal calf serum. Stock solutions (10 mM) of the naltrindole analogs were prepared by dissolving each of the compounds in dimethyl sulfoxide. 12-well plates were utilized to incubate U266 MM cells at 100,000 cells/ml for 72 hours with various concentrations of naltrindole and its analogs. The MM cells were treated with 3 or 4 different concentrations of each drug.

After the 72-hour incubation period, a Beckman Coulter ViCell instrument was used to calculate drug efficacy by determining the number of viable cells/ml in treated samples versus controls. The % control # of viable cells/ml for a drug at a particular concentration was calculated by dividing the sample # of viable cells/ml – 100,000 by the control # of viable cells/ml – 100,000. This value can be interpreted as the % inhibition of proliferation relative to the control. Graph Pad Prism was used to develop dose-response curves for the compounds using non-linear regression. Additionally, the EC\(_{50}\), or the concentration of a drug at which cell proliferation is inhibited by 50% relative to the control, of each drug was calculated using Graph Pad Prism. The concentration of each drug used was modified in subsequent trials based on the drug’s...
potency in order to obtain a more accurate non-linear regression. The data from the several trials was compiled in order to find the mean EC$_{50}$ and mean dose-response curve of each drug. Statistical analysis was performed using the unpaired Student’s t test.

**Summary:**

N1’-Me-7’-Br-Nti had the lowest EC$_{50}$ at 1.417 μM, while naltrindole had the highest EC$_{50}$ at 15.470 μM (Table 1). Since a lower EC$_{50}$ corresponds to a more potent drug, N1’-Me-7’-Br-Nti was the most potent drug we tested. The mean EC$_{50}$ of N1’-Me-7’-Br-Nti, N1’-Et-5’, 7’-Br-Nti, 7’-Cl-Nti, and 7’-Br-Nti was significantly lower (p<0.05) than the mean EC$_{50}$ of naltrindole.

Figures 1-3 depict the dose-response curves of each of the analogs compared to the parent compound naltrindole for the inhibition of multiple myeloma cell proliferation. Figure 1 shows the dose response of 5’-substituted naltrindole derivatives, which in this case was only 5’-MeO. Naltrindole and 5’-MeO showed similar efficacy in inhibiting multiple myeloma cell proliferation (Figure 1). Figure 2 shows the dose response of 7’ halogenated naltrindole derivatives, which includes 7’-F-Nti, 7’-Cl-Nti, and 7’-Br-Nti. The 7’-Cl-Nti and 7’-Br-Nti exhibited similar efficacy, with EC$_{50}$’s of 4-5 μM, while the 7’-F-Nti was slightly less potent with an EC$_{50}$ of 7.4 μM (Figure 2). Figure 3 depicts the dose response of N1’-substituted derivatives. The two compounds that stick out are N1’-Me-7’-Br-Nti and N1’-Et-5’,7’-Br-Nti, which have clearly greater efficacy than naltrindole, with EC$_{50}$’s of 1.4 and 3.5 μM, respectively (Figure 3).

**Conclusion:**

Naltrindole and its structural analogs inhibit U266 MM cell proliferation *in vitro*. N1’-Me-7’-Br-Nti is the most effective drug and is about 10 times more potent than naltrindole in inhibiting human U266 multiple myeloma cell proliferation. The results of this study laid the foundation for several other experiments that can collectively lead to the development of a novel therapeutic treatment for multiple myeloma. First, the analogs can be tested on multiple myeloma cells in combination with therapeutic drugs currently used to treat myeloma patients to determine synergistic efficacy. Second, additional structural analogs of naltrindole should be synthesized, particularly those with different functional groups at the 7’ and N1’ positions, in order to develop the most potent drug as a potential therapeutic drug to treat multiple myeloma. Finally, the new drug or drug combination that proves to be most effective *in vitro* should be tested *in vivo* in order to be used in clinical treatments for multiple myeloma. Ultimately, there is promise that this study along with further experimentation can finally lead to a new therapeutic treatment of multiple myeloma that can extend the life of a patient diagnosed with the dismal disease.
Tables and Figures:

Table 1. Mean EC$_{50}$ of naltrindole and analogs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean EC$_{50}$ (µM)</th>
<th>SEM</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nti</td>
<td>15.470</td>
<td>3.742</td>
<td>3</td>
</tr>
<tr>
<td>7'-F</td>
<td>7.399</td>
<td>1.780</td>
<td>4</td>
</tr>
<tr>
<td>7'-Cl</td>
<td>4.443*</td>
<td>1.124</td>
<td>4</td>
</tr>
<tr>
<td>7'-Br</td>
<td>5.239*</td>
<td>0.321</td>
<td>4</td>
</tr>
<tr>
<td>N1'-Me</td>
<td>13.977</td>
<td>1.650</td>
<td>3</td>
</tr>
<tr>
<td>N1'-Me-7'-Br</td>
<td>1.417*</td>
<td>0.265</td>
<td>3</td>
</tr>
<tr>
<td>N1'-Et</td>
<td>10.29</td>
<td>0.756</td>
<td>3</td>
</tr>
<tr>
<td>5'-MeO</td>
<td>10.224</td>
<td>2.282</td>
<td>3</td>
</tr>
<tr>
<td>N1'-Et-5',7'-Br</td>
<td>3.468*</td>
<td>0.564</td>
<td>3</td>
</tr>
</tbody>
</table>

*The mean EC$_{50}$ of these compounds is significantly lower (p<0.05) than the mean EC$_{50}$ of Nti.

Figure 1. Dose-response of Nti and a 5'-substituted Nti derivative on U266 MM cells
Figure 2. Dose-response of Nti and 7' halogenated Nti derivatives on U266 MM cells

Figure 3. Dose-response of Nti and N1'-substituted Nti derivatives on U266 MM cells

References:


Objective:

Kinases have become a major drug target for treating cancer. Conventional approach attempts to hinder phosphorylation activity of kinases; however, specificity has been a main issue. Fortunately, blocking particular protein-protein interactions has been found to be a promising approach for treating cancer. The interaction between focal adhesion kinase (FAK) and vascular endothelial growth factor receptor 3 (VEGFR3) play a crucial role in signaling for invasion and metastasis; it allows the tumor cell to survive apoptosis. Kurenova et al proposed that a small molecule chloropyramine hydrochloride (C4) can inhibit this interaction specifically by binding to the FAT domain of FAK using the 3D model of C4-FAT interaction by rigid, high-throughput, targeted docking. However, the details of some factors that might affect the docking were not discussed in the paper. Our interest is in reevaluating the effects of charged states of the ligand and the conformation of FAT domain and its N terminal residues on the ligand-FAT domain complex formations. We examined the residues that interact with each docking site, determine the most energetically favorable one using DOCK 6.7, and analyze the FAK-VEGFR3 interaction site.

Methods:

The small molecule, C4, and the crystal structure files for FAK were obtained from ZINC and the Protein Data Bank (1k04 and 1k05), respectively. While the FAT domain clearly exhibits a 4-helix bundle structure in 1k05, 1k04 differs from 1k05 in that the N terminus and helix 1 is at an open form, away from the three other helices. Standard procedure was used with UCSF DOCK 6.7 & Chimera. The receptor file was first prepared by stripping all ligands and solvent molecules, and then hydrogen atoms were added using Chimera. Spheres around the receptor were generated using the sphgen function of DOCK which was then used to draw a box a specific sphere cluster. Using the grid function, the energetics around the specified box and cluster were calculated. Finally, the dock function was used to dock the ligand to the receptor at various positions and orientations; and the most energetically favorable site was recorded. We used both rigid and flexible docking. Flexible docking allows the ligand to change its conformation up to 500 times to allow for the best fit. We determined the docking sites for protein 1k05 as well as its open form, 1k04, using both the protonated and neutral form of C4.
Summary:

For the neutral form of C4, we found three docking sites, two for 1k05 and one for 1k04. Also, for the protonated form of C4, we found four sites, three for 1k05 and one for 1k04. 1k04 had the same docking site for both neutral and protonated forms of C4, although with slightly different conformation of the ligand. Both the neutral and protonated forms of C4 had the same docking sites for chain A, B of 1k05, with only slight change in conformation of the ligand. However, the docking site for chain C differed between the neutral and protonated form of C4. The protonated form of C4 docked close to, but slightly away from the docking site for the neutral form, which had the same site as that of docking site B. The most energetically favorable docking site with docking score of -43.36 between the N terminus and helix 4 for protonated form of C4 on chain C of 1k05. Both forms of C4 docked on the same site for chain A of 1k05, at the tip of the four helices as shown on Figure 2, but was on a completely different location from chain B and C. Interestingly, our proposed binding site in chain B of 1k05 had interacting residues that overlap with several other proteins that interact with FAK, such as paxillin and CD4. Residues TYR 925, HIS 1025, and LEU 1035 are shared with paxillin protein A-D chains of protein 1OW7 and 1OW8. HIS 1025, VAL 1029, LYS 1032, and LEU 1035 also interact with paxillin protein A-D chains of protein 1OW6 and 1OW7. HIS 1025, ALA 1028, and LEU 1035 interact with chains A-D of CD4 protein 3B71.
Table 1. Docking scores for neutral and protonated forms of C4 on FAT domain of FAK.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Neutal</th>
<th>Protonated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>docking site</td>
<td>docking score</td>
</tr>
<tr>
<td>1k04</td>
<td>site 1</td>
<td>-26.31</td>
</tr>
<tr>
<td>1k05_A</td>
<td>site 2</td>
<td>-26.48</td>
</tr>
<tr>
<td>1k05_B</td>
<td>site 3</td>
<td>-33.14</td>
</tr>
<tr>
<td>1k05_C</td>
<td>site 3</td>
<td>-32.43</td>
</tr>
</tbody>
</table>

Figure 2. Docking sites for neutral C4 in chain A, B, C of 1k05 in superimposed view.

Figure 3. 1k05 chain B docking result for neutral C4.
Conclusion:

We found three docking sites for neutral C4 and four docking sites for protonated C4 on protein 1k04 and 1k05. The three chains (A,B,C) found in 1k05 are all FAT domain forming a trimer in crystal, but a small change in number of residues seems to affect the docking results for B and C versus A. Several N terminal residues present in chain B and C involved in ligand binding were absent in crystal structure of chain A. Also, the protonated form of C4 seemed to have affected its interaction with the residues in the vicinity of N terminus and helix 4, thus docking slightly away from the pocket in which C4 docked in chain B. This may suggest an intricate role of near N terminal residue in four helix bundle as well as protonation states of the ligand. In addition, the closed four helix bundle structure (1k05) shows different binding sites from one for the open form (1k04), a presumed minor form with domain exchange, swapping N terminus with first helix in a symmetry related molecule.

Our finding proposes that the neutral form of C4 docks on a different site from the one proposed by Kurenova et al where C4 interacts with Pro 906, The 1022, and His 1025 residues. Rather, results for chain B and C show that C4 docks on the same site as N-[(4-chlorophenyl)methyl]-N-[(2-dimethylamino)ethyl] quinolin-2-amine, compound 29, that was proposed in another paper by the same research group Gogate et al.7 This sharing in docking site is reasonable since compounds C4 and 29 have very similar structures; they only differ by an aromatic ring, with a pyridine in C4 and quinolin in compound 29. Alternate docking sites that we have found can be considered and tested with laboratory experiments in vivo, or with other docking programs to reach a consensus.

Moreover, the docking site of C4 on the N terminus of FAT region of FAK revealed that there were overlapping residues that also interact with paxillin and CD4. Although the interaction between paxillin and FAK has been thought independent from VEGFR3, further computational and experimental verifications could help verify docking sites and bring new insight to protein-protein interactions.

References
Objective:

Pituitary adenomas are benign, slow-growing lesions that arise from the pituitary gland and may secrete one or more pituitary hormones. These lesions constitute 10 to 15% of primary brain tumors operated in the United States and have a higher incidence in African Americans than in other ethnicities. While the majority of these tumors are less than 5 mm in diameter and do not require surgical intervention, larger tumors can lead to compression of important neural and vascular structures surrounding the pituitary, such as the optic chiasm and intercavernous nerves and blood vessels. In such cases, treatment of pituitary adenomas requires surgical resection.

One of the major concerns when considering surgical removal of pituitary adenomas is the possibility of developing postoperative hypopituitarism requiring life-long hormone replacement therapy. Until a few years ago, many neurosurgical institutions along with their endocrine counterparts would routinely medicate their patients with perioperative hormonal supplements, most commonly consisting of cortisol replacement, without thorough consideration of the details of the patients case, such as the size of the tumor, the type of tumor (functional vs. nonfunctional), degree of invasiveness, and preoperative pituitary function. Oftentimes during follow-up these patients would not be weaned off their supplements or challenged to test their natural pituitary function, leaving the question regarding whether these patients actually required these supplements unanswered. These hormonal supplements are not only a nuisance to the patient, but are also costly and may have side-effects, such as hypertension and weight gain, especially in patients with normal postoperative pituitary function.

Today, with the rise of novel techniques like endoscopic transsphenoidal surgery, pituitary adenomas can be removed with higher precision and near total preservation of the pituitary gland, unlike in the past when concerns of intraoperative pituitary damage were prevalent with the microsurgical transsphenoidal technique. Thus, the transition to the endoscopic transsphenoidal approach increases the rate of preservation of pituitary gland function without requirement of hormone replacement therapy. In the present retrospective study performed on patients who underwent endoscopic endonasal transsphenoidal surgery for pituitary adenomas between 2009 and 2015, we investigate the outcomes of surgery, focusing on the incidence of new hypopituitarism (hypocortisolemia and hypothyroidism) following surgery and the need for hormone replacement therapy.

Methods:

A retrospective chart review was conducted on all patients who underwent endoscopic endonasal transsphenoidal surgery for a pituitary adenoma between December 2009 and May 2015. Procedures were performed by the senior authors (JKL and JAE) at University Hospital. Patient charts and electronic medical records were used to collect pre- and post-operative neurological characteristics, hormone lab values, pathology reports of immunostained tumor...
biopsies, CT/MRI characteristics, operative procedures, and neurosurgery, otolaryngology, endocrinology, and ophthalmology follow-up notes. Ultimately, 101 patients were identified for this study, and 19 were excluded because they had preoperative cortisol or thyroid hormone axis dysfunction, such as Cushing’s disease or hypothyroidism. This study was approved by the Institutional Review Board (IRB) at NJMS.

Surgical Technique:

The initial nasal portion of this surgery to expose the sphenoid sinus was performed by the otolaryngologist (JAE). A 30 degree high definition Storz endoscope was used to perform the approach into the sphenoid sinus. The middle turbinates were then lateralized and a sphenoidotomy was performed followed by widening with a high-speed drill and removal of the sellar floor with Kerrison rongeurs. After careful incision of the dura, the tumor was resected. The dead space at the site of tumor excision was packed with Surgicel and gentamicin-soaked Gelfoam pledgets. Secondary repair of the skull base was achieved with AlloDerm patch grafts, fat grafts, and/or a nasoseptal flap with additional bolstering using Surgicel and Gelfoam pledgets.

Hormonal replacement treatment protocol:

The need for hormonal replacement was based on immediate postoperative blood work screening for pituitary hormone values. If the patient demonstrated low serum cortisol along with symptoms of hypocortisolism, such as fatigue, nausea, vomiting, and loss of appetite, cortisol supplements were administered. Likewise, low serum TSH, T4, or T3 values were treated with thyroid hormone supplements (levothyroxine). All patients continued to be monitored prior to discharge with supplements provided as needed. Following discharge patients received careful instruction to report to the ER if any symptoms such as fatigue, dizziness, headache, or nasal discharge developed. They were given follow-up appointments at 2 weeks, 6 weeks, 3 months, 6 months, 1 year, and then yearly with neurosurgery, otolaryngology, and endocrinology.

Summary:

In total, 9.8% of patients (8 out of 82) developed new hypopituitarism following endoscopic endonasal transsphenoidal surgery for a pituitary adenoma. In the group with normal postoperative pituitary function following surgery (N=74) there were 50 non-functional adenomas, 13 prolactinomas, 6 growth hormone (GH) secreting, 3 adrenocorticotropic hormone (ACTH) secreting, 1 thyroid stimulating hormone (TSH) secreting, and 1 gonadotroph-secreting adenoma. Among the patients who developed new hypopituitarism following surgery (N=8), 5 had non-functional adenomas, 2 had prolactinomas, and 1 had a gonadotroph-secreting adenoma (Figure 1).
Comparison of characteristics and surgical factors of the two groups (new hypopituitarism and normal pituitary function following surgery) did not reveal many significant differences. One distinguishing characteristic between the groups was gender, with 51.4% of the normal pituitary function group being female compared to 75% of the new hypopituitarism group being female (Fisher’s exact t-test, P=0.185). The mean tumor volume was 13837 mm$^3$ in the normal pituitary function group compared to 14393 mm$^3$ in the new hypopituitarism group (Mann-Whitney test, P = 0.784), with a mean age of 51 compared to 54 (t-test, P=0.592). Considering other factors among the 2 groups, 38.9% of cases in the normal pituitary group had cavernous sinus invasion compared to 37.5% in the new hypopituitarism group (Fisher’s exact t-test, P=0.627), 74.3% compared to 75.0% for optic chiasm compression (Fisher’s exact t-test, P=0.667), 26% compared to 37.5% for use of fat graft for reconstruction (Fisher’s exact t-test, P=0.374), and 58.9% compared to 50% for use of Alloderm for reconstruction (Fisher’s exact t-test, P=0.451).

Additionally, assessment of the postoperative serum cortisol values revealed that patients who develop new hypopituitarism after surgery had a significantly lower postoperative day 2 (POD2) cortisol level than patients with normal pituitary function after surgery (t-test, P=0.013). While the serum cortisol values were lower in the new hypopituitarism group than in the normal pituitary function group on postoperative day 1 (POD1) and postoperative day 3 (POD3), the differences were not significant (t-test, P=0.149 for POD1 and P=0.107 for POD3).

Figure 2. Percent gender distribution for the two postoperative group

Figure 3. Morning serum cortisol levels on postoperative day 1(POD1), 2 (POD2) and 3 (POD3) of patients with normal pituitary function and those who developed new hypopituitarism following surgery. The groups were significantly different on POD2, represented with *. Black diamonds represent the means of each group.
Conclusion:

Overall, this study supports the utility of withholding postoperative hormonal replacements until the need arises in patients who are carefully monitored and instructed through their postoperative course following endoscopic endonasal transsphenoidal surgery for a pituitary adenoma. Only a small fraction of patients developed new hypopituitarism following surgery and postoperative day 2 cortisol values were a significant indicator of hypocortisolemia in these patients. Thus, this protocol for administration of hormonal replacement therapy is safe, effective, and avoids the potentially dangerous exposure to excess hormones in a patient with normal pituitary function. Moving forward, it is important to further investigate the relationship between intraoperative choices, nuances in surgical techniques, methods of sellar reconstruction, and postoperative outcomes. In addition to the current assessment of anterior pituitary function, it will also be interesting to assess the postoperative incidence of de novo diabetes insipidus, indicating new posterior pituitary dysfunction.

References:

PRIYAL SHAH (NJMS 2018)

PD-L1 INDUCTION BY RECEPTOR TYROSINE KINASES, TYRO3, AXL, AND MER

Stanley Kimani (Department of Microbiology, Biochemistry, and Molecular Genetics)
Raymond B. Birge, Ph.D. (Department of Biochemistry and Molecular Biology)

Objective:

TAM receptors (Tyro3, Axl, Mer) are a family of three homologous receptor tyrosine kinases that are overexpressed in numerous cancer cells. They recognize apoptotic cells through the ligands, Protein S (ProS) and Growth-Arrest-Specific Gene 6 (GAS6), which interact with phosphatidylserine that is exposed on the surface of apoptotic cells. TAM receptors are inhibitory receptors that dampen the inflammatory response. Tumor cells that express TAM receptors also acquire the ability to phagocytose apoptotic cancer cells until professional phagocytes detect them and alert the immune system. Thus, abnormal expression of TAM receptors has been implicated in promoting proliferation of cancer cells and suppressing anti-tumor immunity. Not much is known about the downstream signaling cascades of TAM receptors. However, in immune cells, there is a link between TAM receptors and PD-L1 (programmed death ligand 1). Programmed Death Ligand 1 (PD-L1) is also overexpressed in tumor cells. PD-L1 on tumor cells interacts with PD-1 on T cells to cause a decrease in cytokine production and block T cell proliferation. As a result, the immune system is suppressed and does not destroy cancer cells expressing PD-L1. We hypothesize that TAM receptors induce the expression of PD-L1.

To study ligand-mediated activation of TAMs, we generated a series of reporter cell lines expressing chimeric TAM receptors. Using this system, we probed for PD-L1 to see if TAM receptors led to the up regulation of PD-L1. Overall, these studies demonstrate that TAM receptors induce PD-L1 expression suggesting that TAM receptors may be potential targets for immunotherapy.

Methods:

CHO-derived cells were cultured in HAM’s F12 media. Four different cell lines were used. Parental (control) and three expressing chimeric TAM receptors (Tyro3, Axl, Mer). All cells were cultured at 37°C with 5% CO2. Cells were starved for 24 hours in media containing 1% serum. There were two treatments per cell line, with and without EGF. They were then stimulated with EGF for 24 or 48 hours. Cells were washed with cold PBS and lysed using lysis buffer. Protein concentrations were determined by Bradford method. Proteins were resolved by SDS-PAGE using an 8% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The blots were incubated with the PDL1 antibody overnight and horseradish peroxidase-conjugated secondary antibody for 1 hour followed by detection by chemiluminescence.
Summary:

Figure 2: Proposed model of the relationship of TAM receptors, PD-L1 and immune escape: (A) Dying tumors exhibit highly dysregulated PS. GAS6 and ProS bind to and activate TAM receptors while serving as a bridging molecule between PS and TAM receptors. (B) Activation of TAM receptors induces PDL1 expression on the surface of the tumor cell and an amplification of TAM receptors. PD1 on T cells binds to PDL1 leading to the inactivation of T cells. Blocking PS or TAM will break these immune checkpoints.

Figure 1: Activation of chimeric TAM receptors using EGF for (A) 24 hours and (B) 48 hours increased expression of PD-L1 compared to the Parental (control) cells.
Conclusion:

The data shows a direct relationship between TAM receptors and the expression of PD-L1. Compared to the control, the cells expressing TAM receptors had increased expression of PD-L1, suggesting that TAM receptors may induce PD-L1 expression. Since, increased PD-L1 leads to immune escape of cancer cells, inhibition of TAM receptors would block this pathway and allow immune cells to target tumor cells. Thus, TAM receptors may be a potential target for immunotherapy. Further studies include flow cytometry to test the surface expression of PD-L1 and experiment.

References:

Objective:

Prostatic adenocarcinoma and benign prostatic hyperplasia, disorders of malignant and benign growth of the prostate respectively, are both strongly correlated with age. Benign prostatic hyperplasia (BPH) develops in men above 40 years of age, affecting 50% of men by the age of 60 and 90% of men by the age of 85 (1). Prostatic adenocarcinoma affects 6.4% of men between 60 and 69 years of age and 11.2% of men older than 70 years old resulting in 29,480 deaths per year (2).

As bodies age and cells continue to divide, telomeres shorten over time as a biological clock measuring cell divisions. When cells divide a certain number of times the telomeres become too short and the cells begin to recognize the exposed ends of the chromosomes as damaged DNA, resulting in the suspension of cell division while the cell attempts to repair what it believes to be a double stranded DNA break. This stable arrest in cellular proliferation is considered an important tumor suppression mechanism that prevents cells from continuously dividing and becoming cancerous.

To become cancerous, growing cells must overcome the telomere dysfunction induced senescence and continue dividing unchecked. This can occur either by reactivation of telomerase to extend the telomeres as the cells continue dividing, or by other unknown mechanisms by which senescent cells in a benign tumor become malignant and resume cell division, as shown in Figure 1 (3).

Therefore, we tested for the presence of DNA damage response (DDR) as measured by the presence of 53BP1, Tumor suppressor p53 binding protein 1, using immunofluorescence (4) in two prostatic growth conditions. Additionally we determined DDR co-localization with telomeric sites using immunoFISH with peptide nucleic acid probes to quantify the presence of telomere induced senescence in benign and metastatic prostatic neoplastic lesions. We observed that DDR was more prevalent in benign lesions than in adenocarcinomas, and the foci found in BPH were frequently co-localized with telomeric sites.
Methods:

Immunohistochemical staining was performed for 53BP1 as a marker of DNA damage response in patient tissue samples of benign prostatic hyperplasia and prostatic adenocarcinoma. Co-staining with HP1β, p16, and macroH2A was attempted as senescence markers, with none of the different antibodies providing a significant number of positive cells. All images used for analysis used HP1β co-staining, with the green channel turned off to remove any background caused by the staining procedure. The steps of the procedure were to initially deparaffinize the provided tissue samples, incubate with the primary antibody (polyclonal rabbit anti-53BP1), then incubate with the secondary antibody (Cy3 conjugated polyclonal donkey anti-rabbit) before mounting with DAPI (a blue fluorescent stain for nuclear DNA) and imaged.

ImmunoFISH staining for telomeric PNA was performed along with co-staining using immunohistochemical staining for 53BP1 in benign prostatic hyperplasia tissue samples only. The steps to this procedure began with deparaffinizing the tissue samples, incubating with Cy3 conjugated peptide nucleic acid probes specific to telomeric DNA regions, then incubating with primary antibodies (polyclonal rabbit anti-53BP1) and secondary antibodies (Alexa488 conjugated goat anti-rabbit) before mounting with DAPI and imaging.

Summary:

From five samples each of benign prostatic hyperplasia and prostatic adenocarcinoma, twelve images were taken at 63x magnification. The cells were then counted and classified into four categories: no 53BP1, 1-2 foci, 3+ Foci, and granular cells. Cells of each type were added for all 12 images in each tissue sample and divided by the total number of cells from each sample and the results are displayed in Figure 2.

<table>
<thead>
<tr>
<th>Patient</th>
<th>1-2 Foci</th>
<th>3+ Foci</th>
<th>Granular</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0410750</td>
<td>4.73%</td>
<td>0.24%</td>
<td>40.73%</td>
<td>15.42%</td>
</tr>
<tr>
<td>S046140</td>
<td>12.96%</td>
<td>1.03%</td>
<td>27.98%</td>
<td>16.32%</td>
</tr>
<tr>
<td>S038111</td>
<td>10.38%</td>
<td>1.25%</td>
<td>40.25%</td>
<td>12.34%</td>
</tr>
<tr>
<td>S042849</td>
<td>4.75%</td>
<td>0.26%</td>
<td>32.96%</td>
<td>12.51%</td>
</tr>
<tr>
<td>S046970</td>
<td>7.08%</td>
<td>0.70%</td>
<td>68.20%</td>
<td>10.96%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patient</th>
<th>1-2 Foci</th>
<th>3+ Foci</th>
<th>Granular</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3</td>
<td>1.65%</td>
<td>0.07%</td>
<td>37.36%</td>
<td>9.61%</td>
</tr>
<tr>
<td>L5</td>
<td>0.97%</td>
<td>0.00%</td>
<td>16.50%</td>
<td>10.52%</td>
</tr>
<tr>
<td>L6</td>
<td>1.06%</td>
<td>0.00%</td>
<td>18.67%</td>
<td>10.83%</td>
</tr>
<tr>
<td>L9</td>
<td>0.74%</td>
<td>0.00%</td>
<td>48.94%</td>
<td>19.26%</td>
</tr>
<tr>
<td>L10</td>
<td>2.01%</td>
<td>0.00%</td>
<td>37.59%</td>
<td>8.61%</td>
</tr>
</tbody>
</table>

**Figure 2.** Quantification of immunofluorescence staining. Percentages given are for the total number of each classification of DDR positive cell divided by the total number of cells included in all (n=12) images from each patient sample. A) BPH total totals. B) Prostatic adenocarcinoma totals.

The sums of all types of 53BP1 positive cells from each of the patient samples and in total across all of the patient samples are shown in Figure 3. The totals were compared using a Student's t-test and found to be statistically significant with a p value of 3.37*10^-7.
PNA immunoFISH was performed with 53BP1 immunofluorescence co-staining to determine co-localization of DNA damage response foci and telomeric sites, only for BPH samples. This was due to the fact that the percentage of cells positive for foci amongst each image (in total, 8.51% for BPH and 1.31% for adenocarcinoma) were compared using a Student’s t test. The groups were found to have a statistically significant difference such that $p=6.65\times10^{-11}$. The cells were counted as in the previous assay, as well as counting the number of cells containing 50% or more foci co-localized to telomeric sites. An example image and the results of this assay are shown in Figure 4.
In addition, it was observed across BPH samples in both assays that granular cells were more common on the apical edges of the epithelium in BPH while cells containing foci were more prevalent near basal edges, possibly suggesting that the cells on the apical edge are actively dividing and the basal cells are already senescent.

**Conclusion:**

1. DNA damage foci are significantly more prevalent in the hyperplastic epithelium of BPH tissues than in the cancerous growths of prostatic adenocarcinoma.
2. The observed DDR foci are frequently co-localized (66.52%) with telomeric sites.
3. Cells containing foci were frequently found closer to the basal edge of the epithelium in BPH. Granular cells frequently were found closer to the apical edge.

While cellular senescence could not be determined due to a failure to see results in tissues stained with senescence markers, the co-localization of foci and telomeric sites suggests that the telomeres are shortened and the cell is recognizing them as damaged DNA. Using this fact to assume these cells are senescent, this data is in line with most of the previous data from our lab, using other tissue’s benign and cancerous regions and testing for senescence markers and DNA damage foci, such as in breast cancer and its precursors.

From this we can conclude that telomere dysfunction induced senescence acts to suppress progression of prostate cancer by arresting the division of cells after a limited number of divisions have been reached. However, in adenocarcinoma the cells either have reactivated telomerase or acquired some other oncogenic mutation allowing the cells to bypass this replication barrier and progress mitosis as cancerous tissue.

**References:**

Michael Song (TCNJ 2018 / NJMS 2021)

Effects of Chromatin Regulatory Proteins on Double Strand Breaks at Telomeres

Katsunori Sugimoto, PhD. (Microbiology and Molecular Biology)

Objective:

Telomeres are nucleoprotein complexes located at the ends of linear chromosomes. They function to prevent DNA degradation, as well as recombination and DNA end fusions with adjacent chromosomes. Telomeric sequences can also be found at internal sites of chromosomes. Telomeric sequences at both chromosome ends and intragenic regions act as replication barriers that slow down DNA replication fork movement, largely because of their high GC content and the single-stranded nature of telomeric overhang. This replication fork block has been proposed to generate DNA breakages that result in genome instability, a hallmark of cancer. Telomeric sequences are covered with telomere sequence-specific DNA binding proteins, likely along with histones. We therefore hypothesized that chromatin remodeling including histone modification regulates DNA breakage induction at telomeric sequences. We examined which chromatin regulatory proteins are involved in double strand break (DSB) induction using a gene disruption mutant library of the budding yeast *Saccharomyces cerevisiae.*

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 antidigoxigenin 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:

The telomeric sequence TG250 was introduced into various mutant library strains containing deletions for genes coding for chromatin regulatory proteins (Figure 1). The frequency of DSB induction was then monitored through screening for URA3- colonies on an FOA plate using six deletion library strains transformed with TG250 and one positive control (YCP33). ESC1 and SET2 were comparatively most similar to YCP33 (high quantity of URA3- colonies) and were therefore selected as positive candidates for a quantitative assay (Figure 2).

![Figure 1. Illustration of the integration site of the telomeric sequence. Induction of DSB causes loss of the URA3 marker.](image1)

![TG cassettes](image2)

![Figure 2. Spot test 1-10 dilutions of TG250 transformants (ESC1, SET2, NFI1), and one control (wild type). Fig. 2A is a rich media plate (YPD) and Fig. 2B is a selection plate for URA3- cells (FOA). The ESC1 and SET2 deletion mutants were determined to have more URA3- colonies and therefore a higher incidence of DSBs.](image3)
Figure 3. Southern blot results depicting lengths of telomere ends in mutants and wild type. Both candidates exhibiting higher frequency of DSBs in TG250 integration (ESC1, SET2) exhibit shorter telomere lengths than the wild type.

<table>
<thead>
<tr>
<th>Gene disrupted</th>
<th>Description of gene (related proteins)</th>
<th>Loss of URA3 through integration of TG250</th>
<th>Different telomere end length compared to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIA2</td>
<td>functions in ubiquitylation of silent chromatin structural protein</td>
<td>-</td>
<td>N/T</td>
</tr>
<tr>
<td>NF1I</td>
<td>Promotes chromatin anchoring</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ESC1</td>
<td>Protein localized to the nuclear periphery; involved in telomeric silencing</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ESC2</td>
<td>roles in silencing, lifespan, chromatid cohesion and the intra-S-phase DNA damage checkpoint</td>
<td>-</td>
<td>N/T</td>
</tr>
<tr>
<td>HMT1</td>
<td>modifies hnRNPs</td>
<td>-</td>
<td>N/T</td>
</tr>
<tr>
<td>GCN5</td>
<td>subunit of ADA and SAGA histone acetyltransferase complexes</td>
<td>-</td>
<td>N/T</td>
</tr>
<tr>
<td>SET2</td>
<td>Histone methyltransferase with a role in transcriptional elongation</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 4. Table depicting the effect of losing certain chromatin regulatory proteins on DSB induction at telomeres. The +/- symbols indicate the increased or decreased frequency of DSBs or variation in telomere end length for each mutant strain relative to the control.

Conclusion:

It was determined that the gene disruption mutants for ESC1 (involved in telomeric silencing) and SET2 (involved in histone modification) exhibited the highest frequencies of DSBs after being transformed with TG250, an internal telomeric sequence. Though no direct correlation to variation in telomere length has been established, a southern blot affirmed that both of these strains (in the library background) exhibited a shorter telomere end length than NF11 and the wild type control. This suggests variability among the mutant strains. For further study, a
transfer of deletion cassettes from the library background to our lab background containing TG250 must be performed to eliminate external variables, and thereby confirm that it is the deletion of these genes themselves that are inducing a higher frequency of DSBs at the TG250 sequence. Additionally, the integration of deletion cassettes into a ura3 mutant strain of the same lab background should be conducted for the analysis of the frequency of DSBs at endogenous telomeres (Figure 5).

![Diagram of mutant strain construction](image)

Figure 5. Illustration of the mutant strain construction. A DSB at the telomere of Chr. VII induces DNA repair via homologous recombination with Chr. V. This results in expression of the URA3 marker on Chr. VII.

References:


Objective:

Lung Cancer is the leading cancer killer in both men and women in the US; it causes more deaths than colorectal, breast, and prostate cancers combined. An estimated 158,040 Americans are expected to die from lung cancer in 2015, approximately 27% of all cancer deaths, making research on lung cancer imperative (1). BMP2 is an essential molecule in neonatal development and adult physiology. Increased BMP2 mRNA levels are associated with poor lung prognosis and elevated BMP2 levels in lung tumors appear to promote malignant progression via stimulation of cell proliferation and angiogenesis (2). Dr. Roger's lab and other labs have discovered that an ultra-conserved sequence (UCS) in the 3'untranslated region (UTR) of BMP2 mRNA post-transcriptionally represses BMP2 in normal lung tissue (3-5). Understanding the regulation of this 3'UTR is key as repressive miRNAs inhibit lung tumor BMP2 expression via the UCS (6). Therefore, we hypothesized that UCS mediated mechanisms that prevent BMP2 synthesis are impaired in lung cancer and lung pathologies, and that miRNA replacement therapy may be effecting in clinical lung cancer treatments.

Deletion of the UCS and Phenotyping the new SH/SH and SH/+ Mice:

One of our aims in this project was to study the morphological changes that result from the deletion of the UCS in mice. The UCS is conserved across distantly related species, including mammals and fishes, suggesting that it is crucial for survival. The lab generated two alleles to test UCS function in mice: one that contains the UCS (the Flox allele, FL) and one that does not contain the UCS (the Short allele, SH). The lab used Cre-recombinase/loxP deletion to delete the UCS, resulting in the desired Short allele. We decided to test the relative fitness of these new alleles by analyzing the percent of each genotype present amongst the litters, and hypothesized that the SH/SH mice would be underrepresented in the pup population. Tumors recapitulate embryo processes; ergo studying embryonic regulatory mechanisms can provide insight into tumor environments. We hypothesized that deleting the BMP2 UCS disrupts the development of the heart and other organs and aimed to compare the lung morphology of SH/SH embryos at day 13.5 to that of sh/+ control embryos. We also aimed to follow these morphological and physiological changes into adulthood and began to phenotype these new SH/SH and SH/+ mice. One parameter was the relative adult lung volumes. We hypothesized that deleting the UCS affects lung morphology and aimed to compare the lung volumes of the SH/SH mice to those of SH/+ mice. Another parameter was the weights of these adult mice, as weight can be indicative of lung function. We hypothesized that deleting the UCS influences weight and aimed to compare weights of SH/SH mice to SH/+ mice. A third parameter was relative BMP2 and pSMAD protein concentrations. We hypothesized that BMP2 and BMP2 signaling are induced in SH/SH mice relative to the SH/+ control and aimed to examine levels of [BMP2] and [phosphoSmad1/5/8] (signaling component) by Western Blots.

Klotho Null Mice as a Model of Aging and Lung Pathologies:
In lung cancer, the UCS switches from an activator of BMP2 expression to a repressor. This corresponds with the increase in [BMP2] in lung tumors (3). So we postulated that BMP2 would
be induced in another lung pathology – age induced Emphysema. As klotho null (kl/kl) exhibit lung pathologies, we used them as an in vivo model. We hypothesized that BMP2 is induced in kl/kl mice relative to kl/+ control mice and aimed to compare the expression of a BMP2-driven lacZ reporter transgene with intact 3’ UTR in kl/kl and kl/+ mice.

**Methods:**

**Deletion of the UCS and Phenotyping the new SH/SH and SH/+ Mice:**

In order to analyze the relative fitness of the pups with the new alleles, the number of male and female pups of each genotype was tabulated. In this breeding scheme, mice with one copy of the floxed allele and one copy of the recombined allele (BMP2^{FL/SH}) were bred with one another, resulting in pups with either a BMP2^{FL/FL}, BMP2^{SH/FL}, or BMP2^{SH/SH} BMP2 genotype. A Chi Squared Analysis via Graphpad.com was performed to compare the expected percent in each genotype to the actual percent.

Day 13.5 embryos of multiple genotypes were extracted, such as SH/SH and SH/+. The embryos were genotyped using PCR analysis and Gel Electrophoresis. They were fixed in 3.7% formaldehde, dehydrated in 25%, 50%, 75%, 95% methanol, and stored in 100% methanol. Some embryos were then washed with xylenes, embedded in paraffin, sectioned, mounted on slides, and stained with Hematoxylin and Eosin. Subsequently, the slides were visualized on a dissecting scope, and then captured the images at various magnifications to visually compare the embryo morphology of the lungs of the SH/SH pups to those of the SH/+ control pups.

Eight female mice were set to the Molecular Imaging Center Rutgers University for full analysis-two control C57Bl, three SH/+, and three SH/SH. Components of total lung volume were calculated – such as air space (freely breathing), active moving lung tissue, and dense lung tissue. The values of each component was normalized to the total lung volume to analyze the data. Weights of the fed mice from various dates, plus an additional weight for each animal that was taken after overnight fasting, were also provided.

A Western Blot on lung tissue from pairs of SH/SH, SH/+, and FL/+ male mice was also performed. The tissue was homogenized and lysed in a PBS/Heparin solution via a sonicator. The resulting blots were then probed with anti-BMP2 and anti-phoshoSMAD (1/5/8). The images of the probed blots were captured using a GelDoc apparatus and then the relative protein concentrations were quantified by obtained the relative band intensities using AlphaView Software. The relative band intensities were first normalized to a positive control, and then normalized those values were normalized to Actin levels.

**Klotho Null Mice as a Model of Aging and Lung Pathologies:**

When a klotho null homozygous mouse presented signs of nearing death, this mouse was sacrificed along with a heterozygous control littermates. The lungs, heart, aorta, and 2 kidneys were dissected from each mouse. This procedure was performed multiple times throughout the summer, and multiple sets of tissue were allocated for various assays: calcium assay, Western Blots, and histological sectioning and staining. For the mice allocated for β-Gal staining, as the mice have the BMP2-driven lacZ reporter transgene, the lungs, heart, aortic, and kidney tissue were fixed and stained for β-Gal activity. For this project, the lungs were then taken from a pair of kl/kl and kl/+ mice and hand sectioned into 4 sections of lung tissue from both animals, 3 transverse sections from the middle to lower left lung, and one sagittal section from the posterior midline of the lungs. The sections were then cleared in methyl salicylate for 15 minutes, placed on slides, cover-slipped, and photographed using the dissecting scope. After photography, the sections were then placed back into 100% ethanol.
Summary:

**Deletion of the UCS and Phenotyping the new SH/SH and SH/+ Mice:**
The Chi Squared analysis revealed that the resulting pup genotypes were in fact significantly skewed from expected Mendelian Genetics, particularly with the male BMP2^{FL/FL}, male BMP2^{SH/SH}, and female BMP2^{SH/SH}. Chi squared = 22.240 with 5 degrees of freedom; 2 tailed P value = 0.0001 (Table 1).

**Table 1: Variable Fitness of these New Alleles**

<table>
<thead>
<tr>
<th>Expected Genotype</th>
<th>Male</th>
<th>Female</th>
</tr>
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<tbody>
<tr>
<td>BMP2^{FL/FL}</td>
<td>12.50%</td>
<td>12.50%</td>
</tr>
<tr>
<td>BMP2^{FL/SH}</td>
<td>25%</td>
<td>25%</td>
</tr>
<tr>
<td>BMP2^{SH/SH}</td>
<td>12.50%</td>
<td>12.50%</td>
</tr>
<tr>
<td>Expected %</td>
<td></td>
<td></td>
</tr>
<tr>
<td># of Pups (n=25)</td>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Actual %</td>
<td>4%</td>
<td>12%</td>
</tr>
<tr>
<td></td>
<td>24%</td>
<td>16%</td>
</tr>
<tr>
<td></td>
<td>44%</td>
<td>4%</td>
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Morphological embryo analysis revealed that deleting the UCS affects overall development. Whole mount analysis revealed stark differences amongst some SH/SH and SH/+ embryos, including neural tube defects in some of the SH/SH embryos (Fig. 1A) (6). Analysis of the stained embryo slides revealed that the lungs at E13.5 might be denser (Fig. 1B).

**Fig. 1A.** Evident rostral NTD in SH/SH E11.5 embryos (7)  
**1B.** H&E stained E13.5 sagittal sections.

Analysis of the relative lung volumes in adult female mice revealed that SH/SH mice might have smaller air space while freely breathing, and inversely, a larger volume of dense lung tissue. Lung volume component values were normalized to total lung volume, ave ± SEM (C57Bl n=2, SH/+ and SH/SH n=3, Fig. 2) Additionally, weight analysis of the same mice revealed the trend that the female SH/SH weigh less, both chronically (Fig. 3A) and after fasting (Fig. 3). Weights graphed as ave ±SEM (C57Bl n=2, SH/+ & SH/SH n=3)
Western Blot analysis revealed that BMP2 and pSMAD decreased in SH/SH mice relative to SH/+, but the results are not conclusive. [BMP2] (Fig. 4A) and [pSMAD] (Fig. 4B) graphed as relative band intensities (normalized to a positive control, then normalized to actin) ±SEM (SH/SH n=4, SH/+ n=6, & FL/+ n=4).

**Fig. 4. A. Relative [BMP2]. B. Relative [pSMAD]**

Klotho Null Mice as a Model of Aging and Lung Pathologies:
Examination of the lung sections under the dissecting scope revealed that 5/5 examined kl/kl panels had some β-Gal expression, while only 2/5 kl/+ sections had visible β-Gal expression.

**Conclusion:**
In terms of embryo morphology, the whole mount embryos and sectioned slides demonstrate that deleting the UCS affects overall development. However, as only slides from 2 embryos were compared, additional sections need to be cut from more embryos are needed to confirm any defect.

At the adult state, deleting the UCS continues to affect morphology and physiology. Regarding lung volumes, the altered ratio of dense tissue to air space in SH/SH mice is consistent with a functional or structural effect of the UCS. The increased density observed previously at E13.5 embryos is consistent with a developmental effect on lung structure. Additionally, SH/SH mice weigh less, both over time & after fasting. The difference is more noticeable after fasting, suggesting differences in ability to retain food. After fasting, the SH/SH weights differed
significantly from the SH/+ & C57Bl combined weights, P<0.05, implying that something is different about these SH/SH mice. However, as before, more samples are needed before making definitive conclusions. This is important because weight reflects on lung health and could potentially provide insight into lung pathologies that are observed in these animals. For the Western Blot analysis, the hypotheses failed; [BMP2] and [pSMAD] appeared to be higher in SH/+ mice compared to the SH/SH. There is precedence for an autoregulatory loop where BMP2 induces the Noggin antagonist. Next, we will measure Noggin in these samples and perform this same assay on samples from female mice to see if there is a gender difference. And again, more samples are needed, as well as analysis on the female mice. Regarding the β-Gal analysis, since the kl/kl lungs may have more β-Gal expression than kl/+ lungs, Klotho null mice model pathologically induced BMP2 in lungs. Next, lungs will be cryostat sectioned, staining quantified via ImageJ, and [BMP2] measured via Western Blot analysis. In the future, we will study miRNAs that are predicted to bind the BMP2 3'UTR and other proteins in the BMP2 signaling pathways, and that are reported to be present in lung pathologies, such as miR-34b and -34c miR-486 (6). Once we identify miRNAs that reduce BMP2 expression, we can test their efficacy as therapeutics for lung cancer on in vivo models. Pre-clinical miRNAs can be tested for efficacy on kl/kl mice via β-Gal analysis.
References:

7. Shaikh, N. and MB Rogers, unpublished data.
8. E13.5 Embryo textbook image from Kaufman’s The Atlas of Mouse Development